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Efficacy of favipiravir (T-705) and T-1106 pyrazine derivatives in phlebovirus disease models

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ABSTRACT

Several studies have reported favipiravir (T-705) to be effective in treating a number of viral diseases modeled in rodent systems. Notably, the related pyrazine derivative, T-1106, was found to be more effective than T-705 in treating yellow fever virus infection in hamsters. Based on these findings, we hypothesized that T-1106 may be more effective in treating hepatotropic Punta Toro virus (PTV, *Phlebovirus*) infection in rodents. In cell culture, the inhibitory concentrations of the compounds against various phleboviruses ranged from 3 to 55 μM for T-705 and from 76 to 743 μM for T-1106. In PTV-challenged hamsters, a model that generally presents with high liver viral loads, T-1106 was more effective at reducing mortality. However, in mice infected with PTV, a model wherein systemic infection is more prominent, the greater efficacy exhibited by T-1106 in the hamster system was not apparent. In contrast, T-705 was superior in preventing mortality in hamsters challenged with Pichinde virus (PICV, *Arenavirus*), an infection characterized as diffuse and pantropic. Remarkably, T-1106 has proven more active *in vivo* than would have been expected from our cell culture results, and our *in vivo* findings suggest that it is more effective in infections characterized predominantly by high levels of hepatic viral burden.

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1. Introduction

Rift Valley fever (RVF) is a viral zoonosis that primarily affects ungulates, and to a lesser extent, humans (Morrill and McClain, 1996). The etiological agent, Rift Valley fever virus (RVFV), is a mosquito-borne RNA virus member of the *Bunyaviridae* family, genus *Phlebovirus*. It is classified as "Category A Pathogen" by the NIAID and meets selection criteria for dual DHHS and USDA "Select Agent" status, underscoring the importance of RVFV in the context of national security and global public health. Punta Toro virus (PTV) is a phlebovirus related to the highly pathogenic RVFV, which causes a severe hepatotropic disease in mice and hamsters (Anderson et al., 1990; Fisher et al., 2003; Pifat and Smith, 1987) similar to that reported for RVF (Peters and Meegan, 1981). PTV infection in humans is generally asymptomatic or limited to a mild febrile illness (Peters and LeDuc, 1984). Small animal and cell culture model systems based on infection with the less biohazardous PTV and RVFV vaccine strain, MP-12, respectively, are often employed to evaluate experimental therapies early in the preclinical development process (Gowen et al., 2009, 2006a,b, 2008b, 2007a,b; Sidwell et al., 1988; Smee et al., 1991). Ultimately, advanced studies employing authentic RVFV strains in rodent or nonhuman primate models are required for continued preclinical development (Gowen and Holbrook, 2008; Peters et al., 1986).

There are currently no FDA-approved antivirals for the treatment of RVF. Ribavirin has shown some efficacy in animal model systems of RVF (Huggins, 1989; Peters et al., 1986), but due to concerns regarding its toxicity and limited data in humans, it is only indicated under compassionate use guidelines in the event of emergency (Borio et al., 2002). Recently, we reported on the activity of favipiravir (T-705, 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) against PTV infection in mice and hamsters, and against several other bunyaviruses including the RVFV vaccine strain in cell culture (Gowen et al., 2007a). Moreover, T-705 has also been shown to be effective in treating orthomyxovirus (influenza viruses), arenavirus (Pichinde virus), and flavivirus (West Nile and yellow fever viruses) infections in animal models (Furuta et al., 2002; Gowen et al., 2008a; Julander et al., 2009; Morrey et al., in press; Sidwell et al., 2007), and is currently in clinical trials for the treatment of influenza virus infections.

T-1106 (3,4-dihydro-3-oxo-4-β-D-ribofuranosyl-2-pyrazinecarboxamide), a related pyrazine derivative, has been shown to be more efficacious than T-705 for treating yellow fever virus (YFV) infection in hamsters (Julander et al., 2009). YFV

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infection causes a severe hepatic disease in hamsters, suggesting that T-1106 may be a better option for treating viral syndromes that principally target the liver. Because PTV infection causes a severe liver disease in rodents, we hypothesized that T-1106 may be a better option for treating PTV-infected animals. Thus, in the present study, we compared the inhibitory effects of T-1106 and T-705 in the mouse and hamster models of acute PTV disease. In addition, the antiviral activities of the pyrazine derivatives were compared in the hamster Pichinde arenavirus (PICV) infection model, as well as several phlebovirus and arenavirus cell culture model systems.

2. Materials and methods

2.1. Animals

Female 6-week-old Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA). Female 6-week-old C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Hamsters and mice were acclimated to the Laboratory Animal Research Facility at Utah State University for 6 days prior to use. Animals were 7–8 weeks of age at time of challenge. All procedures complied with USDA guidelines and were approved by the Utah State University Institutional Animal Care and Use Committee.

2.2. Viruses

PTV, Adames strain, was received from Dr. Dominique Pifat of the U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick (Frederick, MD). The virus used was from a stock prepared following four passages of the original virus through LLC-MK_2 monkey kidney cells (ATCC; American Type Culture Collection, Manassas, VA) and one passage in hamsters. Clarified viral stocks made from pooled hamster liver homogenates containing high titers of infectious PTV were diluted in minimal essential medium (MEM, Hyclone, Logan, UT) just prior to infectious challenge by the subcutaneous (s.c.) route. Sandfly fever virus, Naples strain (SFNV, *Phlebovirus*) and Tacaribe virus (TCRV, *Arenavirus*), strain TRVL 11573, were from ATCC. The RVFV and Junin virus (JUNV, *Arenavirus*) vaccine strains, MP-12 and Candid 1, respectively, were provided by Dr. Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston, TX). PICV (*Arenavirus*), strain An 4763, was provided by Dr. David Gangemi (Clemson University, Clemson, SC). The virus was passed once through hamsters and a stock made from pooled hamster liver homogenates. PICV was inoculated (0.2 ml) via the intraperitoneal (i.p.) route.

2.3. Test articles

T-705 (MW 157.1) and T-1106 (MW 269.2) were provided by the Toyama Chemical Company, Ltd. (Tokyo, Japan). The structures and basic properties of these pyrazine derivatives are described elsewhere (Furuta et al., 2009). Both T-705 and T-1106 were suspended in 0.4% carboxymethylcellulose (CMC) for oral administration (p.o.). For cell culture studies, the compounds were dissolved in minimal essential medium.

2.4. Cell culture antiviral assays

The monkey kidney cell lines, Vero and Vero 76 were obtained from ATCC and maintained in MEM supplemented with 0.18% $NaHCO_3$ and 10% fetal bovine serum (FBS, Hyclone). Viruses were diluted in culture medium containing 2% FBS to a cell culture 50% infectious dose ($CCID_{50}$) that produced maximal cytopathic effect (CPE) by visual examination in preliminary virus titration experiments. Varying concentrations of the pyrazine derivatives were added to test wells at the time of infection. Vero and Vero 76 cells were ~70% and 90% confluent at time of infection and treatment. For toxicity determinations, drugs were added in the absence of viral challenge. Plates were incubated at 37 °C, 5% CO_2, until virus-infected control wells were observed to have maximal viral CPE (4–5 days for phleboviruses, 7–8 days for the arenaviruses), at which time the plates were processed to assess cell viability by neutral red (NR) vital dye uptake as previously described (Gowen et al., 2007a). The mean effective drug concentration (EC_{50}) and the concentration that reduced cell viability by 50% (CC_{50}) were determined by regression analysis. Virus yield reduction (VYR) data were determined as the concentration of drug reducing virus yield by 1 $\log_{10}$ (EC_{90}) based on regression analysis. Selectivity index (SI) values were calculated as the CC_{50}/EC_{50} for the CPE reduction (CPER) NR-based assays, and as CC_{50}/EC_{90} for the VYR assays.

2.5. In vivo challenge studies

For the PTV studies, hamsters or mice were weighed on the morning of infection and grouped so that the average weight per group across the entire experiment varied by less than 5% between groups. Animals in each group ($n = 15$) were treated orally with pyrazine derivatives (25–100 mg/kg/day) or 0.4% CMC placebo ($n = 25$) for 5–6 days beginning 24–48 h after challenge. Hamsters were inoculated with 50 plaque-forming units (PFU) of PTV and mice were inoculated with 5×10^3 PFU. Five animals from each treatment group were sacrificed on day 3 (mice) or 4 (hamsters) of infection to measure viremia, liver virus burden, and serum alanine aminotransferase (ALT) activity. Serum was collected for assaying systemic viral burden and ALT activity and livers were harvested, homogenized, and clarified for viral titer determination as described below. The remaining 10–20 animals were observed 21 days for mortality. Three sham-infected controls were included for comparison to establish baselines for all test parameters. The PICV hamster challenge (~2 PFU by intraperitoneal route) efficacy study was conducted similarly, except that treatment was initiated on day 4 and lasted 7 days. Improvement in survival outcome, and reduction in viral burden and ALT levels were used to measure therapeutic efficacy.

2.6. Determination of liver and serum virus titers

Virus titers were assayed by infectious cell culture assay as previously described (Gowen et al., 2007a). Liver homogenates or sera were serially diluted and added in triplicate to Vero cell monolayers in 96-well microplates. Viral CPE was determined 7–8 days after exposure to the samples and the 50% endpoints were calculated as described (Reed and Muench, 1938). The assay detection range was 2.75–9.5 $\log_{10}$ $CCID_{50}$/g of liver or 1.75–8.5 $CCID_{50}$/ml of serum. In samples presenting with no detectable liver or serum virus, a value of <2.8 $\log_{10}$ or <1.8 $\log_{10}$, respectively, was assigned. In cases wherein virus exceeded the detection range, a value of >9.5 $\log_{10}$ or >8.5 $\log_{10}$ was assigned. For the purpose of statistical analysis, values of 1.8, 2.8, 8.5, or 9.5 $\log_{10}$ were assigned as needed for samples with undetectable or saturated virus levels.

2.7. Measurement of serum ALT activity

Serum ALT activity, a robust marker indicative of liver disease (Amacher, 1998), was measured in serum samples using the ALT (SGPT) Reagent Set (Pointe Scientific, Lincoln Park, MI). The reagent volumes were adjusted for analysis on 96-well microplates for large sample numbers.

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