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Short Communication

Successful treatment of advanced Ebola virus infection with T-705 (favipiravir) in a small animal model

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ABSTRACT

Outbreaks of Ebola hemorrhagic fever in sub-Saharan Africa are associated with case fatality rates of up to 90%. Currently, neither a vaccine nor an effective antiviral treatment is available for use in humans. Here, we evaluated the efficacy of the pyrazinecarboxamide derivative T-705 (favipiravir) against Zaire Ebola virus (EBOV) in vitro and in vivo. T-705 suppressed replication of Zaire EBOV in cell culture by 4 log units with an IC_{90} of 110 μM. Mice lacking the type I interferon receptor ($IFNAR^{-/-}$) were used as in vivo model for Zaire EBOV-induced disease. Initiation of T-705 administration at day 6 post infection induced rapid virus clearance, reduced biochemical parameters of disease severity, and prevented a lethal outcome in 100% of the animals. The findings suggest that T-705 is a candidate for treatment of Ebola hemorrhagic fever.

Filoviruses of the genus *Ebolavirus* comprise four virus species that cause hemorrhagic fever in humans with case-fatality rates of up to 90%. Since the first documented outbreak of Zaire Ebola virus (EBOV) in 1976 in the Democratic Republic of Congo (formerly Zaire), EBOV has caused outbreaks in several sub-Saharan African countries. Ebola hemorrhagic fever (EHF) is characterized by host immunosuppression, high viremia, and multiorgan failure resembling septic shock. Several vaccine candidates including DNA vaccines, vectored vaccines, and virus-like particles-based vaccines have shown efficacy in non-human primate models of EHF. However, no EBOV vaccine is currently licensed for application in humans (for a review, see Feldmann and Geisbert, 2011).

Drug candidates such as rNAPc2 (Geisbert et al., 2003), estrogen receptor modulators (Johansen et al., 2013), siRNA (Geisbert et al., 2010), interferon (IFN) (Smith et al., 2013), or neutralizing monoclonal antibodies (Olinger et al., 2012; Qiu et al., 2012) have shown protection when administered shortly after infection. However, none of them had a therapeutic benefit beyond the time window of 2 days post infection. The vesicular stomatitis virus-vectored vaccine expressing the EBOV glycoprotein demonstrated a similar post exposure protection (Feldmann et al., 2007). Combination of monoclonal antibodies with adenovirus-vectored IFN-alpha has recently extended the treatment window to 3 days post-exposure when early viremia and symptoms were already detectable (Qiu et al., 2013). Despite the major success so far, the therapeutic window to treat EBOV disease is still narrow highlighting the necessity to develop strategies for clinical management of symptomatic EHF beyond supportive therapy.

The pyrazinecarboxamide derivative T-705 (favipiravir) was published in 2002 by Toyama Chemicals (Japan) as an inhibitor of influenza virus replication (Furuta et al., 2002) and is currently in late stage clinical development for the treatment of flu (for a review, see Furuta et al., 2013). It is converted by host enzymes to T-705-ribofuranosyl-5′-triphosphate and presumably acts as a nucleotide analog that selectively inhibits the viral RNA-dependent RNA polymerase or causes lethal mutagenesis upon incorporation into the virus RNA (Baranovich et al., 2013; Furuta et al., 2005; Jin et al., 2013; Naesens et al., 2013; Smee et al., 2009). Besides influenza virus (Furuta et al., 2002), T-705 has shown potent antiviral activity against other segmented negative-strand RNA viruses such as arena- and bunyaviruses in vitro and in vivo (Gowen et al., 2007; Safronetz et al., 2013). In addition, T-705 has also demonstrated activity against positive-strand RNA viruses such as noro- and flaviviruses (Morrey et al., 2008; Rocha-Pereira et al., 2012).

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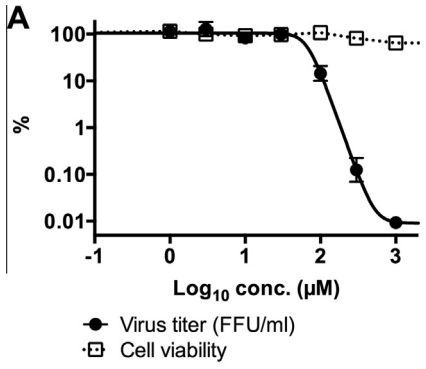
Following up on these previous reports, in this study we sought to determine the effect of T-705 against EBOV, a negative strand RNA virus belonging to the order of *Mononegavirales*. The T-705 compound (favipiravir; CAS No. 259793-96-9; PubChem CID 492405) was custom synthesized by BOC Science, Creative Dynamics, USA, dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml, and stored at −20 °C. All experiments were conducted in the biosafety level 4 (BSL-4) laboratory at the Bernhard-Nocht-Institute for Tropical Medicine in Hamburg using the wild-type Zaire EBOV Mayinga 1976 strain. It had been provided around 1980 by the Center for Disease Control, Atlanta, Georgia. The passage history since 1976 is not documented. To define our strain precisely, it has been completely sequenced. The sequence is identical to that of Zaire EBOV Mayinga 1976 strain (GenBank accession No. NC_002549) except of two differences: C1751T changing nucleoprotein codon CCC (Pro) to TCC (Ser), and G6175A changing glycoprotein codon AGT (Ser) to AAT (Asn). The virus stock was grown on Vero E6 cells (ATCC No. CRL-1586), quantified by immunofocus assay (see below), and stored at −70 °C until use in in vitro and in vivo experiments.

First, we evaluated the antiviral activity of T-705 in cell culture. The compound was added to Vero E6 cells infected one hour before with EBOV at a multiplicity of infection of 0.01. Final DMSO concentration in the cell culture supernatant was 0.1%. Five days post infection (p.i.) the concentration of infectious EBOV particles in cell culture supernatant was determined by immunofocus assay using polyclonal monkey anti-EBOV (kindly provided by Pierre Rollin) for detection of infected foci as described (Günther et al., 2004). Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) method as described (Günther et al., 2004). T-705 was able to suppress EBOV replication by 4 $\log_{10}$ units (Fig. 1A) with an inhibitory concentration that reduced the virus titer by 50% (IC_{50}) of 67 μM (10 μg/ml) (Fig. 1B), which is in the same order of magnitude than the IC_{50} for the only member of the order *Mononegavirales* tested so far, namely respiratory syncytial virus (RSV; IC_{50} = 41 μg/ml) (Furuta et al., 2002). T-705 treatment did not affect cell viability in the test range as measured by MTT assay (cytotoxic concentration that reduced cell growth by 50% > 1000 μM).



B

		95% CI
IC_{50}	67 μM (10.5 μg/ml)	56 – 75 μM
IC_{90}	110 μM (17 μg/ml)	83 – 143 μM
IC_{99}	186 μM (29 μg/ml)	132 – 265 μM

Fig. 1. Antiviral activity of T-705 against EBOV in cell culture. (A) Vero E6 cells were infected with EBOV and T-705 was added 1 h p.i. After 5 days, the concentration of infectious viral particles in the cell culture supernatant was measured by immunofocus assay. A sigmoidal dose–response curve was fitted to the data using Prism GraphPad 6.0 (GraphPad Software). Cell viability was measured by the MTT method. (B) The IC_{50}, IC_{90} and IC_{99} values for T-705 with 95% confidence interval (95% CI) were calculated from the sigmoidal function.

Next, we addressed the therapeutic efficacy of T-705 in a small animal model. To this end, we used mice lacking the type I IFN-alpha/beta receptor ($IFNAR^{-/-}$), which have been shown to be susceptible to infection with wild-type Zaire 1976 EBOV (Bray, 2001; Lever et al., 2012). To rule out effects related to the mouse genetic background, both $IFNAR^{-/-}$ C57BL/6 and $IFNAR^{-/-}$ 129/Sv mice were used. All animal experiments were carried out in the BSL-4 animal facility in strict accordance with the recommendations of the German Society for Laboratory Animal Science under supervision of a veterinarian. The protocol had been approved by the Committee on the Ethics of Animal Experiments of the City of Hamburg (Permit No. 44/11). All staff carrying out animal experiments has passed an education and training program according to category B or C of the Federation of European Laboratory Animal Science Associations. The experimental groups contained 5–10 age-matched female mice. Natural mucosal exposure to EBOV was mimicked by intranasal inoculation of 1000 focus-forming units (FFU) of EBOV diluted in 50 μl phosphate-buffered saline (PBS). T-705 was administered in 0.5% methylcellulose twice daily per os using a stomach probe.

In $IFNAR^{-/-}$ C57BL/6 mice, treatment [300 mg/(kg × d)] was initiated 6 days p.i. or later. As expected, EBOV infection of untreated mice resulted in 100% lethality within 10 days after infection (Fig. 2, left panel). All mice lost weight rapidly and some of them reached the limit of humane endpoint established in our approved animal protocol (20% weight loss). Strong elevation of serum aspartate and alanine aminotransferase (AST and ALT) levels up to 10,000 U/l — a hallmark of EHF in humans (Rollin et al., 2007) — high viremia up to 6 $\log_{10}$ FFU/ml, and substantial decrease of body temperature below 32 °C consistent with shock were observed in untreated mice (Fig. 2, left panel). Until the first day of treatment (day 6 p.i.), all mice in the T-705 group lost weight similarly to control mice, developed viremia, and showed elevated serum levels of AST and ALT indicating symptomatic EBOV infection (Fig. 2, middle panel). Already two days after initiation of treatment (day 8 p.i.), levels of AST, ALT, and viremia dropped significantly in treated mice relative to the controls ($p = 0.0025$, $p = 0.0043$, and $p = 0.0025$, respectively; Mann–Whitney U test) (Fig. 2, middle panel). Within four days of T-705 treatment (day 10 p.i.), the animals had cleared the virus from blood. Treatment was continued until day 13, and at the end of the 3-weeks observation period, all mice recovered (100% survival vs. 100% fatality rate among controls, $p = 0.00033$; 2-tailed Fisher's Exact Test). All surviving mice developed EBOV-specific antibodies as tested by immunofluorescence assay and CD8 T cells specific for the viral nucleoprotein (data not shown), suggesting that suppression of virus replication by T-705 allowed the host to mount a virus-specific adaptive immune response, even in the absence of functional type I IFN signaling.

To determine the therapeutic window for T-705 treatment, 300 mg/(kg × d) were also administered to a group of mice beginning at day 8 p.i. (Fig. 2, right panel). At this time, all animals showed peak levels of AST, ALT, and viremia, and reduced body temperature. Loss of body weight was evident and close to the humane endpoint. T-705 treatment delayed death and reduced virus load and biochemical correlates of disease in 1/5 (20%) mice even at this terminal stage, but did not prevent death. Thus, in the $IFNAR^{-/-}$ C57BL/6 mouse model, T-705 was 100% effective in the treatment of Zaire EBOV infection up to 6 days p.i. when high viremia and overt signs of disease are present, but was hardly beneficial at the terminal stage of the disease.

$IFNAR^{-/-}$ 129/Sv mice received either 30 mg/(kg × d) from days 2 to 9 or 300 mg/(kg × d) from days 2 to 9, days 4 to 11, or days 6 to 13. Surprisingly, despite clear clinical and biochemical signs of

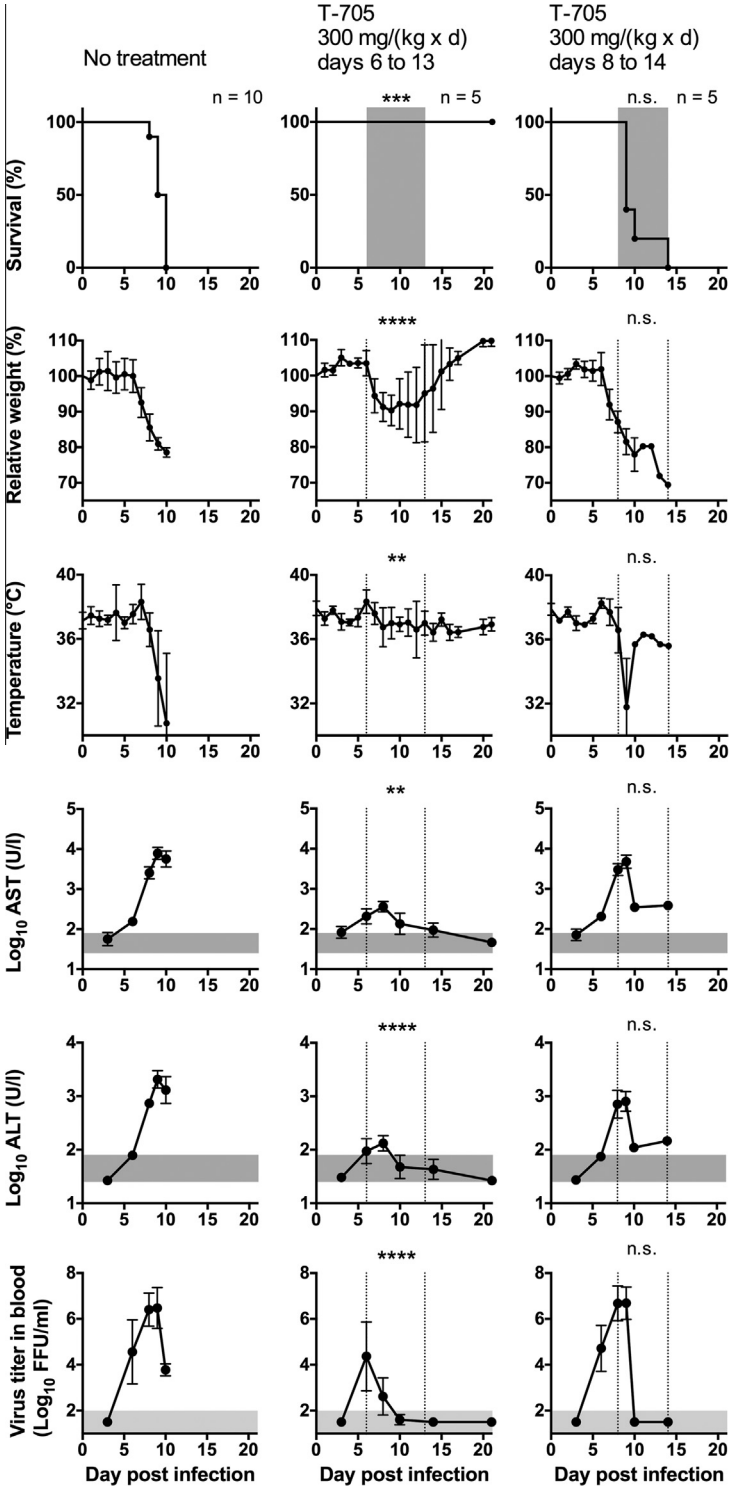


Fig. 2. Treatment of EBOV-infected IFNAR$^{-/-}$ C57BL/6 mice with T-705. Groups of 5 mice were inoculated intranasally with 1000 FFU of EBOV. Mice were treated with a T-705 dose of 300 mg/(kg × d). The drug was administered twice daily per os using a stomach probe. Placebo mice received no treatment. Treatment was performed for the period indicated above the columns (shaded in gray with the survival curves). Survival was determined using Kaplan–Meier analysis. The humane endpoint established in our animal protocol was reached by 10/10 of the control group and 4/5 of the day-8 treatment group; one animal was found dead. Relative weight, temperature, AST, ALT, and log-transformed virus titers in blood are shown as mean with standard deviation. Treatment duration, normal ranges for AST and ALT, and the range of viremia below the limit of detection of the immunofocus assay are shaded in gray. Statistical analysis for treatment at days 6–13 vs. controls: (i) weight and temperature were tested with two-way ANOVA (treatment [yes and no] vs. time [days 7, 8, and 9]) (weight: treatment $p = 0.02$, time $p = 0.0001$, interaction $p < 0.0001$; temperature: treatment $p = 0.13$, time $p = 0.001$, interaction $p = 0.007$) and t-test for day 9 (weight $p = 0.00017$; temperature $p = 0.03$), (ii) AST, ALT, and log-transformed viremia were tested at day 8 by Mann–Whitney U test ($p = 0.0025$, $p = 0.0043$, and $p = 0.0025$, respectively) and, as the values were found to be normally distributed, also by t-test ($p = 0.001$, $p < 0.0001$, and $p < 0.0001$, respectively), and (iii) survival was tested by Fisher's Exact Test ($p = 0.00033$) and log-rank (Mantel–Cox) test ($p = 0.0008$). Statistical analysis for treatment at days 8–14 vs. controls did not reveal any significant associations. The p values of the ANOVA interaction, the t-test, and the log-rank test are shown in the figure ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n.s., not significant).

disease, nearly 80% of untreated mice survived the infection (Fig. 3). This is in contrast with previous results indicating that aerosolized Zaire 1976 EBOV is uniformly lethal for IFNAR$^{-/-}$ 129/Sv mice (Lever et al., 2012). Differences in the virus strain used (Zaire EBOV E718 1976 strain compared to Mayinga strain in our study) may account for these contradictory findings. In addition, the study by Lever et al. used a Collison nebulizer to generate particles predominantly 1–3 μm in diameter, which may result in deposition of virus particles deeper in the respiratory tract compared to intranasal inoculation. As shown in Fig. 3, untreated mice showed serum AST levels of 352–9500 U/l and ALT levels of 210–2,400 U/l at day 8 p.i. Conversely, all T-705-treated mice had significantly reduced AST and ALT levels ($p = 0.0016–0.0031$ and $p = 0.001–0.002$ respectively; Mann–Whitney U test). In addition, while the mean virus load in blood at day 8 p.i. was 5.1×10^5 FFU/ml for untreated mice, no virus was detectable in

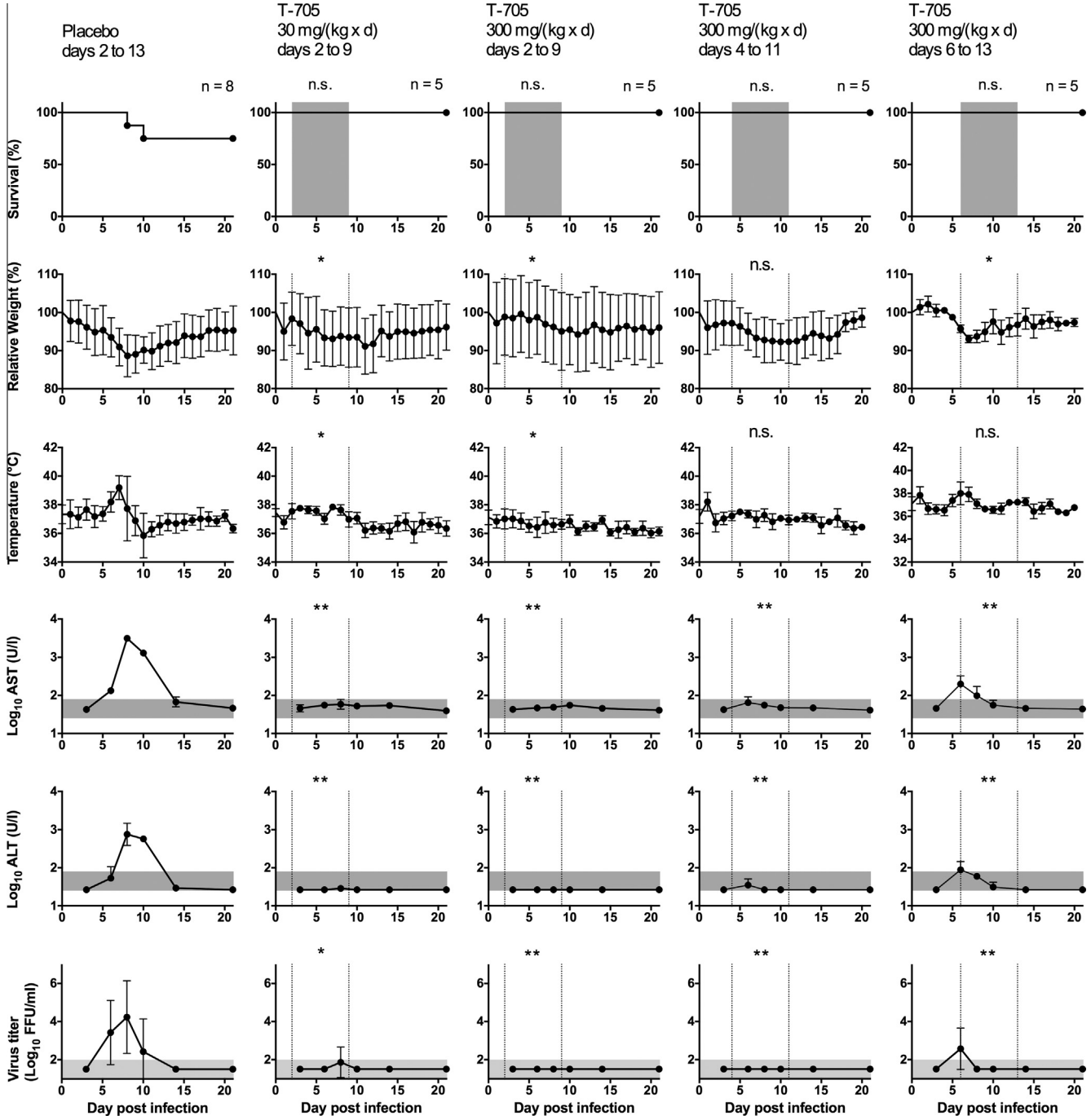


Fig. 3. Treatment of EBOV-infected IFNAR$^{-/-}$ 129/Sv mice with T-705. Groups of 5–8 mice were inoculated intranasally with 1000 FFU of EBOV. Mice were treated with a T-705 dose of 30 or 300 mg/(kg × d). The drug was administered twice daily per os using a stomach probe. Placebo treated mice received 100 μl 0.5% methylcellulose twice daily. Treatment was performed for the period indicated above the columns (shaded in gray with the survival curves). Survival was determined using Kaplan–Meier analysis. The humane endpoint established in our animal protocol was reached by 2/8 of the control group. Relative weight, temperature, AST, ALT, and log-transformed virus titers in blood are shown as mean with standard deviation. Treatment duration, normal ranges for AST and ALT, and the range of viremia below the limit of detection of the immunofocus assay are shaded in gray. Statistical analysis for treatment vs. controls: (i) weight and temperature were tested with two-way ANOVA (treatment [yes and no] vs. time [each day under treatment until day 8 p.i.]), (ii) AST, ALT, and log-transformed viremia were tested at day 8 by Mann–Whitney *U* test, and (iii) survival was tested by Fisher's Exact Test (n.s.) and log-rank (Mantel–Cox) test (n.s.). The *p* values of the ANOVA interaction, the *t*-test, and the log-rank test are shown in the figure ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n.s., not significant).

mice treated with T-705 irrespective of the dose or the time point for initiation of treatment ($p = 0.02–0.0055$; Mann–Whitney U test). The differences in survival, weight loss, and body temperature at day 8 p.i. were not statistically significant between the experimental groups. Similarly to C57BL/6 mice, all surviving 129/Sv mice developed EBOV-specific antibodies and CD8 T cells (data not shown).

To the best of our knowledge, T-705 represents the first effective therapeutic agent for advanced Zaire EBOV infection in an animal model. It reduces viremia, ameliorates clinical and biochemical signs of disease, and prevents lethal outcome in 100% of the animals if treatment is commenced 6 days after infection, which corresponds to 2–4 days before the time of death in control animals. Our study is consistent with and extends a parallel study just in press showing that T-705 prevents death of IFNAR$^{-/-}$ 129/Sv mice when given 1 h after aerosol challenge with EBOV (Smither et al., 2014). T-705 has the additional advantage that it is capable of reducing EBOV viremia following oral administration. This is comparable to ribavirin, which can be given orally to treat other hemorrhagic fevers such as Lassa fever (McCormick et al., 1986), but is ineffective against filoviruses (Feldmann and Geisbert, 2011). The data further indicate that T-705 is active in vivo against viruses of the order *Mononegavirales*, which comprise a multitude of human pathogens. The only previous example, the paramyxovirus RSV, was shown to be less susceptible to T-705 than segmented negative strand RNA viruses in vitro, which somewhat reduced optimism regarding the putative use of T-705 against non-segmented negative strand RNA viruses (Furuta et al., 2002). The IC_{50} of T-705 for EBOV is also higher than that for influenza or arenaviruses (Furuta et al., 2002; Gowen et al., 2007). Nevertheless, the drug shows clear effects in vivo which holds promise for in vivo efficacy of T-705 against other viruses, which are inhibited in vitro only at rather high drug concentration. In the case of EBOV infection, we speculate that the suppression of virus replication by T-705 in vivo allows the adaptive arm of the host immune system to tackle viral infection effectively and thus to contribute to the therapeutic effect of T-705. This is in line with previous data indicating an association between the level of EBOV viremia and the strength of the host immune response (Leroy et al., 2001). Further experiments to determine the involvement of immune responses in the context of T-705 treatment are under way. T-705 has been shown to be effective at inhibiting replication of RNA viruses in various small animal models (Furuta et al., 2002; Gowen et al., 2007; Morrey et al., 2008; Safronetz et al., 2013). This, together with our data generates cautious optimism about the translation of our findings into more realistic EHF models such as non-human primates, and eventually clinical practice.

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