DEVELOPMENT AND USE OF AN EMBRYONIC STEM CELL-DERIVED SPINAL MOTONEURON ASSAY FOR THE EVALUATION OF POTENTIAL TREATMENTS AGAINST ALPHAVIRUSES

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TABLE OF CONTENTS

Page

ABSRACT

Venezuelan (VEEV), eastern (EEEV), and western (WEEV) equine encephalitis viruses are positive-stranded RNA viruses transmitted by mosquitos and cause disease in horses and humans. Current methods to evaluate potential therapeutics *in vitro* use immortalized cell lines, but their tumorigenic properties result in genetic and physiologic differences from the primary cells they mimic. Primary cells while physiologically relevant, are difficult to produce in large quantities. Stem cells can be produced in larger quantities than primary cells and are more physiologically relevant than immortalized cell lines. For this work, embryonic stem (ES) cell-derived motoneurons resembling native motor neurons both physiologically and morphologically were utilized to develop a screening method to allow for better down selection of compounds with anti-alphavirus efficacy prior to *in vivo* experiments Several compounds were screened in the VEEV ES cell-derived motoneurons assay and one compound was down selected and evaluated for *in vivo* efficacy.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BoNTs	Botulinum neurotoxins
CC_{50}	half maximal cytotoxic concentration
CNS	central nervous system
CPE	cytopathic effect
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EBME	Eagle's basal medium with Earl's salts
EEEV	eastern equine encephalitis virus
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
ES	embryonic stem
FBS	fetal bovine serum
FDA	Federal Drug Administration
HI	heat inactivated
HRP	horseradish peroxidase
HTS	high-throughput screening
IACUC	Institutional Animal Care and Use Committee
IC_{50}	half maximal inhibitory concentration
LD_{50}	lethal dose 50% or median lethal dose
MEF	mouse embryonic fibroblast
MEM	minimum essential medium

MOI	multiplicity of infection
NBF	10% neutral buffered formalin
PBS	phosphate buffered saline
PFU	plaque forming unit
pK	pharmacokinetics
SC	subcutaneous
VEEV	venezuelan equine encephalitis virus
WEEV	western equine encephalitis virus

INTRODUCTION

Venezuelan (VEEV), eastern (EEEV), and western (WEEV) equine encephalitis viruses are positive-stranded RNA viruses that belong to the *Alphavirus* genus in the family *Togaviridae*. These viruses are transmitted by mosquitos and cause disease in horses and humans. Due to their high infectivity, potential for aerosolization, and lack of medical countermeasures, they are of concern as both biowarfare and bioterrorism agents (Steele & Twenhafel 2010).

The VEEV complex consists of eight closely related viruses. The epizootic strains of IAB and IC subtypes are highly pathogenic for equids and humans, and are responsible for epidemics in the Americas. These subtypes lead to a high incidence of encephalitis along with high rates of morbidity and mortality in horses. VEEV IAB and IC subtype viruses amplify to high viral titers in horses increasing the transmission to mosquitos then to horses and humans thus driving the epizootics. The majority of human epidemics are caused by IAB and IC viruses following outbreaks in horses. No human to human transmission has ever been reported (Steele & Twenhafel 2010). The enzootic strains include Everglades (formerly subtype II), Mucambo (formerly subtype IIIA), Pixuna (formerly subtype IV), Cabassou (formerly subtype V), Rio Negro (formerly subtype VI), Mosso das Pedras (formerly subtype IF) and strains ID and IE. The enzootic strains may cause disease in humans but are not as virulent to equids as the epizootic stains. Enzootic VEEV strains tend to cycle mainly between mosquitos and small mammals. Human infections with these strains tend to be individual cases or small outbreaks in endemic areas (Honnold *et al.* 2007; Steele & Twenhafel 2010).

VEEV infections in humans typically present as an acute illness with symptoms of fever, headache, malaise, myalgia, sore throat, and vomiting. A small percentage of naturally acquired cases show an apparent central nervous system (CNS) infection (0.5% of adults and up to 4% of children). The neurological disease has a range of symptoms and severity from mild confusion to coma. Mortality in neurological cases has been as high as 10% for adults and 35% for children. Long term neurological effects have been documented in survivors. Limited information exists for VEEV infection pathogenesis in humans, but it appears to be consistent with VEEV infection pathogenesis in several animal models. This includes lesions in the brain caused by edema, congestion and mild meningitis (Steele & Twenhafel 2010).

The most well studied animal model for VEEV is the mouse model. The median lethal dose (LD_{50}) is between 1 to 30 plaque forming units (PFU) depending on route of infection and mouse strain and age used (Ludwig *et al.* 2001; Pratt *et al.* 2006; Stephenson *et al*. 1988). Subcutaneous (SC) or footpad inoculations are used in mice to mimic mosquito-transmitted infection. By this route of infection VEEV has been documented to replicate within four hours post-infection and enter the bloodstream by twelve hours post-infection (Grieder *et al.* 1995). Within 36-48 hours post-infection VEEV infection is evident in the brain. VEEV enters the brain primarily by crossing from capillaries in nasal tract to the axons of the olfactory nerves and then passing into the olfactory bulb. By the aerosol route of infection, VEEV appears to directly infect the olfactory nerves in the nasal tract and thus infection reaches the brain in as little as 16 hours. By both routes of infection, VEEV spreads rapidly from the olfactory bulb to the rest of the brain. Neurons are a main target of VEEV infection in both the brain and the spinal cord. Virus antigens

and mature virions have both been detected in neurons. Both necrosis and apoptosis of the neurons have been detected (Steele *et al.* 1998).

EEEV has been isolated in the eastern regions of North and South America from small mammals, birds, horses, and humans. It is most commonly found along the east coast of the United States, but has been isolated as far west as Michigan. Many species of birds are susceptible to infection and are the primary reservoir host. Most of them have prolonged viremia, but remain asymptomatic. EEEV has caused localized outbreaks in horses, pheasants, and humans (Griffin 2007; Honnold *et al.* 2007; Steele & Twenhafel 2010). Other animals known to have been infected include emus, penguins, sheep, dogs, and deer (Griffin 2007). Human infections with Madariaga virus (formerly South American EEEV) which circulates along the north and east coasts of South America and the Amazon River basin are typically mild or subclinical (Griffin 2007; Honnold *et al.* 2007). EEEV strains found in North America are the most virulent of encephalitic alphaviruses and have the highest mortality rates ranging from 36-75% with children being more susceptible. In children, 1 in 8 infections results in encephalitis, but in adults the rate is only 1 in 23 infections (Griffin 2007; Steele & Twenhafel 2010). In humans, most infections are asymptomatic, but neurological signs, lesions, and sequelae are more severe when there is CNS involvement, compared to other arboviral encephalitides. Symptoms of acute illness from EEEV are similar to acute illness caused by VEEV. Neurological symptoms include paralysis, respiratory impairment, and seizures. Encephalitis cases often have meningismus, cranial nerve palsies, hemiparesis, and hyponatremia. Edema of the face and extremities also often occurs. A large number of survivors, 35%-80%, have significant long-term impairment including paralysis and seizures (Griffin 2007; Steele &

Twenhafel 2010). Lesions in the brain of fatal cases have included edema, hemorrhage, malacia, and meningeal congestion. These lesions are often widespread, but the basal nuclei, thalamus, and brainstem are particularly affected. The spinal cord does not typically have lesions. (Honnold *et al.* 2007; Steele & Twenhafel 2010).

WEEV has been isolated in the western regions of North and South America from mammals, wild birds, humans, and horses. Like human cases of VEEV and EEEV, early phase of WEEV infection is a flu-like illness. A significant proportion of cases results in CNS signs which include seizures, coma, somnolence, and motor neuron dysfunction. Like with VEEV and EEEV, children are more susceptible to severe symptoms and higher mortality rates than adults. While the mortality rate depends on several factors including age, the mortality rate for WEEV infection is estimated to be 3%-15%. Much like with EEEV infection, survivors of WEEV infection can have neurological symptoms that persist for months or are even permanent. Also, similar to EEEV cases lesions in the brain are often widespread with the most severely affected areas being the basal nuclei, thalamus, and brainstem. Lesions in the spinal cord have also been documented (Steele & Twenhafel 2010).

There are no FDA-approved therapeutics for the treatment of alphavirus infections. Current methods to evaluate potential therapeutics *in vitro* use immortalized cell lines. The immortalized cell lines commonly used are Vero 76, U87MG, or HeLa cells. While these cells can be cultured for a prolonged period of time, immortalization can result in disruption of protein expression and essential cellular pathways. These disruptions can alter virus infection or host responses to infection. The interferon pathway provides an example of an important pathway in viral pathogenesis that can be deficient in immortalized cells lines

(Desmyter *et. al.* 1968). Vero and baby hamster kidney (BHK-21) cells are examples of cell lines with disruptions in interferon production or the response pathway. VEEV infection has been shown to induce the production of interferon *in vivo* (Gardner *et. al.* 2008; White *et. al.* 2001). Interferon can have a protective effect against VEEV infection providing a slower onset of disease in immuno-competent mice compared to immuno-compromised mice (White *et. al.* 2001). Based on the sensitivity of VEEV to interferon, the disruption of this pathway results in high titer virus yield in these cells; however, use of these cells in screening assays would not result identify compounds exhibiting antiviral effects through this pathway.

For many screening assays, cells are often pretreated with the compound of interest for 2 hours, compound is removed for virus infection after which compound is added back to the cells for a final incubation of 24-48 hours. Some methods evaluate virus production in supernatants by plaque assay which is very labor intensive and limits the number of compounds that can be investigated (Amaya *et al.* 2014; Amaya *et al.* 2015). Other methods compare cell viability in the presence or absence of compound using assays such as Cell Titer Glo (Chung *et al.* 2016; Madsen *et al.* 2014). These methods increase throughput; however, are not virus-specific. Methods that combine both of these use antibodies that bind to viral proteins to monitor virus infection of the cells and microscopy to evaluate cell characteristics simultaneously (Panchal *et al.* 2012).

The downside of all of these methods is that they rely on immortalized cell lines and their tumorigenic properties result in genetic and physiologic differences compared to the primary cell they are meant to mimic. This often leads to promising therapeutics *in vitro* that fall drastically short of expectations *in vivo*. Compound BAY-11-7082, a known

inhibitor of IKKβ, was shown to reduce VEEV TC-83 viral titers by 2 logs compared to the DMSO control on U87MG cells. However, in C3H/HeN mice infected intranasally with 2 x 10^7 PFU/ml VEEV TC-83 and pretreated one day prior with either BAY-11-7082 (10 mg/kg) or DMSO (100%) and then daily for 10 days subcutaneously, there was a trend towards increased survival in the BAY-11-7082 treated mice, but it lacked statistical significance. Serum sampling taken at 3, 7, and 10 days post-infection showed a 3-log reduction in titers in treated mice on day 7, but only slight reductions on days 3 and 10, 33.3% and 20% respectively. Brain samples taken from mice sacrificed at the same time points showed a 1 log reduction on day 3 post-infection only (Amaya *et al.* 2014). Acriflavine (ACF), an Ago2 inhibitor, at a concentration of 2.5 μM, resulted in an approximately 7 log drop in VEEV TC-83 titers compared to DMSO treated U87MG cells. ACF also proved to be effective against VEEV Trinidad reducing titers in Vero cells treated with 2.5 μM ACF by approximately 4 logs compared to a DMSO control. In BALB/c mice administered 5mg/kg ACF orally two hours prior to infection with 1 x 10^5 PFU/ml VEEV Trinidad by aerosol and then treated daily, there was no increased survival or decrease in viral load compared to mice treated with PBS intraperitoneally. C3H/HeN mice treated daily with 10 mg/kg of ACF or PBS by oral gavage and exposed intranasally to a 90% lethal dose of VEEV TC-83 (2 x 10^7 PFU) showed some increase in survival, 60% ACF treated versus 10% PBS treated. However, some of the ACF treated mice succumbed earlier than the PBS treated mice (Madsen *et al.* 2012). Both of these compounds were very promising in immortalized cell lines, but fell short in *in vivo* studies suggesting a more physiologically relevant method is needed for evaluating potential therapeutic compounds

in vitro to reduce these failures. The use of more physiologically natural cell types may provide greater rates of hit to lead therapeutic transition.

Primary cells while physiologically relevant can be difficult to produce in large quantities. One compromise is to use stem cells which can be produced in larger quantities than primary cells and are more physiologically relevant than immortalized cell lines. Embryonic stem (ES) cell-derived spinal motoneurons have been used to evaluate the efficacy of therapeutics against Botulinum neurotoxins (BoNTs), which like alphaviruses target neurons in the brain and spinal cord. ES cell-derived spinal motoneurons resemble native motor neurons both physiologically and morphologically, to include expression of motoneuron-specific markers and formation of cell processes, making them very useful in the study of BoNTs (Kiris *et al.* 2011; Kiris *et al.* 2014). These factors should make these cells ideal for use in evaluating therapeutics against alphavirus infection. However, mouse ES-derived spinal motoneurons, while more physiologically relevant, are still more difficult to obtain and grow and thus are not ideal for HTS screening. To overcome the issues of both immortalized cell lines and mouse ES-derived spinal motoneurons, a semi-HTS assay was used to first screen therapeutics for efficacy against alphaviruses. In this thesis research, I developed an assay using mouse ES-derived spinal motoneurons to screen compounds with demonstrated efficacy in the semi-HTS assay. The ability to screen therapeutics in a mouse ES-derived spinal motoneurons based assay should allow for better assessment of potential therapeutics for *in vivo* studies.

MATERIALS AND METHODS

Cell-based ELISA HTS Assay

Vero 76 cells (CRL-1587, ATCC) were seeded at 4 x 10^4 cells per well in 100 µl of EMEM + 10% Heat Inactivated (HI) FBS, 1% L-Glutamine, and 1% penicillin/streptomycin and placed in an incubator at 37ºC with 5% CO_2. The next day media was removed and compounds were added at 2X concentration in 100 µl in duplicate or triplicate on 2 plates. One plate was to evaluate efficacy of the compound in the presence of virus infection and the other plate was to evaluate compound toxicity by cell viability assay. For the cell viability assay, 100 µl of media was added for mock infection and then plates incubated at 37ºC and 5% CO_2 for 24 hours ± 2 hours. Plates for infection were moved to BSL3 for virus infections. Plates were infected at a multiplicity of infection (MOI) of 0.01 with VEEV, EEEV, or WEEV in 100 µl in all wells except column 1. Column 1 served as background and 100µl of media was added. Following infection, plates were placed in an incubator at 37ºC and 5% CO_2. After 24 hours ± 2 hours, media was removed from the plates and the cells fixed using 10% neutral buffered formalin (NBF) at 4ºC for a minimum of 24 hours.

Cell Viability Assay

Following incubation at 37ºC and 5% CO_2 for 24 hours ± 2 hours of the cell viability plates, the Cell Titer-Glo Luminescent Cell Viability Kit (Promega, Madison, WI) was used to determine the number of viable cells, following the manufacturer's protocol. Briefly, all reagents were equilibrated to room temperature, the Cell Titer-Glo buffer was

added to the Cell Titer-Glo substrate to make the Cell Titer-Glo reagent mixture. Media/compounds were removed from the plates, then Cell Titer-Glo reagent mixture (100 µl) and EMEM (100 µl) were added to each well. Plates were covered with foil and gently mixed using an orbital shaker for 2 minutes at room temperature and then incubated for 10 minutes at room temperature to stabilize the luminescence signal. Luminescence was measured using a SpectraMax M5 (Molecular Devices). Determination of cell viability was made by first subtracting background and calculating percent inhibition of the signal relative to no compound control cells on the plate.

Cell-based ELISA

Following fixation, formalin was removed and plates were washed with phosphate buffered saline (PBS) at least 3 times and then incubated with 100µl blocking buffer (3% BSA) for 1 hour at room temperature. Blocking buffer was removed following the 1 hour incubation. Virus-specific anti-mouse monoclonal antibodies (Table 1) were prepared in blocking buffer at indicated dilution, and 65 µl of diluted antibody was added to each well. Plates were incubated at room temperature for 2 hours. Plates were washed 3 times with PBS. Following washing, horseradish peroxidase (HRP) goat anti-mouse secondary antibody (KPL) was added to each well at dilution listed in Table 1 (65 µl/well) and plates were incubated for 1 hour at room temperature. The plates were washed 3 times with PBS, subsequently 100 µl of SuperSignal ELISA Pico Chemiluminescent Substrate (1:1 mixture of luminol/enhancer and the stable peroxidase buffer solution; Pierce Biotechnology, Rockford, IL) was added to each well. Plates were examined for luminescence following

a 5-minute incubation at all wavelengths using a SpectraMax M5 or Gemini EM (Molecular Devices).

Table 1: Cell-based ELISA Parameters for Screening Assay

Virus	**MOI**	**Ab**	**Primary**	**Secondary**
VEEV	0.01	1A4A	1:4000	1:10000
EEEV	0.01	6G8-1-2	1:4000	1:4000
WEEV	0.01	9F12-1-1	1:4000	1:4000

Analysis

Analysis of cell viability and viral inhibition were performed as described previously (Johansen *et.al.* 2013). Briefly, raw phenotype measurements **T** from each treated well were converted to normalized fractional inhibition **I** = **1-T/V** relative to the median V of vehicle-treated wells arranged around the plate. After normalization, median activity values were calculated between replicate measurements at the same treatment doses along with σ_1, the accompanying standard error estimates. Drug response curves were represented by a logistic sigmoidal function with a maximal effect level (A_{max}), the concentration at half-maximal activity of the compound (EC_{50}), and a Hill coefficient representing the sigmoidal transition. All curve fits were through the 0 concentration. These fitted curve parameters were used to calculate the concentration (IC_{50}) at which the antiviral drug response reached an absolute inhibition of 50%, limited to the maximum tested concentration for inactive compounds.

Directed Differentiation of Mouse Embryonic Stem Cells into Spinal Motoneurons

Mouse Embryonic Stem (ES) cells differentiated into spinal motoneurons were prepared by Dr. Erkan Kiris as previously described and provided for testing as cells were available (Kiris *et al.* 2011). Briefly, pluripotent HBG3 cells were maintained on mitomycin-inactivated primary mouse embryonic fibroblast (MEF) cells in DMEM for ES cells (Chemicon, Billerica, MA) with 15% ES-cell tested FBS (Hycole, Waltham, MA), 1% Nucleosides (Chemicon), 1000 Unit/ml Leukemia Inhibitory Factor (LIF) (Millipore, Billerica, MA), β-mercaptoethanol (final 0.1 mM), 1% penicillin/streptomycin, 1% Glutamax, and 1% nonessential amino acids. ES cells were trypsinized and suspended in 1:1 Advanced DMEM/F12 plus Neurobasal medium supplemented with β-mercaptoethanol (final 0.1 mM), 1% penicillin/streptomycin, 1% Glutamax, and 15% Knockout serum replacer (differentiation medium). Removal of MEFs was achieved by plating the single cell mixtures onto tissue culture-treated plates for 30 minutes. MEFs adhered to the plates more tightly than the ES cells which allowed for the ES cells to be collected in the supernatant. The ES cells were counted using a Cellometer AutoT4 (Nexcelom Biosciences, Lawrence, MA), and seeded at a density of 500 cells per well onto AggreWell 400 (Stem Cell Technologies, Vancouver, Canada) plates to form embryoid bodies (EBs). The next day (Day 1) EBs were harvested, placed in fresh differentiation medium, and maintained in suspension for another 24 hours. To induce neutralization and caudalization, retinoic acid (1μM, Sigma, St. Louis, MO) was added on Day 2. On Day 3, Sonic Hedgehog protein (100 ng/ml, R&D Systems, Minneapolis, MN) was added to the cultures induce motoneuron specification and on Day 4 purmorphamine (Calbiochem, Gibbstown, NJ) was added to the cultures at a concentration of 1 μM. The next day (Day

5) EBs were transferred to fresh medium comprised of 1:1 1:1 Advanced DMEM/F12 plus Neurobasal medium supplemented with 1% penicillin/streptomycin, 1% Glutamax, 2% B27 serum-free supplement, brain-derived neurotrophic factors (10 ng/ml, Chemicon), Glial cell-derived neurotrophic factors (100 ng/ml, R&D Systems), Cliary-derived neurotrophic factors (10 ng/ml, Chemicon), and Neurotrophin 3 (10 ng/ml, Chemicon). The EBs were dissociated on Day 7 by using a paspain dissociation system (Worthington, Lakewood, NJ) and plated onto Matrigel coated dishes (BD Biosciences, San Jose, CA) for an additional 3 days to allow for neurite elongation. Unless otherwise stated above all reagents were obtained from Invitrogen (Carlsbad, CA).

Infection of Mouse ES Differentiated Cells

Confluent cultures of mouse ES-derived spinal motoneurons in 24 well plates were infected with virus diluted to the desired concentration in Neurobasal Media (Gibco). Media was removed from plates and cells infected with 0.5 ml of diluted virus. All mock-infected wells received 0.5 ml of Neurobasal Media only (if relevant to assay). Cells were incubated at 37ºC and 5% CO_2 for one hour, with rocking approximately every 15 minutes. Following incubation, media was removed from the wells and the cells washed twice with sterile PBS (Gibco). Neurobasal Media containing test compounds or controls (0.5 ml) was added to each well in a minimum of duplicate wells. Bafilomycin A1 at a concentration of 1 µM was used as a positive control. Infection proceeded for 24 hours at 37ºC and 5% CO_2. Following incubation, cells were observed microscopically for signs of cytopathic effect (CPE). Cell supernatants were collected from each well and stored at -80ºC for further analysis.

Plaque Assay

Confluent cultures (85-100% confluency) of ATCC Vero 76 cells in 6 well plates were used. Supernatant samples collected from mouse ES-derived spinal motoneurons infections (samples) were thawed at ambient temperature. Samples were diluted by performing a series of 1:10 dilutions in MEM + 5% Heat Inactivated (HI) FBS + 2% Penicillin, Streptomycin + 1% 1M HEPES (MEM Complete). Most experiments had 6-9 dilutions. Media was removed from plates and cells were infected with 100 µl of diluted sample in duplicate. Cells were incubated at 37ºC and 5% CO_2 for one hour with rocking approximately every 15 minutes. Following incubation, media-agarose overlay (2ml of a 1:1 mixture of 1.2% agarose and 2X EBME + 10% HI FBS + 2% Penicillin, Streptomycin (2X EBME Complete)) were added to each well. Once overlay was solidified, plates were placed in incubator at 37ºC and 5% CO_2 for 24 hours ± 4 hours. Following incubation, second media agarose overlay containing 5% neutral red in a 1:1 mixture of 1.2% agarose and 2X EBME complete was added to each well. Once overlay solidified, plates were placed in incubator at 37ºC and 5% CO_2 for 24 hours ± 4 hours. Following incubation PFU were counted and the virus yield (PFU/ml) for each sample calculated.

Analysis:

The average virus yield was determined as an average PFU/ml for each replicate from the mouse ES-derived spinal motoneurons infection. The average virus yield for each compound, was compared to the average virus yield of the negative control (DMSO) to determine the efficacy as a reduction in virus yield. Positive control wells were included

to evaluate assay performance and reproducibility. Standard deviation was determined and plotted. For optimization assays viral titers at 24- and 48-hour time points as well as viral titers for virus only and Bafilomycin A1 treated controls were analyzed for statistically significant differences using a one-tailed t-test. For evaluation of compounds in the optimized VEEV ES-derived spinal motoneuron assay viral titers from compounds were compared to DMSO control for statistical significance using a one-tailed t-test.

In Vivo Efficacy Evaluation

Tolerability Study

The best lead compound was advanced for efficacy evaluation in the mouse model. All animal studies were completed under an USAMRIID approved Institutional Animal Care and Use Committee (IACUC) protocol. A tolerability study was conducted in BALB/c mice. The doses for the tolerability evaluation were chosen based on the *in vitro* data and a pK study conducted by a contract research organization. Mice given a single dose of Cmpd 6 at 10 mg/kg showed no clinical signs and the half-life ($t_{1/2}$) was about 12 hours determined from plasma sampling. For the tolerability study three groups of five mice each were administered 10 mg/kg, 5 mg/kg, or 2.5 mg/kg of Cmpd 6 in a volume of 200 µl by intraperitoneal injection once daily for ten days. A fourth group was administered 200 µl of PBS (diluent) by intraperitoneal injection once daily for ten days. Mice were observed daily for clinical signs of toxicity from the compound to include ruffled fur, slowed activity, hunched posture, and group weight loss. Group body weight was compared to that of the diluent group for signs of increased weight loss due to toxicity of the compound.

Efficacy of Compound 6 in VEEV Mouse Models

To test the efficacy of Cmpd 6 in infected mice, two studies were conducted. One study was in the BALB/c mouse model infected with VEEV INH-9813 (subtype IC strain) and the other study was in the C3H-HeN mouse model using VEEV TC-83 vaccine strain virus (attenuated subtype IAB strain). For either model, mice were divided into four groups of ten mice each. Two groups were administered 2.5 mg/kg or 1.25 mg/kg of Cmpd 6 in a volume of 100 µl by intraperitoneal injection two hours prior to infection and then daily for nine more days. A third group was administered 100 µl of PBS (diluent) by intraperitoneal injection two hours prior to infection and then daily for nine more days. The remaining group was not treated. BALB/c mice were infected subcutaneously with 8.0×10^3 PFU of VEEV INH-9813. C3H-HeN mice were infected by intranasal route with 8.0×10^7 PFU of VEEV TC-83. Mice were observed and weighed once daily until signs of infection were present and then observations were increased to a minimum of twice daily until signs of infection were no longer present and then were reduced back to once daily. Moribund mice were humanely euthanized. Surviving mice were evaluated for 22 days post-infection and then humanely euthanized. Efficacy was determined by survival, extension of mean survival time, and overall health post-infection.

RESULTS

Cell-based ELISA HTS Assay

Screening of a library of over 2000 FDA-approved, former US approved, and previously existing drugs were screened against VEEV was completed previously. From an initial 3-point dose screen, 69 compounds were identified as hits. A hit was defined as inhibiting viral inhibition >50% at any concentration tested with corresponding anti-proliferation <30%. These 69 compounds were then further tested against VEEV in an 8-point dose range and were also screened against EEEV and WEEV to identify pan-alphavirus compounds. Thirty-five compounds were identified as being the most effective based on IC_{50} and CC_{50} values as well as effectiveness against different alphaviruses (Table 2).

Forty-eight compounds tested against VEEV were confirmed for activity in a secondary cell line, U87MG (human glioblastoma cells line). While this cell line is considered a more relevant cell type, it is still an immortalized cell line and has differences from the glial cells it is intended to mimic. Four compounds were exclusively active in Vero cells and 13 compounds were only active in U87MG cells.

Table 2: Compounds with Antiviral Efficacy Against Alphaviruses Identified In Cell-based ELISA HTS Assay

	U87 cells		Vero cells					
Drug	**VEEV-IC50**	**CC50**	**VEEV-IC50**	**EEEV-IC50**	**WEEV-IC50**	**CC50**	**Max [ ] tested**	**Min [ ] tested**
Cmpd A	12.400	ND	29.300	25.600	24.200	37.100	39.9	0.62
Cmpd B	0.050	ND	0.044	0.052	0.053	ND	3.97	0.06
Cmpd C	0.646	ND	1.530	1.850	1.650	6.030	7.97	0.06
Cmpd D	6.750	ND	19.200	6.880	4.530	ND	39.9	0.62
Bafilomycin A1	0.654	ND	1.040	4.830	4.230	ND	12	0.19
Cmpd E	<MCT	0.017	<MCT	<MCT	<MCT	ND	7.95	0.004
Cmpd F	4.210	7.010	15.300	13.000	8.440	17.200	39.9	0.62
Cmpd G	<MCT	0.006	0.005	0.003	0.002	ND	3.98	0.002
Cmpd H	<MCT	ND	<MCT	<MCT	<MCT	ND	3.97	0.06
Cmpd I	0.211	0.640	<MCT	<MCT	<MCT	ND	7.98	0.13
Cmpd J	ND	ND	0.745	0.666	0.465	ND	7.98	0.13
Cmpd K	0.125	0.456	0.251	0.331	0.199	ND	3.99	0.03
Cmpd L	ND	ND	0.483	0.441	0.239	ND	3.98	0.03
Cmpd M	ND	ND	5.610	4.420	4.000	ND	19.9	0.31
Cmpd N	16.200	ND	14.300	13.000	8.200	ND	39.9	0.62
Cmpd 6	0.064	ND	0.133	0.096	0.097	ND	3.97	0.06
Cmpd O	<MCT	ND	2.990	2.970	1.390	ND	27.9	0.44
Cmpd P	<MCT	ND	0.151	0.089	0.124	ND	3.97	0.06
Cmpd Q	0.275	ND	0.464	0.296	0.387	ND	14	0.22
Cmpd R	5.120	ND	24.000	11.600	8.220	ND	39.9	0.62
Cmpd S	0.434	ND	1.860	1.600	1.800	ND	19.9	0.31
Cmpd T	3.380	15.500	9.460	7.210	7.570	13.100	19.9	0.31
Cmpd U	ND	ND	0.246	0.198	0.144	ND	3.97	0.06
Cmpd V	6.810	ND	15.100	11.800	11.800	ND	19.9	0.31
Cmpd W	6.820	30.400	21.700	23.700	29.100	ND	39.9	0.62
Cmpd X	<MCT	0.210	<MCT	<MCT	<MCT	ND	3.97	0.06
Cmpd Y	14.100	60.900	41.300	30.300	44.900	68.400	119	0.94
Cmpd Z	<MCT	0.006	<MCT	<MCT	<MCT	ND	3.98	0.002
Cmpd AA	<MCT	ND	1.670	1.300	1.410	ND	39.9	0.62
Cmpd AB	0.725	ND	2.420	1.270	1.870	ND	3.98	0.03
Cmpd AC	3.280	12.600	7.810	5.190	6.120	ND	19.9	0.31
Cmpd AD	7.390	ND	12.900	13.800	12.100	ND	19.9	0.31
Cmpd AE	ND	ND	26.200	17.100	25.600	28.600	39.9	0.62
Cmpd AF	0.086	0.297	<MCT	<MCT	<MCT	ND	3.97	0.06
Cmpd AG	13.700	ND	32.800	23.800	26.900	ND	39.9	0.62

All concentrations are in µM; [ ], concentration; ND, not determined; <MCT, less than minimum concentration tested

Development of Mouse ES-Derived Spinal Motoneurons Screening Assays

VEEV Infection of Mouse ES-Derived Spinal Motoneurons Optimization

For the development and optimization of the VEEV screening assay, several multiplicities of infection (MOIs) and incubation times post-infection were evaluated. Mouse ES-derived spinal motoneurons were infected at MOIs of 0.1, 1, and 5. Supernatant samples were harvested at 24- and 48-hours post-infection for determination of virus yield by plaque assay (Figure 1). The average virus yield in the virus only controls ranged from 1.22×10^7 PFU/ml to 8.38×10^7 PFU/ml. The average virus yield in the Bafilomycin A1 controls varied more from 7.3×10^3 PFU/ml to 3.45×10^6 PFU/ml. The only MOI that had a statistically significant difference between the virus only and Bafilomycin A1 controls was an MOI of 1 and this was at both the 24 hour (one tail t-test, p value = 0.001) and 48 hour (one tail t-test, p value = 0.008) time points. Additionally, when comparing the same MOI at 24 and 48 hours, only the virus only controls at an MOI of 1 had a statistically significant difference (one tail t-test, p value = 0.004). Based on these results, the parameters of this assay for compound testing utilized VEEV infection at a MOI of 1 and collection of supernatants at 24 hours post-infection.

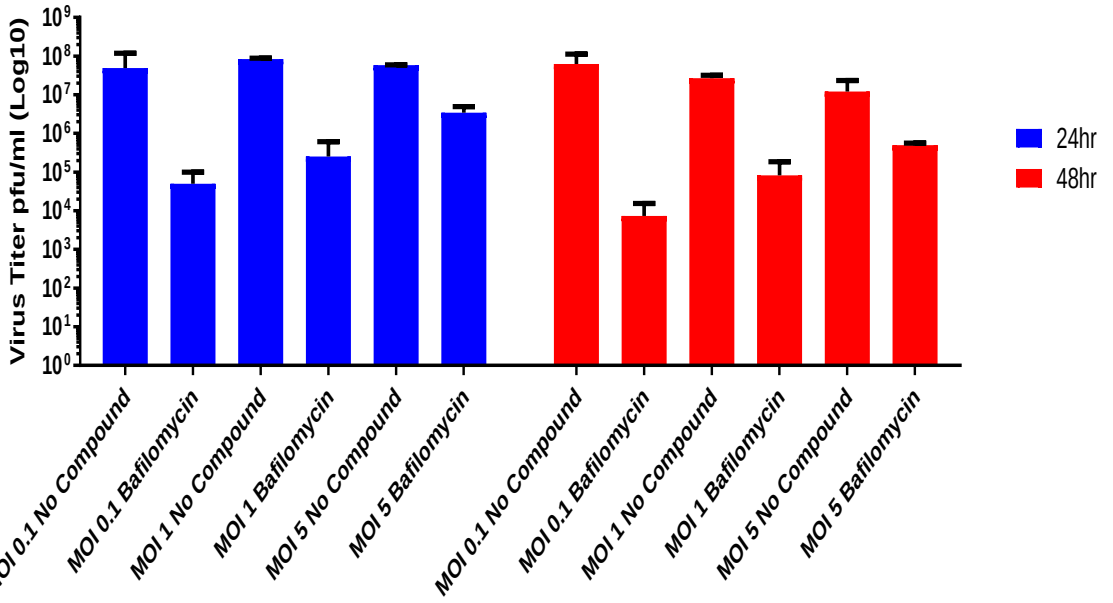


Figure 1: Optimization of VEEV Infection of mouse ES-derived spinal motoneurons. Mouse ES-derived motoneurons were infected with VEEV at MOIs of 0.1, 1, and 5. Supernatants were collected at 24 (blue) and 48 (red) hours post-infection. Virus yield (pfu/mL) in the supernatant samples was determined by plaque assay on Vero 76 cells. The optimal assay conditions were determined to be a time point of 24 hours and a MOI of 1. Data represents an n=2 with the errors representing standard deviation.

EEEV Infection of Mouse ES-Derived Spinal Motoneurons Optimization

For the development and optimization of the EEEV screening assay, several MOIs and incubation times post-infection were evaluated. Mouse ES-derived spinal motoneurons were infected at MOIs of 0.1, 1, and 5. Supernatant samples were harvested at 24- and 48-hours post-infection for determination of virus yield by plaque assay (Figure 2). The virus yield for all three MOIs was about a log higher at 24 hours (1.28×10^8 PFU/ml to 2.10×10^8 PFU/ml) compared to 48 hours (2.45×10^7 PFU/ml to 4.03×10^7 PFU/ml) in the virus only controls which was statistically significant (one tail t-test, p value < 0.05). The same was true for the Bafilomycin A1 controls which ranged from 1.45×10^6 PFU/ml to 7.15×10^6 PFU/ml at 24 hours and 2.73×10^5 PFU/ml to 1.08×10^6 PFU/ml at 48 hours. However, for the Bafilomycin A1 controls only an MOI of 0.1 (one tail t-test, p value = 0.009) and an MOI of 1 (one tail t-test, p value = 0.03) were statistically significant. At 24 hours post-infection had statistically significant difference between the virus only controls and Bafilomycin A1 controls (one tail t-test, p value < 0.05). At 48 hours there was only a statistically significant difference between the virus only control and Bafilomycin A1 controls at a MOI of 0.1 (one tail t-test, p value =0.004) and an MOI of 5 (one tail t-test, p value = 0.006). Based on these results a time point of 24 hours was chosen since the higher titer was statistically significant and would allow for a larger range of reduction in titer from compounds which could differentiate a highly effective compound from a moderately effective compound better allowing for more effective down selection prior to in vivo studies. Since all MOIs at 24 hours were statistically significant an MOI of 1 was chosen to remain consistent with the VEEV assay.

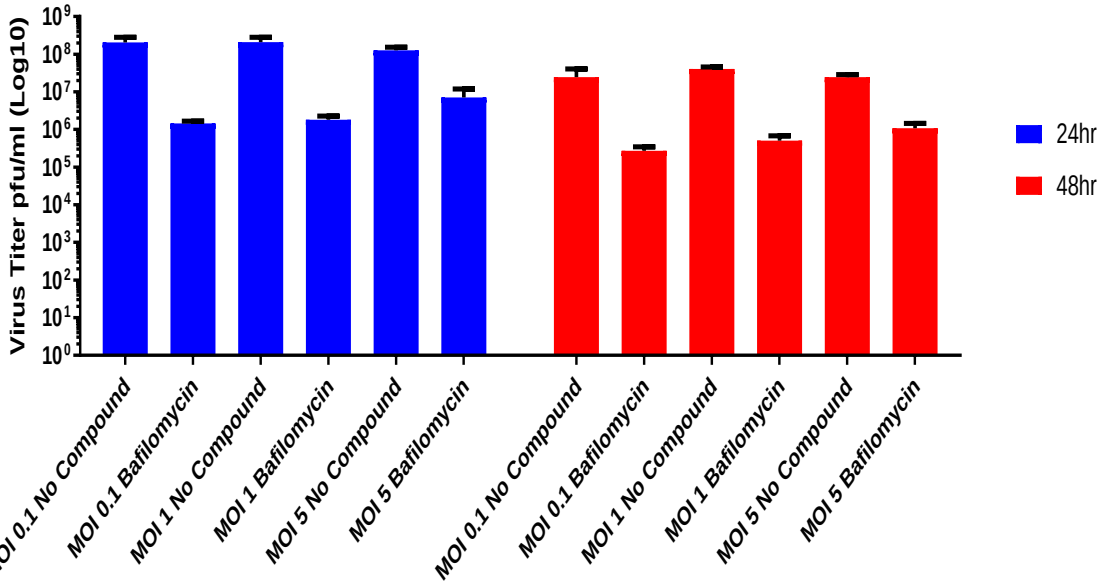


Figure 2: Optimization of EEEV Infection of mouse ES-derived spinal motoneurons. Mouse ES-derived motoneurons were infected with VEEV at MOIs of 0.1, 1, and 5. Supernatants were collected at 24 (blue) and 48 (red) hours post-infection. Virus yield (pfu/mL) in the supernatant samples was determined by plaque assay on Vero 76 cells. The optimal assay conditions were determined to be a time point of 24 hours and a MOI of 1. Data represents an n=2 with the errors representing standard deviation.

WEEV Infection of Mouse ES-Derived Spinal Motoneurons Optimization

For the development and optimization of the WEEV screening assay, several MOIs and incubation times post-infection were evaluated. Mouse ES-derived spinal motoneurons were infected at MOIs of 0.1, 1, and 5. Supernatant samples were harvested at 24- and 48-hours post-infection for determination of virus yield by plaque assay (Figure 3). There was no statistically significant difference between the virus only controls and Bafilomycin A1 controls except at 48 hours post-infection and an MOI of 1. No virus was detected in the Bafilomycin A1 treated wells following infection at a MOI of 0.1 collected at 48 hours post-infection. This was likely due to an experimental error since Bafilomycin A1 was not very effective in reducing virus yield in the 24 hour post-infection samples or any of the other 48 hour post-infection samples. However, this assay was unable to be repeated or further optimized, because we were unable to obtain additional cells from our collaborator. There were statistically significant differences between virus only control samples at 24- and 48-hours post-infection at a MOI of 1 (one tail t-test, p value = 0.002) and a MOI of 5 (one tail t-test, p value = 0.01). Additionally, there were statistically significant differences between Bafilomycin A1 control samples collected at 24- and 48-hours post-infection at a MOI of 0.1 (one tail t-test, p value = 0.001) and a MOI of 1 (one tail t-test, p value = 0.0004). Though not completely conclusive, it seems that Bafilomycin A1 was more effective in reducing VEEV and EEEV virus yields, but it was not as effective against WEEV in this assay. Based on these results, the parameters of this assay for compound testing against WEEV appear to be a MOI of 1 and collection of supernatants at 24 hours post-infection. However, further confirmation would be needed before moving forward with the WEEV screening assay.

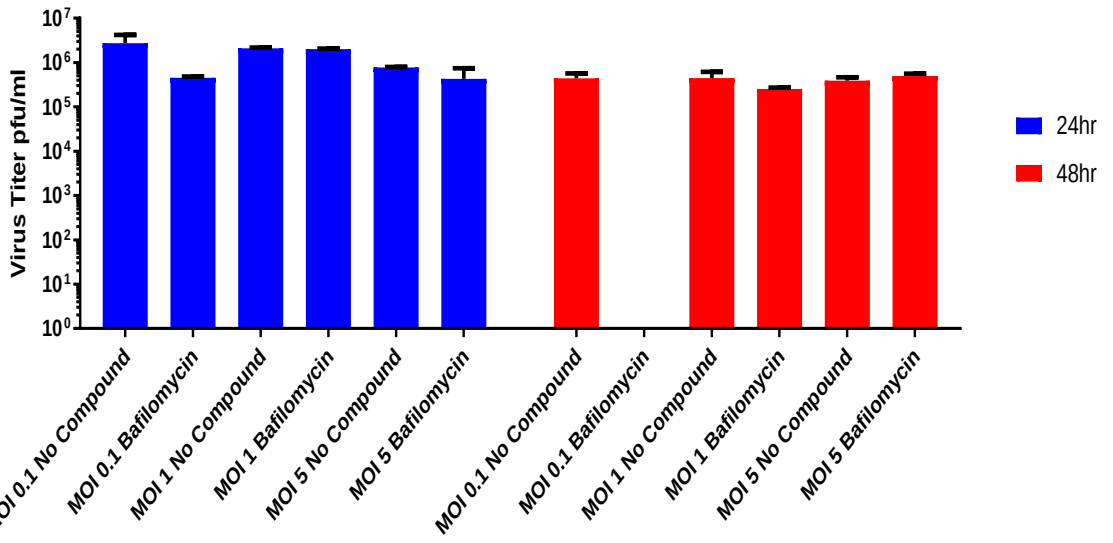


Figure 3: Optimization of WEEV Infection of mouse ES-derived spinal motoneurons. Mouse ES-derived motoneurons were infected with VEEV at MOIs of 0.1, 1, and 5. Supernatants were collected at 24 (blue) and 48 (red) hours post-infection. Virus yield (pfu/mL) in the supernatant samples was determined by plaque assay on Vero 76 cells. The optimal assay conditions were determined to likely be a time point of 24 hours and a MOI of 1 but further confirmation is needed. Data represents an n=2 with the errors representing standard deviation.

Compound Testing in the VEEV Mouse ES-Derived Spinal Motoneurons Assay

A subset of compounds were selected for testing in this assay based on results from the HTS. Several additional compounds were evaluated directly in this assay, because based on their hypothesized mechanism of action they would only be effective in a more native or primary cell line. Twenty-three compounds (Cmpd 1-23) were tested at a concentration of 50 µM in the optimized VEEV assay (Figure 4). Mouse ES-derived spinal motoneurons were infected with VEEV at a MOI of 1. Following infection, media was replaced with fresh media containing compounds of interest at a concentration of 50 µM. Supernatants were collected at 24 hours post-infection and virus yield was determined by plaque assay. Samples were evaluated in duplicate and the average virus yield and deviation was calculated. Virus infection in the presence of ten compounds resulted in significant reductions in virus yield compared to the Negative Control (DMSO) (one tail t-test, p value < 0.05, denoted by asterisks in Figure 4). The presence of six of these compounds resulted in no detectable virus produced. Cmpd 22 and Cmpd 23 were only able to be tested in a single replicate due to a limited number of cells and therefore were not included in the statistical analysis. However, treatment of virus infected cells with Cmpd 22 resulted in an over 2 log reduction in virus yield compared to the Negative Control (DMSO). Cmpd 23 treated VEEV infected cells generated no detectable virus at concentration of 50 µM. True cytotoxicity evaluations were not completed in these cells due to the limited availability of cell numbers. These compounds were known to have low cytotoxicity in other cell lines as determined by our laboratory or our collaborators. However, cell monolayers were monitored microscopically for integrity. Based on these observations, it was noted that those wells with lower virus yield had a higher percentage

of the monolayer intact. Cmpds 2-7, Cmpd 13, Cmpd 16, Cmpd 17, Cmpd 21, Cmpd 23, and the Bafilomycin control had more attached cells than the other compounds and DMSO control. While not an absolute determination, this observation suggested these cells were not highly toxic at 50 µM. From this testing, seven compounds were identified which reduced virus yields to below detectable levels at a concentration of 50 µM.

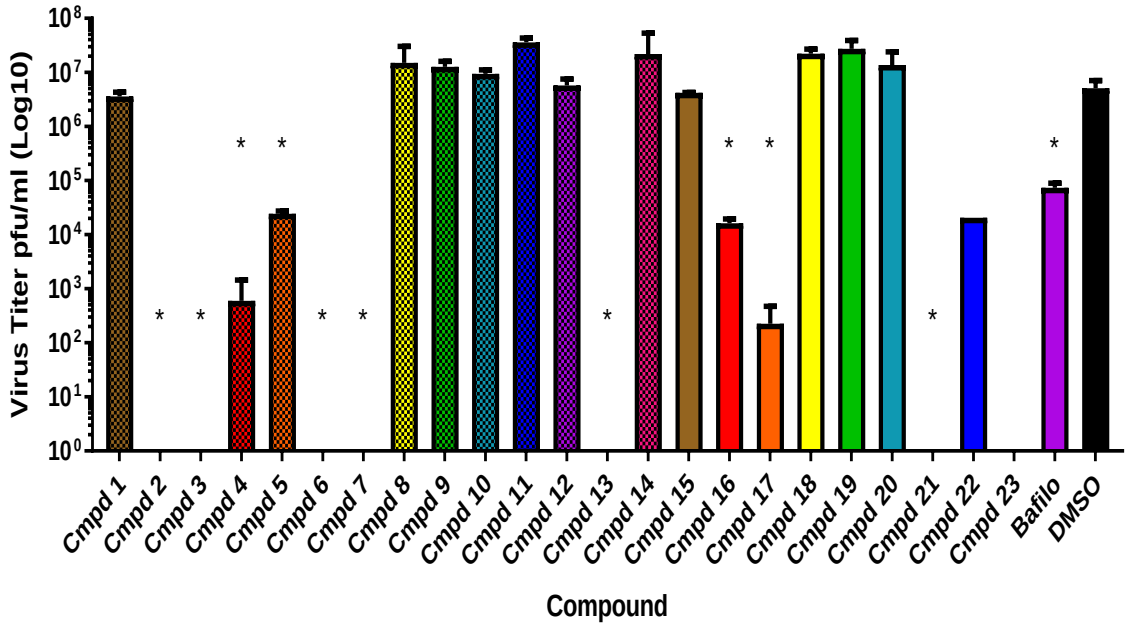
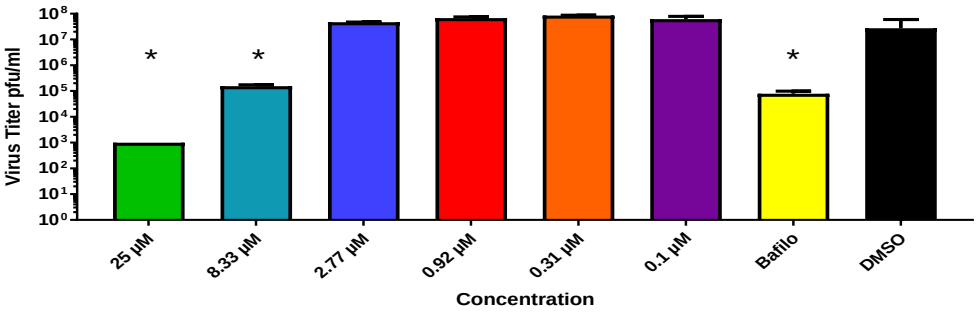


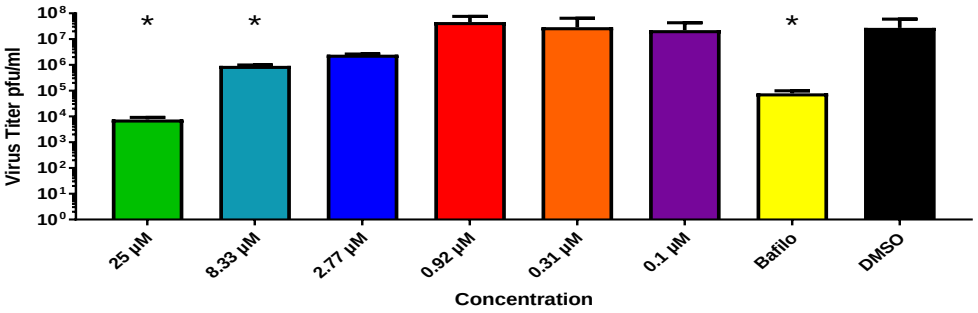
Figure 4: Efficacy of compounds against VEEV in the mouse ES-derived motoneuron Assay. Cells were infected with VEEV for 1 hour after which compounds were added to the media at a concentration of 50 µM. Supernatants were harvested at 24 hours post-infection and virus yield (pfu/mL) was determined by plaque assay. Bafilomycin A1 was included as a positive control and DMSO was included as the virus only control. Data represents an n=2 with the errors representing standard deviation except for Cmpd 22 and Cmpd 23 which were only an n=1. Asterisks represent a significant reduction in virus yield compared to the Negative Control (DMSO) (one tail t-test, p value < 0.05).

These seven compounds (Cmpd 2, Cmpd 3, Cmpd 6, Cmpd 7, Cmpd 13, Cmpd 21, and Cmpd 23) were further evaluated at concentrations of 25 µM, 8.33 µM, 2.77 µM, 0.92 µM, 0.31 µM, and 0.1 µM. While the compounds were all tested at the same time, there were two different sets of mouse ES-derived spinal motoneurons cells provided. Due to potential differences, controls were included for each set and the dilutions for Cmpd 7 were split across the two cell sets. The presence of Cmpd 2, Cmpd 3, Cmpd 7, and Cmpd 13 resulted in significant virus yield reductions at concentrations of 25 µM and 8.33 µM (one tail t-test, p value < 0.05) (Figure 5). The presence of Cmpd 21 and Cmpd 23 both resulted in significant reductions in virus yield at concentrations of 25 µM, 8.33 µM, and 2.77 µM (one tail t-test, p value < 0.05) (Figure 6). Cmpd 6 was the most promising compound generating significant virus reductions at concentrations of 25 µM, 8.33 µM, 2.77 µM, and 0.92 µM (one tail t-test, p value < 0.05) (Figure 7). As noted in the previous assay, those wells with lower virus yields had a greater number of intact, attached cells suggesting the virus yield reduction was not due to the toxicity of the compound. Based on these results, Cmpd 6 was chosen to be advanced for *in vivo* evaluation.

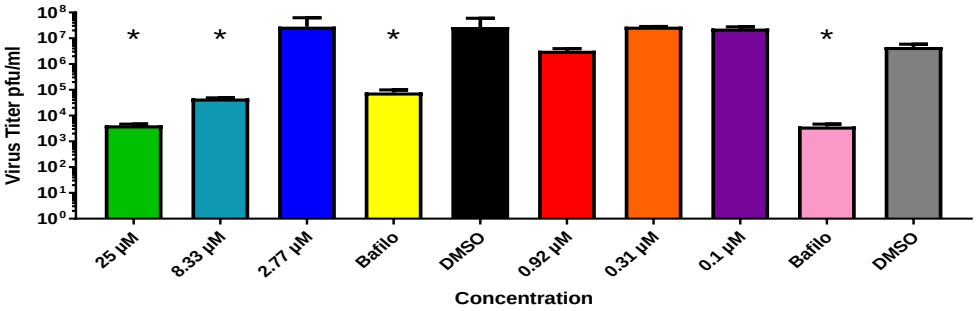
A


Virus Titer pfu/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
*
25 µM
8.33 µM
2.77 µM
0.92 µM
0.31 µM
0.1 µM
Bafilo
DMSO
Concentration

B


Virus Titer pfu/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
*
25 µM
8.33 µM
2.77 µM
0.92 µM
0.31 µM
0.1 µM
Bafilo
DMSO
Concentration

C


Virus Titer pfu/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
*
*
25 µM
8.33 µM
2.77 µM
Bafilo
DMSO
0.92 µM
0.31 µM
0.1 µM
Bafilo
DMSO
Concentration

D

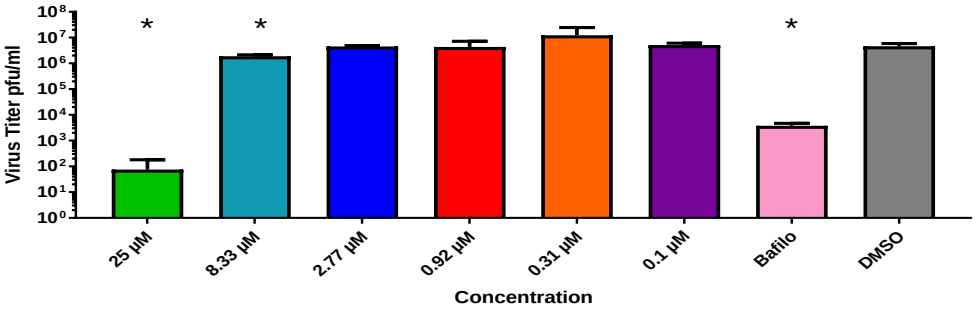

Virus Titer pfu/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
*
*
*
25 µM
8.33 µM
2.77 µM
0.92 µM
0.31 µM
0.1 µM
Bafilo
DMSO
Concentration

Figure 5: Efficacy of dose range testing of Cmpd 2 (A), Cmpd 3 (B), Cmpd 7 (C), and Cmpd 13 (D) against VEEV in the mouse ES-derived motoneuron Assay. Cells were infected with VEEV for 1 hour after which compounds were added to the media at concentrations of 25 µM, 8.33 µM, 2.77 µM, 0.92 µM, 0.31 µM, and 0.1 µM. Supernatants were harvested at 24 hours post-infection and virus yield (pfu/mL) was determined by plaque assay. Bafilomycin A1 was included as a positive control and DMSO was included as the virus only control. These four compounds had statically significant reductions in virus at concentrations of 25 µM and 8.33 µM. Data represents an n=2 with the errors representing standard deviation. Asterisks represent a significant reduction in virus yield compared to the Negative Control (DMSO) (one tail t-test, p value < 0.05).

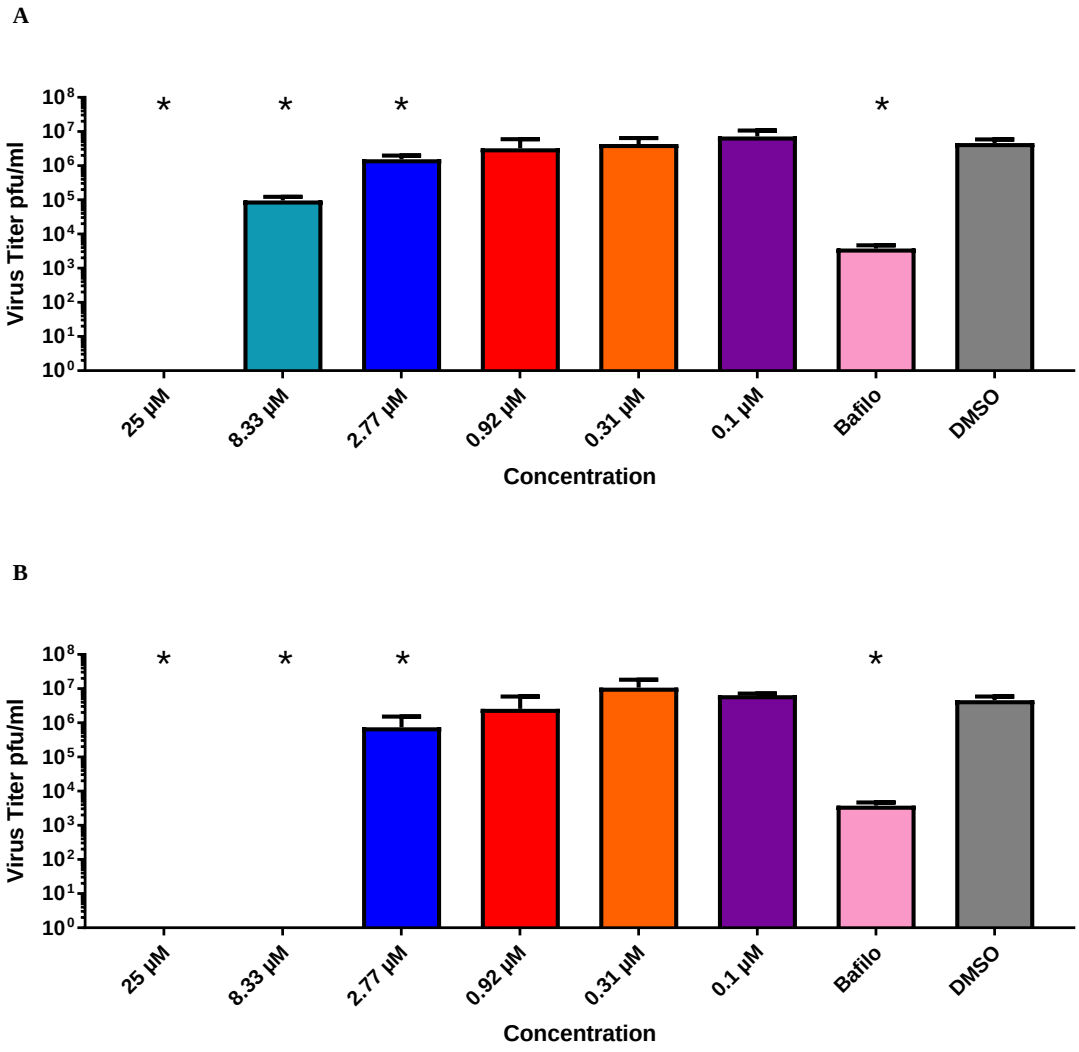


Figure 6: Efficacy of dose range testing of Cmpd 21 and Cmpd 23 against VEEV in the mouse ES-derived motoneuron Assay. Cells were infected with VEEV for 1 hour after which compounds were added to the media at concentrations of 25 µM, 8.33 µM, 2.77 µM, 0.92 µM, 0.31 µM, and 0.1 µM. Supernatants were harvested at 24 hours post-infection and virus yield (pfu/mL) was determined by plaque assay. Bafilomycin A1 was included as a positive control and DMSO was included as the virus only control. These two compounds had statically significant reductions in virus at concentrations of 25 µM, 8.33 µM and 2.77 µM. Data represents an n=2 with the errors representing standard deviation. Asterisks represent a significant reduction in virus yield compared to the Negative Control (DMSO) (one tail t-test, p value < 0.05).

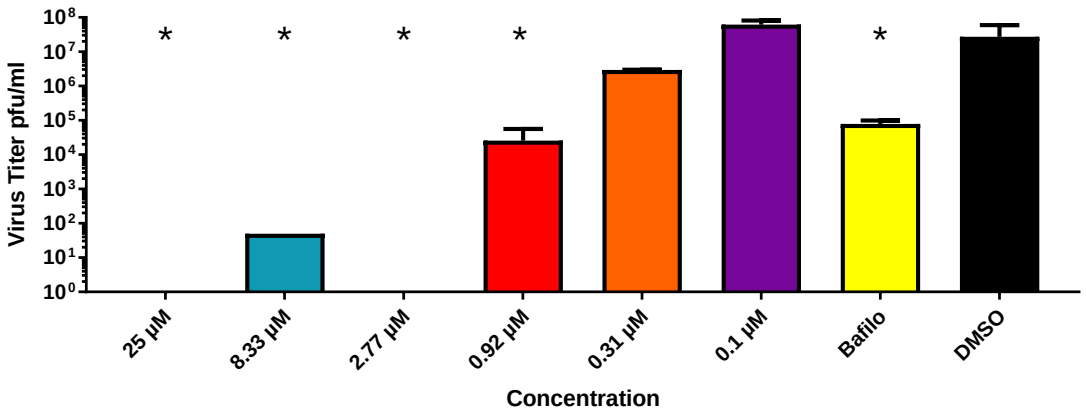


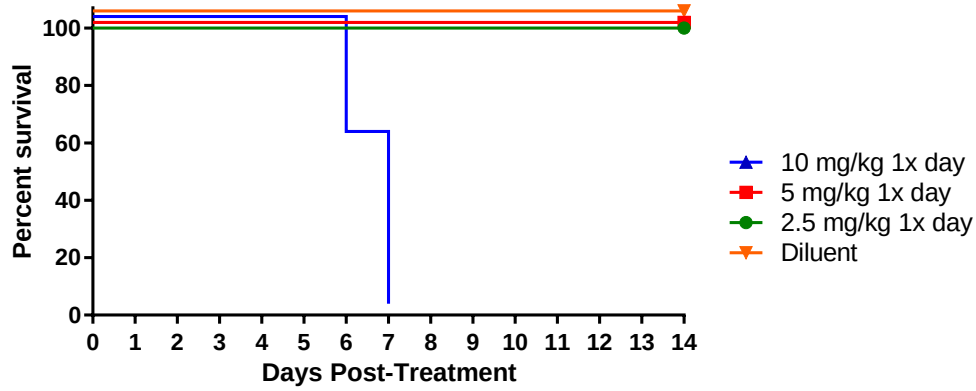
Figure 7: Efficacy of dose range testing of Cmpd 6 against VEEV in the mouse ES-derived motoneuron Assay. Cells were infected with VEEV for 1 hour after which compounds were added to the media at concentrations of 25 µM, 8.33 µM, 2.77 µM, 0.92 µM, 0.31 µM, and 0.1 µM. Supernatants were harvested at 24 hours post-infection and virus yield (pfu/mL) was determined by plaque assay. Bafilomycin A1 was included as a positive control and DMSO was included as the virus only control. Cmpd 6 had significant reductions in virus compared to the control at concentrations at or above 0.92 µM. Data represents an n=2 with the errors representing standard deviation. Asterisks represent a significant reduction in virus yield compared to the Negative Control (DMSO) (one tail t-test, p value < 0.05).

In Vivo Efficacy Evaluation

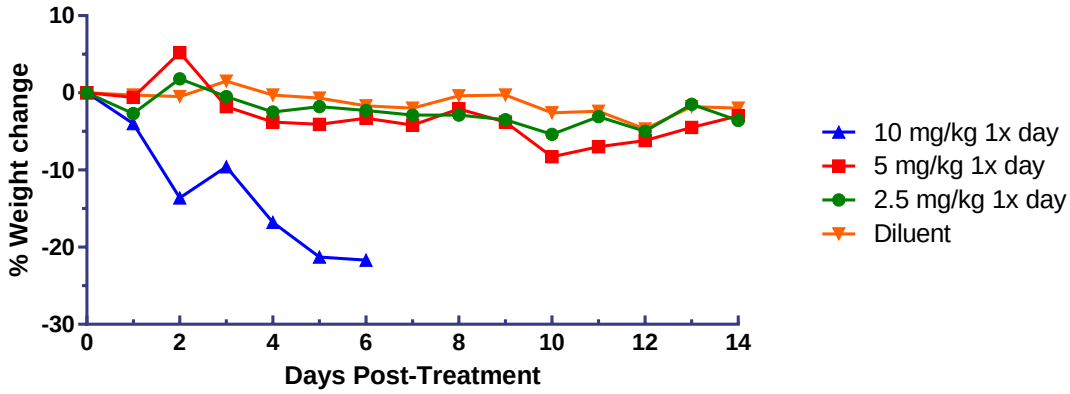
Tolerability Study

The tolerability study revealed that 10mg/kg of Cmpd 6 given daily was not well tolerated. Mice administered this dose repeatedly had higher clinical scores, group weight loss over 20%, and all mice succumbed or met euthanasia criteria by day 7 (Figure 8). All mice in the 5 mg/kg, 2.5 mg/kg, and diluent groups survived to the end of the study. The 5 mg/kg dosage was better tolerated with mice showing no significant difference in weight loss when compared to the diluent group, but did have slightly elevated clinical scores on days 7-12. Mice in the 2.5 mg/kg group showed no adverse effects with no elevation in clinical scores and no significant difference in weight loss when compared to the diluent group.

A


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post-Treatment
10 mg/kg 1x day
5 mg/kg 1x day
2.5 mg/kg 1x day
Diluent

B


% Weight change
10
0
-10
-20
-30
0 2 4 6 8 10 12 14
Days Post-Treatment
10 mg/kg 1x day
5 mg/kg 1x day
2.5 mg/kg 1x day
Diluent

C

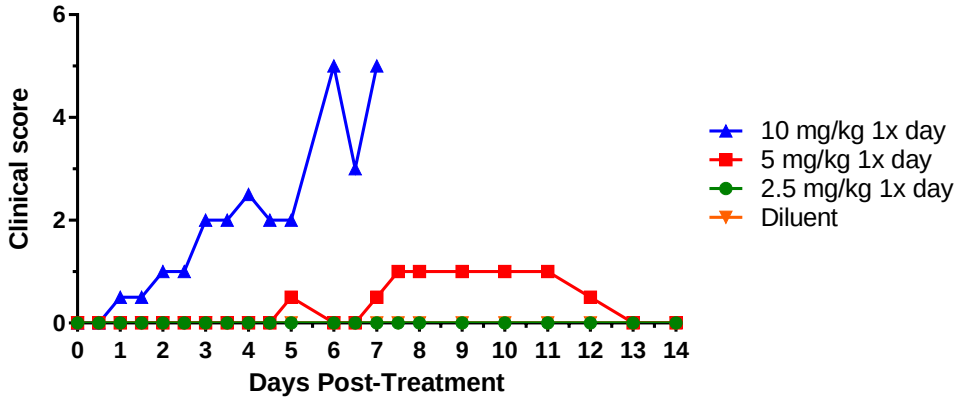

Clinical score
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post-Treatment
10 mg/kg 1x day
5 mg/kg 1x day
2.5 mg/kg 1x day
Diluent

Figure 8: Tolerability of Cmpd 6 in mice. Survival (A), percent weight change (B), and clinical scores (C) are shown for mice administered doses of 10 mg/kg, 5 mg/kg, or 2.5 mg/kg daily for 10 days. Mice in the 10mg/kg group exhibited weight loss and increased clinical scores until succumbing or meeting euthanasia criteria at 6 or 7 days of treatment. Mice in the 5 mg/kg group exhibited increased clinical scores, but otherwise tolerated the treatment. Mice in the 2.5 mg/kg group had no reductions in weight or increase in clinical scores compared to controls.

Efficacy of Compound 6 in VEEV Mouse Models

In the efficacy experiments, mice were administered 1.25 mg/kg or 2.5 mg/kg of Cmpd 6 by intraperitoneal route daily for 10 days starting two hours prior to infection. Cmpd 6 was not effective against VEEV INH-9813, but did show some promise in the VEEV TC-83 mouse model. All mice in the VEEV INH-9813 mouse model met euthanasia criteria by day 7 post-infection (Figure 9). In the VEEV TC-83 mouse model, 70% of the mice treated with 2.5 mg/kg and 60% of the mice treated with 1.25 mg/kg survived to the end of the study. However, since 50% of both the diluent and untreated mouse groups survived to the end of study as well, the survival of the treated mice is not statistically significant. Mice in both the diluent and untreated mouse groups first met euthanasia criteria at 5.5 days post-infection and mice in both treated groups first met euthanasia criteria at 10.5 days post-infection, however there was no significant difference in mean time to death. In both studies, there was no difference in weights between treated and untreated groups (Figure 10). There was no difference in clinical scores in the VEEV INH-9813 mouse model between treated and untreated groups (Figure 11). The only difference in clinical scores observed in the VEEV TC-83 mouse model was that the untreated mice had elevated clinical scores out to day 18 post-infection while the other groups no clinical scores after day 14 post-infection. The results of the VEEV TC-83 mouse model experiment showed that there is some promise for Cmpd 6. The VEEV TC-83 mouse model is a partially lethal model which can confound study results. The route of inoculation likely plays a role in this variability as mice may swallow rather than absorb virus via the nares. Further work on this model is planned to identify other markers to

monitor infection. Additionally, some refinement of the dose and schedule regimen may further increase survival in the treated groups.

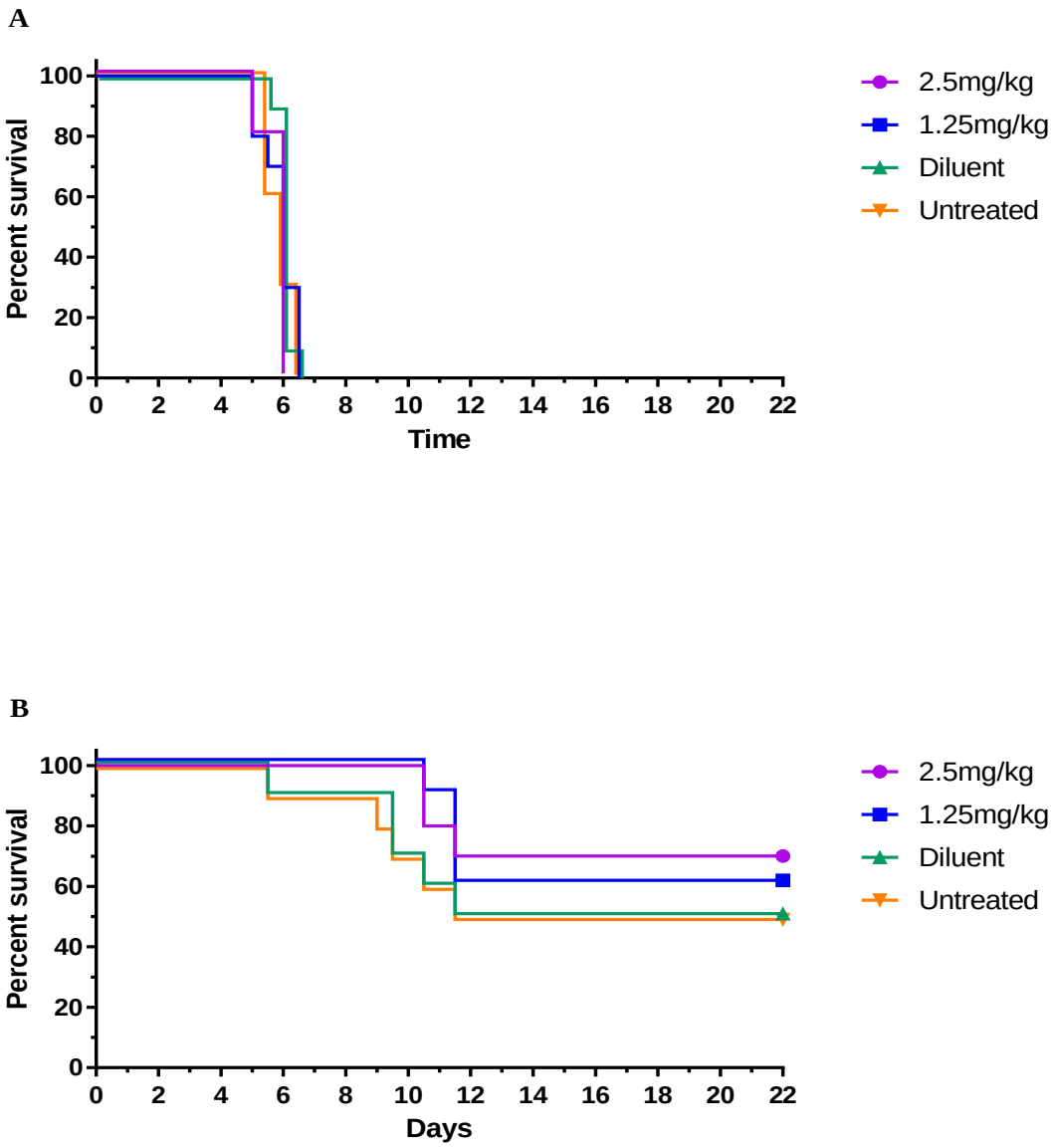


Figure 9: Efficacy of Cmpd 6 in VEEV mouse models as determined by survival. Mice were administered 2.5 mg/kg or 1.25 mg/kg by intraperitoneal route daily for 10 days starting two hours prior to infection. All mice in the VEEV INH-9813 mouse model (A) met euthanasia criteria by day 7 post-infection. In the VEEV TC-83 mouse model (B), mice treated with 2.5 mg/kg and 1.25 mg/kg had 70% and 60% survival respectively but 50% of both the diluent and untreated mouse groups survived. While there appeared to be a slight delay in mean time to death in the treated groups it was not statistically significant.

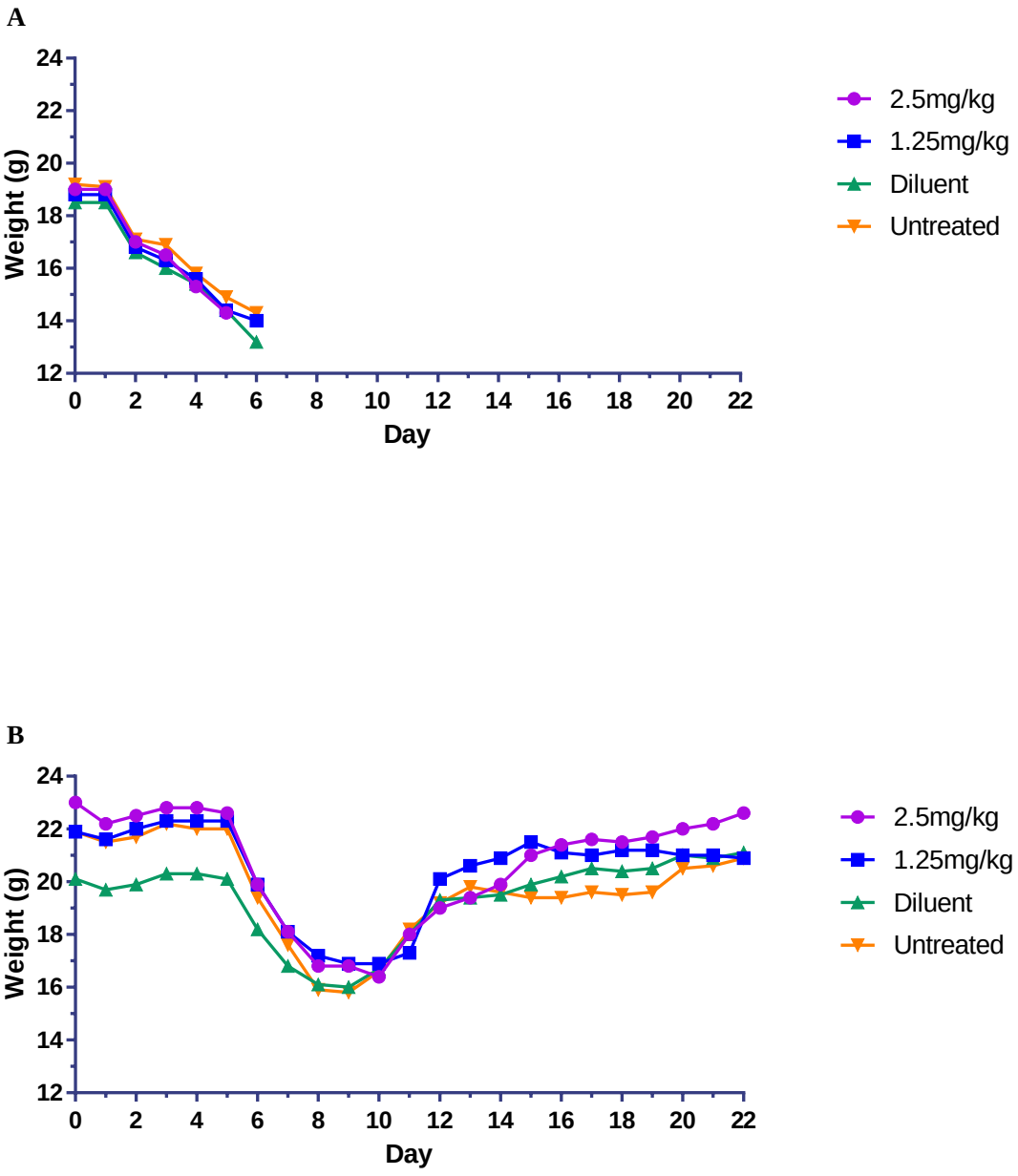


Figure 10: Efficacy of Cmpd 6 in VEEV mouse models as determined by weights. Mice were administered 2.5 mg/kg or 1.25 mg/kg by intraperitoneal route daily for 10 days starting two hours prior to infection. The weights of treated mice in both the VEEV INH-9813 (A) and VEEV TC-83 (B) models had no statistical difference when compared to the diluent treated and untreated mice.

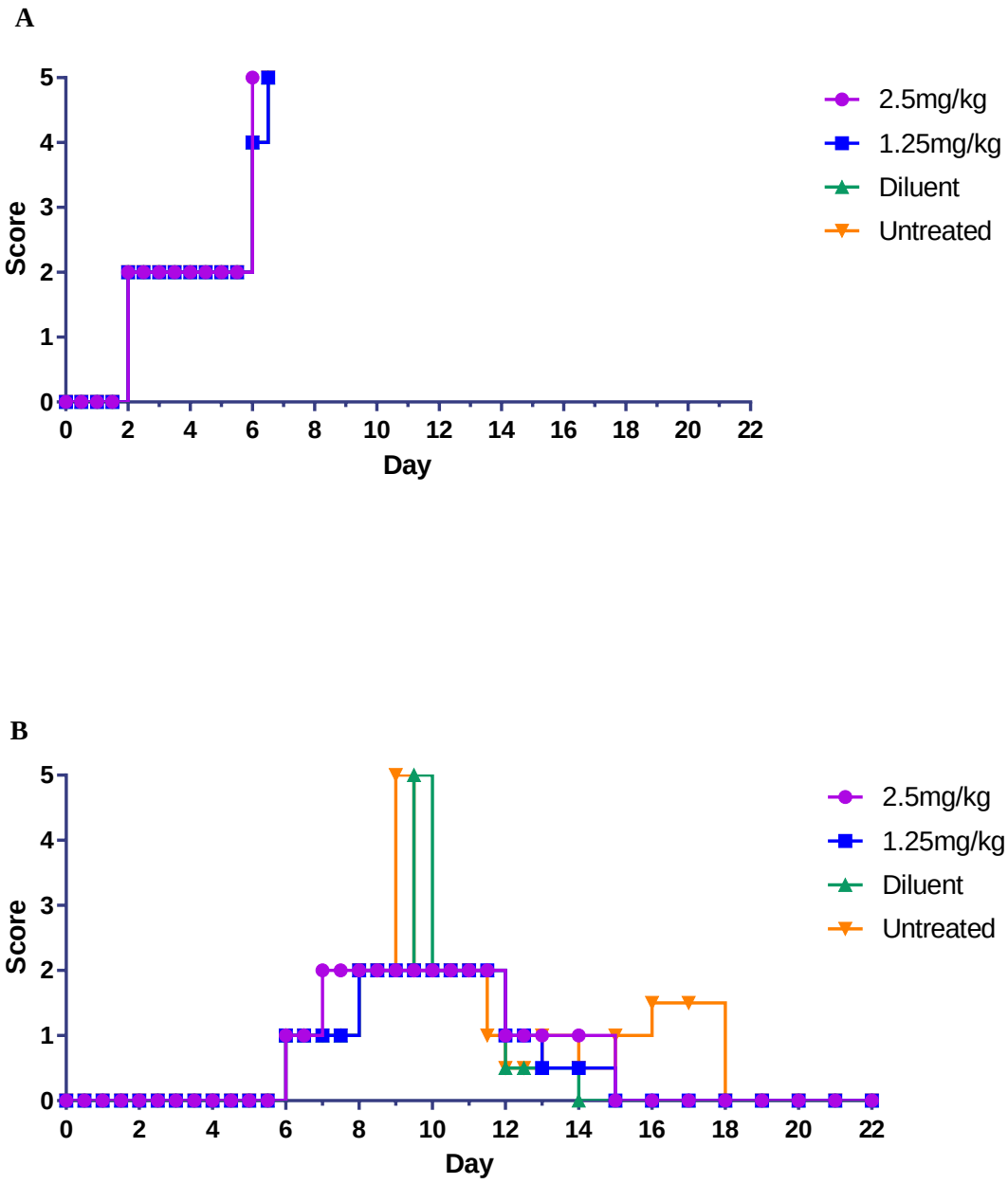


Figure 11: Efficacy of Cmpd 6 in VEEV mouse models as determined by clinical score. Mice were administered 2.5 mg/kg or 1.25 mg/kg by intraperitoneal route daily for 10 days starting two hours prior to infection. There was no difference in clinical scores between groups in the VEEV INH-9813 model (A). In the VEEV TC-83 model (B) the most notable difference was that the untreated mice had elevated clinical scores out to day 18 post-infection while the other groups no clinical scores after day 14.

DISCUSSION

Screening assays utilizing more relevant cell types, such as ES-derived spinal motoneurons, can be used to assist in the down selection of potential therapeutic compounds for *in vivo* studies in alphaviruses. Development of similar assays using primary cells could allow for further down selection prior to compounds proceeding to *in vivo* studies. The assay developed for screening compounds against alphaviruses in ES-derived spinal motoneurons was able to identify several potential therapeutic compounds and further focus the compounds to be evaluated for efficacy *in vivo*. Used in conjunction with HTS screening assays there is the potential to screen thousands of compounds and subsequently down select to a handful of compounds that are most likely to have therapeutic benefit.

It is important to note that compounds are added to the cell-based ELISA HTS assay and the VEEV ES-derived spinal motoneurons assay at different times in the completion of the assay. In the cell-based ELISA HTS assay, the compounds are added to the cells prior to virus addition and remain throughout the course of infection. Based on the fast replication cycle of VEEV, this practice could skew the assay toward identification of compounds which block virus entry or viral replication. For the VEEV ES-derived spinal motoneurons assay, cells were infected and then the virus was removed prior to the compound being added. The assay developed in mouse ES cell-derived motoneurons added compounds after virus infection, since the mechanism of action of these compounds was expected to impact steps later in the virus replication cycle. Compounds identified as most effective in this assay likely block viral replication or viral budding since virus would have already entered the cells. Evaluating the effect of compounds added at different times

pre- or post-infection can help to determine the mechanism of action of a compound and could lead to the identification of additional effective compounds.

Further study altering the ES-cell derived assay may also improve the selection of compounds for *in vivo* evaluation. Alterations could include adding compounds prior to virus addition similar to the cell-based ELISA HTS assay or evaluating different time points of compound addition post-infection. Additionally, evaluating other stem cell lines or primary cells may prove to be a better evaluation tool than ES-cell derived motoneurons. Both granule cell neurons of the cerebellum and cortical neurons from the cerebral cortex have been shown to be infected by VEEV and could be possible alternatives (Cho *et. al.* 2013). These methods could be completed in addition to the VEEV ES-derived spinal motoneurons assay to assist in selecting the best possible candidates for *in vivo* evaluation.

While Cmpd 6 did not fully protect mice against VEEV INH-9813, it partially protected against VEEV TC-83. The VEEV INH-9813 mouse model is a more stringent model for therapeutic evaluation due to the rapid progression of the disease. It is for this reason that many groups report efficacy in the VEEV TC-83 mouse model as there is a slower onset of symptoms. Further developments of the VEEV TC-83 mouse model evaluating various virus stock preparations and exposure doses are needed. Alphaviruses have an inherently high mutation frequency and repeated passage can alter infectivity and virulence in animal models. Preparing a virus stock directly from the VEEV TC-83 vaccine preparation with limited passages may increase virulence in the model. Alternatively, the use of a stock prepared from a cDNA clone may provide reproducibility in the stock preparation. Another benefit of a cDNA-derived VEEV TC-83 is the ability to include a luminescent marker which could be monitored in all mice regardless of the lethality of the

virus. A marker would allow for not only determining differences in viral load, but could potentially reveal differences in virus dissemination in the presence of a potential therapy. If these alterations in the animal model were not sufficient to achieve statistical significance, increases in group sizes may be required.

A change in dosing regimen for Cmpd 6 *in vivo* may provide additional survival as well. A pK study completed by a collaborator, after *in vivo* studies described here were completed, administered 0.1 mg/kg, orally to mice and then monitored levels of Cmpd 6 in plasma, liver, lung, and spleen. They found the concentrations of Cmpd 6 in most plasma samples evaluated to be below the limit of quantification. In the tissues, however there was an accumulation of Cmpd 6 and the half-life ($t_{1/2}$) in the tissues was 35 hours. This indicates that our dosing strategy may have been too high or frequent and a reduction in dose or dosing schedule to every other or every three days may be required to increase survival. Cmpd 6 accumulation in the tissues may have created a toxic effect, leading to decreased survival in infected mice. Additionally, a different route of administration may also be more beneficial. This combined with further developments of the VEEV TC-83 mouse model could lead to effectiveness that was expected from this compound.

Ultimately, there were several compounds identified as potential therapeutics using the VEEV ES-derived spinal motoneurons assay and even more identified in HTS screening that were not evaluated in the ES-derived spinal motoneuron assay. Evaluation of additional compounds with potential efficacy may lead to the identification of compounds with greater *in vivo* efficacy. Also, an examination of host and/or viral targets of these compounds may lead to the discovery of other potential candidates or may provide information on the mechanisms involved in VEEV infection.

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