COMBINED TREATMENT OF A NOVEL ADENOSINE ANALOGUE INHIBITOR AND HISTONE DEACETYLASE INHIBITOR REPRESENTS AN IMMUNOTHERAPY STRATEGY TO AMELIORATE WEST NILE VIRUS INFECTION

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By

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Chapter 1

Background

1. West Nile virus and disease

1.1 West Nile virus

West Nile virus (WNV) is an arthropod-borne virus (arbovirus) belonging to the family *Flaviviridae*. It is in the Japanese encephalitis virus (JEV) group of flaviviruses that also includes other mosquito-borne neurotropic viruses such as Murray Valley fever and St. Louis encephalitis virus. WNV first emerged in the United States in 1999 and since then, it has become one of the leading causes of viral encephalitis and the most prevalent arboviral encephalitis [1].

1.2 Virus structure

WNV has a 10.7-kb positive-sense single-stranded RNA genome, which is transcribed as a single polyprotein that is then cleaved post-translation via viral proteases into 7 smaller proteins. Structural proteins (C, PrM/M, E) form the capsid, matrix and envelope of the 50 nm spherical virions. Non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) have a range of functions including helicase and proteases (NS3), RNA-dependent RNA polymerase (NS5) [2], transmembrane proteins and rearrangement of cytoplasmic membranes for replication (NS4A and NS4B) [3], and secreted membrane binding proteins that can bind to host cells and interact with viral transmembrane proteins (NS1) [2]. The host antibody response primarily targets the E protein and the NS1 protein, and

anti-NS1 antibodies treated mice are protected against lethal WNV challenge.

1.3 Epidemiology

WNV is a mosquito-borne old world virus that causes febrile illness. In 1999, WNV emerged in the United States and caused neuroinvasive disease in addition to febrile illness [4]. In the initial outbreak in New York in 1999, 62 patients developed encephalitis and seven died. This is a higher mortality rate than was previously observed in outbreaks in Africa, suggesting that the virus had mutated and now presented a higher risk of neuroinvasive disease. Since its entry in New York in 1999, the virus has spread across the United States and is now the most prevalent arbovirus in the United States. In 2002 and 2003, there were large outbreaks that required hospitalization of almost 3000 patients with encephalitis. Year 2003 had the most number of patients with WNV infection while 2002 saw the highest number of patients with neurological symptoms. There was sustained transmission throughout the following decade with numbers declining to 712 total cases with 486 cases with neurological symptoms in 2011. However, in 2012, the number of cases rose sharply, with 5,674 confirmed cases and 2,873 cases showing neurological symptoms. There were 286 deaths attributed to WNV in 2012, making it the deadliest year for WNV infection in the United States. Currently, every state in the 48 contiguous United States has reported cases of WNV, varying from 1 case in Maine to 4,994 cases in Colorado [5].

1.4 Transmission

The cycle of transmission for WNV uses mosquitoes to transmit the virus to vertebrates that are either dead end hosts, such as humans and horses, or amplifying hosts, such as birds [6]. Thus far, Passeriformes, Charadriiformes, Strigiformes and Falconiformes are genera of birds that are able to generate a viremia that is infectious to mosquitoes. Corvids, finches, grackles and house sparrows develop a very high viremia, but also exhibit mortality in excess of 40% [6]. Mosquitoes in the genus *Culex* are the most competent for WNV infection and are responsible for most of the transmission of the virus [7]. Infection in mosquitoes most commonly occurs horizontally when they feed on infected birds, however, there have been noted instances of vertical transmission [8, 9].

1.5 Pathology

Approximately 80% of WNV infections are asymptomatic, the other 20% experiencing mild disease symptoms. Common symptoms from West Nile infection include fever, malaise, joint pain and gastrointestinal problems. Less than 1% of infections results in neuroinvasive West Nile disease, which is characterized by meningitis, encephalitis and ascending flaccid paralysis. Symptoms begin approximately 1 week after exposure to the virus and can persist months after infection [10].

The disease progression and pathology in C57BL/6 mice is similar to that seen in humans, therefore becomes an ideal model to study WNV

infection and disease pathogenesis. In the mouse model of WNV infection, the viremia peaks at days 3-5 post-infection and neurological symptoms begin between days 6-8 post-infection. Mortality is observed between 8-12 days post-infection [11]. The virus in the periphery is cleared by the time the mice start to exhibit severe neurological symptoms. The mechanism by which WNV enters the brain are not very clear but WNV is proposed to invade the CNS by crossing the blood-brain barrier through gaps created by infected astrocytes or by infected leukocytes via a Trojan horse mechanism [12].

1.6 Immunology

During the early stage of infection, inflammation is important to clear the virus and eliminate infection in the periphery. Production of pro-inflammatory cytokines such as IL-1β, TNFα and type-1 interferons mediate several functions such as recruitment of leukocytes to the site of infection, APC activation and appropriate T cell responses. Neurons secrete chemokines such as CXCL10 in response to WNV infection, which recruits effector CD8+ T-cells in the brain. The infiltrating leukocytes are critical in clearing virus in the brain, however, these leukocytes may further enhance the neuroinflammation by activating microglia and astrocytes that contribute to the bystander death of naïve neurons, thereby enhancing neuronal damage at the later stages in the brain [12].

1.7 Treatment strategies and vaccines

There are currently no vaccines or antiviral compounds available to inhibit or prevent WNV infection in humans. However, there are approved vaccines for other flaviviruses such as JEV and YFV. ChimeriVax-WN vaccine is licensed for use in horses and consists of a yellow fever (YF 17D) vaccine strain that expresses WNV prM-E glycoproteins [13]. It is currently being developed further for use in humans and elicits a neutralizing antibody response that remains effective at 12 months after a single dose. There are a number of WNV and dengue vaccine candidates in either preclinical stages or clinical trials for use in humans [14]. The discovery of small molecule inhibitors of viral replication and novel anti-inflammatory compounds is an important research focus to control flavivirus infections. In dengue virus and JEV, a promising antiviral target is the NS5 protein, which functions as an RNA-dependent RNA polymerase and methyl transferase [14]. RNA-dependent RNA polymerases in other viruses, such as hepatitis B and HIV, have been shown to be blocked by nucleotide analogue inhibitors. Recently, Novartis, Singapore have developed an adenosine analog NITD-008 that potently inhibits DENV in vitro and in vivo. In vitro studies demonstrated that NITD-008 also had antiviral effect against WNV; however, the efficacy of NITD-008 in attenuating WNV replication in vivo has not been evaluated so far.

Another strategy of managing WNV disease is to target the host responses to viral infection. Immune modulators that have shown

promising effects in controlling virus replication include type I IFN therapy [15], and administration of monoclonal anti-WNV antibodies in animal models [16]. Since inflammation is one of the major causes of neuropathology, several novel drugs targeting inflammatory pathways are being discovered. The inability to clear the virus in the periphery can lead to increased pathology, as evidenced by patients with hypertension. Many broad-spectrum anti-inflammatory drugs such TNF-a inhibitors have shown improved disease outcome of neuroinflammatory disorders such as Alzheimer's disease [16].

1.8 Epigenetics

Epigenetic control of histone proteins in the chromatin is a mechanism by which a cell regulates production of rapid, inducible and reversible pathway for gene transcription. Histone modifying enzymes such as histone deacetylase (HDAC) and histone acetyltransferase (HAT) are responsible for adding and removing acetyl groups from histone tails that ultimately controls the coiling of chromatin and allow the transcription of genes including cytokines such as TNFα and IL-1β [17-19]. Alteration in the activity and expression of these enzymes, specifically HDACs has been reported in several inflammatory diseases. For example, in Alzheimer's disease and tumor genesis, HDACs are upregulated and contribute to potentiating [17, 20]. Since HDACs play such an important role in regulating inflammation, they have become an attractive target to attenuate inflammation.

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Chapter 2

Hypothesis and Aims

Gap:

Given the spread of WNV disease, it is critical to evaluate novel treatment strategies to improve disease severity. The major gaps in the field of WNV therapeutic research are:

i. There are no antiviral compounds currently in trials for WNV [21]. While NITD-008 has shown promise as an antiviral for DENV, the ability of this drug to block WNV replication is only limited to in vitro studies. There are no in vivo studies testing the therapeutic efficacy of any drug in improving WNV disease outcome.
ii. Since inflammation is a major cause of the pathology associated with neuroinflammation resulting from WNV infection, evaluation of novel anti-inflammatory compounds would be another promising strategy to attenuate inflammation and improve WNV encephalitis symptoms. However, so far no anti-inflammatory drugs have been tested for WNV in vivo.
iii. HDAC inhibitors have shown promise treating other inflammatory diseases, but no studies have investigated their effects in modulating inflammation in acute viral disease models including flaviviruses.

Objective:

The objective of this study is to test the effect of the treatment of a novel antiviral NITD-008 and the newly characterized anti-inflammatory drug vorinostat

(suberanilohydroxamic acid; SAHA), on WNV replication kinetics and disease progression using a well-established mouse model of infection.

Overall Hypothesis:

We hypothesize that treatment with NITD-008 will block WNV replication in vivo, which will prevent associated mortality and morbidity in a dose- and time-dependent manner. We further hypothesize that treatment with SAHA during the CNS phase of infection will further improve WNV disease outcome by limiting neuroinflammation.

Specific Aims:

Specific Aim 1: To determine the effect of NITD-008 on WNV replication and overall disease outcome in vivo.

Specific Aim 2: To assess the effect of SAHA on neuroinflammation associated with WNV infection.

Specific Aim 3: To evaluate the effect of combined treatment of SAHA and NITD-008 during the CNS-stage of disease, on the WNV disease outcome in mice.

Rationale and experimental plan

Specific Aim 1: To determine the effect of NITD-008 on blocking WNV replication in vivo and disease outcome.

Hypothesis:

NITD-008 will protect against lethal WNV infection by reducing viral replication in a dose- and time-dependent manner.

Rationale:

NITD-008 is an adenosine analogue inhibitor that interrupts the RNA-dependent RNA polymerase of flaviviruses. In vero cells, NITD-008 treatment immediately after infection has been shown to reduce the titer of WNV from 10 log PFU/ml to 2 log PFU/ml. Likewise, NITD-008 also reduced replication of other flaviviruses such as powassan virus, dengue virus-2, hepatitis C virus and yellow fever virus; however, it did not inhibit replication of alphaviruses such as western equine encephalitis virus and Venezuelan encephalitis virus, suggesting that the antiviral effect of NITD-008 is specific to flaviviruses. In the AG129 mouse model of dengue virus infection, when administered at up to 48 hours after infection, 10 mg/kg of NITD-008 significantly reduced the viremia and prevented mortality [22]. It is not clear whether NITD-008 can block WNV replication in vivo and whether the efficacy of NITD-008 would be dependent on the stage of WNV disease. Lack of such information limits our understanding of the potency of NITD-008 in controlling WNV infection. This aim would be the first step to characterize the

antiviral effect of NITD-008 against WNV in vivo. The rationale is that if NITD-008 shows promise in preventing mortality due to WNV infection in vivo, this study might lay the groundwork for future studies to develop NITD-008 as an effective antiviral in preventing WNV disease in humans.

Objectives:

- Determine the dose dependent response of NITD-008 in protecting against lethal WNV infection
- Establish a therapeutic window of efficacy of NITD-008 by administering drug at different time points after WNV infection.
- *Experimental Plan:* Infect 9-10 week old C57BL/6 mice with 1000 PFU WNV/NY/99 and then administer NITD-008 at different concentrations (25 mg/kg, 10 mg/kg, 5 mg/kg, 1 mg/kg) via oral gavage, twice per day, starting the day after infection. Mice will be observed daily for 14 days post infection to monitor disease progression and severity.
- Infect 9-10 week old C57BL/6 mice with 1,000 PFU WNV/NY99 and then administer the lowest concentration of NITD-008 that was protective from earlier experiments via oral gavage, twice per day, starting 3 and 5 days after infection. Mice will be observed daily for 14 days after infection to monitor disease progression and severity.
- Harvest brains, spleen and kidneys at 3, 5 and 8 days after infection to measure the levels of inflammatory cytokines in the tissues and serum using RT-PCR.
- Assess viral titers in tissues and serum using viral plaque assay.

Specific Aim 2: To assess the effect of SAHA on neuroinflammation associated with WNV infection.

Hypothesis:

Treatment with SAHA will reduce the neuroinflammation associated with WNV infection by reducing the level of inflammatory cytokines produced in the CNS. Further, SAHA will improve the disease outcome of WNV infection in vivo when administered in the CNS phase of disease progression.

Rationale:

Histone deacetylase inhibitors are already approved for the treatment of diseases such as cancer, Alzheimer's disease and HIV [23-25]. There is evidence that HDAC inhibitors are effective in treating the inflammation associated with neurodegenerative diseases. In an in vivo model of Alzheimer's disease, daily injections of SAHA improved cognitive function in mice by reducing associated inflammation[26]. In a rat model of septicemia, valproic acid is able to reduce TNFα and IL-1β and improve survival following cecal ligation puncture. Further, in vitro treatment with HDAC inhibitor TSA decreased the production of cytokines in alveolar cell cultures infected with *L. pneumophila* [27]. Suberanilohydroxamic acid (SAHA) is an FDA approved pan-HDACi marketed under the name Vorinostat was chosen for this study because of its ability to cross the blood-brain barrier. There is limited information on the role of HDACs in regulating inflammation associated with acute virus infection. The rationale is that based on

the findings of the previous studies, it is likely that SAHA will be able to reduce the inflammation caused by WNV infection in vivo.

Objective:

- Assess the ability of SAHA to modulate WNV-associated inflammation in vivo.
- Investigate whether SAHA can improve the disease outcome of WNV disease in mice.

Experimental Plan:

- Administer 100 mg/kg SAHA IP daily at an early (day 3 p.i.) and late stage (day 5 p.i.) of WNV infection and monitor the morbidity and survival for 17 to 20 days.
- Harvest brains at day 8 p.i. from different groups of mice and determine the level of inflammatory cytokines using qRT-PCR.
- Measure markers of WNV-associated encephalitis (neuronal death and infiltration of leukocytes) in the brains from different group of mice by using H&E staining, CD45 staining and TUNEL assay.

Specific Aim 3: ***To evaluate the effect of combined treatment of SAHA and NITD-008 during the CNS-stage of disease on the WNV disease outcome in mice.***

Hypothesis:

WNV replication and associated neuroinflammation will be reduced following combined treatment with NITD-008 and SAHA resulting in improved overall disease outcome.

Rationale:

Combination therapy is a successful approach to treat many different diseases including HBV and HIV [28, 29]. For example, lamivudine is the preferred reverse transcriptase inhibitor used to treat HBV, but resistance to lamivudine is common. When adefovir, another reverse transcriptase inhibitor, is added to the treatment regimen there is a decrease in resistance to either drug resulting in improved disease management [29]. Another area where combined therapy is used is HIV disease management. Combined anti-retroviral therapy (cART) with the addition of HDAC inhibitors results in an increase in the apoptosis of infected cells and improved virus clearance [28]. Rationale for this aim is that it is possible that NITD-008 may not be effective at preventing pathology during all phases of disease progression. In that case, the addition of an anti-inflammatory compound would be beneficial in order to reduce the pathology associated with WNV neuroinflammation, even though there may not be an effect on the replication of the virus.

Objective:

- Determine the combined effects of treatment with SAHA and NITD-008 during late phase of WNV infection on viral replication and neuroinflammation in the brain.

Experimental Plan:

- Administer 100 mg/kg SAHA (once daily, IP) and 10 mg/kg NITD-008 (twice daily, P.O.) at a late stage (day 5 p.i.) of WNV infection to determine the effect of combined therapy on disease outcome.
- Harvest brains from mice on day 8 p.i. that have been treated with 100 mg/kg SAHA and 10 mg/kg NITD-008 at a late stage of infection and determine the level of inflammatory cytokines via qPCR and neuropathology via H&E staining, CD45 staining and TUNEL.

Chapter 3

Results

(Manuscript draft)

Title: Combined treatment of adenosine nucleoside inhibitor NITD-008 and histone deacetylase inhibitor vorinostat represents an immunotherapy strategy to ameliorate West Nile virus infection

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1. Introduction

West Nile Virus (WNV) is a positive sense single-stranded RNA ((+) ssRNA) flavivirus that is one of the most common causes of arthropod-borne encephalitis globally. It is closely related to Japanese Encephalitis Virus (JEV) and dengue virus (DENV) and has pathology similar to that of JE, tick-borne encephalitis, St. Louis encephalitis and California encephalitis viruses, and alphaviruses such as Venezuelan equine encephalitis virus. The viral life cycle shifts between mosquitoes and birds but humans are a dead end host for the virus [30, 31].

WNV has been spreading in the United States since the first reported cases in 1999 and is now a leading cause of viral encephalitis. In Africa, where the WNV was first detected, it is not a major source of encephalitis and causes arthralgia, low grade fever and myalgia. However, a new strain that is more neurotropic and neurovirulent was introduced into the United States in 1999 and is responsible for serious cases of meningoencephalitis, which causes severe headache, high fever, confusion, tremors, convulsions, paralysis and muscle weakness [1]. This is illustrated by the outbreak in 2012, which had 5,674 total cases including 2,873 neuroinvasive cases reported with 286 deaths. Since the introduction of the virulent strain of WNV into the United States, there have been 39,557 cases with 17,463 cases having neurological involvement resulting in 1,668 deaths [32]. WNV infection poses the greatest threat to the elderly, immunocompromised individuals and people with high blood pressure and diabetes [22, 33].

WNV has a 10.7-kb positive-sense single-stranded RNA genome that is transcribed as a single polyprotein that is then cleaved post-translation via viral proteases into seven smaller proteins. Structural proteins (C, PrM/M, E) form the capsid, matrix and envelope of the 50 nm spherical virions [34, 35]. Non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) have a range of functions including helicase and proteases (NS3), RNA-dependent RNA polymerase (NS5) [2], transmembrane proteins and rearrangement of cytoplasmic membranes for replication (NS4A and NS4B) [3], and secreted membrane binding proteins that can bind to host cells and interact with viral transmembrane proteins (NS1) [2]. The host antibody response primarily targets

the E protein and NS1 protein, and anti-NS1 antibodies introduced to mice can protect against lethal WNV challenge [36].

WNV first infects skin Langerhans cells, which then migrates and seed into the lymph nodes and spleen where primary viremia is established. The virus is initially detectable in the blood shortly after infection and persists until the onset of symptoms in humans [37]. There is a correlation between serum IgM and clearance of the virus, illustrating the importance of a strong humoral immune response that is facilitated by a robust innate immune response immediately after infection. In immunocompromised patients, the viremia is prolonged and there is an elevated risk of neuroinvasive disease [37].

The mouse is a good model to study WNV infection and mimics human disease pathogenesis. In mice, the onset of neurological symptoms is exhibited between days 6-8 with mortality occurring between days 8-12. The virus is cleared from the periphery by the time the mice start to exhibit severe neurological symptoms and the virus is cleared from the brain by the time mortality occurs. The innate immune responses to WNV in the periphery includes the release of pro-inflammatory cytokines such as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and type-1 interferon (IFN) which contribute to the ability to clear the infection, [12]. In the CNS, neurons are the primary target of WNV replication, however other CNS cells such as astrocytes and microglia can also support WNV replication and are major source of cytokines and chemokines production [12]. The peripheral leukocytes, specifically CD8+ T cells are recruited in the CNS to

clear WNV, however later uncontrolled inflammation becomes cause of bystander neuronal injury.

There are no vaccines or antiviral compounds available to inhibit or prevent WNV infection in humans. However, there is an approved vaccine for other flaviviruses such as JEV and YFV. Approaches to identify antiviral inhibitors include small molecule based inhibitors targeting viral proteins critical for virus replication. In flaviviruses, a promising antiviral target is the NS5 protein, which functions as an RNA dependent RNA polymerase (RdRp) and as a methyl transferase that serves to cap the 5' end of viral RNA [38]. Recently Yin and colleagues have developed an adenosine analogue inhibitor NITD-008 with potent antiviral activity against DENV in vivo [21]. NITD-008 was able to inhibit virus replication by causing the termination of RNA chain synthesis, resulting in incomplete RNA replication. In AG129 mice (Interferon α/β and γ receptor deficient) model of DENV infection, NITD-008 completely protected mice from DENV infection and mortality in a dose dependent manner. This study also demonstrated that NITD-008 could block replication of WNV in Vero cells; however, the efficacy of NITD-008 in blocking WNV infection has not been evaluated.

Another therapeutic strategy to improve encephalitis symptoms is to target the host inflammatory response in the CNS [21]. Epigenetic control of histone proteins in the chromatin is a mechanism by which a cell regulates production of rapid, inducible and reversible pathway for gene transcription. Histone modifying enzymes such as histone deacetylase (HDAC) and histone acetyltransferase (HAT) are responsible for adding and removing acetyl groups from histone tails

that ultimately controls the coiling of chromatin and allow the transcription of genes including cytokines such as TNFα and IL-1β [17-19]. Alteration in the activity and expression of these enzymes, specifically HDACs has been reported in several inflammatory diseases [17, 20]. Since HDACs play such an important role in regulating inflammation, they have become an attractive target to attenuate inflammation.

In light of these studies, we first examined whether NITD-008 can block WNV replication *in vivo* and improve disease outcome. We further tested the hypothesis that treatment with a novel anti-inflammatory compound, suberanilohydroxamic acid (SAHA or vorinostat), an HDAC inhibitor, during the CNS phase of infection will further improve WNV disease outcome by limiting neuroinflammation.

2. Materials and Methods

2.1 Animal experiments

This study was approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC), (protocol # 12-1521) and was conducted in strict accordance with guidelines established by the National Institutes of Health and the University of Hawaii IACUC. All WNV inoculation experiments were approved by the Institutional Biosafety Committee and were conducted in the ABSL-3. Wild type C57BL/6 mice purchased from the Jackson Laboratory were bred in the vivarium of John A. Burns School of Medicine. Nine- to eleven-weeks old mice were inoculated with 1,000 PFU WNV/NY/99 (isolated from crow brain passaged

once in Vero cells) via footpad injection. NITD-008 was a generous gift from Dr. P-Y Shi and Novartis Institute for Tropical Diseases. A stock solution of NITD-008, (1.25 mg/m) prepared daily by dissolving in 6 N HCL with final pH 3.5 (using 0.1 N NaOH and citric acid buffer) was administered via oral gavage twice per day as described previously[22]. The SAHA (Cayman Chemical; 10009929) was dissolved to 12.5 mg/mL in DMSO and sterile PBS was administered via intraperitoneal route (IP) once per day. Blood samples were collected through tail vein sampling or cardiac puncture and serum was separated via centrifugation and stored at -80 °C. Mice were anesthetized and perfused with 20 mL cold PBS before tissue harvesting (brain, kidney, spleen) and the tissues were flash frozen and stored at -80 °C.

2.2 Viral quantitation in blood and tissues

Mice were perfused with 10 to 15 mL of PBS, whole brains harvested, tissue homogenized and total RNA was extracted using RNeasy kit (Qiagen). Virus titers were determined using qRT-PCR on samples of the brain, spleen and kidneys. qRT-PCR was conducted using the Applied Biosystems 7500 Real-Time PCR system with primers and FAM- and TAMRA-labeled probes specific for WNV env region and the standard curve was generated by using RNA extracted from previously titrated WNV dilutions (10000 to 0.1 PFU) as previously described [39]. The data is expressed as WNV PFU equivalents $\mu g^{-1}$of RNA. At days 3 and 5 after infection 100 – 200 µL of blood was collected via tail vein nick or blood was collected or by exsanguinations at terminal sacrifice. Serum was separated via centrifugation at 2500x g for 10 min and frozen for subsequent

analyses. Virus in the serum was quantitated by plaque assay using Vero cells, as previously described [39].

2.3 Detection of mRNA levels using quantitative real-time PCR

The mRNA levels of inflammatory cytokines (MIP-1α and TNFα) were determined by using the Applied Biosystems 7500 Real-Time PCR system with Bio-Rad IQ SYBR Green supermix and cytokine specific primers [40]. Fold change in infected tissues, as compared to uninfected controls, were calculated after normalizing to the GAPDH gene as previously described [40].

2.4 Immunohistochemistry

Mice were transcardially perfused with 20 ml PBS followed by 20 ml of 4% paraformaldehyde (PFA), brains were harvested, cryoprotected in 30% sucrose (Sigma) for 3 days at 4°C and frozen in Optimal Cutting Temperature solution (Tissue-Tek). Horizontal sections of 10 µm thickness were fixed in 2% PFA for 15 min, permeablized by incubating 0.3% Triton X-100 (Sigma) in PBS for 1 hr at room temperature and then blocked for 1 h using 5% goat serum (Jackson ImmunoResearch) in PBS. Tissue preparation and staining with monoclonal anti-CD45 antibodies (Santa Cruz) were performed as described previously. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed using an in situ cell death detection kit (Roche) according to the manufacturer's instructions. Tissue sections were also stained with haematoxylin and eosin and examined for pathological changes.

2.5 Statistical analysis.

All mRNA quantitation data is reported as SEM. Either unpaired Student's t-test or Mann–Whitney U test for single-mean comparison was conducted using GraphPad Prism 5.0 (GraphPad software). Survival curve data was compared using a Mantel-Cox Log- Rank test using GraphPad Prism 5.0 (GraphPad software). $P<0.05$ was considered as statistically significant for all analyses.

3. Results

3.1 NITD-008 protects mice against WNV infection in a dose-dependent manner

Although NITD-008 has been shown to inhibit WNV replication in vitro and is protective against DENV infection in the AG129 mouse model, we know little about the efficacy of NITD-008 in limiting WNV infection in vivo [22]. In order to determine if NITD-008 could protect against WNV infection in vivo, 10 to 11-week old C57BL/6 mice were inoculated with 1,000 PFU of WNV (NY99) via footpad injection and then administered with 25, 10, 5 or 1 mg/kg of NITD-008 via oral gavage on days 1 to 6 day after infection (Fig. 1). . As seen in Fig. 1, infected mice exhibited clinical signs typical of WNV disease, such as ruffled fur, ataxia, hunchback, etc. and resulted in 67% mortality. In contrast, mice treated with 25 and 10 mg/kg of NITD-008 were completely protected and did not show any signs of clinical disease (Fig. 1A and 1B). At the lower dose of 5 mg/kg of NITD-008, infected mice exhibited a reduced mortality rate ($P<0.05$) with very mild disease signs, as compared to untreated mice (Fig. 1C). However, treatment with 1 mg/kg NITD-008 did not have any significant difference in the mortality or

clinical score as compared to the control group. This data collectively indicates that NITD-008 is highly protective against WNV infection in vivo when administered during early stage of the infection in a dose dependent manner. The data suggests that NITD-008 is able to prevent the replication of WNV and correlates with previous in vitro studies demonstrating inhibition of WNV replication in Vero cells (Yin et. al 2009).

3.2 NITD-008 loses efficacy in WNV infection when administered during late phase of infection

We next investigated whether NITD-008 can protect mice from WNV infection at later stages of viral infection, specifically when the virus has entered the brain. WNV-infected mice were treated with 10 mg/kg of NITD-008 during the peak viremia stage (days 3-6 after infection) or during the central nervous system invasion phase (days 5-8 after infection). As seen in Fig. 2, delay of NITD-008 treatment by two days (days 3 to 6), albeit not completely protective, was able to significantly reduce the mortality of WNV-infected mice. However, treatment on days 5 to 9 days after infection exhibited a marked decrease in the survival of infected mice, which correlated with no significant difference in the clinical score of these mice when compared to mice treated on days 1 to 6. This data illustrates that NITD-008 loses its protective effect when treated during CNS-stage of WNV disease.

3.3 NITD-008 blocks WNV replication at different stages of infection

To understand the correlation of NITD-008 treatment with virus replication, we determined the levels of WNV in different groups of mice. As shown by our previous studies, WNV is in found in different peripheral tissues such as serum, spleen and kidney during days 2-6 after infection, while in the brain peak WNV replication can be detected between days 7-10 after infection. As seen in Fig. 3, at day 5 after infection WNV was not detected in the spleen and kidney of NITD-008-treated mice (10 mg/kg dose on days 1 to 6), which correlated well with significant reduction of virus replication in the serum. Further, no virus was detected in the brains of mice treated with NITD-008 starting day 1 after infection, suggesting that reduced viremia completely restricts WNV neuroinvasion in this mouse model.

3.4 Combined treatment with SAHA increases the therapeutic window of NITD-008

Having demonstrated that NITD-008 loses its protective effect when WNV has established infection in the CNS, we next evaluated the impact of the anti-inflammatory drugs on neuroinflammation and WNV disease outcome. Histone deacetylase inhibitor suberanilohydroxamic acid (SAHA) has been shown to reduce the transcription of inflammatory genes in several neuroinflammatory scenarios. Mice were treated with 100 mg/kg of SAHA for either days 3-6 or days 5-9 after infection and as seen in figure 4, SAHA treatment on days 3 to 6 after infection had no significant effect on the mortality rate of WNV-infected mice. However, when SAHA was administered during the CNS stage of infection, there was a trend of decreased mortality, but it was not significant. Since, SAHA is not

an antiviral, as expected we did not observe any significant difference in the levels of WNV in brains of mice at day 8 after infection as compared to vehicle control. The ability for SAHA to increase survival during late stage of infection without affecting WNV replication suggested that the improved disease outcome was a result of attenuated inflammation in the brain.

3.5 SAHA increases the therapeutic window of NITD-008 when treatment is combined with NITD-008

Our results demonstrated that independently both NITD-008 and SAHA had slight but not significant effect on the survival of infected mice when treated during the CNS phase of the disease, suggesting that reducing virus replication or inflammation alone is not enough to improve disease outcome. Therefore, we tested the efficacy of combined therapy of these two drugs in protecting mice from progressing to encephalitis. As seen in Fig. 5, mice receiving both 10 mg/kg NITD-008 and 100 mg/kg SAHA (days 5 to 9) showed a significant reduction in the mortality rate (10%) when compared to groups treated with SAHA and NITD-008 alone (Fig. 4B, P=0.002).This finding supports our hypothesis that combination therapy would be effective in improving WNV disease outcome.

3.6 Co-treatment with SAHA and NITD-008 attenuates the mRNA levels of inflammatory cytokines in the brain

Since pan-HDAC inhibitors are shown to exert multiple neuroprotective properties, we examined the specific effect of SAHA treatment on WNV replication and neuroinflammation in the brain. WNV load in the brains of

different groups of treated mice demonstrated that SAHA treatment had no effect on virus replication in the brains when given alone or in combination with NITD-008 (Fig 2B). Increased inflammatory cytokines in the brain are markers for disease severity in neuroinflammatory disorders and viral encephalitis [41]. WNV infection causes the increase of pro-inflammatory cytokines by activating pattern recognition receptors, which in turn increase the transcription of cytokines such as TNF-α and IL-1β. Using quantitative real time PCR we analyzed the mRNA transcripts of pro-inflammatory cytokine TNF-α and chemokine MIP1α in the brain of mice of different groups. As seen in Fig 5, we observed a significant increase in TNF-α and MIP1α in animals infected with WNV. When NITD-008 treatment was started at day 5 after infection, there was a slight decrease in the transcripts TNF-α and MIP1α. However, the group that received SAHA alone or SAHA plus NITD-008 starting day 5 after infection exhibited significantly lower levels of TNF-α and MIP1α transcripts. This data suggests that the approach of combined therapy results better in preventing inflammation and WNV replication, thus improving the overall disease outcome as compared to independent treatment.

3.7 Treatment with NITD-008 and SAHA reduce WNV-associated neuropathology markers in the brain

Markers of WNV-associated brain injury were further compared in mice treated with SAHA and NITD-008 to understand the correlation between these drugs and neuropathology. Infiltrating leukocytes are the catalyst and primary cause of inflammation in the brain of WNV-infected mice. H&E staining of brain sections of

untreated mice demonstrated infiltration of leukocytes in the brain accompanied with astrogliosis and degenerating neurons as evident by loss of nuclei in Fig 6. However, treatment with 10 mg/kg of NITD-008 starting day 1 after infection did not exhibit any injury of the neurons (Fig 6). Immunostaining of CD45, a marker of infiltrating leukocytes demonstrated an increase in CD45+ cells in the brains, especially around microvasculature in the untreated mice, which reduced significantly in mice treated with NITD-008 beginning day 1 after infection. However, when NITD-008 treatment began at day 5 after infection, infiltration of CD45+ cells into the brain was clearly visible, which decreased significantly in the mice treated with both SAHA and NITD-008.

4. Discussion

The continuous expansion of WNV-associated neurological disease in the United States underscores the need for effective antiviral strategies. Effective therapy to manage cases of WNV is proposed to target either viral proteins that are essential for virus replication or host molecules that mediate neuronal damage. The objective of this study was to test the effect of the small molecule-based viral inhibitor NITD-008 developed for DENV and a newly characterized anti-inflammatory compound on the WNV replication kinetics and disease progression using a mouse model of infection. The highlights of this study are (i) the treatment of mice with NITD-008 significantly attenuates viremia and prevents neuroinvasion, resulting in complete protection from WNV-associated mortality and morbidity (ii) NITD-008 loses its protective effect when administered during

CNS phase of the disease and (iii) co-treatment with SAHA during the later stages of WNV disease improves disease outcome by reducing inflammation.

NS5 polymerase is the most conserved protein among flavivirus and comprises the RNA-dependent RNA polymerase and methyltransferase domains. Therefore, NS5 is an ideal target for the development of small molecule inhibitors. NITD-008 is a nucleoside analog inhibitor that has recently shown promising antiviral effects against DENV [18]. Based on the encouraging results of nucleoside analog inhibitors against other viruses such as HIV and HCV, nucleoside analogue inhibitors remain the most promising class of antiviral compounds against flaviviruses. To date, there is no report of the use of a nucleoside analogue inhibitor against WNV in vivo. However, since NS5 is a highly conserved flavivirus protein and based on the fact that NITD-008 blocked WNV replication in Vero cells in vitro, we tested the antiviral efficacy of NITD-008 against WNV infection in mice. Our findings show that NITD-008 significantly reduced viral load (Fig. 3) and was protective against WNV mortality in mice in a dose dependent manner (Fig. 1). Further, we determined that 10 mg/kg is the lowest dosage of NITD-008 that completely protects WNV-infected mice from disease symptoms and associated mortality when administered 24 hours after infection (Fig. 1). This is similar to the in vivo efficacy of NITD-008 observed in DENV infected AG129 mice. Interestingly, in addition to flaviviruses, recent studies demonstrate that NITD-008 has potent antiviral activity *in vitro* and *in vivo* against enterovirus 71 [42].

The time of administration of any antiviral is a critical factor when determining the efficacy of novel compounds. Our data indicate that NITD-008 remained moderately effective in protecting mice from WNV-associated mortality when the treatment of NITD-008 was delayed by 3 days. However, when NITD-008 treatment began at day 5 after infection, there was no substantial difference in disease outcome as compared to untreated animals. There can be several reasons for the failure of NITD-008 protection at late phase. It is possible that although the 10 mg/kg dosage of NITD-008 reduced virus replication but was not enough to block virus-CNS invasion. On the other hand it is also likely that by day 5, WNV infection in neurons is already established resulting in a high level of replication and inflammation. Further, the ability of NITD-008 to cross the BBB is not yet characterized, therefore there is a possibility that NITD-008 is not able to completely diffuse into the CNS and block WNV replication in the brain. This loss of protection in delayed NITD-008 treatment is not surprising and was also seen with DENV. Yin and colleagues reported that delayed start of treatment with NITD-008 at 48 hr after infection did not protect DENV-associated mortality in AG129 mice. Similarly, previous studies using disease models of Ebola virus, influenza virus, HIV, and tuberculosis support the fact that the time of treatment is critical for the disease outcome [43-45]. Oestereich et al. showed that antiviral favipiravir that was protective against Ebola infection in mice lost its efficacy when the treatment was delayed by 2 days [46]. Liao et al. determined that the addition of Germacrone or Ribavirin in MDCK and A549 cell cultures could inhibit viral replication, but only if added within the first 2 hours of infection [47].

Therefore, because of this limitation of timing of antiviral drug treatment, they can be proposed as effective prophylactic treatment to control virus spread, but are not the best option to treat acute disease conditions. Another complication with neurotropic viruses including WNV is that by the time patients exhibit symptoms of severe disease such as encephalitis, WNV is almost cleared from the periphery and brain. In this scenario, administration of antivirals might not be enough to prevent disease progression. In a mouse model of infection, WNV replication in the brain is detected between days 6-10 after infection. At day 7 after infection, WNV infection in neurons and glial cells results in the production of pro-inflammatory cytokines and chemokines such as TNFα. Chemokines such as MIP-1α, are crucial in recruiting peripheral leukocytes such as CD8+ T cells that are critical in clearing WNV. However, exacerbated inflammation later becomes the cause of neuronal damage. The symptoms of severe disease are typically observed in mice between days 9-12, when inflammation is at its peak. Therefore, by mitigating the inflammation or disruption of the blood-brain barrier (BBB), WNV disease outcome is shown to be improved [48].

In our study, we chose SAHA as an anti-inflammatory drug to attenuate neuroinflammation because this drug is able to cross the BBB and has shown promise in treating inflammatory encephalopathy [49-52]. Ge and colleagues showed that treatment with SAHA reduced clinical severity of autoimmune encephalomyelitis in mice by reducing inflammation in the brain [50]. A recent study by Singh and colleagues demonstrated that SAHA reduced the levels of pro-inflammatory cytokines, inducible nitric oxide and activation of NF-κB in

mouse primary astrocytes with induced X-ALD neuroinflammation [49]. Further, Fang et al. utilized SAHA to reduce neuronal apoptosis due to sepsis-associated encephalopathy in the cecal ligation puncture rat model of sepsis. Our results further support the effect of SAHA in attenuated WNV-associated inflammation in the brain. Our results clearly demonstrate that when NITD-008 treatment begins during late stage of infection, the addition of 100 mg/kg SAHA can prevent the progression of WNV disease (Fig. 4). This is a novel finding, as SAHA has not been previously used as an anti-inflammatory to treat any acute viral infection.

The production of pro-inflammatory cytokines is responsible for the activation of microglia cells, which can contribute to the death of naïve neurons via bystander injury and by causing glutamate excitotoxicity in neurons [53-55]. While this response is damaging during the CNS phase of WNV infection, robust inflammation is essential in clearing the virus from the peripheral organs and fine-tuning the innate-adaptive interface during earlier phases of disease [12]. Therefore, we theorized that the treatment of SAHA during early phase of WNV disease (days 3-5 after infection) would not improve the disease outcome. Epigenetic regulation of gene expression is cell type specific and several recent studies support the fact that brain cells have very different activities of HDACs and HATs [56]. Although more detailed study is warranted to carefully delineate the role of epigenetic mechanisms in WNV-associated immune responses, our results indicate that epigenetic machinery is impaired during CNS infection of WNV and plays a role in modulating neuroinflammation.

A major limitation of targeting host machinery is the possibility of undesirable side effects in long-term treatments, because these factors also play important role(s) in cell survival. However, the approach of short-term treatment for controlling acute diseases, such as encephalitis associated with WNV and related neurotropic pathogens such as JEV, may not face similar shortcomings as compared to long-term blocking of inflammatory molecules in chronic diseases, such as AD or HIV infections [57]. In conclusion, our results for the first time provide conclusive evidence that NITD-008 is an effective antiviral compound that protects mice from encephalitis when administered during early stage of disease. Current research is actively addressing the toxicity associated with long-term usage of this drug, which might lead to the development of NITD-008 as the most effective prophylactic antiviral against WNV. Our study acknowledges the limitation of NITD-008 to treat acute WNV-associated disease and therefore we propose that advantage of combined therapy of this drug with effective anti-inflammatory drug to manage clinical cases of WNV-encephalitis. These findings are significant because they lay the platform for future studies in mice and humans to develop novel therapeutic options to manage WNV and other neurotropic viruses.

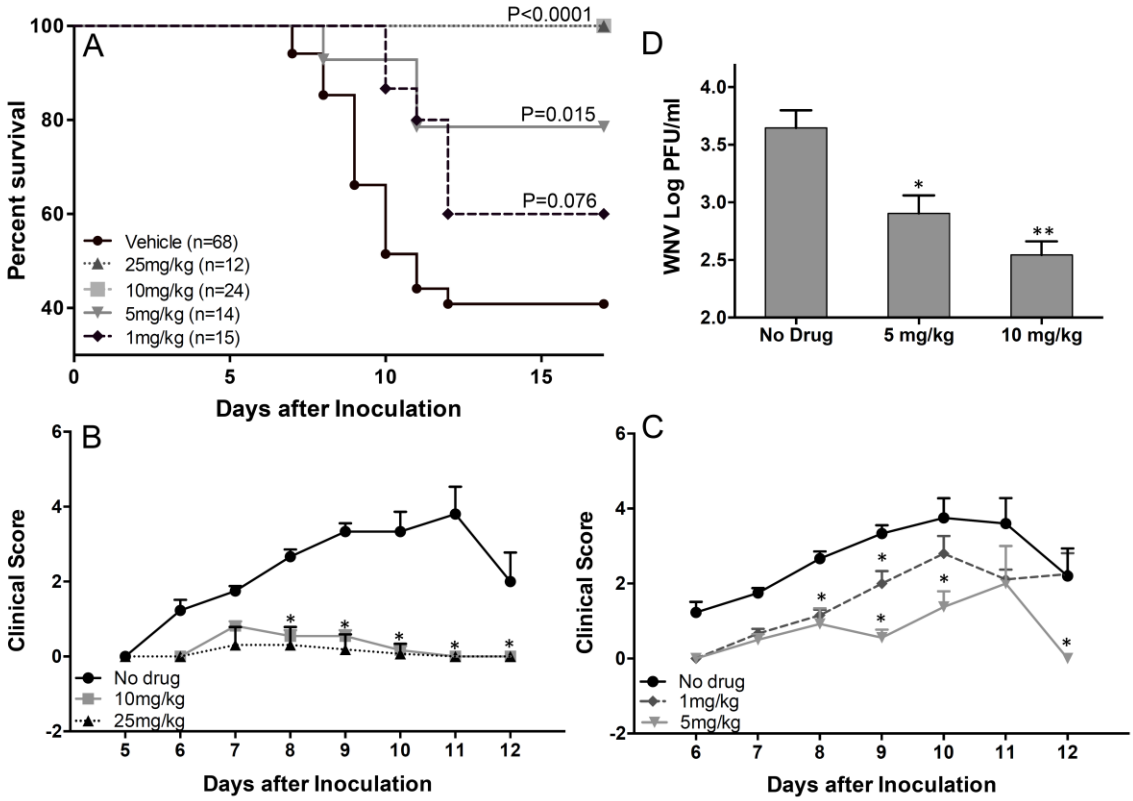

Figure 1: NITD-008 protects against lethal challenge with WNV. (A-C) C57BL/6 mice inoculated with 1000PFU WNV (NY99) via footpad injection were administered with different doses of NITD-008 via oral gavage twice per day from day 1 to 6 after inoculation and observed for clinical symptoms and mortality. Clinical score criteria: 1, ruffled fur; 2, ruffled fur with distinct hunched back; 3, paresis/difficulty walking; 4, paralysis; 5, moribund/euthanized; (D) WNV titers measured in the serum at day 3 after infection using plaque assay. Error bar represent ±SEM from at least 4 mice/group. ** p< 0.005, * p<0.05

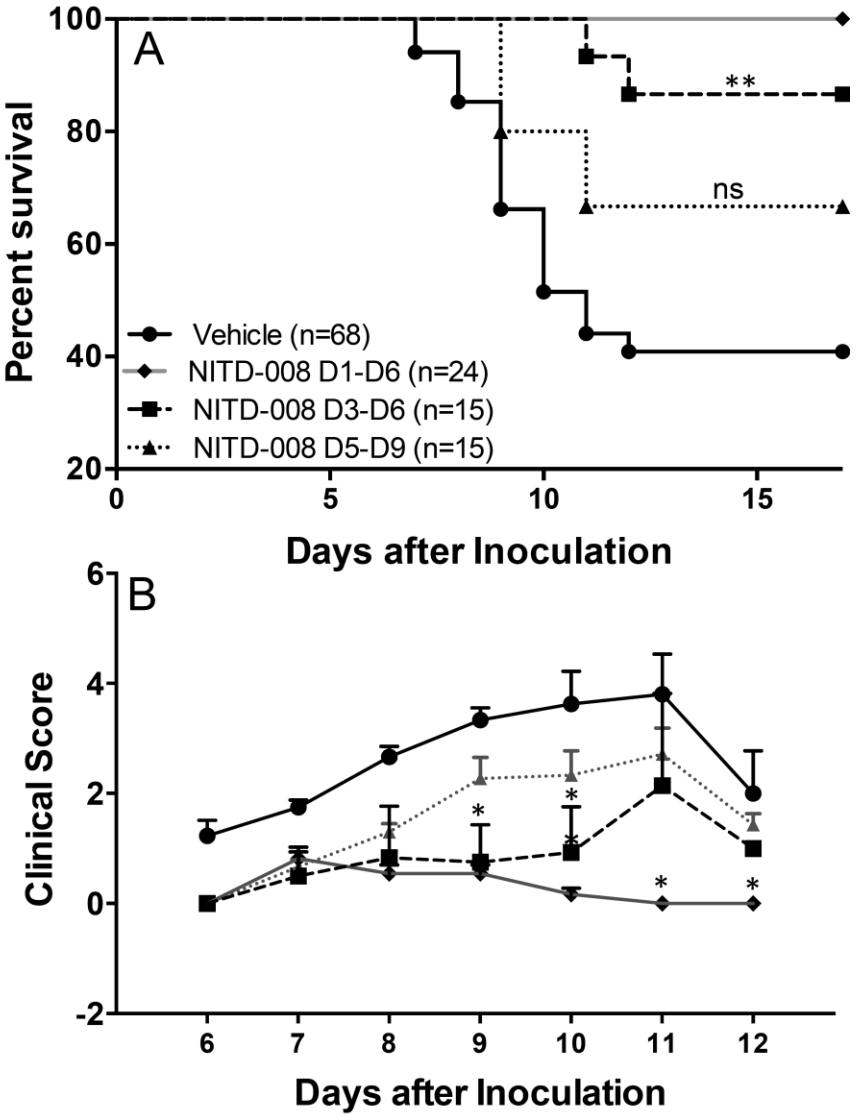

Figure 2: Protective effect is lost when NITD-008 treatment is delayed. WNV-infected mice were administered with vehicle or 10 mg/kg NITD-008 on either days 1-6, 3-6 or 5-9 and observed for (A) mortality and (B) clinical symptoms. Error bar represent ±SEM. **p< 0.005, *p<0.05

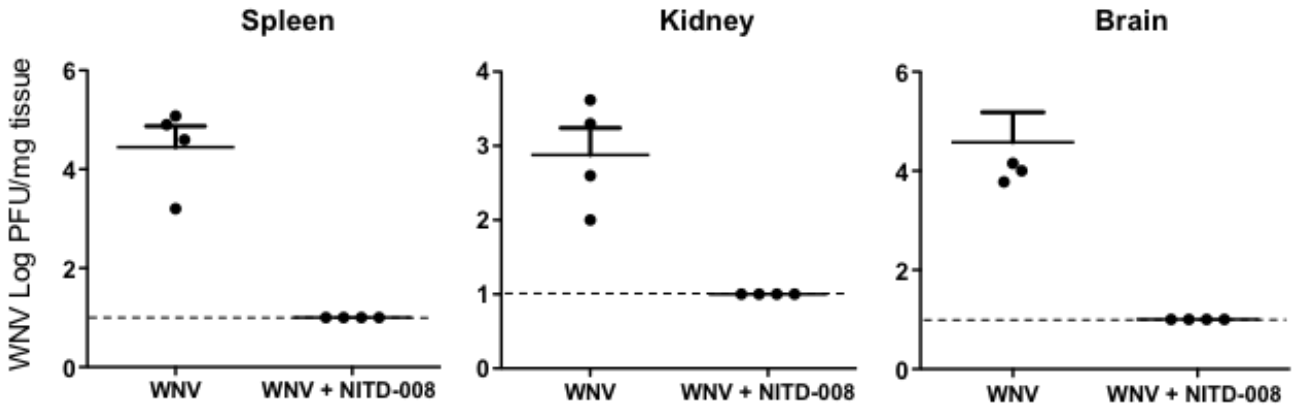

Figure 3. NITD-008 blocks WNV dissemination to peripheral tissues and brain Nine to ten week-old C57BL/6 mice were inoculated with 1,000 PFU WNV (NY99) via footpad injection and treated with vehicle or 10 mg/kg NITD-008 on days 1-6 after inoculation. Mice were sacrificed at day 5 (spleen and kidney) and day 8 (brain) after infection and WNV titers were analyzed in tissue homogenates using plaque assay. Dotted line-detection limit of plaque assay and *n*= at least 4 animals per group.

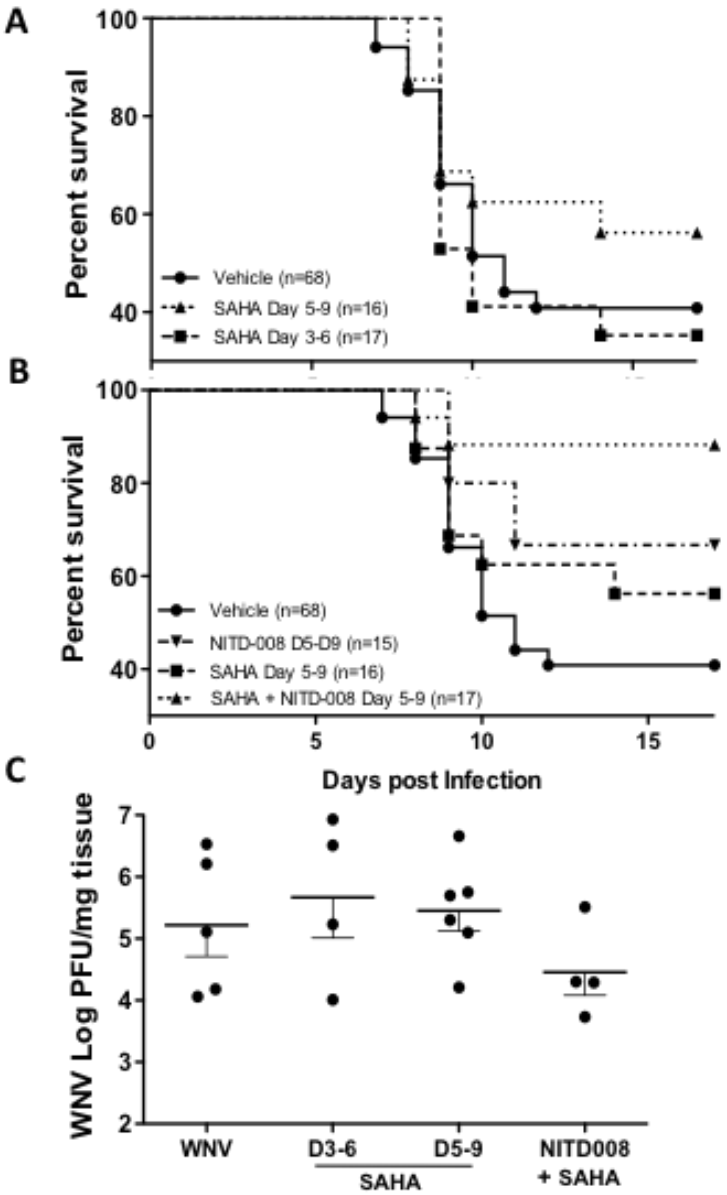

Figure 4. Co-treatment of SAHA with NITD-008 improves outcome of lethal challenge with WNV: (A) Effect of SAHA treatment on WNV disease outcome. Survival of 10 week-old C57BL/6 mice infected with 1,000 PFU of WNV following treatment with 100 mg/kg of SAHA. SAHA was administered on days 3 to 6 or 5 to 9 after inoculation, once per day via IP injection. (B) Survival of 10 week-old C57BL/6 mice infected with 1,000 PFU of WNV following treatment with either 100 mg/kg of SAHA, 10 mg/kg NITD-008 or co-treatment of 100 mg/kg of SAHA and 10 mg/kg NITD-008 on days 5 to 9 after inoculation. (C) WNV titers in the brain from infected mice treated with SAHA (days 3 to 6), SAHA (days 5 to 8) and SAHA plus NITD-008 (days 5 to 8). Brains were harvested at day 8 after inoculation and WNV titers were analyzed in the total RNA extracted using qRT-PCR.

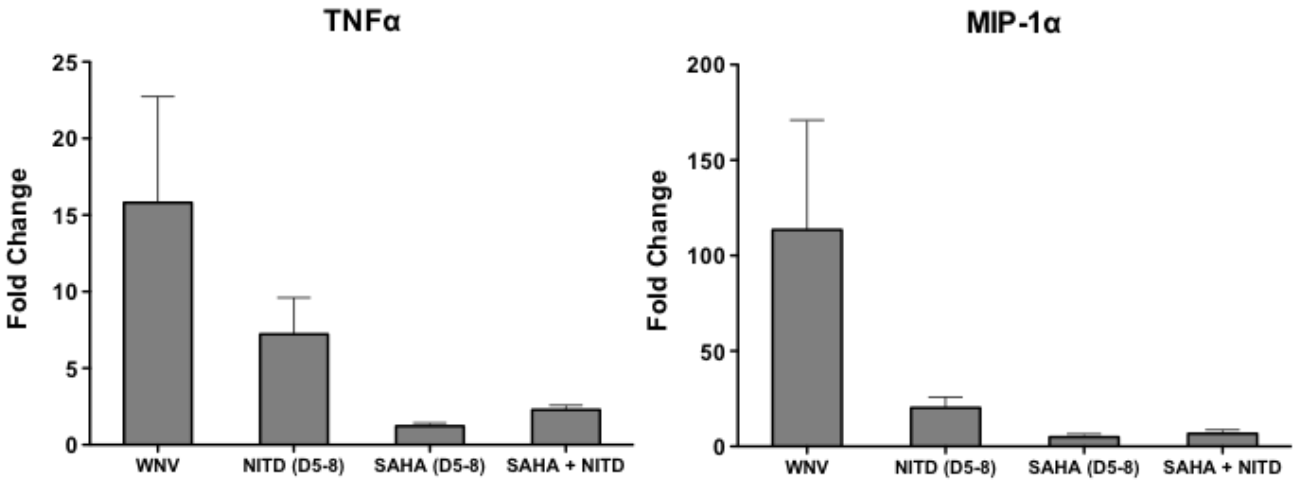

Figure 5. Treatment with NITD-008 and SAHA attenuates inflammatory cytokines in infected mouse brains. Brains were harvested from mice at day 8 after infection treated with 10 mg/kg NITD-008, 100 mg/kg SAHA or both compounds on days 5 to 8 after inoculation. Total RNA extracted was used to analyze the levels of mRNA of TNF-a and MIP1-a using specific mouse primers and qRT-PCR. The data was normalized to GAPDH and is represented as fold change as compared to un-infected controls. n= at least 4 animals per group

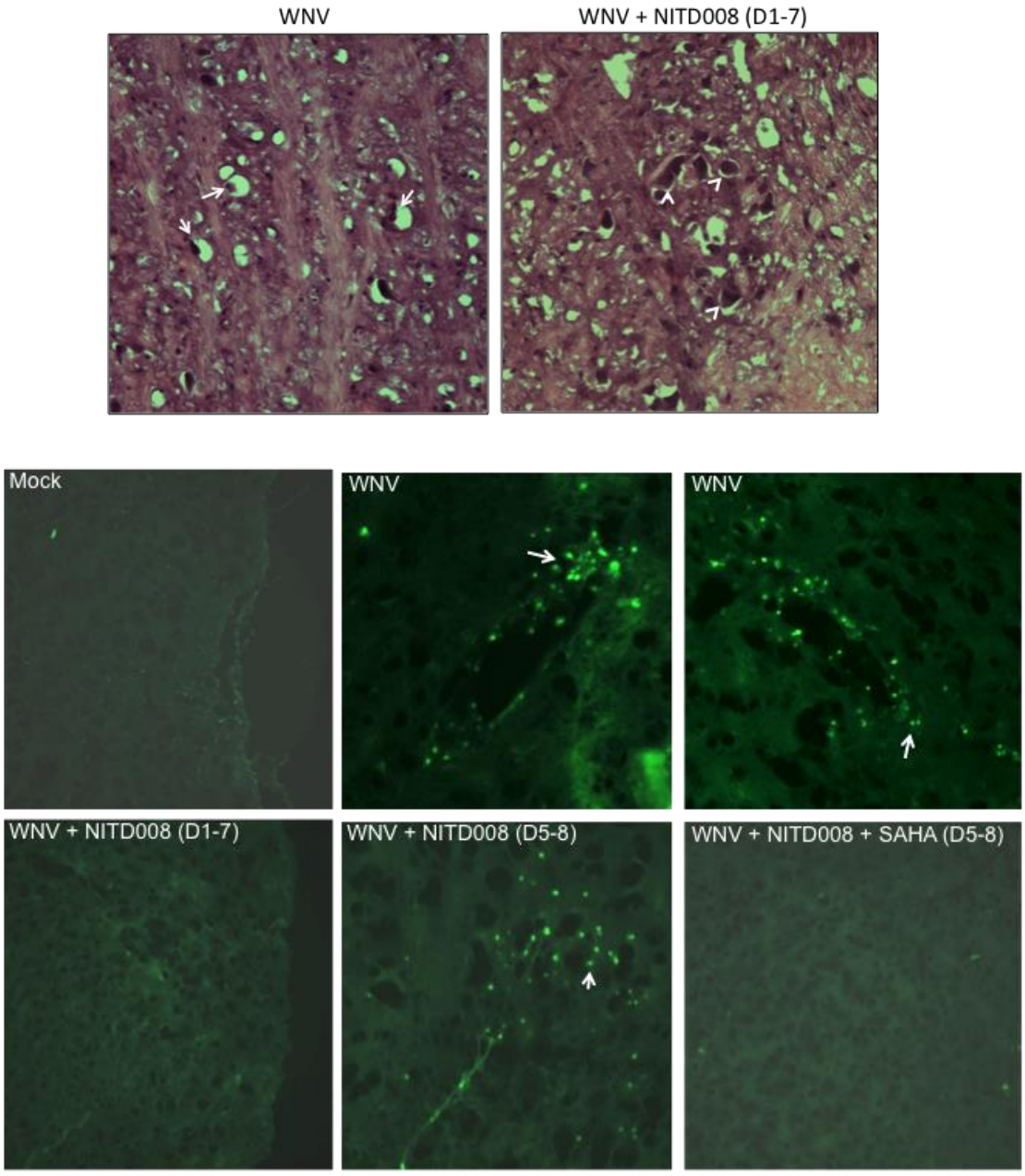

Figure 6. Treatment with NITD-008 and SAHA reduces markers of WNV-associated neuropathology in the brain: (A) H&E staining of PFA perfused brains depicts shrunken neuron body (white arrows) with light pink cytoplasmic staining representing degenerating neurons in the brain of WNV infected mice at day 8 after inoculation. NITD-008 treatment demonstrated significant improvement in the neuron body (white arrow head). (B) Infiltration of immune cells (white arrows- Green CD45+ cells, marker for leukocytes) was prominent in the brain of WNV-infected mice at day 8 after infection, specifically near brain microcapillaries. The staining of anti-CD45 was significantly reduced in the brain from mice treated with early NITD-008 and combined treatment of NITD-008 + SAHA. Figure represents data from at least two mice in duplicate.

Chapter 4

Summary

Summary

West Nile virus (WNV) has proven to be a major cause of viral encephalitis since emergence in the United States in 1999. Despite efforts to control the spread of the virus, clinical cases are now seen in 48 states. In 2013, there were 2,469 cases of WNV infection, resulting in 119 deaths, bringing the total number of cases and deaths to 39,557 and 1,668, respectively. While the year-to-year number of infections has been unpredictable, it continues to remain a major public health concern.

There is no vaccine available for humans and no approved antiviral for prophylactic treatment or for clinical management of WNV infection. There is an urgent need to develop an effective antiviral for treating WNV infection. However, since much of the pathology is due to inflammation generated by the host response to WNV infection, other methods of treatment also need to be investigated. In this study, we investigated the efficacy of a novel antiviral compound (NITD-008 generated by Novartis Institute for Tropical Disease) in the treatment of WNV for the first time *in vivo* and an anti-inflammatory agent as a method of preventing WNV-associated neuroinflammation.

The development of NITD-008 was intended to be a preventative measure for DENV infection. Yet due to high homology between the NS5 protein of DENV and WNV that is targeted by NITD-008, we proposed that this drug might be effective at blocking the replication of WNV as well. We showed

that not only is it effective in blocking WNV replication in mice, it is also able to completely prevent death due to WNV. We determined a dosage and treatment window for NITD-008 in mice that would protect against WNV induced mortality. This is a novel finding and an important step in developing an antiviral to treat WNV infection.

The inflammation that is seen during WNV infection is a contributing factor to the severity of WNV disease. Infiltration of CD45+ cells along with production of pro-inflammatory cytokines such as TNFα contribute to the destruction of neurons. SAHA has been shown previously to lower inflammation in other neuroinflammatory diseases, but our study for the first time has used SAHA to decrease the inflammation associated with virus infection. Further, this study marks the first time that any anti-inflammatory agent has improved WNV disease outcome *in vivo*.

The results of this study are significant as they lay a platform for future studies in mice and then humans to ultimately develop these drugs as novel therapy options to treat WNV and other neurotropic flaviviruses such as JEV.

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