Efficacy of Favipiravir (T-705) in Rabies Postexposure Prophylaxis

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Rabies is a fatal encephalitis caused by rabies virus (RABV), and no antiviral drugs for RABV are currently available. We report for the first time the efficacy of favipiravir (T-705) against RABV in vitro and in vivo. T-705 produced a significant, 3–4 log10 reduction in the multiplication of street and fixed RABV strains in mouse neuroblastoma Neuro-2a cells, with half-maximal inhibitory concentrations of 32.4 µM and 44.3 µM, respectively. T-705 significantly improved morbidity and mortality among RABV-infected mice when orally administered at a dose of 300 mg/kg/day for 7 days, beginning 1 hour after inoculation. T-705 significantly reduced the rate of virus positivity in the brain. Furthermore, the effectiveness of T-705 was comparable to that of equine rabies virus immunoglobulin for postexposure prophylaxis. Collectively, our results suggest that T-705 is active against RABV and may serve as a potential alternative to rabies immunoglobulin in rabies postexposure prophylaxis.

Keywords. rabies; T-705; antiviral drug; postexposure prophylaxis.

T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide), also known as favipiravir, was initially discovered as an antiviral compound against influenza virus [1] and was subsequently shown to be active against a broad range of RNA viruses [2]. T-705 is a purine analog that is converted to T-705-ribofuranosyl-5’-triphosphate (T-705-RTP) by host-cell enzymes; this compound is predicted to act on viral RNA-dependent RNA polymerase as a chain terminator or mutagen [2]. Recently, it was reported that postexposure administration of T-705 was effective against encephalitis viruses, West Nile virus (WNV), and western equine encephalitis virus (WEEV), in small-animal models [3, 4]. T-705 was also effective against Ebola virus (EBOV) in a mouse model [5, 6].

Rabies is a zoonotic disease caused by rabies virus (RABV), which is usually transmitted by animal bites and causes lethal encephalitis. The annual number of deaths due to rabies globally has been estimated to be approximately 59,000 [7]. The case-fatality rate for rabies is almost 100%, and there is still no cure for this disease after the onset of symptoms. It was reported that a patient survived rabies by the so-called Milwaukee protocol, which includes an artificially induced (therapeutic) coma and the administration of some antiviral drugs, without the use of antirabies biologics [8]. However, this protocol has failed to prevent death in numerous subsequent cases, and therefore it has been suggested that its use should be discontinued [9]. Although symptomatic rabies is still incurable, post-exposure prophylaxis (PEP) is available to prevent the onset of symptoms during the incubation period, which is usually between 30 and 90 days. For rabies PEP, the World Health Organization (WHO) recommends administering rabies immunoglobulin (RIG) along with 4 or 5 doses of rabies vaccine, especially in cases with severe exposure (category III) [10]. However, RIG is not always used in these cases, because of economic reasons and a limited supply [11–15].

Similar to EBOV, RABV has nonsegmented negative-sense RNA genomes and belongs to the Mononegavirales order. Therefore, T-705 may also be effective against RABV infection. In the present study, we evaluated the antiviral efficacy of T-705 against RABV infection. We found that T-705 was able to effectively suppress viral growth in mouse neuroblastoma cells. It also inhibited the viral invasion from the peripheral site to the central nervous system in mice after postexposure administration, which implies that T-705 has the potential to be used in rabies PEP.

METHODS

Viruses and Cells

Mouse neuroblastoma cell lines, NA and Neuro-2a (N2a) cells, were maintained in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). The street RABV strain, 1088, which was originally isolated from a racid woodchuck in North America [16], was propagated in suckling...
mouse brains, and the supernatant from a 10% brain homogenate in phosphate-buffered saline (PBS) supplemented with 2% FCS was used [17]. The entire genome sequence of 1088 has been determined (GenBank Accession no. AB645847) [18], and phylogenetic analysis showed that 1088 was similar to strains isolated from skunks in North America (data not shown). The fixed (laboratory-adapted) RABV strain, challenge virus standard (CVS), was propagated in NA cells.

**Compound**

T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) was synthesized at Toyama Chemical. For cell culture-based experiments, T-705 was dissolved in MEM as a 10 mM stock solution. For animal experiments, T-705 was suspended in 0.5% methylcellulose (viscosity, 400 cP; Sigma-Aldrich). The T-705 solutions were stored at 4°C and used within 1 week.

**Plasmid Construction and Transfection**

The mouse hypoxanthine guanine phosphoribosyltransferase (hprt) gene was amplified by reverse-transcription polymerase chain reaction, using total RNA extracted from N2a cells. The DNA fragment was cloned into the pCI vector (Promega), using the EcoRI and XbaI sites. The insert was sequenced, and it corresponded to the hprt sequence of the C57BL/6 mouse (GenBank accession no. NM_013556). Plasmids were transfected into NA cells, using TransIt-Neural reagent (Mirus Bio) according to the manufacturer’s instructions.

**Western Blotting**

Preparation of the cell lysates and Western blots were performed as described previously [18, 19]. The β-actin and HPRT proteins were detected using an anti-β-actin mouse monoclonal antibody (mAb; G043, Applied Biological Materials) and an anti-HPRT rabbit polyclonal antibody (ab10479, Abcam), respectively.

**Virus Titration**

The viral titer was determined in NA cells using a focus assay, as described previously [18]. The viral titer was expressed as focus-forming units (FFU).

**Evaluation of the Antiviral Activity of T-705 Against RABV in Neuroblastoma Cells**

Each virus solution was inoculated into the indicated cells on a 24-well plate at a multiplicity of infection (MOI) of 0.01. After virus adsorption for 1 hour, the inoculum was removed, and 1 mL of medium (MEM supplemented with 5% FCS) was added per well, with or without T-705. After a 96-h incubation period, the culture medium was collected, and viral titers were determined using the focus assay.

**Evaluation of the Antiviral Activity of T-705 Against RABV in Mice**

Six-week-old female ddY mice (Kyudo, Japan) were intramuscularly inoculated (in the right hind limb) with 10^5 FFU of the 1088 RABV strain. The inoculated mice were administered T-705 (30, 100, or 300 mg/kg/day) or 0.5% methylcellulose (as the control) daily for 7 or 14 days. These treatments were administered by oral gavage (20 mL/kg) under isoflurane anesthesia twice daily (in the morning and in the afternoon) with a 6-h interval between doses. Administration commenced 1 hour, 1 day, 2 days, or 4 days after inoculation. In addition, other groups of inoculated mice were administered 40 international units (IU)/kg of body weight of equine RIG (ERIG; Thai Red Cross Society, lot no. E0246P) intramuscularly at the virus inoculation site at 1 hour, 1 day, or 2 days after infection. The 40-IU/kg dose is the dose recommended by the WHO [10]. The inoculated mice were monitored (twice per day) and weighed for 28 days. We considered the inoculated mice to be sick when clinical signs, such as significant weight loss (ie, a 2-g reduction from the day before), piloerection, a foot fault (foot slip) on a stainless steel wire top clip of a mouse cage, and/or paralyses, were observed. The animal experiments were approved by the Animal Experiment Committee of Oita University (approval no. Q010003), and mice that were moribund (ie, in a deep coma) were euthanized.

**Titration of Viral Load in the Brain**

Brain samples were obtained from the infected mice after inducing euthanasia with an isoflurane overdose. Each brain was homogenized (20% w/v) in phosphate-buffered saline supplemented with 2% FCS. After centrifugation (at 1800 g for 10 minutes at 4°C), each supernatant was collected and stored at −80°C until use. Virus titers were determined using the focus assay, as described above.

**Rapid Fluorescent Focus Inhibition Test (RFFIT)**

Sera were collected from the surviving mice and subjected to titration of virus neutralizing antibody (VNA), using RFFIT, as described previously [20, 21]. The VNA titer was expressed as IU per milliliter.

**Statistical Analyses**

Unpaired 2-tailed Student t tests, 2-way analyses of variance (ANOVAS), log-rank (Mantel–Cox) tests, Fisher exact tests, and Tukey multiple comparisons tests were performed using GraphPad Prism (version 6.0). The half-maximal inhibitory concentration (IC_{50}) of T-705 against RABV was also determined using GraphPad Prism (version 6.0).

**RESULTS**

**T-705 Efficiently Suppressed RABV Multiplication in Mouse Neuroblastoma N2a Cells**

It has been reported that human HPRT converts T-705 into ribose-5’-monophosphate (T-705-RMP) prior to forming T-705-RTP [22]. Both mouse neuroblastoma cell lines, NA and N2a cells, have been widely used in RABV studies. NA cells were derived from a subclone of N2a cells; moreover, NA cells were selected as 8-azaguanine-resistant cells and found to lack HPRT activity [23]. We confirmed the absence of HPRT expression in NA cells (Figure 1A). Then, we evaluated T-705 activity...
against both strains (street and fixed) of RABV in both NA and N2a cells (Figure 1B). Although T-705 slightly suppressed multiplication of the 1088 street strain in NA cells, this effect was not statistically significant. However, T-705 significantly suppressed multiplication of this strain by 3 log_{10} units in N2a cells. Similarly, T-705 suppressed multiplication of the CVS fixed strain by only 1 log_{10} unit in NA cells but suppressed it by 4 log_{10} units in N2a cells. A 2-way ANOVA detected a significant interaction between the T-705 treatment and the type of cell line, indicating that T-705 was more active in N2a cells than it was in NA cells. We then evaluated T-705 anti-RABV activity in NA cells transfected with a vector encoding the mouse hprt gene or an empty vector (Figure 1C). As shown in Figure 1D, T-705 significantly inhibited 1088 replication by 0.75 log_{10} units in NA cells transfected with the hprt vector but not in cells transfected with the empty vector. However, T-705 showed less antiviral activity in the hprt-transfected cells than it did in the N2a cells. The IC_{50} values for T-705 against RABV in N2a cells were 32.4 µM for the 1088 strain and 44.3 µM for the CVS strain (Figure 2).
First, mice were inoculated intramuscularly with 10⁵ FFU of 1088 and orally administered T-705 (30, 100, or 300 mg/kg/day) for 7 days (days 0–6). As shown in Figure 3A, T-705 improved the survival rate of mice infected with 1088 in a dose-dependent manner, although the only significant difference was detected between the control and T-705 300 mg/kg/day groups. Notably, it appeared that the rate of surviving and apparently asymptomatic mice was higher in the T-705 300 mg/kg/day group (5 of 9). The VNA titers of surviving asymptomatic mice were clearly elevated, compared with those of noninfected mice, but they were lower than the VNA titers of surviving symptomatic mice (Figure 3B). Furthermore, all symptomatic mice exhibited neurological signs after T-705 administration was complete (Figure 3A), and the onset day of the signs was significantly delayed by an average of 3.5 days in the T-705 300 mg/kg/day group, compared with the control group (Supplementary Table 1). We also determined the viral loads in the brains of inoculated mice with or without T-705 administration (300 mg/kg/day; days 0–6). As shown in Figure 4, on day 11 after inoculation, infectious viruses were detected in only 1 of 5 mice that received T-705 but were found in all 4 mice that did not receive T-705; all of the virus-positive mice showed clinical signs, while the virus-negative mice did not. However, the mouse with the highest titer was one of the mice that received T-705.

Next, we assessed the effect of longer-term T-705 administration (300 mg/kg/day for 14 days) and the effect of delaying the initiation of treatment. Mice inoculated intramuscularly with 10⁵ FFU of 1088 were orally administered T-705 at 1 hour or 4 days after inoculation and on days 0–13 or 4–17, respectively. T-705 administration on days 0–13 did not completely prevent the lethal infection, but significant differences in the survival curves and onset days were detected between the control and T-705 (days 0–13) groups (Figure 5 and Supplementary Table 2). In addition, it appeared that this long-term administration did not improve the survival rate (mortality rate, 5 of 10 mice) or prevent the onset of illness (incidence, 4 of 10 mice) when compared with the 7-day treatment regimen (6 of 9 mice and 5 of 9 mice, respectively; Figure 3). On the other hand, no statistical differences were detected between the control group and the delayed T-705 administration (days 4–17) group in terms of their survival curves and onset days (Figure 5A and Supplementary Table 2), with P values of .96 (by the log-rank test) and .94 (by the Tukey multiple comparison test), respectively. Moreover, in the delayed administration group, mortality was observed during the treatment period. Consistent with this, no significant efficacy was observed in the infected mice when the oral administration of T-705 (300 mg/kg/day) was initiated on the onset day of neurological signs (data not shown). We also confirmed that all surviving mice had higher VNA titers than noninfected mice (Figure 5B), while surviving asymptomatic mice showed lower titers than surviving symptomatic mice.

**Comparison of the Efficacy of T-705 and ERIG as Postexposure Treatments**

We conducted an additional experiment to compare the efficacy of T-705 and ERIG as postexposure treatment, using the mouse model. In this experiment, we also evaluated how long the initiation of the administrations can be delayed after infection with the virus, and we did not use rabies vaccines to simply estimate the efficacy. Mice that had been intramuscularly inoculated with 1088 were orally administered T-705 (300 mg/kg/day) for 7 days, beginning 1 hour, 1 day, or 2 days after inoculation, or they were intramuscularly injected with 40 IU/kg of ERIG at the virus inoculation site at 1 hour (on day 0), 1 day, or 2 days after inoculation. As shown in Figure 6, we detected a tendency for the survival rate to decrease when the administration was delayed. Notably, T-705 administration for days 0–6 significantly improved the survival of inoculated mice, whereas ERIG administration on day 0 reduced the mortality rate (50%), compared with the control (90%), but no significant difference was detected between the 2 groups by the log-rank test.
Figure 3. Efficacy of postexposure T-705 administration for 7 days in mice infected with 1088. A. Mice were intramuscularly inoculated with 1088 and orally administered T-705 (30, 100, or 300 mg/kg/day) or 0.5% methylcellulose (as a control) daily for 7 days (days 0–6) beginning 1 hour after inoculation. Mice were monitored for 28 days. “Sick” indicates that mice showed significant body weight loss or neurological signs. Surviving sick mice had begun to gain body weight but with sequelae, such as limb paralysis. For the survival curves, a significant difference was observed between the control and T-705 (300 mg/kg/day) groups (P < .01, by the log-rank test) but not between the control and T-705 (30 or 100 mg/kg/day) groups (P ≥ .05, by the log-rank test). B. The VNA titers in sera of surviving mice. Sera were collected at 28 days after inoculation, and the titers were determined by a rapid fluorescent focus inhibition test.
DISCUSSION

T-705 has previously been shown to have potent antiviral activity against a broad range of RNA viruses [2], including Mononegavirales [1, 5, 6]. T-705 efficiently suppressed replication of EBOV, which causes a highly lethal hemorrhagic fever in humans, in cell culture and prevented mortality in EBOV-infected mice [5]. We therefore expected T-705 to be effective against RABV, as well.

In this study, we initially found that T-705 had efficient antiviral activity against RABV in vitro. In mouse neuroblastoma N2a cells, T-705 produced a 3–4 log10 reduction in RABV growth, with IC50 values of 32.4 µM for the street strain (1088) and 44.3 µM for the fixed strain (CVS); these values were lower than the values reported for other Mononegavirales viruses, including EBOV (67 µM) [5] and respiratory syncytial virus (261 µM) [1]. We also found that T-705 activity was lower in NA cells than it was in N2a cells. NA cells were selected as 8-azaguanine-resistant cells from N2a cells and found to be deficient in HPRT activity [23], which is required to convert T-705 to its active form, T-705-RTP [22]. We confirmed the lack of HPRT expression in NA cells and predicted that the antiviral activity of T-705 in NA cells would be restored by transfection with an expression vector encoding the hprt gene. However, the antiviral activity of T-705 was not fully restored in NA cells by the complementary expression of the hprt gene, indicating that our NA cells might lack other factor(s) involved in the conversion of T-705-RMP to T-705-RTP. These factors have not yet been identified, although it has been suggested that guanine monophosphate kinase and nucleoside-diphosphate kinase may be involved [22]. Comparative analysis of the NA and N2a cells might help to clarify the mechanisms involved in the conversion of T-705-RMP to T-705-RTP within cells.

Since T-705 showed antiviral effects against RABV in cultured cells, we next evaluated its efficacy in a mouse model. We chose the intramuscular route for inoculation of the street virus strain, to mimic the natural route of RABV infection. The street virus strain 1088 has been used in several of our previous studies [17–19, 24–27], and we found that it caused animals to slowly develop progressive clinical signs and induced efficient lethal infection, compared with the fixed strains, in mice after peripheral inoculation and, thus, that it represents typical natural RABV infection. Therefore, we consider that it is rational to use this strain for evaluating the efficacy of an antiviral compound against rabies in a mouse model. In the 7-day regimen, administration of T-705 300 mg/kg/day on days 0–6 reduced the morbidity and mortality of mice infected with RABV but did not completely prevent its lethality. As all symptomatic mice exhibited neurological signs after T-705 administration was complete (after day 7), we then assessed the efficacy of longer-term T-705 administration (300 mg/kg/day for 14 days; days 0–13). However, the 14-day regimen did not completely prevent the lethality of the diseases and did not appear to improve survival, compared with the 7-day regimen. We also assessed the efficacy of delayed T-705 administration (days 4–17 after inoculation), but this regimen did not improve the animals’ survival time or rate as compared to control (vehicle-treated) mice. A similar result was previously observed in mice infected with WNV, where T-705 administration could be delayed for 2 days but not for 3 days after the viral challenge [4]. However, in our study, T-705 (300 mg/kg/day) had little effect against RABV infection 1 or 2 days after inoculation. A higher dose of T-705 might be required to prevent rabies in delayed or ultimately therapeutic administration. Collectively, these findings indicate that T-705 (300 mg/kg/day) might be ineffective against viruses located within the nervous systems but may be effective before neuroinvasion. In support of this notion, we found that T-705 (300 mg/kg/day) reduced the virus-positive rate in the brains of intramuscularly inoculated mice, but the highest titer was detected not in a control mouse, but in a mouse that had received T-705. This situation was not reported in the cases of WNV and WEEV infection [3, 4]. This might be due to different infection modes (ie, arboviruses multiply robustly in multiple organs prior to central nervous system infection); therefore, the brain titer might be directly reflected by the peripheral virus titers [3, 4], which would be affected by an antiviral drug. In contrast, the brain titer in RABV infection might not directly depend on the titers in nonnervous peripheral tissues, although this is important for neuroinvasiveness [28].

The major findings of this study are that early preventive T-705 administration prolonged the incubation period and
reduced the morbidity and mortality associated with RABV infection in mice, indicating that T-705 has the potential to be used in rabies PEP. Indeed, we also showed that T-705 was as effective as ERIG for PEP. The VNA elevation observed in surviving mice treated with T-705 suggested that T-705 might inhibit the spread of the virus from the initial inoculation site and may assist in RABV clearance by the host immune system in these mice, although it was not be able to prevent viral infection. For rabies PEP, the WHO recommends administering the rabies vaccine and RIG for severe exposures (category III) [10]. RIG can provide a patient with instant passive immunity, which will neutralize RABV and inhibit viral spreading. Since vaccination requires a given period to induce an adequate humoral immunity, administering RIG is important for immediately
preventing rabies after severe exposures. However, RIG is often not administered in these cases, owing to a global production shortage [12, 13, 15]. RIG is available for <1% of patients who require it in developing countries, and its global availability is 2%–5% [12]. Since the high cost and short supply of RIG have hampered efforts to reduce the rabies death toll in developing countries, alternatives to RIG are required [15]. Several experiments using human mAbs to replace RIG are emerging, and some of these have entered clinical trials [29, 30]. However, these potential RIG alternatives are still a long way from clinical use. Unlike biological products such as RIG and human mAbs, T-705 can be mass produced industrially as tablets that can be shipped and stored without a cold chain, which can provide a stable supply and reduce therapeutic costs. Therefore, we suggest that T-705 may be a viable alternative to RIG for PEP in circumstances when RIG is not available.

Figure 6. Comparison of the efficacy of T-705 and equine rabies immunoglobulin (ERIG) as postexposure treatments. Mice were intramuscularly inoculated with 1088 and orally administered T-705 (300 mg/kg/day) daily for 7 days (days 0–6) beginning 1 hour (0 days), 1 day, or 2 days after inoculation, or were intramuscularly injected with 40 IU/kg of ERIG at the virus inoculation site at 1 hour (on 0 day), 1 day, or 2 days after inoculation. Inoculated mice without any treatments were used as controls. Mice were monitored for 28 days. "Sick" indicates that mice showed significant body weight loss or neurological signs. Surviving sick mice had begun to gain body weight but with sequelae, such as limb paralysis. For the survival curves, log-rank tests were performed, and the $P$ values are indicated in the figure. A $P$ value of <.0056 (after Bonferroni correction) was considered significant.
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 copiedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes
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Potential conflicts of interest. T. K. and Y. F. are employees of Toyama Chemical, which is the producer of T-705. A. N. received funding to perform this study from Toyama Chemical. All other authors report no potential 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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