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BRIEF REPORT

Efficacy of Favipiravir Alone and in Combination With Ribavirin in a Lethal, Immunocompetent Mouse Model of Lassa Fever

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We studied the therapeutic potential of favipiravir (T-705) for Lassa fever, both alone and in combination with ribavirin. Favipiravir suppressed Lassa virus replication in cell culture by 5 $\log_{10}$ units. In a novel lethal mouse model, it lowered the viremia level and the virus load in organs and normalized levels of cell-damage markers. Treatment with 300 mg/kg per day, commenced 4 days after infection, when the viremia level had reached 4 $\log_{10}$ virus particles/mL, rescued 100% of Lassa virus–infected mice. We found a synergistic interaction between favipiravir and ribavirin in vitro and an increased survival rate and extended survival time when combining suboptimal doses in vivo.

Keywords. Lassa fever; favipiravir; ribavirin; mouse model; drug interaction.

Lassa fever (LF) is a viral hemorrhagic fever caused by Lassa virus (LASV), an arenavirus endemic in West Africa. Standard of care for LF is the nucleoside analogue ribavirin [1]. However, because of the small therapeutic window [1] and the fact that still one third of patients with LF die despite ribavirin treatment [2], there is a need for more-effective drug therapies against LF. A promising candidate is the broad-spectrum antiviral drug favipiravir (Toyama Chemical) [3], which is approved for emerging influenza virus in Japan. Besides influenza virus, favipiravir shows potent antiviral activity in vitro and in vivo against a wide range of negative-strand RNA viruses, including arenaviruses [3, 4]. We have tested the efficacy of favipiravir alone and in combination with ribavirin in a novel, chimeric mouse model, which is based on lethally irradiated type I interferon receptor knockout (*Ifnar*$^{-/-}$) mice transplanted with wild-type bone marrow progenitor cells (termed "*Ifnar*$^{-/-B6}$ mice"). *Ifnar*$^{-/-B6}$ mice feature a fully functional immune system and are susceptible to wild-type LASV infection (Oestereich et al, unpublished data).

METHODS

Antiviral and Toxicity Testing In Vitro

Vero E6 cells (4×10^4 cells per well in a 24-well plate) were inoculated with LASV Ba366 at a multiplicity of infection of 0.01, and compound was added 1 hour after infection. Concentration in cell culture supernatant of infectious virus particles was measured 3 days after infection, using an immunofocus assay. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) method. The concentrations that reduced the virus titer by 50% (IC_{50}), 90% (IC_{90}), and 99% were calculated from dose-response curves, using Prism GraphPad 6.0 (GraphPad Software). Drug interaction was tested in a 8×8 matrix; both favipiravir and ribavirin were tested at concentration of 0, $IC_{90}/4$, $IC_{90}/2$, $IC_{90}/1.5$, IC_{90}, $IC_{90} \times 1.5$, $IC_{90} \times 2$, and $IC_{90} \times 4$ in all possible combinations. The data were analyzed according to the Bliss independence model [5]. Effects of compounds on the LASV replication complex were tested in the context of the cellular RNA polymerase I–based LASV replicon system.

Antiviral Testing In Vivo

Chimeric *Ifnar*$^{-/-B6}$ C57BL/6 mice and *Ifnar*$^{-/-}$ A129 mice were infected intraperitoneally with 1000 focus-forming units of LASV Ba366 and monitored daily for signs of disease, body weight, and body temperature. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in blood were analyzed using a colorimetric assay (Reflotron, Roche Diagnostics). Organs were collected from randomly chosen mice 7 or 8 days after infection and from mice that died from the infection. Infectious virus particles in blood and organs were determined by immunofocus assay. Organs were fixed in 4% formaldehyde–phosphate-buffered saline (PBS), embedded in paraffin, and stained with hematoxylin-eosin. Ribavirin in 0.9% NaCl was administered intraperitoneally; 0.9% NaCl served as placebo. Favipiravir was suspended in methylcellulose-PBS and was administered twice daily per os, using a stomach probe; 0.5% methylcellulose-PBS served as placebo.

See Supplementary Methods for an extended description of methods.

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RESULTS

Favipiravir and Ribavirin Show Comparable Antiviral Activity Against LASV In Vitro

Favipiravir suppressed LASV replication by ≥5 $\log_{10}$ units with IC_{50} of 29 μM and IC_{90} of 43 μM (Supplementary Figure 1). Ribavirin displayed comparable inhibitory activity with IC_{50} of 26 μM and IC_{90} of 33 μM. Neither drug significantly affected cell viability. The antiviral activity of favipiravir was confirmed in the LASV replicon system; the IC_{50} was approximately 15 μM.

Favipiravir Increases Survival Rate Among LASV-Infected Mice

All LASV-infected and placebo-treated *Ifnar*$^{-/-B6}$ chimeras died within 10 days after infection (Figure 1 and Supplementary Figure 2*A*). They showed high levels of virus in blood and organs, rapidly lost weight, and displayed features of severe human LF, such as elevated AST and ALT levels, with AST levels increasing by a greater percentage than ALT levels, and hypothermia [2, 6]. To test the therapeutic efficacy of favipiravir, treatment was commenced at day 4 after infection, when viremia had reached a level of about 10^4 infectious virus particles/mL. Oral doses of 75 mg/kg per day and 150 mg/kg per day did not prevent or significantly delay death (Figure 1). However, treatment suppressed the viremia level by 2 log units between days 4 and 8, and it reduced virus levels in organs around the time of death. In addition, AST and ALT levels were significantly lower and terminal hypothermia was absent. Increasing the favipiravir dose to 300 mg/kg per day from days 4 to 11 after infection rescued 100% of LASV-infected mice (Figure 1). Viremia decreased within the first 3 days of treatment to levels below the limit of detection of the assay, and after 4 days of treatment the virus levels in organs were up to 6 log units lower than those in organs from placebo-treated animals. Decrease in body weight stopped after 4 days of treatment, hyperthermia was absent, and transaminase levels were hardly elevated or returned to normal during treatment. However, histopathological analysis revealed that widespread liver tissue damage remained even after 4 days of treatment with the high dose of favipiravir (Supplementary Figure 3*A*).

To confirm the antiviral activity of favipiravir in a nontransplant model, we performed the same set of experiments in *Ifnar*$^{-/-}$ mice on the A129 genetic background (Supplementary Figure 4). These mice represent a nonlethal model for LASV infection [7]. As in the chimeric mice, favipiravir showed a dose-dependent beneficial effect on body weight, transaminase levels, viremia, and virus load in organs.

Moderate Effect of Ribavirin In Vivo

Ribavirin was tested at a dose of 80 mg/kg per day, with commencement of treatment at day 0 (Supplementary Figure 5*A*) and day 4 (Figure 2 and Supplementary Figure 2*B*) after infection. None of the treatment regimens increased the survival rate, compared with the rates in the respective placebo groups, and only treatment starting at day 0 after infection prolonged the survival duration. No significant effect was seen on viremia, although the virus levels in organs were somewhat decreased. However, both treatment regimens prevented increases in AST and ALT levels. Treatment from days 4 to 11 with a high dose of 160 mg/kg per day significantly prolonged the survival duration, and 1 of 5 animals (20%) survived (Figure 2). No reduction in levels of viremia or virus in organs was seen, while transaminase levels were suppressed as in the low-dose treatment groups. At day 8 after infection, widespread liver tissue damage was present in mice treated with ribavirin, although this finding seemed less pronounced than that for animals treated with placebo or favipiravir, suggesting a cell-protective effect of ribavirin (Supplementary Figure 3*B*).

In analogy to the favipiravir experiments, we tested ribavirin in *Ifnar*$^{-/-}$ A129 mice (80 mg/kg per day during days 0–7 after infection; Supplementary Figure 5*B*). Consistent with the findings in the chimeric mice, the drug reduced signs of LASV-associated disease such as weight loss and elevated aminotransferase levels, while it failed to influence the levels of viremia and virus in organs.

Beneficial Effect of Combination Treatment of Favipiravir With Ribavirin In Vitro and In Vivo

As ribavirin is the standard of care for LF, clinical trails may consider a combination of both drugs. Therefore, we first tested the antiviral activity of 64 combinations of ribavirin and favipiravir in cell culture (Supplementary Figure 6). The 8 × 8 concentration matrix was designed around the IC_{90} values of both drugs. The dose-response surface demonstrated that all combinations of ribavirin and favipiravir exhibited strong antiviral effects, with suppression of virus replication by ≥5 log units. Possible antagonistic or synergistic effects were evaluated according to the Bliss independence model [5]. This analysis revealed clear synergistic effects when the drugs were combined in concentrations of around $IC_{90}/2$ to IC_{90}. In this area of the matrix, the experimental virus titer was 1–2 log units lower than the titer predicted by the Bliss independence model for additive effect. The MTT test did not reveal drug toxicity over the whole matrix.

To explore whether combination of 2 subeffective doses may result in effective treatment in the lethal mouse model, we administered a ribavirin dose of 80 mg/kg per day plus a favipiravir dose of 150 mg/kg per day from days 4 to 11 after infection (Figure 2 and Supplementary Figure 2*B*). While none of the single-dose treatments improved the survival rate or prolonged the survival duration (Figure 1 and Figure 2), the combination treatment prolonged the survival duration by 6 days, compared with both single-drug treatments ($P < .01$; see Supplementary Table 1 for all statistical comparisons of combination vs single-drug treatments). The combination treatment led to a decline in viremia level until end of the treatment at day 11. However, a rebound in viremia level occurred following

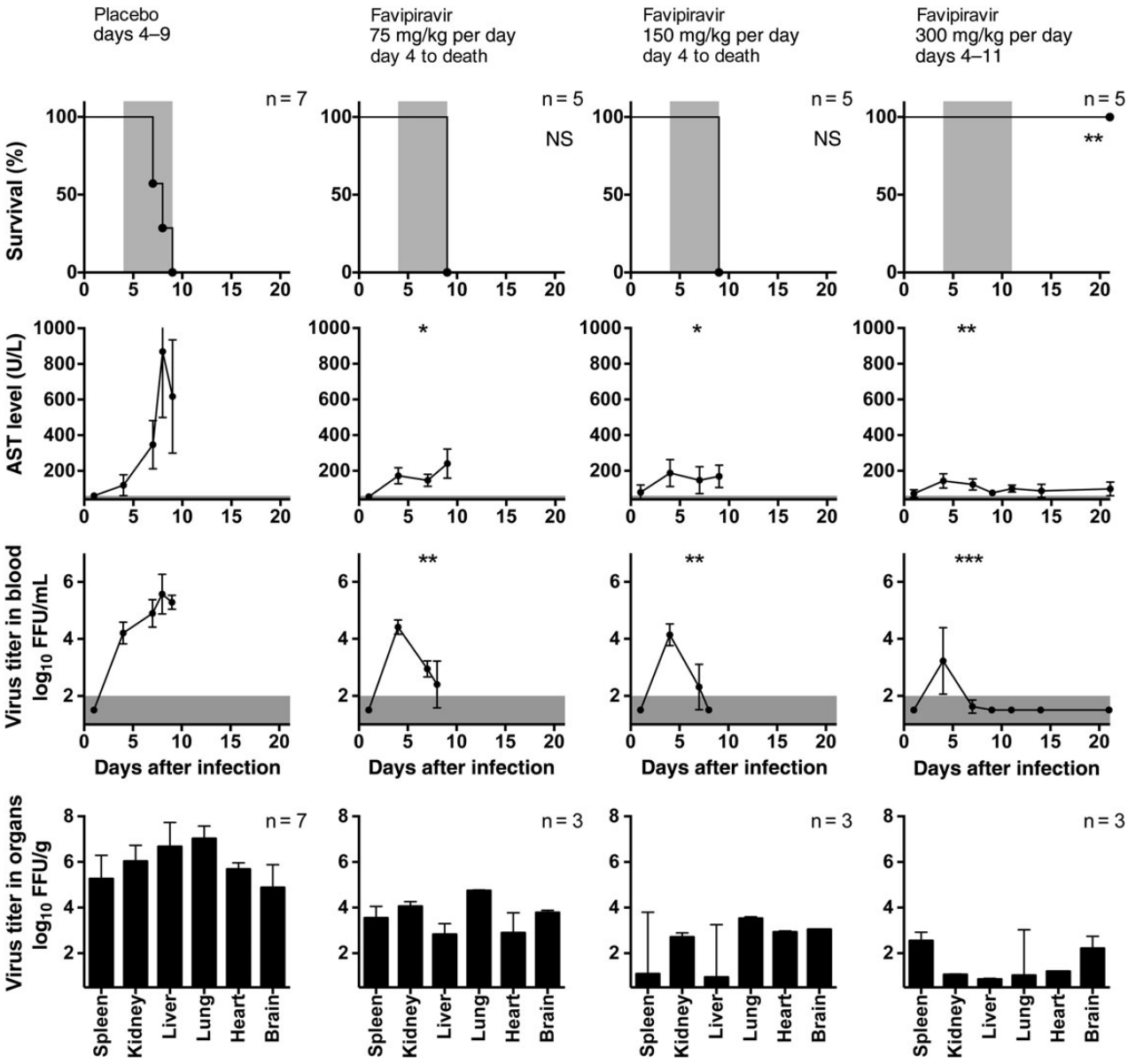

Figure 1. Treatment of Lassa virus (LASV)–infected chimeric *Ifnar*$^{-/-B6}$ mice with different doses of favipiravir. Chimeric *Ifnar*$^{-/-B6}$ mice were inoculated intraperitoneally with 1000 focus-forming units (FFU) of LASV. Favipiravir was administered twice daily per os, using a stomach probe. Treatment was commenced 4 days after infection and continued until death or day 11. Organs were collected from 2 randomly chosen mice per group at day 7 after infection and from mice that died from infection between days 7 and 9 after infection and analyzed for infectious virus titers. The duration of treatment in the survival plots, the range of the viremia level below the detection limit of the immunofocus assay, and the normal reference range of the aspartate aminotransferase level (AST) in mice are shaded in gray. The corresponding values for weight, body temperature, and alanine aminotransferase level of the animals are shown in Supplementary Figure 2*A*. Mean values and standard deviations are shown. Favipiravir treatment groups were compared with the placebo group after 3 days of treatment (day 7 after infection), using statistical tests as indicated in the Supplementary Methods. $^{*}P \leq .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$. Abbreviation: NS, not significant.

cessation of therapy, which may have caused the death of the animals around day 15 after infection Therefore, the combination treatment was extended until day 15 after infection (Figure 2), which resulted in survival of 3 of 5 mice (60%), with surviving mice effectively clearing virus from blood and regaining weight. Alternatively, the ribavirin dose was doubled to 160 mg/kg per day (for the single-drug experiment, see Figure 2) and administered in combination with a favipiravir dose of 150 mg/kg per day from days 4 to 11 after infection (Figure 2). This treatment led to survival of 4 of 5 mice (80%) during the observation period. However, a rebound in viremia level was not prevented. Surviving animals did not completely clear the virus from blood and still showed weight loss with biochemical evidence of disease at day 21 after infection. Increased AST and ALT levels, as seen in placebo controls, were effectively suppressed by all combination treatments. Histological analysis at day 8 after

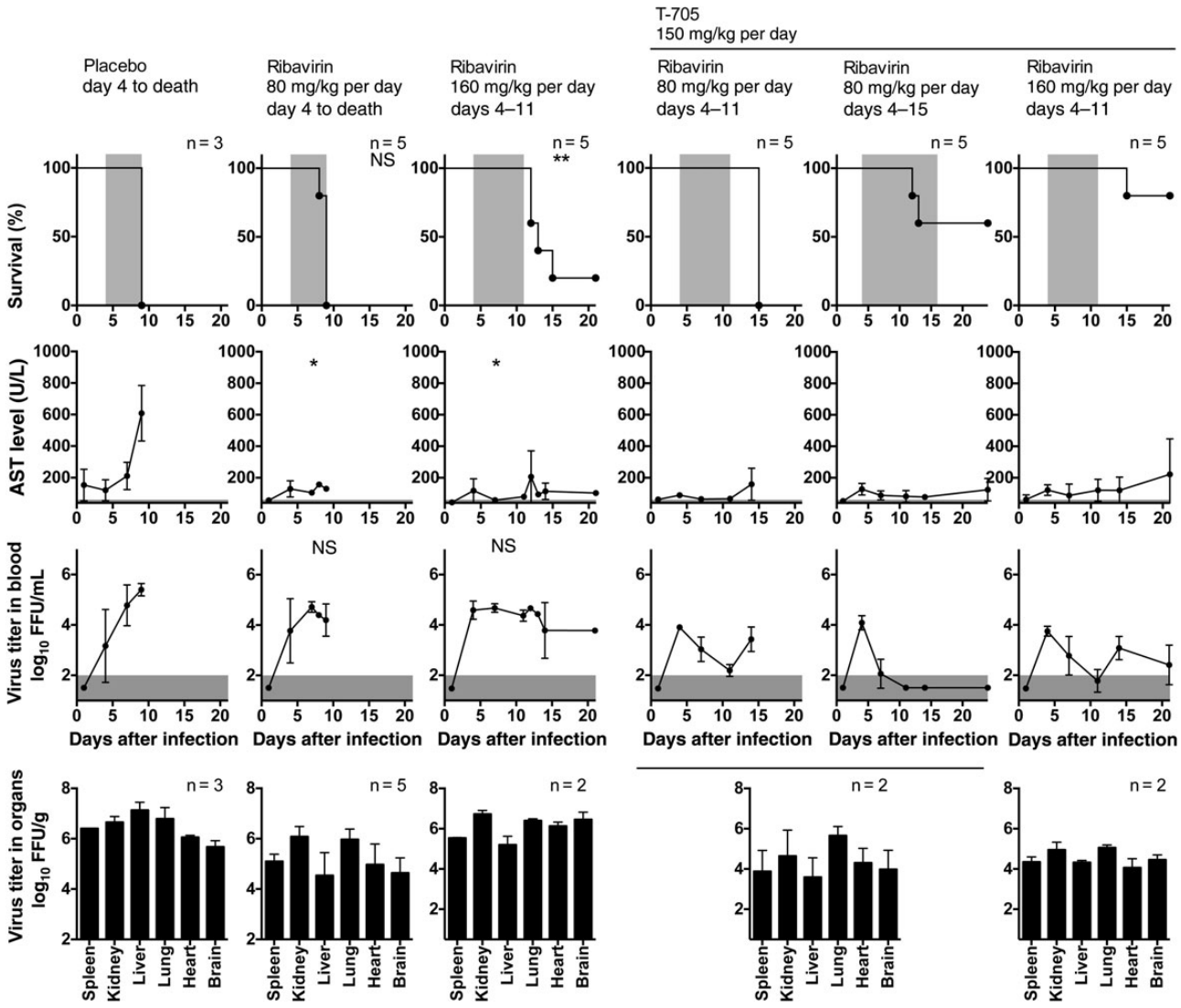

Figure 2. Treatment of Lassa virus (LASV)–infected chimeric *Ifnar*$^{-/-B6}$ mice with different doses of ribavirin or a combination of favipiravir and ribavirin. Chimeric *Ifnar*$^{-/-B6}$ mice were inoculated intraperitoneally with 1000 focus-forming units (FFU) of LASV. Ribavirin was administered once daily (80 mg/kg per day) or twice daily (160 mg/kg per day) by the intraperitoneal route, and favipiravir was administered twice daily per os, using a stomach probe. Treatment was commenced 4 days after infection and continued until death, day 11, or day 15. Organs were collected from 2 randomly chosen mice per group at day 7 after infection and from mice that died from infection between days 7 and 9 after infection and were analyzed for infectious virus titers. The duration of treatment in the survival plots, the range of the viremia level below the detection limit of the immunofocus assay, and the normal reference range of the aspartate aminotransferase level (AST) in mice are shaded in gray. The corresponding values for weight, body temperature, and alanine aminotransferase level among the animals are shown in Supplementary Figure 2*B*. Mean values and standard deviations are shown. Ribavirin treatment groups were compared with the placebo group after 3 days of treatment (day 7 after infection), using statistical tests as indicated in Supplementary Methods. Favipiravir-ribavirin combination treatment groups were compared with the 2 respective single-drug groups after 3 days of treatment (day 7 after infection). The significance levels are shown in Supplementary Table 1. *$P \leq .05$ and **$P \leq .01$. Abbreviation: NS, not significant.

infection supported again a cell-protective effect of ribavirin (Supplementary Figure 3C). Liver tissue damage was still abundant but was slightly ameliorated in mice that received combined treatment, compared with those treated with favipiravir alone.

DISCUSSION

Favipiravir strongly suppressed LASV replication in vitro and in vivo, consistent with its potent antiviral effect against other negative-strand RNA viruses [3]. Even if treatment was commenced 4 days after infection, the drug led to a rapid decline in viremia level, ameliorated disease, and increased survival rate. The replicon data indicate that the drug is targeting the LASV polymerase complex, consistent with previous findings for influenza virus and lymphocytic choriomeningitis virus [4, 8]. We found a synergistic interaction between favipiravir and ribavirin in vitro and increased protection from mortality and extended survival durations when combining suboptimal doses as compared to treatment with each drug alone.

Despite the marginal antiviral effect of ribavirin in the mouse model, which may not be explained by insufficient metabolization of the drug in mouse cells [9], we observed a clear cell-protective effect. This resembles observations in influenza virus–infected mice [10]. Here, ribavirin improved survival rate and lung pathology, while it hardly reduced the virus titer in lungs. These findings led the authors to conclude that "it seems unlikely that the activity of ribavirin (in vivo) is based primarily upon its antiviral properties" [10, p. 1070]. Several hypotheses have been put forward to explain in vivo effects of ribavirin, including inhibition of induction of proinflammatory cytokines in virus-infected macrophages [11] and induction of interferon-stimulated genes [12].

An important finding of our study is the synergistic interaction of favipiravir and ribavirin in vitro and in vivo. Various scenarios may explain the synergism. First, depletion of the intracellular GTP pool by ribavirin [9] may facilitate the incorporation of favipiravir into viral RNA, as the favipiravir-nucleotide complex competes against GTP and ATP during RNA synthesis and exogenous elevation of the GTP and ATP levels reverses the inhibitory effect of favipiravir [4, 8, 13]. Second, it may be that both ribavirin and favipiravir are incorporated into the viral RNA and hamper progeny RNA synthesis and/or cause lethal mutagenesis in a synergistic manner [14, 15]. Third, the synergistic effect in vivo may also stem from combination of direct suppression of virus replication by favipiravir with modulation of pathophysiological or immunological host pathways by ribavirin [11, 12].

In conclusion, our data hold promise that favipiravir is of value in the treatment of LF and provide a rationale for clinical trials evaluating favipiravir in combination with ribavirin.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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L. O., C. M.-F., and S. G. conceived and designed the experiments. L. O., T. R., A. L., P. R., S. W., E. P., S. B., S. K., and C. M.-F. performed experiments. L. O., C. M.-F., and S. G. analyzed the results and wrote the manuscript. All authors reviewed the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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