Therapeutic effects of favipiravir against severe fever with thrombocytopenia syndrome virus infection in a lethal mouse model: dose-efficacy studies upon oral administration

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Running Head: Treatment of SFTSV infection with favipiravir

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Abstract

Background

Severe fever with thrombocytopenia syndrome (SFTS), caused by SFTS virus (SFTSV), is a viral hemorrhagic fever with a high case fatality rate. Favipiravir was reported to be effective in the treatment of SFTSV infection *in vivo* in type I interferon receptor knockout ($IFNAR^{-/-}$) mice at treatment dosages of both 60 mg/kg/day and 300 mg/kg/day for a duration of 5 days.

Methods

In this study, the efficacy of favipiravir at dosages of 120 mg/kg/day and 200 mg/kg/day against SFTSV infection in an $IFNAR^{-/-}$ mouse infection model was investigated. $IFNAR^{-/-}$ mice were subcutaneously infected with SFTSV at a 1.0×10^6 50% tissue culture infectious dose followed by twice daily administration of favipiravir, comprising a total dose of either 120 mg/kg/day or 200 mg/kg/day. The treatment was initiated either immediately post infection or at predesignated time points post infection.

Results

All mice treated with favipiravir at dosages of 120 mg/kg/day or 200 mg/kg/day survived when the treatment was initiated at no later than 4 days post infection. A decrease in body weight of mice was observed when the treatment was initiated at 3–4 days post infection. Furthermore, all control mice died. The body weight of mice did not decrease when treatment with favipiravir was initiated immediately post infection at dosages of 120 mg/kg/day and 200 mg/kg/day.

Conclusions

Similar to the literature-reported peritoneal administration of favipiravir at 300 mg/kg/day, the oral administration of favipiravir at dosages of 120 mg/kg/day and 200 mg/kg/day to IFNAR$^{-/-}$ mice infected with SFTSV was effective.

Author summary

Severe fever with thrombocytopenia syndrome (SFTS), which is caused by SFTS virus (SFTSV), is a generalized infectious disease with a high case fatality rate. Currently, no effective therapeutics for SFTS is available; therefore, the development of effective

51 antiviral drugs is needed. Favipiravir exhibits antiviral activity against various RNA
52 viruses, including SFTSV. The present study demonstrated the efficacy of favipiravir in
53 the treatment of SFTSV infection in a lethal mouse model, when the dose was set
54 similar to that approved for anti-influenza drug in humans by the Ministry of Health,
55 Labour and Welfare, Japan. The present study suggests that favipiravir is a promising
56 drug for the treatment of SFTSV infection.
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Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is caused by SFTS virus (SFTSV), belonging to the family *Phenuiviridae* (genus *Phlebovirus*). SFTS is a viral hemorrhagic fever with a high case fatality rate; it was first reported as a novel infectious disease in China [1, 2], followed by discovery in South Korea and Japan [3, 4]. It is characterized by marked reduction in platelet, white blood cell, and total blood cell counts in patients. Hemorrhagic symptoms, such as gingival oozing, bloody diarrhea, and hematuria, are commonly observed in patients with severe and fatal SFTS [3, 5, 6]. Because of the associated high mortality rate, it is critical to develop specific and effective therapy for SFTS. Unfortunately, no such treatment has been developed yet. The inhibitory effect of ribavirin on the replication of SFTSV has been elucidated *in vitro* as well as *in vivo* [7, 8]. Although ribavirin inhibited the replication of SFTSV *in vitro* in a dose-dependent manner, therapeutic effect *in vivo* was limited in comparison with that of favipiravir. Thus, an anti-SFTSV effect of ribavirin is limited or absent in the clinical setting [9, 10]. Favipiravir is an RNA-dependent RNA polymerase inhibitor and a potent broad-spectrum antiviral drug. It inhibits the

76 replication of multiple families of RNA viruses *in vitro* and *in vivo* [11, 12]. Favipiravir
77 is a therapeutic antiviral drug against influenza virus approved in Japan. However,
78 during the 2014–2015 Ebola outbreak in West Africa, it was also considered as a
79 candidate agent against Ebola virus infection [13, 14]. In addition, favipiravir was
80 demonstrated to have antiviral effects against the newly discovered emerging viruses
81 SFTSV and Heartland virus (HRTV) [15]. HRTV is an emerging tick-borne virus,
82 which, similar to SFTSV, belongs to the genus *Phlebovirus* in the family *Phenuiviridae*.
83 Patients infected with HRTV show similar symptoms as SFTS patients. The efficacy of
84 favipiravir against HRTV infections was demonstrated in animal infection models using
85 STAT2 knockout hamsters [15].
86 Reportedly, favipiravir is effective when administered even after symptoms appeared.
87 The antiviral effects of favipiravir against SFTSV were confirmed in a mouse model as
88 well as STAT2 knockout hamster model [16]. We have previously demonstrated the
89 antiviral effects of favipiravir against SFTSV in a lethal mouse model using IFNAR$^{-/-}$
90 mice. In the study, the highest dose of favipiravir used in mice experiments, at which
91 side effects did not appear, was 300 mg/kg/day via intraperitoneal (i.p.) route. All mice

92 treated with favipiravir at 300 mg/kg/day survived without showing any symptoms
93 upon SFTSV infection. In the mouse model, all mice also survived when treated i.p.
94 with favipiravir at 60 mg/kg/day. However, their body weight decreased by
95 approximately 10% [8]. In the present study, the efficacy of favipiravir in the mouse
96 lethal model was evaluated at dosages of 120 mg/kg/day via oral administration (p.o.)
97 and 200 mg/kg/day p.o. The two doses of favipiravir were selected in clinical trials to
98 evaluate the efficacy of favipiravir against influenza virus infections in humans.
99 Favipiravir dosages of 120 mg/kg/day p.o. and 200 mg/kg/day p.o. have been applied
100 for approval in Japan, and the phase III study conducted in the USA. The aim of this
101 study was to assess the efficacy of favipiravir at dosages of 120 mg/kg/day p.o. and 200
102 mg/kg/day p.o. in the treatment of SFTSV infection in the lethal mouse model using
103 IFNAR$^{-/-}$ mice.
104

105 **Materials and methods**

106 **Ethics statement**. All animal experiments were performed in biological safety level 3
107 (BSL-3) containment laboratories at the National Institute of Infectious Diseases (NIID)

in Japan and adhered to NIID regulations and guidelines on animal experimentation. Protocols were approved by the Institutional Animal Care and Use Committee of the NIID (No. 215024).

Cells, viruses, and antiviral compounds. Vero cells obtained from American Type Culture Collection (Summit Pharmaceuticals International, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (DMEM-10FBS). The SFTSV Japanese strain SPL010 was used in this study [8]. Pseudotyped vesicular stomatitis viruses (VSV) possessing SFTSV-GP or VSV-G, designated SFTSVpv or VSVpv, respectively, were used [17]. SPL010 virus stocks were stored at −80°C until use. All work with SFTSV was performed in BSL-3 containment laboratories in the NIID in accordance with the institutional biosafety operating procedures. Favipiravir (Toyama Chemical Co., Ltd., Toyama, Japan) was suspended in 0.5% (*w*/*v*) methylcellulose solution.

123 **Animal experiments.** IFNAR$^{-/-}$ C57BL/6 mice were produced as described previously
124 [8]. IFNAR$^{-/-}$ C57BL/6 mice were bred and maintained in an environmentally
125 controlled specific pathogen-free animal facility of the NIID. Eight- to 10-week-old
126 male mice were used. Favipiravir was administered in mice using a stomach probe after
127 subcutaneous inoculation (s.c.) with 1.0×10^6 50% tissue culture infectious dose
128 ($TCID_{50}$) of SFTSV in 100 µl DMEM. Treatments were commenced at 1 h post
129 infection or at 1, 2, 3, 4, or 5 days post infection and continued for 5 days.
130 To determine the efficacy of favipiravir in the treatment of SFTSV infection, the mice
131 were treated with favipiravir at dosages of either 120 mg/kg/day p.o. or 200 mg/kg/day
132 p.o. [60 or 100 mg/kg/bis in die (BID), p.o.] for 5 days starting at various time points as
133 described above (Fig.1). Blood samples (20 µl/animal) were obtained via tail vein
134 puncture at intervals of 2–4 days over a period of 14 days (<4 blood drawings in total)
135 for the measurement of viral RNA levels. Body weight was recorded daily for 2 weeks,
136 and each mouse was monitored daily for the development of clinical symptoms such as
137 hunched posture, ruffled fur, activity, response to stimuli, and neurological signs. When

138 mice showed serious clinical symptoms or weight loss of more than 30 %, they were
139 considered to be reached the humane endpoint so that they were anesthetized.
140
141 **Viral RNA quantification.** The concentration of SFTSV genomic RNA in blood was
142 determined as previously described [18]. Total RNA was prepared from 20 μl of blood
143 samples using High Pure Viral RNA Kit (Roche Diagnostics K.K., Tokyo, Japan). Gene
144 expression was estimated using QuantiTect Probe RT-PCR kit (Qiagen, Hilden,
145 Germany) according to the manufacturer's protocol. Fluorescent signals were estimated
146 using LightCycler 96 (Roche Diagnostics K.K., Tokyo, Japan). Statistical analyses were
147 performed using GraphPad Prism6 Software. One-way analysis of variance (ANOVA)
148 with Bonferroni's multiple comparison test was used.
149
150 **Neutralization assay.** The day of SFTSV infection was considered as Day 0 and days
151 post infection were subsequently counted. Sera from the mice at a convalescent phase
152 were obtained at Day 14. To examine the neutralization antibody responses against
153 SFTSV of the mice at a convalescent-phase, pseudotyped VSV system was employed.

154 SFTSVpv and VSVpv were pre-incubated with serially diluted sera of the mice at a
155 convalescent-phase for 1 h at 37°C. Then, Vero cells were inoculated with each of the
156 virus–serum mixtures. After 2 h of adsorption at 37°C, cells were washed with DMEM-
157 10FBS and infectivity was determined by measuring luciferase activity after 24 h of
158 incubation.
159
160
161 **Results**
162 **Therapeutic efficacy of favipiravir against SFTSV infection in IFNAR$^{-/-}$ mice.**
163 Consistent with the results of a previous study, the optimal lethal infectious dose of
164 SFTSV strain SPL010 in mice was determined to be 1.0×10^6 $TCID_{50}$ [8]. All mice
165 treated with favipiravir at dosages of 120 mg/kg/day or 200 mg/kg/day survived from a
166 lethal SFTSV infection when treatment was initiated within 3 days and 4 days post
167 infection, respectively (Fig. 2B and 2C). All control mice, infected with SFTSV died
168 within 8 days post infection [8] (Fig. 2A). When treatment was initiated on Day 4, the
169 mice treated with favipiravir at dosages of 120 mg/kg/day and 200 mg/kg/day exhibited

170 67% and 100% survival, respectively. However, under these conditions, the health of
171 mice was highly deteriorated, with more than 15% weight loss. A few mice treated with
172 favipiravir at a dosage of 200 mg/kg/day dose initiated on Day 5 survived even with
173 30% weight loss (Fig. 2C).

174 The RNA levels in the blood of mice gradually decreased upon administration of
175 favipiravir at dosages of 120 mg/kg/day and 200 mg/kg/day, respectively (Fig. 2B and
176 2C). There was no significant difference in the RNA levels between the two treatment
177 groups. The viral RNA in blood was undetectable by Day 14 in most mice treated with
178 favipiravir at dosages of 120 mg/kg/day and 200 mg/kg/day (Fig. 2B and 2C).

179

180 **Neutralizing antibody responses against SFTSV in the mouse sera at a**
181 **convalescent-phase.** To examine whether neutralizing antibodies were induced in the
182 mice at a convalescent-phase, serum samples collected on Day 14 were tested for
183 neutralizing activity with an assay using a pseudotyped VSV system. Sera of
184 convalescent-phase mice neutralized SFTSVpv infection at a dilution of 1 in 800 (Fig.
185 3) and in a dilution-dependent manner (data not shown), whereas no significant

186 neutralization of VSVpv infection was observed (Fig. 3). The induction of neutralizing
187 antibody responses in mice wherein treatment was initiated on Days 0 or 5 seemed
188 lower than the induction of neutralizing antibody responses in mice wherein treatment
189 was initiated on Day 1 at a dosage of 200 mg/kg/day (Fig. 3B).
190
191

192 **Discussion**

193 We have previously demonstrated the protective efficacy of favipiravir in the treatment
194 of SFTSV infection at dosages of 300 mg/kg/day i.p. in the lethal mouse model [8].
195 Since favipiravir is approved for anti-influenza drug as a formula of p.o. drug in Japan,
196 we have tested the efficacy of favipiravir at dosages of 120 mg/kg/day and 200
197 mg/kg/day p.o. against SFTSV in the lethal mouse model. The results demonstrated
198 favipiravir at both dosages were effective via oral administration. The dosages were the
199 standard dose applicable in humans. For utilizing favipiravir as an anti-influenza drug in
200 humans, a dosage of 120 mg/kg/day p.o. has been set for clinical use in Japan and a
201 dosage of 200 mg/kg/day p.o. has been set for phase III studies in the USA,

202 respectively. With regard to the Ebola virus disease (EVD) outbreak that occurred in
203 West Africa in 2013–2015, favipiravir was required to be administered at a higher dose
204 for the treatment of EVD than that required for the treatment of influenza. This was
205 based on the higher IC_{50} values of favipiravir for Ebola virus *in vitro* and *in vivo* [14,
206 19, 20].
207 The effective concentration of favipiravir in blood is considered to be similar when
208 administered p.o. and when administered i.p. [21]. Here the therapeutic effect of
209 favipiravir in the treatment of SFTSV infection was observed both when administered
210 p.o. as well as when administered i.p. In contrast to the previous reports, where
211 favipiravir was administered once a day, favipiravir was administered twice a day (BID)
212 in the present study. The antiviral effects of favipiravir when administered orally at the
213 tested doses might be higher than those when administered via the intraperitoneal route
214 quaque die [8]. This difference may be attributed to the maintenance of effective
215 favipiravir concentration in blood. Furthermore, the observed therapeutic effect was
216 obtained not only due to a direct inhibition of viral replication by favipiravir but also
217 due to the production of neutralizing antibodies against SFTSV in the later phase of the

218 disease (Fig. 3). The neutralizing antibody responses were higher in mice wherein
219 treatment was initiated on Days 1 and 2 than in those wherein treatment was initiated on
220 Day 0. This may be attributed to the amount of replicated virus as an antigen.
221 Conversely, the production of neutralizing antibodies was weak in mice wherein
222 treatment was initiated on Day 5, suggesting that neutralizing antibody producing cells
223 were more heavily damaged in mice wherein the treatment was initiated in the later
224 stages of the disease.
225 The therapeutic effect of favipiravir is remarkably higher against SFTS in animal
226 models than other reported viral infectious diseases [19, 22, 23]. Administration of
227 favipiravir after the onset of the disease did not show any efficacy in the treatment of
228 EVD or Crimean-Congo hemorrhagic fever viral infection in animal models [19, 22,
229 23]. Conversely, the administration of favipiravir in the mice infected with SFTSV
230 within 4 days post infection showed efficacy even at a dosage of 120 mg/kg/day, which
231 is the dosage approved to be prescribed to humans (Fig. 2). Therefore, favipiravir was
232 effective not only for prophylactic use but also for treating SFTS in the mouse model.
233 However, it was too late to initiate the administration of favipiravir at Day 5 in the mice

model (Fig. 2). The results obtained in the present study indicate that favipiravir should be administered as early as possible post infection. This also indicates that favipiravir should be administered as early as possible from disease onset for the treatment of patients with SFTS.

Currently, there is no antiviral therapy available for the treatment of SFTSV infection. Here, we studied the efficacy of favipiravir at dosages of 120 mg/kg/day p.o. and 200 mg/kg/day p.o. in the treatment of mice infected with SFTSV. These dosages can also be applied to humans. Currently, clinical trials are underway for evaluating the efficacy of favipiravir in the treatment of patients with SFTS in Japan [24]. We hope that favipiravir will not only be used as a prophylactic drug against SFTS in the near future but also as a therapeutic drug in clinical practice.

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359

360 **Figure Legends**

361 **Fig 1.** Schematic experimental design. Six mice in each group were administered
362 favipiravir at either 120 mg/kg/day or 200 mg/kg/day starting at 1 h or 1, 2, 3, 4, or 5
363 days post infection and continued for 5 consecutive days. Placebo control mice were
364 treated with an equal volume of 0.5% (w/v) methylcellulose solution administered at 1 h
365 post infection and continued for 5 consecutive days.

366

367 **Fig 2.** Effects of treatment with favipiravir against SFTSV infection in IFNAR$^{-/-}$ mice.
368 (A) Ten mice in the placebo control group were inoculated s.c. with 1.0×10^6 $TCID_{50}$ of
369 SFTSV strain SPL010. Control mice received 0.5% (w/v) methylcellulose solution via
370 the p.o. route. (B, C) Six mice in each group were inoculated s.c. with 1.0×10^6 $TCID_{50}$
371 of SFTSV strain SPL010. Mice were treated with favipiravir at a dose of 120 mg/kg/day
372 (B, 60 mg/kg/BID, p.o.) or 200 mg/kg/day (C, 100 mg/kg/BID, p.o.). Treatment was
373 commenced at 1 h or 1, 2, 3, 4, or 5 days post infection. Favipiravir was administered
374 twice daily p.o. using a stomach probe until death or for 5 days as indicated in the upper
375 columns (shaded in gray with survival curves). Survival was determined using Kaplan–

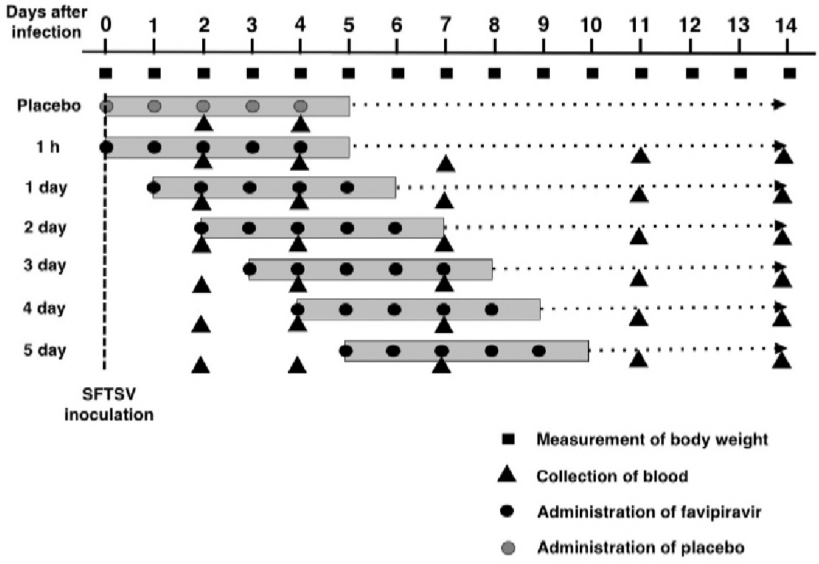
376 Meier analysis and GraphPad Prism6 (GraphPad Software) and shown in the upper
377 columns. Relative weights are shown as means with standard deviations (middle
378 columns). SFTSV RNA levels in blood samples collected at 2, 4, 7, 11, or 14 days post
379 infection were determined by quantitative RT-PCR assays (lower columns). One way
380 ANOVA with Bonferroni's multiple comparison test was used to determine statistical
381 significance. Dashed lines indicate the detection limits of the assay in blood samples.
382 Significance was determined in comparison to the results of the placebo group (for
383 survivals) or Day 2 blood samples (for RNA copies): ****, $P < 0.0001$; ***, $P < 0.001$;
384 **, $P < 0.01$; * $P < 0.05$; N.T., not tested.
385
386 **Fig 3.** Neutralization of SFTSVpv by convalescent-stage mouse sera. SFTSVpv were
387 preincubated with 800-fold diluted mouse sera collected on Day 14 (120 mg/kg/day
388 treatment group [(A) left columns] and 200 mg/kg/day treatment group [(B) right
389 columns]). Subsequently, Vero cells were infected with SFTSVpv. Infectivity of
390 SFTSVpv was determined by measuring luciferase activities at 24 h post infection.
391 Results from three independent assays are shown, with error bars representing standard

392 deviations. Significance was determined in comparison to the results from non-serum

393 treatment or infectivity of VSVpv. ****, $P < 0.0001$; **, $P < 0.01$; * $P < 0.05$.

394

Tani *et al.*, Fig. 1



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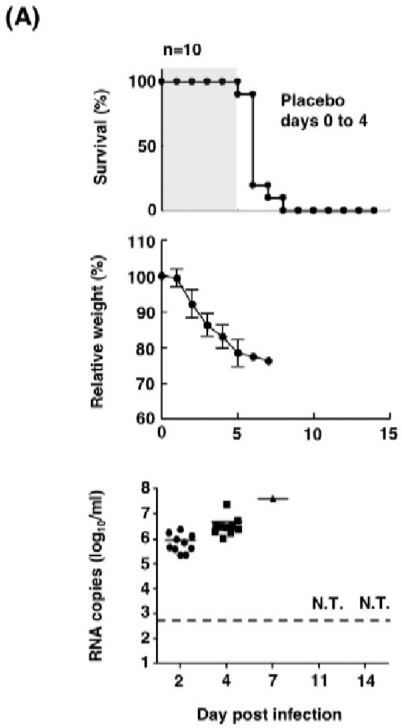
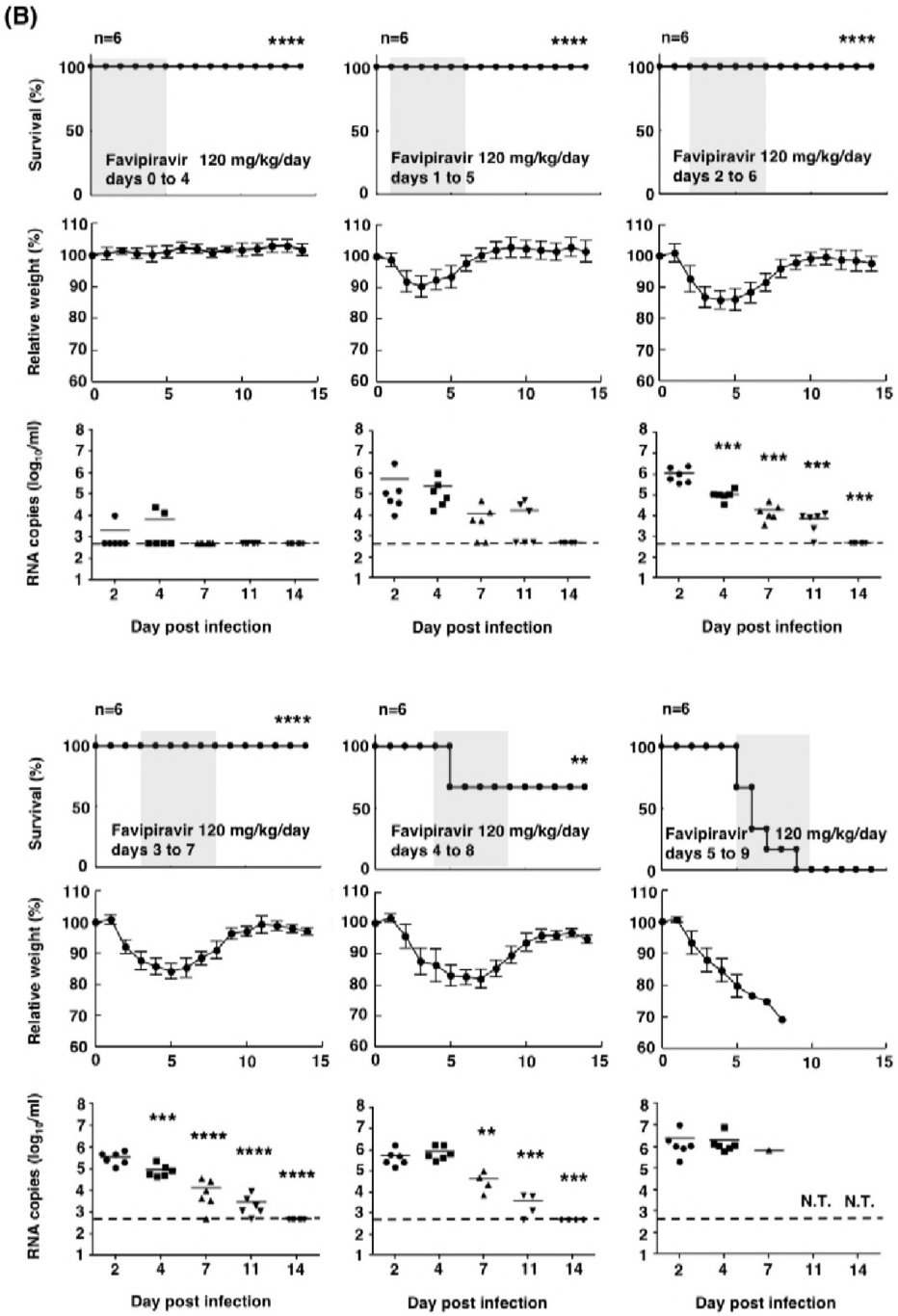
Tani *et al.*, Fig. 2



Tani *et al.*, Fig. 2



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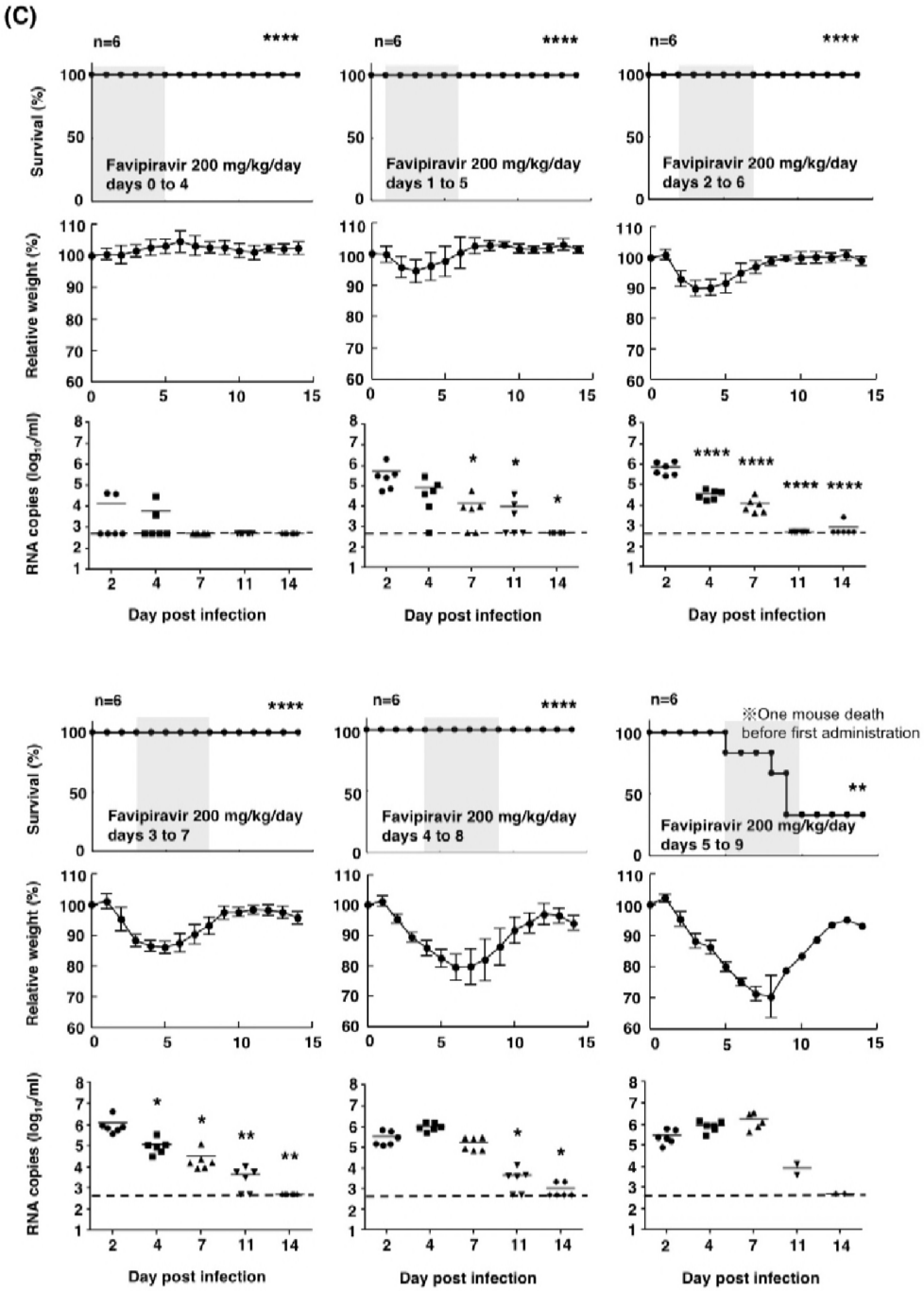
Tani *et al.*, Fig. 2



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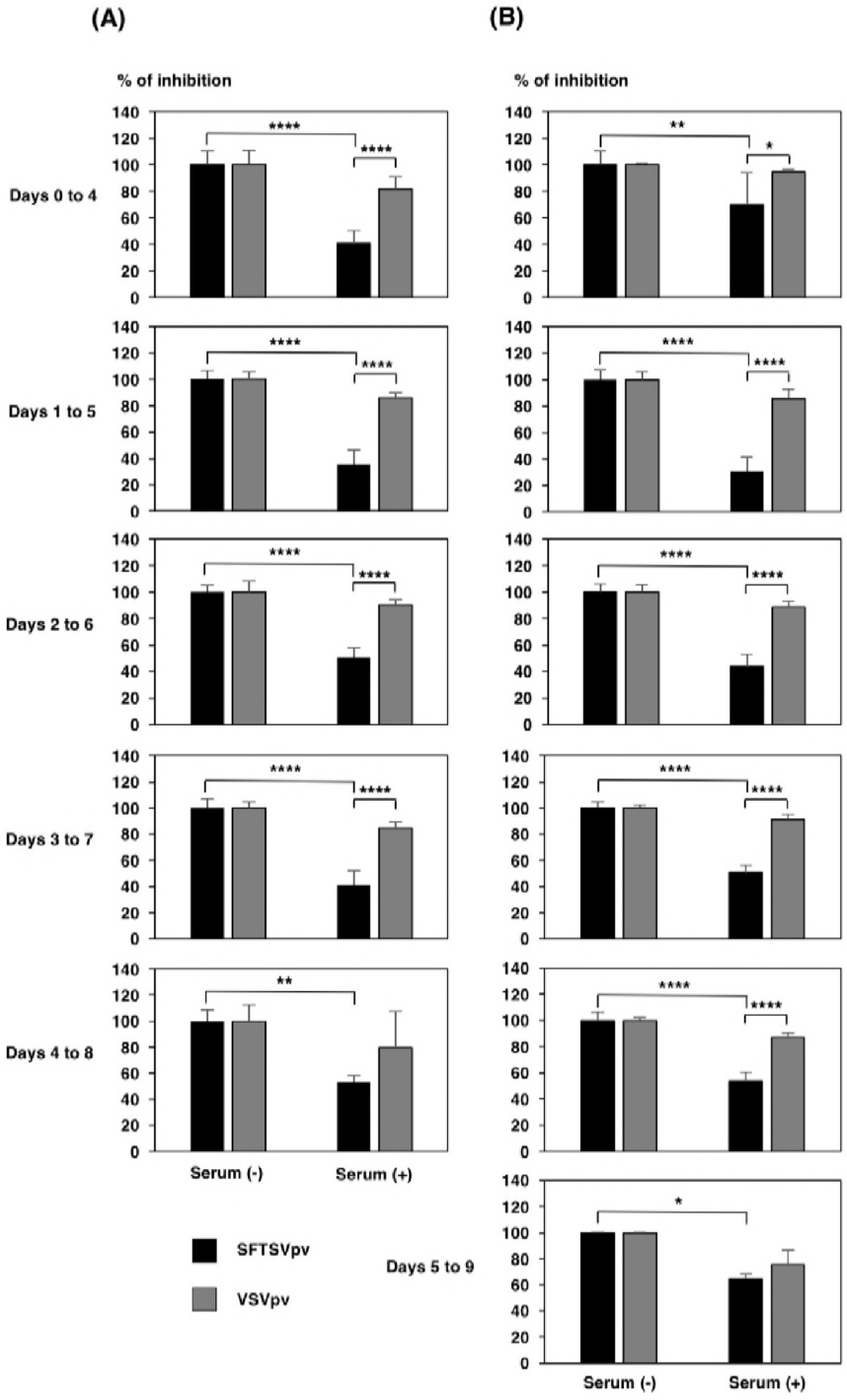
Tani *et al.*, Fig. 3



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