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The prophylactic and therapeutic activity of a broadly active ribonucleoside analog in a murine model of intranasal venezuelan equine encephalitis virus infection

George R. Painter[a,b,c], Richard A. Bowen[d], Gregory R. Bluemling[a,b], John DeBergh[a,1], Vindhya Edpuganti[a], Prabhakar R. Gruddanti[a,1], David B. Guthrie[a,1], Michael Hager[a], Damien L. Kuiper[a], Mark A. Lockwood[a], Deborah G. Mitchell[a], Michael G. Natchus[a], Zachary M. Sticher[a], Alexander A. Kolykhalov[a,b,*]

[a] Emory Institute for Drug Development (EIDD), Emory University, Atlanta, GA, USA
[b] Drug Innovation Ventures at Emory (DRIVE), Atlanta, GA, USA
[c] Department of Pharmacology, Emory University School of Medicine, Atlanta, GA, USA
[d] Department of Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

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ABSTRACT

The New World alphaviruses Venezuelan, Eastern, and Western equine encephalitis viruses (VEEV, EEEV and WEEV, respectively) commonly cause a febrile disease that can progress to meningoencephalitis, resulting in significant morbidity and mortality. To address the need for a therapeutic agent for the treatment of Alphavirus infections, we identified and pursued preclinical characterization of a ribonucleoside analog EIDD-1931 (β-D-N^4-hydroxycytidine, NHC), which has shown broad activity against alphaviruses in vitro and has a very high genetic barrier for development of resistance. To be truly effective as a therapeutic agent for VEEV infection a drug must penetrate the blood brain barrier and arrest virus replication in the brain. High plasma levels of EIDD-1931 are rapidly achieved in mice after oral dosing. Once in the plasma EIDD-1931 is efficiently distributed into organs, including brain, where it is rapidly converted to its active 5′-triphosphate. EIDD-1931 showed a good safety profile in mice after 7-day repeated dosing with up to 1000 mg/kg/day doses. In mouse model studies, EIDD-1931 was 90–100% effective in protecting mice against lethal intranasal infection when therapeutic treatment was started as late as 24 h post-infection, and partial protection was achieved when treatment was delayed for 48 h post-infection. These results support further preclinical development of EIDD-1931 as a potential anti-alphavirus drug.

1. Introduction

Three viruses in the genus *Alphavirus* (family *Togaviridae*) endemic to the Americas, Eastern, Western and Venezuelan equine encephalitis viruses (EEEV, WEEV and VEEV, respectively), can cause severe outbreaks of encephalitis in equine species and in humans (Beck and Wyckoff, 1938; Zacks and Paessler, 2010). All three viruses are arthropod-borne pathogens that can be transmitted to humans from equine (or avian) intermediate hosts by the bite of an infected mosquito. Most infections in adult humans are asymptomatic or produce a mild illness characterized by fever, chills, headache, nausea, vomiting and myalgia. Although the frequency of neuroinvasive disease (encephalitis and/or meningitis) due to mosquito transmitted EEEV, WEEV and VEEV is low, the disease symptoms can be devastating (Steele and Twenhafel, 2010). EEEV is the most virulent with a mortality rate ranging between 50 and 75% in those developing neurologic disease. Patients who survive suffer from neurologic sequelae including convulsions, tremors, seizures and intellectual disability. In the case of WEEV, mortality ranges between 3 and 7% with 15–50% of the encephalitis survivors, especially young children, suffering permanent

Abbreviations: b.i.d., twice a day; PO, (*per os*) oral dosing; $t_{1/2}$, terminal half-life; AUC, area under the concentration-versus-time curve; PFU, plaque forming units

* Corresponding author. Emory Institute for Drug Development (EIDD), Emory University, Drug Innovation Ventures at Emory (DRIVE), USA.
E-mail address: a.kolykhalov@emory.edu (A.A. Kolykhalov).

[1] Present addresses: JD, AstraZeneca, Frederick, MD; PRG, Recro Pharma, Inc., Gainesville, GA; DBG, Defense Threat Reduction Agency, Aberdeen Proving Ground, MD.

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neurological damage including mental retardation and emotional instability. Incidences of VEEV associated encephalitis are infrequent, however the virus has caused serious morbidity and mortality following explosive epizootic outbreaks in horses. A 1995 outbreak of VEEV in Columbia and Venezuela, in which horses served as a critical disease-amplifying host, affected 75,000 people with 3000 people developing neurologic conditions and 300 people dying (Weaver et al., 1996, 2004).

EEEV, WEEV and VEEV are also considered significant public health threats due to their potential use as bioweapons (Reichert et al., 2009; Steele and Twenhafel, 2010). The National Institute of Allergy and Infectious Diseases and the Center for Disease Control and Prevention include EEEV and VEEV on their Category B Pathogen and Select Agent lists because they can easily be grown to high titers in cell culture, are stable in the environment, have a high attack rate and are highly infectious via the aerosol route (Torrence, 2005). In addition, alphaviruses are readily susceptible to genetic manipulation, a property that can, in principle, be used to amplify their virulence and effectiveness as an aerosol delivered bioweapon. There is a dearth of clinical experience with human infections via the aerosol route even though airborne VEEV has been responsible for 94% of exposures due to laboratory accidents involving alphaviruses. Animal studies with aerosol VEEV infection have demonstrated accelerated neuroinvasion via the olfactory nerve and faster onset of incapacitating and potentially fatal encephalomeningitis (Hunt et al., 2011; Pratt et al., 2012; Ryzhikov et al., 1995; Vogel et al., 1996).

There are currently no therapeutic agents for the prophylaxis and treatment of alphavirus infections. Given the significant success rate in the development of nucleoside/nucleotide analogs as antiviral drugs (De Clercq and Li, 2016), the development of this class of inhibitors as potential therapeutic agents is extremely promising. The intent of treatment with the appropriately designed ribonucleoside analog is to selectively shutdown the primary pathway of genetic information flow for the virus, the copying of RNA from RNA, by inhibiting the RNA-dependent RNA polymerase (RdRp) encoded by alphaviruses. A ribonucleotide analog can potentially interrupt replication at multiple points in the viral replication cycle: 1) full length negative-strand RNA synthesis; 2) full length positive-strand genomic RNA synthesis; and 3) synthesis of 26S mRNA. RdRps are also exceptional antiviral targets because they are the most highly conserved of all proteins in RNA viruses. Crystal structures to date have shown that all RdRps adopt a canonical “right hand shape” with three conserved sub-domains referred to as fingers, thumb and palm (Ollis et al., 1985; van der Heijden and Bol, 2002). All RdRps contain a series of eight conserved primary sequence motifs, three of which are located in the palm domain and are critical to catalysis (Shatskaya and Dmitrieva, 2013). The overall structural similarity and the conservation of primary, secondary and tertiary structural elements in the palm and thumb domains has led to speculation that these enzymes may be the ideal target for a broadly acting antiviral drug.

There have been multiple studies demonstrating the activity and favorable cytotoxicity profile of the ribonucleoside analog EIDD-1931 (Fig. 1) against RNA viruses in cell culture models of infection (Barnard et al., 2004; Costantini et al., 2012; Ehteshami et al., 2017; Ivanov et al., 2006; Pyrc et al., 2006; Reynard et al., 2015; Stuyver et al., 2003). We have previously reported the activity of EIDD-1931 against the New World encephalitic alphaviruses EEEV, WEEV and VEEV and against the New World alphavirus chikungunya virus (Painter, 2015; Urakova et al., 2017). Here we report the prophylactic and therapeutic activity of orally administered EIDD-1931 in a murine model of lethal VEEV infection in which infection was initiated by intranasal administration of a lethal dose of VEEV strain Trinidad donkey (TrD). Intranasal exposure is meant to mimic disease resulting from an aerosol exposure. Prior to initiating the in vivo efficacy studies, the tissue distribution profile of EIDD-1931 was examined in ICR (CD-1) mice, the strain of mouse in the animal efficacy studies, to establish that after oral administration the parent ribonucleoside is distributed into tissues key in the pathogenesis of VEEV associated disease, including the brain, where it is anabolized to its 5′-triphosphate, the active antiviral agent. The reported results of these pharmacokinetic/tissue distribution and in vivo efficacy studies strongly support the continued development of EIDD-1931 for the prophylaxis and treatment of VEEV, and possibly other alphavirus infections.

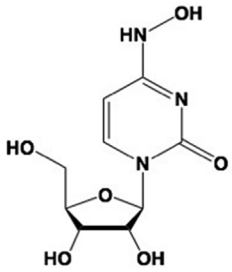


Fig. 1. The structure of EIDD-1931, N^4-hydroxycytidine.

2. Materials and Methods

2.1. Preparation of EIDD-1931 and EIDD-2061

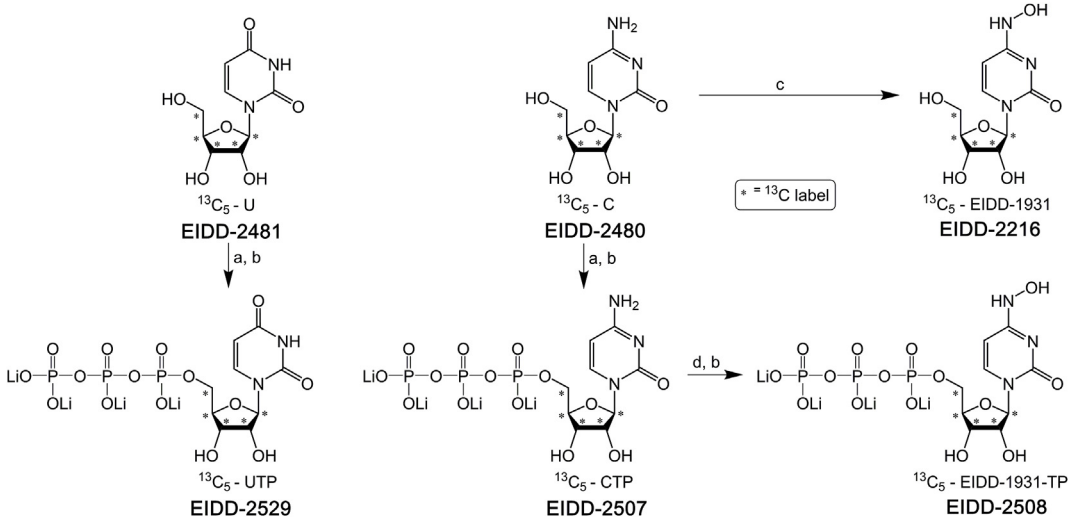
EIDD-1931 was prepared as previously described (Painter et al., 2016; Yoon et al., 2018). The 5′-triphosphate of EIDD-1931 (EIDD-2061) was made as described in Scheme 1 starting with cytidine.

2.2. Preparation of ^{13}C-labeled standards of EIDD-1931 and EIDD-1931 metabolites

$^{13}C_5$-labeled uridine and $^{13}C_5$-labeled cytidine were purchased from Omicron Biochemicals, with each position in the riboside ring labeled with atom ^{13}C at > 99%. Isotopically labeled EIDD-1931 (EIDD-2216) was synthesized in one step on 100 mg scale by treating $^{13}C_5$-labeled cytidine with a mildly acidic aqueous hydroxylamine solution, followed by reverse phase column chromatography as described elsewhere (Purohit et al., 2012). Labeled $^{13}C_5$-cytidine-5′-triphosphate ($^{13}C_5$-CTP, EIDD-2507) was synthesized as shown in Scheme 1 from commercially available ^{13}C-labeled cytidine by treatment with phosphoryl chloride in the presence of Proton Sponge® to provide the 5′-monophosphate, followed by addition of tributylammonium pyrophosphate in the presence of excess amine base. The mixture was purified by ion-exchange chromatography on DEAE Sephadex-25 using aqueous triethylammonium bicarbonate as the mobile phase to afford 5′-triphosphate as the triethylammonium form. Ion-exchange chromatography on Dowex (Li^+ form) afforded the 5′-triphosphate as the tetralithium salt, which is more stable and consequently more easily handled than the triethylammonium salt form. Labeled $^{13}C_5$-EIDD-1931-5′-triphosphate (EIDD-2508) was synthesized by heating $^{13}C_5$-CTP (EIDD-2507) in mildly acidic aqueous hydroxylamine, followed by ion-exchange columns on DEAE Sephadex-25 and Li^+ Dowex to isolate the tetralithium salt.

2.3. Pharmacokinetics and tissue distribution in mice

Female ICR (CD-1®) mice (from Envigo, NJ), 6–8 weeks of age, were used in the studies (to match mice used in efficacy studies). EIDD-1931 was administered by oral gavage (PO) in 240 mM citrate buffer pH 3 ± 0.3 or intraperitoneally (IP) in saline. The oral doses tested were 50, 150 and 500 mg/kg of body weight, and the IP doses were 10 and 50 mg/kg of body weight. Blood samples were collected at 0.08, 0.25, 0.5, 1, 2, 4, 8, and 24 h post IP administration, and at 0.25, 0.5, 1, 2, 3, 4, 8, and 24 h post oral administration. Plasmas were prepared within 30 min after collection by centrifugation at 2000*g* for 10 min at 4 °C and stored at −80 °C before processing for analysis by LC-MS/MS. Mouse



Scheme 1. a) $P(O)Cl_3$, $P(O)(OMe)_3$, Proton Sponge, then tributylammonium pyrophosphate, tributylamine, DMF; b) Li + Dowex; c) aq. NH_2OH, 37 °C, pH = 5; d) aq. NH_2OH, 55 °C, pH = 5. Location of ^{13}C atoms are indicated by (*).

organs (lung, spleen liver, kidney, heart and brain) were collected from all mice immediately following blood collection starting from 0.5 h post dose. The tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C before processing for analysis by LC-MS/MS.

Aliquots of mouse plasma were extracted with 70% acetonitrile in water that included EIDD-2216 as an Internal Standard. Samples were then vortexed and centrifuged at 2000 g for 10 min. The supernatants were transferred to microcentrifuge tubes and centrifuged again for 10 min at 14,000 g. The resulting supernatants were then transferred to HPLC vials for analysis by LC-MS/MS.

Frozen mouse tissues (~50 mg) were extracted with 0.45 ml of cold 70% acetonitrile in water by homogenization in an Omni Bead-Ruptor (Omni International, Kennesaw, GA). The extracts were centrifuged for 10 min at 2000 g. The supernatants were transferred to micro-centrifuge tubes and centrifuged again for 10 min at 14,000 g. The clarified supernatants were transferred to HPLC vials and Internal Standards EIDD-2216 and EIDD-2508 were added.

2.4. LC-MS/MS conditions

HPLC separation was performed on an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA). A SeQuant ZIC-pHILIC 5-µm, 100 by 4.6 mm column (The Nest group, USA) was used for the separation of EIDD-1931, EIDD-2061 and their $^{13}C_5$ labeled counterparts (internal standards EIDD-2216 and EIDD-2508, respectively). HPLC separation of plasma was performed using a linear gradient mode of acetonitrile (75–30%) in 25 mM ammonium bicarbonate buffer, pH 9.4 at a flow rate of 0.75 ml/min over 6 min. HPLC separation of tissue extracts was performed using a linear gradient mode of acetonitrile (85–40%) in 25 mM ammonium bicarbonate buffer, pH 9.4 at a flow rate of 0.75 ml/min over 9 min. Mass spectrometry analysis was performed on a QTrap 5500 Mass Spectrometer (AB Sciex, Framingham, MA) using Negative Mode Electrospray Ionization (ESI) in Multiple Reaction Monitoring (MRM) Mode. Analyte quantitation was based on twelve-point calibration curves of standards in blank plasma spanning the range of 10–30,000 ng/ml. Separately prepared quality-control samples of 30, 500 and 900 ng/ml in blank plasma were analyzed at the beginning of each sample set to ensure accuracy and precision. For organ tissue analysis seven-point standard curves were prepared in blank tissue lysates spanning concentrations from 1.49 to 1490 ng/ml. Calibration in each matrix showed linearity with an R^2 value of > 0.99. Data analysis was performed using Analyst Software (AB Sciex, Framingham). Pharmacokinetic parameters were calculated using Phoenix WinNonlin Non-compartmental analysis software (Certara, Princeton, NJ).

2.5. Dose range finding (DRF) toxicology and toxicokinetic study

This study was conducted in two phases: Phase A (single dose, acute toxicity) and Phase B (multiple doses). During Phase A, two groups of 6 mice (3 males and 3 females each) were administered EIDD-1931 once via oral gavage at 500 and 1000 mg/kg dose levels, and following a four-day washout period the same animals were administered 1500 and 2000 mg/kg doses. The compound was delivered at 10 ml/kg volumes in sodium citrate vehicle (0.24M sodium citrate, pH 3 ± 0.3). After dosing, the animal's weight, food consumption, general physical appearance and behavior were monitored twice daily for four days. During Phase B, EIDD-1931 was administered once daily for 7 consecutive days. Ten male and ten female mice per dose (80 mice total) were tested at dose levels of 200, 500, and 1000 mg/kg/day administered at a dose volume of 10 mL/kg. The Phase B study control group (0 mg/kg) received the vehicle at the same dose volume and in the same manner as the compound treated groups. Individual doses were based on the animals' most recently determined body weight. Animals were observed twice daily for morbidity, mortality, injury, and availability of food and water. Daily evaluations of the skin, fur, eyes, ears, nose, oral cavity, thorax, abdomen, external genitalia, limbs and feet, respiratory and circulatory effects, salivation, nervous system effects including tremors, convulsions, reactivity to handling and unusual behavior were also conducted. Body weights and food consumption were measured daily. All main study animals were housed in stainless steel metabolism cages and urine was collected for at least 12 h. Clinical pathology evaluations were conducted on all Phase B main study animals at the terminal necropsy on day 8 of the study (24 h after the last dose). Blood samples were taken from the vena cava. Samples were collected into tubes containing K_3EDTA for evaluation of hematology parameters. A serum separator with no anticoagulant was used for the clinical chemistry samples. Hematology samples were collected from the first 5 animals/sex/group, while clinical chemistry samples were collected from the last 5 animals/sex/group. Necropsy examinations were performed on all Phase B main study animals. Body weights and organ weights were recorded for all animals and the organ weight ratios were calculated relative to the body and brain weights.

Animals in the toxicokinetic (TK) arm of the study received EIDD-1931 at the same doses and dose volumes and in the same manner as the main study groups at doses of 200, 500, and 1000 mg/kg/day. Thirty six male and 36 female mice per dose level were used in the TK

arm. Blood samples were collected from TK animals for determination of the plasma concentrations of EIDD-1931. Samples were collected from cohorts of 3 TK animals/sex/group/timepoint at 1, 2, 4, 6, 8, and 24 h postdose on Day 1 and at predose and 1, 2, 4, 6, and 8 h postdose on Day 7. Samples were collected in tubes containing lithium heparin as an anticoagulant and kept on ice. Plasmas were prepared within 1 h of blood collection and stored at −80 °C until processed for analysis by LC-MS/MS as described above. Following the blood collections on Days 1 and 7 from the female Phase B TK animals at 4 h postdose, the animals were exsanguinated and the spleen, brain, lung, heart, kidney, and liver tissues were collected, flash frozen in liquid nitrogen, and stored frozen at −80 °C until processed for analysis by LC-MS/MS as described above.

2.6. Murine models of intranasal VEEV infection

Seven to eight-week-old ICR (Crl:CD1) female mice were used in all studies. The Trinidad donkey (TrD) strain of VEEV was originally obtained from the Centers for Disease Control and Prevention (CDC) and had the following history of passaging: the 1943 TrD isolate was passaged (i) once on guinea pig brains, then (ii) six times on Vero cells (including one plaque purification), and (iii) once in BHK21 cells. The last virus was additionally passaged once on Vero cells to expand the virus and was titrated by plaque assay.

Nasal challenge consisted of intranasal application of ~100 PFU of virus, corresponding to ~100 LD_{50}, in 25 µl volume of PBS split into two nostrils and delivered under ketamine-xylazine anesthesia. The residual inoculum used for each experiment was back titrated after the challenge to confirm the dose delivered. The back-titration showed 92 PFU/dose in the prophylactic study and 111 PFU/dose in the time-of-treatment delay study.

The dose dependency of EIDD-1931 was determined in a prophylaxis study. Four groups of mice were dosed via gavage with 150, 300 or 500 mg/kg EIDD-1931 in 240 mM sodium citrate buffer pH 3 ± 0.3 or mock-treated with vehicle only, all at 10 ml/kg dose volume, starting at 2 h before infection. The second treatment was delivered at +2 h post-infection (PI), and then the treatment was continued twice daily (b.i.d.) for 6 days. In a second (therapeutic) study, treatment with 500 mg/kg EIDD-1931 was initiated starting at 6, 12, 24 or 48 h post-infection and the treatment was compared to a vehicle (mock) treated group. For the +6 h group, the second treatment was performed at 12 h post-infection and then, for all groups, the treatment was continued every 12 h (b.i.d.) for 6 days.

Disease parameters measured in all studies were: animal survival, time to death/euthanasia, body weight decline, characteristic signs of disease (scores), day 3 serum virus titers; virus titer in the brain (either after death/euthanasia or on day 14 at the end of the study). The disease symptoms included rough coat, unkempt appearance, hunched posture, decreased activity, lethargy, etc. The disease scores were defined as follows: 0, normal; −1, questionable illness; −2, mild but definite illness; −3, moderate illness; −4, severe illness, moribund – mice are euthanized; −5, found dead.

2.7. Infectious virus titration in cell culture

The virus titers in serum and brain were assayed using a standard double-overlay plaque assay (Fine et al., 2007) in which 0.1 ml volumes of serial dilutions of serum or brain homogenate were inoculated onto Vero cells cultured in 6-well plates. Plaques were counted ~48 h after inoculation and titers calculated on the basis of mL of serum or gram of brain. The limit of detection of these assays was 100 plaques (PFU) per mL of serum or per gram of tissue.

2.8. Statistical analysis

Animal survival was analyzed using a Log-rank (Mantel-Cox) test for groups comparison (Prism 6, GraphPad Software, Inc.). Fisher's exact test was used for statistical analyses of viral titer differences. When appropriate, experimental variabilities are identified by error bars representing standard deviations from the mean (SD).

2.9. Facilities

Animal experiments were conducted in the Association for Assessment and Accreditation of Laboratory Care (AAALAC)-accredited and Public Health Services Animal Welfare Assurance-approved animal care suites at the Yerkes National Non-Human Primate research facility (PK/distribution studies), at the laboratories of Colorado State University (mouse models of VEEV infection), and at the Concord Biosciences research facility (7-day DRF toxicology/toxicokinetics study). All facilities are registered with the United States Department of Agriculture (USDA) in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.10. Ethics statement

All animal protocols using mice were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) at the facilities where the studies were performed, and were reviewed and approved by the Animal Care and Use Review Office (ACURO) of the USAMRMC Office of Research Protections (ORP) before the initiation of studies.

3. Results

3.1. Pharmacokinetics and tissue distribution of EIDD-1931 and EIDD-2061 in ICR (CD-1) mice after oral dosing

A pharmacokinetic and tissue distribution study was conducted to inform dose selection in dose ranging toxicology and in vivo efficacy studies. Time-concentration curves of EIDD-1931 in mouse plasma and plasma PK parameters were previously presented in Yoon et al. (2018) (Yoon et al., 2018). High plasma concentrations of EIDD-1931 were achieved after oral dosing. C_{max} and $AUC_{0-\infty}$ values increased in a dose dependent, though less than dose-proportional manner. Peak plasma levels of EIDD-1931 were reached rapidly (T_{max} < 1 h) and terminal $t_{1/2}$ values ranged from 2.7 to 5.2 h. Bioavailability of EIDD-1931 after oral dosing was relatively high and decreased with increasing dose from 56 to 36%.

For a ribonucleoside analog to be effective in treating an acute onset VEEV infection it must be quickly distributed into tissues critical in the pathogenesis of disease and anabolized to its active 5′-triphosphate form. EIDD-1931 and EIDD-2061 levels were determined in tissue samples collected from mice as described in the Materials and Methods section. The free ribonucleoside was rapidly distributed into all tissues examined including brain and spleen, two tissues critical in the development of VEEV associated disease, with T_{max} values ranging between 0.5 and 2 h depending on dose (Fig. 2A). Higher oral doses resulted in longer T_{max} values. Similar to plasma exposure, organ exposure to the parent ribonucleoside is dose dependent. In the spleen, EIDD-1931 C_{max} values were 10–20X higher than those measured in the brain and appear to be relatively independent of dose, while AUC values tend to be dose dependent (Table 1). In brain tissue both C_{max} and AUC of EIDD-1931 are dose-dependent. Accumulation of EIDD-2061 in organs is somewhat delayed compared to the parent ribonucleoside (Fig. 2B). C_{max} levels were achieved 1–8 h post-dose. In brain tissue the EIDD-2061 level builds up more slowly than in the spleen with T_{max} values ranging between 3 h at the low dose to 8 h at the highest dose tested. C_{max} values in the brain are 3–5X lower than those observed in the spleen. The terminal half-life ($t_{1/2}$) of EIDD-2061 in tissues is quite short, 2–5 h, suggesting twice-daily (b.i.d.) oral dosing with EIDD-1931 in mice is necessary. Data on additional tissues (lung, kidney, heart and liver) are shown in the Supplementary Data section, Fig. S1.

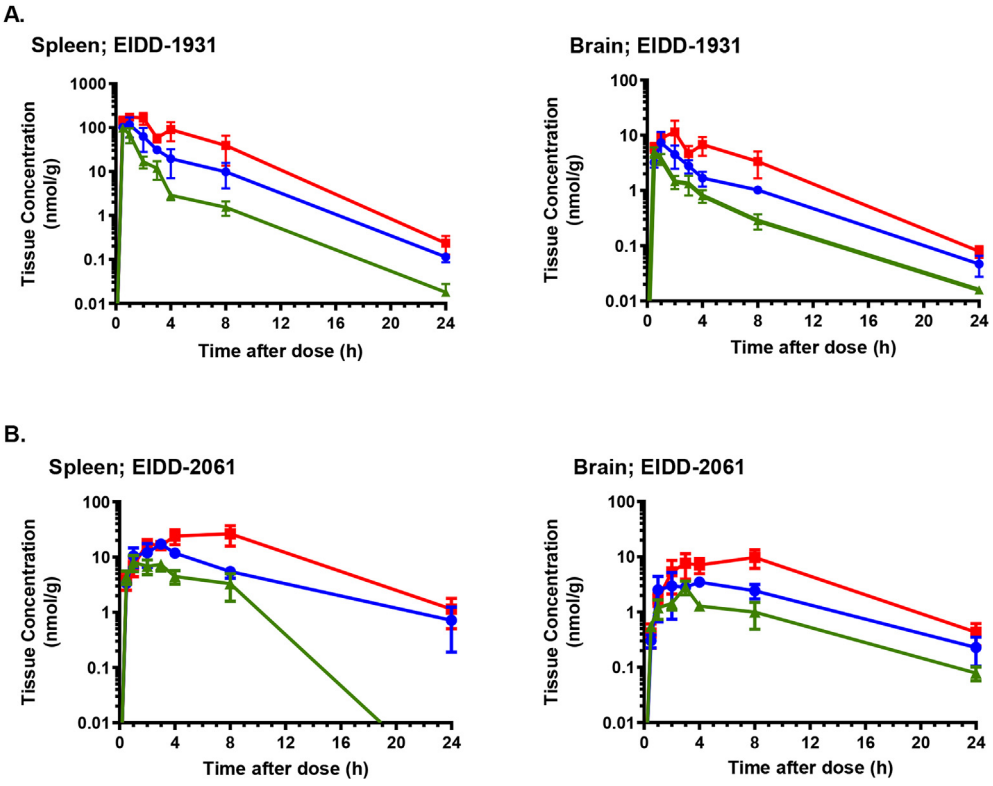


Fig. 2. Concentration versus time plots of accumulation of nucleoside EIDD-1931 (A) and of triphosphate EIDD-2061 (B) in mouse Spleen and Brain tissues after single oral doses at 50 mg/kg (green triangles), 150 (blue circles), or 500 (red squares) mg/kg dose levels. Data shown as Mean ± SD (n = 3).

3.2. Dose-range finding toxicology/toxicokinetic study in mice

A dose range finding toxicology study was conducted to determine if there was background toxicity that could confound efficacy results and/or limit dose. In Phase A (the single dose acute toxicity phase), a suspected test article related decrease in food consumption on Day 2 post dose was noted at the two highest doses tested, 1500 and 2000 mg/kg, with no accompanying change in body weight. Food consumption returned to normal by day 3 post-dose. Based on these results, doses of 200, 500 and 1000 mg/kg/day were selected for oral administration for seven consecutive days in Phase B of the study. All animals in Phase B survived to scheduled necropsy with no test article related clinical signs and no effects on body weight or food consumption. No test article related macroscopic observations were made at necropsy and no organ weight changes compared with the control (vehicle dosed) mice were noted. Additionally, no test article related changes in hematology, clinical chemistry or urinalysis parameters were observed compared with control mice.

Blood for toxicokinetic evaluation was collected at various time points on Days 1 and 7 of Phase B as described in Materials and Methods. C_{max} values were dose dependent and $AUC_{0\text{-}24}$ values were dose proportional. Comparison of Day 1 and Day 7 data shows that there was minimal accumulation of EIDD-1931 in plasma after repeated dosing (less than 2-fold, see Supplementary Fig. S2 and Table S1). Levels of EIDD-1931 and EIDD-2061 were measured in spleen and brain on Day 1 and on Day 7 (Table 2). EIDD-1931 levels in both tissues were dose dependent on Days 1 and 7 of the study and no accumulation was observed after repeated dosing. There was also a dose-dependent exposure to EIDD-2061 on Day 1 and Day 7 of the study with minimal accumulation (less than 2-fold) observed in both tissues.

3.3. Efficacy of EIDD-1931 in a mouse model of intranasal VEEV infection

Peripheral infection of mice with VEEV usually results in a biphasic disease (Grieder et al., 1995). The initial peripheral phase lasts for 4–5 days, during which virus titers rise, decline, and eventually disappear from blood and peripheral organs by days 4–6 post-infection (PI). The second, neurotropic phase of the disease starts after virus invasion of

Table 1
PK parameters in mouse Spleen and Brain after single PO doses of EIDD-1931. T_{max}, time to maximum concentration of drug in tissue; C_{max}, maximum tissue concentration; $AUC_{(0\text{-}\infty)}$, area under the tissue concentration-time curve from time zero to infinity; $t_{1/2}$, half-life.

		EIDD-1931				EIDD-2061			
Tissue	Dose Level	T_{max}	C_{max}	$AUC_{(0\text{-}\infty)}$	$t_{1/2}$	T_{max}	C_{max}	$AUC_{(0\text{-}\infty)}$	$t_{1/2}$
		(h)	(nmol/g)	(hr*nmol/g)	(h)	(h)	(nmol/g)	(hr*nmol/g)	(h)
Spleen	50 mg/kg	0.5	97.7	152.7	1.3	1	8.1	64.4	1.8
	150 mg/kg	1	115.5	380.6	5.1	3	17.2	133.8	4.8
	500 mg/kg	1	172.8	1043.7	3.5	8	26.4	380.9	3.5
Brain	50 mg/kg	0.5	5.0	13.1	3.6	3	2.8	19.8	4.4
	150 mg/kg	1	7.3	29.3	3.7	4	3.5	44.3	5.0
	500 mg/kg	2	11.4	77.2	3.1	8	9.8	135.4	3.6

Table 2
Accumulation of nucleoside EIDD-1931 and its 5′-triphosphate EIDD-2061 in Spleen and Brain tissues of mice dosed PO with EIDD-1931 at 200, 500 or 1000 mg/kg/day either once (Day 1) or daily for seven consecutive days (Day 7). Tissues were collected at 4 h post-dose. Values shown as Mean ± SD (n = 3).

Dose level		200 mg/kg	500 mg/kg	1000 mg/kg
		EIDD-1931 (nmol/g)		
Brain	Day 1	2.2 ± 0.7	6.3 ± 3.1	12.2 ± 4.7
	Day 7	2.5 ± 0.6	10.5 ± 4.0	11.9 ± 3.3
Spleen	Day 1	6.8 ± 1.7	31.7 ± 18.2	106.4 ± 62.2
	Day 7	9.6 ± 3.3	43.5 ± 20.5	56.6 ± 17.7
		EIDD-2061 (nmol/g)		
Brain	Day 1	1.9 ± 1.0	3.1 ± 1.2	7.6 ± 1.8
	Day 7	2.2 ± 0.7	7.7 ± 0.4	9.6 ± 2.2
Spleen	Day 1	2.5 ± 0.2	8.4 ± 3.8	16.8 ± 7.7
	Day 7	3.3 ± 1.2	10.4 ± 4.1	23.2 ± 7.5

the brain on days 2–3 post-infection, presumably through the olfactory bulb route, and ends up with death occurring 5–7 days post-infection. An aerosol or intranasal route of infection with VEEV bypasses the initial phase of disease and provides a more rapid direct route to the olfactory bulb and the brain (Ryzhikov et al., 1995). The intranasal model is considered more stringent for evaluating antivirals or vaccines, and more relevant to potential use of the virus as a bioweapon. Therefore, we have tested the ability of EIDD-1931 to protect mice after intranasal challenge with lethal doses of VEEV.

In an initial prophylactic treatment study, EIDD-1931 treatment was initiated 2 h before intranasal challenge with ~100 LD_{50} of VEEV TrD. Three dose levels were tested (150, 300 and 500 mg/kg; b.i.d.) and compared to vehicle-treated controls. All vehicle-treated, VEEV infected mice developed characteristic disease symptoms including significant weight loss and were euthanized or died 6–7 days post-challenge (Fig. 3). On an intent-to-treat basis, 90% of mice survived the lethal intranasal challenge when treated with 500 mg/kg and 300 mg/kg b.i.d. doses (Fig. 3A). The low dose treatment group (150 mg/kg b.i.d.) showed 80% survival. Survival in each treated group was statistically different from the vehicle treated control group, with $p < 0.001$, but there was no statistically significant difference between the treatment groups. All VEEV infected animals showed initial weight decline; the decline was smaller in treated groups (Fig. 3B), and all treated mice that survived had reversal of the decline during or after the 6-day treatment period (Supplementary Fig. S3). Despite significant weight decline all treated and surviving mice showed little or no clinical signs of disease (Supplementary Fig. S4). All treated mice showed some level of viremia on Day 3 PI (Fig. 3C), which did not correlate well with survival. There was also no correlation of average virus titer with treatment dose level, although there was a statistical difference between each treated group versus mock-treated control ($p < 0.05$).

All mock-treated animals eventually succumbed to the disease and all had high virus titers in the brain at the time of euthanasia/death, ~10^9 PFU/g. All mice treated with EIDD-1931 had undetectable or very low (~10^4 PFU/g) virus titers in the brain (Fig. 3D).

To determine the therapeutic potential of EIDD-1931 against intranasal VEEV challenge, treatment with the highest effective dose (500 mg/kg b.i.d.) was initiated 6, 12, 24 and 48 h after virus challenge with ~100 LD_{50} and continued twice a day for 6 days. All vehicle-treated mice developed characteristic disease symptoms and were euthanized 5.5–6 days PI. All animals survived when treatment with EIDD-1931 was initiated at 6 and 12 h after virus challenge (Fig. 4A). Ninety percent of animals survived when the treatment was initiated 24 h post infection, and 40% of mice survived when treatment was delayed until 48 h post-challenge. Survival in each treated group was statistically different from the mock-treated control group, with $p < 0.01$. All VEEV infected animals showed initial weight decline; the decline was significantly smaller when treatment was initiated before 48 h post-challenge (Fig. 4B). All treated mice that have survived had reversal of the weight decline during or after the 6-day treatment period (Supplementary Fig. S5). With the exception of weight loss, all surviving mice in the 6, 12 and 24 h groups showed little or no clinical signs of disease (Supplementary Fig. S6). This outcome was similar to what was observed in the prophylactic treatment study. In the 48 h delay group, all mice, including those that survived, exhibited definite signs of illness (Score −2; mild but definite illness) on Days 5–7 post-challenge; these signs disappeared in all surviving animals by Day 9 post-challenge.

All mice infected intranasally with VEEV and mock-treated were viremic on Day 3 post-infection, with titers ranging from 8×10^3 - 9×10^5 PFU/ml (Fig. 4C). In the three groups in which EIDD-1931 treatment was initiated at or before 24 h PI, 22 out of 30 mice had detectable viremia (above the 10^2 PFU/ml detection limit). Twenty-one of them had very low virus titers ranging between 2×10^2 and 10^3 PFU/ml and all survived to the end of the study. The mouse that eventually succumbed to the disease had the titer of ~10^4 PFU/ml. Mice in the 48 h delay of treatment group, in which blood was collected one day after the first treatment dose, had higher viremia on Day 3 PI, though the titers in the drug treated mice were lower than in the mock treated mice: 4.1 ± 0.7 $\log_{10}$ PFU/ml in treated versus 5.2 ± 0.7 $\log_{10}$ PFU/ml in control mice ($p < 0.01$). There was no clear correlation between Day 3 viremia levels and mouse survival in this group. Two mice with viremia at ~10^4 PFU/ml survived while 3 mice with similar levels died. Two other survivors in this group had low viremia on Day 3 (~10^3 PFU/ml).

Brain virus titers were analyzed in all mice in all treatment groups after death/euthanasia or in the survivors at the end of the study (Day 14). All infected mock-treated mice were euthanized 5.5–6 days post infection due to moribund condition and all had high brain virus titers, 3.6×10^8 to 1.5×10^9 PFU/g of brain tissue (Fig. 4D). None of the infected animals in which treatment with EIDD-1931 was initiated at 6 or 12 h PI had detectable virus in the brain (limit of quantitation is 10^2 PFU/g of tissue). One mouse out of 10 was euthanized in the 24 h delay of treatment group with a brain virus titer ~100-fold lower (2.5×10^6 PFU/g) than in the mock-treated group. In the 48 h delay of treatment group, six of the ten mice had to be euthanized due to severe disease. All of them had measurable levels of virus in brain tissue (9.0×10^5 to 7.8×10^7 PFU/g).

4. Discussion

The mouse model of VEEV infection has been used extensively to study the pathogenic course of disease resulting from both systemic infection (intended to mimic the course of infection after vector transmission) and intranasal/aerosol infection (intended to model the course of disease after aerosol exposure to weaponized virus), and to establish the efficacy of vaccine candidates and potential therapeutic agents. The pathogenesis of infection in mice inoculated by aerosol has been thoroughly reviewed in the literature, and although the pathogenesis of VEEV infection in humans is not well described, available information suggests that some of the key features of human disease are found in mice (Steele et al., 2007). Of particular significance is neuroinvasion, and the resulting encephalitis. Infection of the brain via the aerosol or intranasal route is very direct, and consequently, somewhat faster than that observed after systemic infection (Hunt et al., 2011; Ryzhikov et al., 1995; Vogel et al., 1996). VEEV in inspired air directly contacts the cilia on the dendritic processes of bipolar olfactory neurons and moves along the axons via orthograde axonal transport directly into the brain. At 20 h post respiratory infection with VEEV, severe necrotizing lesions are observed that involve the full thickness of the olfactory neuroepithelium and virus is detectable in the main olfactory bulb. By 48 h post infection virus is widespread throughout the brain. Neurodegeneration is extensive by 72 h post-infection and animals die between days 5 and 7 post infection. Virus infection of neurons in the

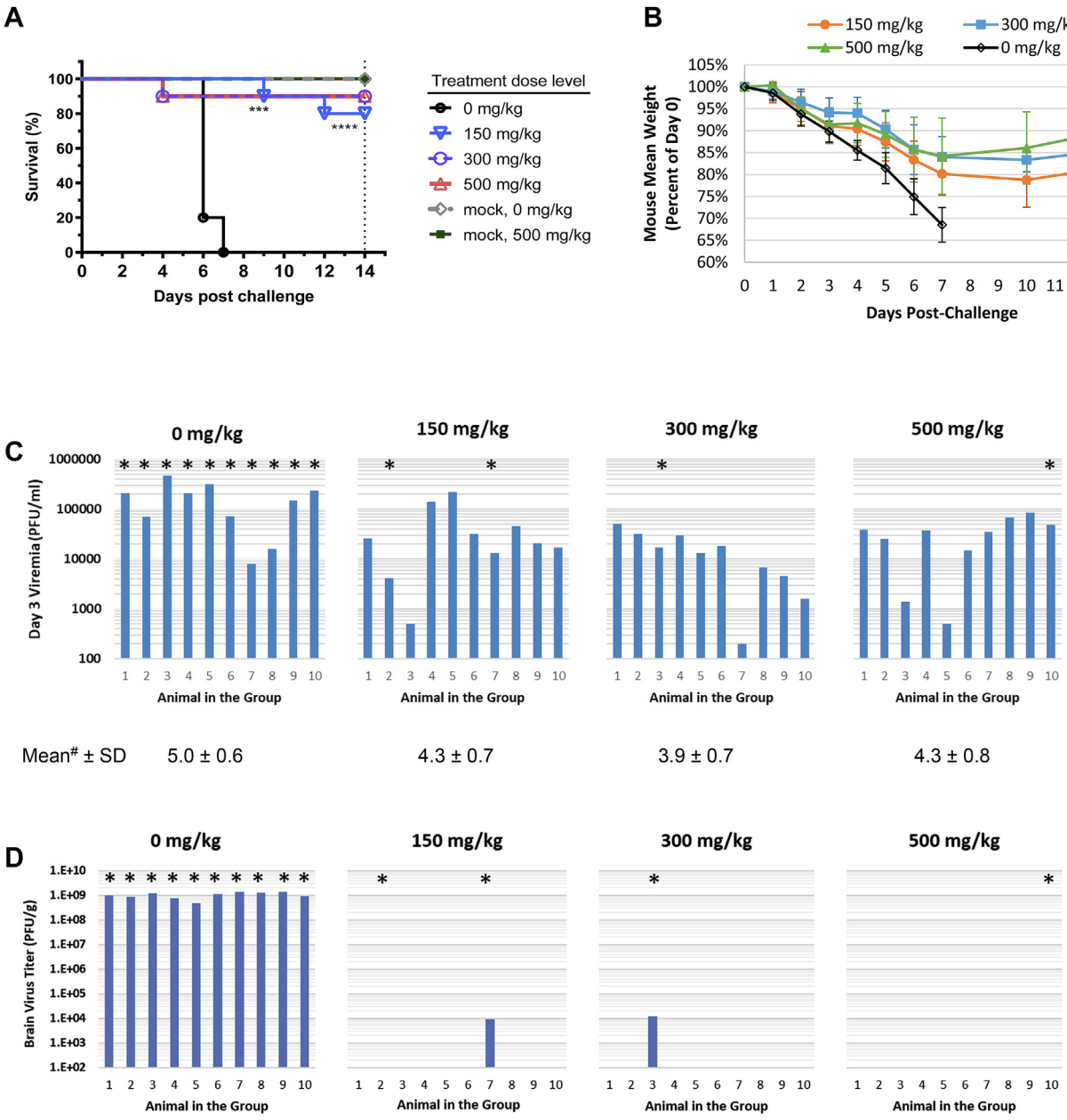


Fig. 3. (A) Effect of treatment with EIDD-1931 on animal survival. Mice were challenged via intranasal infection with ~100 LD_{50} of VEEV on day 0. EIDD-1931 oral treatment was initiated 2 h before the challenge and continued b.i.d. for 6 days. Mock, mock-challenged mice. ***p < 0.001, ****p < 0.0001 relative to 0 mg/kg control, n = 10. (B) Average mouse weight dynamics over 14-day study period. Data shown as Mean ± SD, n = 10. (C) Day 3 viremia in mice. (*) in panels indicates animals that died or had to be euthanized due to severe disease. # average group virus titer calculated as average of individual titers, log_{10} PFU/ml. (D) Effect of treatment with EIDD-1931 on brain virus titers in individual mice. Brain tissues were collected from animals either on the day of euthanasia/death or on day 14 (end of the study). LLOQ = 10^2 PFU/g of tissue. (*) in panels indicates animals that died or had to be euthanized due to severe disease. Note: mouse #10 death in the 500 mg/kg group was considered not virus-related (see text for details).

brain and spinal cord are responsible for the majority of clinical signs and symptoms and the nearly uniform lethality of the disease in this model. It is very clear that if EIDD-1931 is to be effective for the prophylaxis and treatment of intranasal/aerosol VEEV infection, therapeutic levels of its 5′-triphosphate must be achieved in the CNS to provide benefit in mortality in the mouse model.

After oral administration EIDD-1931 is quickly absorbed as evidenced by plasma T_{max} values ranging between 0.5 and 1.0 h. Exposures are high (C_{max} values range between 30 and 40 μM) and are dose dependent, but significantly less than dose proportional. The observation of decreasing bioavailability with increasing dose may indicate capacity limited absorption, a phenomenon that has been reported for other nucleosides (de Miranda et al., 1981). EIDD-1931, like most endogenous nucleosides and xenobiotic nucleoside analogs, is a highly polar, hydrophilic molecule (cLogP = −2.2) and therefore likely to require functional transporters to cross cell membranes. This dependence would explain the capacity limited uptake seen in the pharmacokinetic studies done using the CD-1 mice. Earlier reports also indicated that nucleoside uptake into mouse intestinal epithelial cells is primarily mediated by sodium dependent concentrative nucleoside transporters (Cass et al., 1999; Vijayalakshmi and Belt, 1988).

Onset of antiviral activity is dependent on the time required to distribute EIDD-1931 from the plasma into key organs in the pathogenesis of disease, and the time required for its anabolism to the active 5′-triphosphate. Maximum levels of drug are achieved rapidly in all organs examined, including the spleen and the brain, between 0.5 and

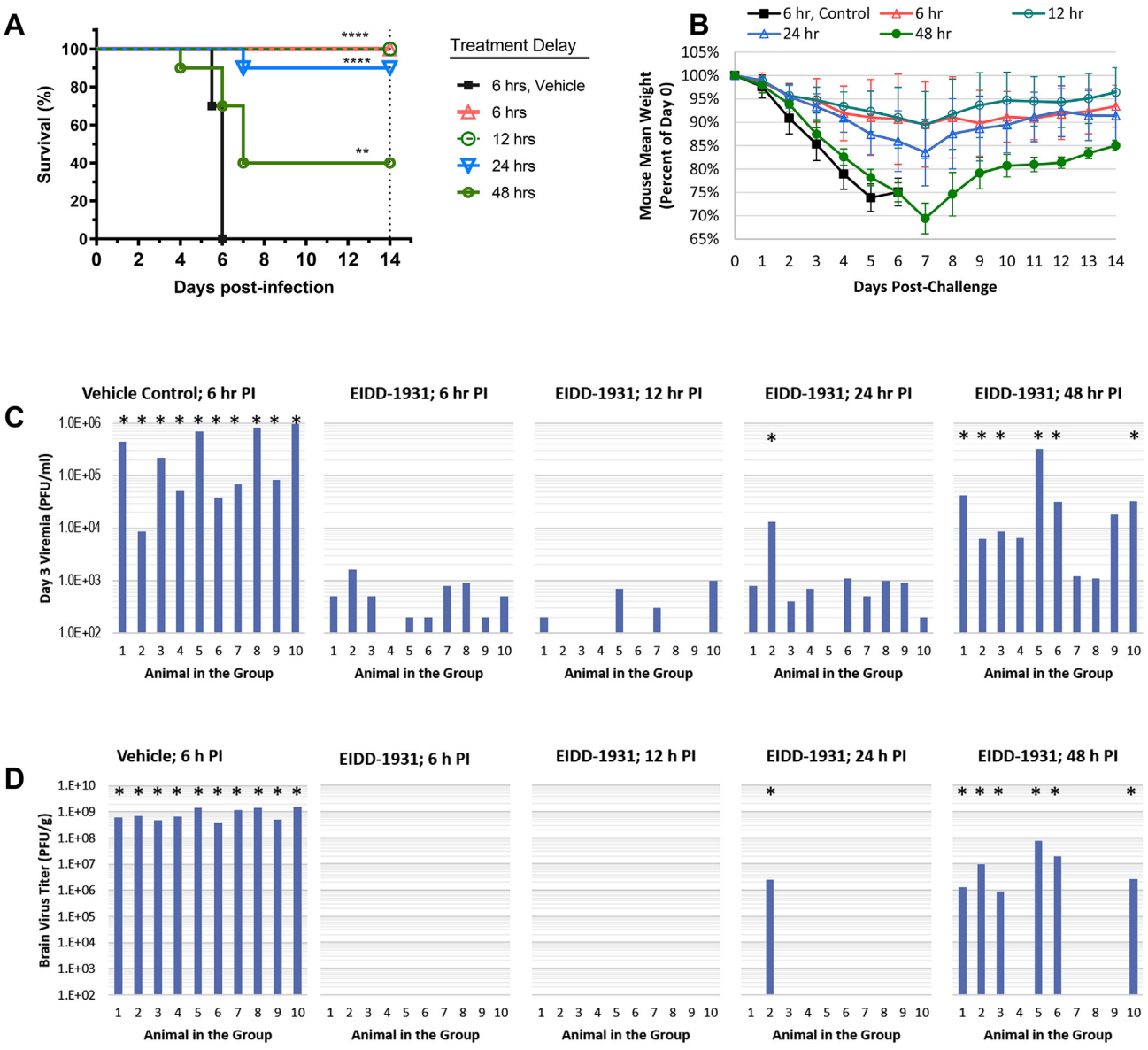


Fig. 4. (A) Effect of time of treatment initiation with EIDD-1931 on animal survival. Mice were challenged via intranasal infection with ~100 LD_{50} of VEEV TrD on day 0. Animals were treated with EIDD-1931 by oral gavage b.i.d. for 6 days starting at indicated time. **p < 0.01; ****p < 0.0001 versus Vehicle Control group; n = 10. (B) Average mouse weight dynamics over 14-day study period. Data shown as Mean ± SD, n = 10. (C) Day 3 viremia in mice. Time of treatment initiation with EIDD-1931 is indicated above each panel. Low limit of quantitation (LLOQ) = 10^2 PFU/ml of plasma. (D) Brain virus titers in individual mice. Brain tissues were collected from animals either on the day of euthanasia/death or on day 14 (end of the study). Low limit of quantitation (LLOQ) = 10^2 PFU/g of tissue. * indicates animals that died or had to be euthanized due to severe disease.

2 h post-dose. Concentration of free ribonucleoside in tissue increases in a dose-dependent but not dose proportional manner. This observation is again in accord with uptake into tissue being modulated by concentrative nucleoside transporters. Because of the solute-impermeant tight junctions between the endothelial cells lining of brain capillaries and the compound's hydrophilic nature, trans- and paracellular routes of entry into the brain generally are not operative for nucleoside analogs (Abbott et al., 2010; Tamai and Tsuji, 2000). Specialized carrier proteins are required to facilitate crossing the blood brain barrier. Uptake of pyrimidines into the brain is mediated by two specialized nucleoside transporters, a low affinity equilibrative system (ENT) that contributes to brain uridine and cytidine uptake when plasma levels of these compounds are elevated, and a high affinity concentrative system (CNT) that contributes principally to uridine uptake. Both of these transport systems have been demonstrated to be involved in the transport of anticancer and antiviral cytidine analog drugs into the brain, e.g. gemcitabine and zalcitabine (Parkinson et al., 2011). Efficient but capacity limited uptake of EIDD-1931 into the brain observed in the mice (as evidenced by a saturable C_{max}) suggests that these transporters likely facilitate uptake of EIDD-1931. Once the nucleoside is in the tissue, micromolar levels of 5′-triphosphate are reached within 4 h in all organs tested with the exception of the brain where T_{max} increases with increasing dose from 3 h at 50 mg/kg to 8 h at 500 mg/kg.

CNS infection is sufficient to explain the majority of clinical signs and symptoms in mice and to account for the uniform lethality of virulent strains of VEEV. Given the aggressive nature of the disease after infection and the exceptionally high level of virus observed in the brains of untreated animals, the decision was made to utilize dosing regimens that could maintain levels of EIDD-1931 in the brain at or above the EC_{90} value determined in cell culture models of VEEV infection (1.04 μM) (Urakova et al., 2017). Based on C_{max} levels of EIDD-1931 determined in the brain after oral dosing and the attendant $t_{1/2}$

values, it was determined that the minimum dose should be 150 mg/kg given b.i.d. The maximum dose of 500 mg/kg b.i.d. was based on the outcome of the 7-day dose-ranging toxicology/toxicokinetic study in which 1000 mg/kg/day was the maximum dose at which no clinical signs/symptoms (such as diminished appetite and weight loss) were observed that could confound the analysis of effectiveness of treatment of disease. The relatively high doses required to achieve the desired levels of EIDD-1931 in the mouse brain may in large part be due to rapid and extensive metabolic extraction in the mouse liver where the N^4–OH group is presumably reduced by the mitochondrial amidoxime reducing component (mARC) to yield cytidine (Krompholz et al., 2012; Ott et al., 2015). The cytidine is subsequently phosphorylated and/or deaminated to yield high levels of cytidine- and uridine-5′-triphosphates. Comparison of metabolism in mouse primary hepatocytes, versus non-human primate and human primary hepatocytes, suggests that rapid catabolism by the liver will not be as robust in monkeys and humans (Supplementary Fig. S7) and that should subsequently result in higher systemic drug levels at lower doses in monkeys and humans.

The results of the prophylactic efficacy study clearly demonstrate that EIDD-1931 is effective in protecting mice from lethal VEEV challenge when treatment is initiated pre-infection. In this study, the three doses chosen to maintain concentration of the ribonucleoside analog at or above the EC_{90} value for activity against VEEV, were 80- to 90% effective in protecting mice from mortality. Results in each of the dose groups were statistically distinguishable from controls (0% survival). Neuroinvasion is a key event in the pathogenesis of VEEV infection (Grieder et al., 1995), and infected untreated animals develop extremely high viral titers in their brain, an event that has been correlated with mortality. Drug treatment initiated 2 h before infection almost completely suppresses virus replication in the animal's brains. Of the three VEEV-related deaths in the study two animals had virus in their brains. A third animal in the low dose group was euthanized on Day 12 with signs of severe disease (as indicated by clinical scores) but with no detectable virus in the brain, leaving open the possibility that the death resulted from another virus associated effect, yet not known. After intranasal infection, virus has been shown to appear first in the olfactory neuroepithelium followed by the blood, presumably due to uptake of virus through fenestrated capillaries in the neuroepithelium (Ryzhikov et al., 1995). In this study, all animals developed viremia including the three animals that died. However, in agreement with multiple reports in the literature, there was no correlation observed between the level of viremia and death (Steele and Twenhafel, 2010). All infected animals exhibited weight loss, although the rate and extent of weight loss in all treatment groups was significantly less than that observed in untreated control. All surviving animals in the two upper dose groups had clinical scores of 0 (no signs of illness) while the animals in the lowest dose group that survived had clinical scores of no worse than −2 (mild but definite illness). These results strongly support the idea that the pharmacodynamic relationship relevant to mortality is in the CNS. Given the aggressive course of disease in the mouse model, an effective drug must be rapidly transported into the brain and, in the case of ribonucleoside analogs, be quickly anabolized to its corresponding 5′-triphosphate. Delays in accumulating adequate drug level in the brain or in its activation may allow the pathology of disease to progress beyond a point that is treatable.

To determine how late antiviral treatment intervention would be beneficial in the context of the pathological progression of VEEV associated disease, the highest dose in the prophylaxis study was carried into a therapeutic study. Initiating treatment up to 12 h post infection was 100% effective, and 90% effective at 24 h post infection. Protection from mortality decreased at 48 h delay with a 40% survival rate. In this study there was complete correlation between virus brain titers and death. Additionally, delay of therapy had a time of treatment associated impact on weight loss and clinical scores. Weight loss became more severe as time of treatment was delayed. Delaying treatment between 6 and 24 h post-infection had no statistically significant impact on survival between treatment groups. In these groups the weight loss was no more than in prophylactic 500 mg/kg group. The rate and extent of weight loss after a 48-h delay of initiation of treatment was almost identical to that of the untreated control group. However, the four surviving animals in the 48-h group began gaining weight and had regained a significant portion of their loss by the termination of the study. Clinical scores paralleled degree of weight loss. Animals in the 6, and 12-h delay of treatment groups and all surviving animals in the 24-h delay of treatment group (9 out of 10) had clinical scores of 0 or -1, displaying no or very mild (questionable) symptoms of disease. Survivors in the 48-h group displayed mild but definite signs of disease (clinical score of −2). This drop off in efficacy is not surprising since by 48 h there is significant virus replication in the brain and evidence of neurodegeneration (Ryzhikov et al., 1995; Vogel et al., 1996).

Currently there are no approved drugs or vaccines for VEEV infection. Given the potential for periodic epizootic/epidemic outbreaks of VEEV infection in the Americas, some of which have involved thousands of people and horses, and the Select Agent status of VEEV owing to its weaponization during the Cold War, there is a critical need for medical countermeasures. EIDD-1931 is an extremely promising development candidate for the prophylaxis and treatment of VEEV infection. In a preliminary dose-ranging study in mice it was well tolerated. It is orally bioavailable and after uptake is rapidly distributed into all organ systems involved in the pathogenesis of VEEV infection, including the CNS. Once distributed into critical organ systems the drug is rapidly anabolized to its corresponding 5′-triphosphate, the active antiviral agent. Rapid anabolism assures a rapid onset of antiviral activity, which is of critical importance in intercepting and arresting the progression of such an aggressive disease. In the mouse model of VEEV infection, treatment with EIDD-1931 results in improved survival and decreased morbidity even when administered as late as 48 h post infection. Of particular consequence is the ability of the drug to arrest viral replication in the brain, which has been correlated with survivability. The efficacy, safety, and pharmacokinetic/distribution profile described here in combination with the previous observation of a high barrier to resistance in VEEV (Urakova et al., 2017) strongly supports the continued development of EIDD-1931 for the prophylaxis and treatment of VEEV infection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104597.

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