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An iminosugar with potent inhibition of dengue virus infection *in vivo*

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ABSTRACT

The aim of the present study was to evaluate the ability of the iminosugar drug UV-4 to provide *in vivo* protection from lethal dengue virus (DENV) challenge. This study utilized a well-described model of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS)-like lethal disease in AG129 mice lacking the type I and II interferon receptors. Herein, we present UV-4 as a potent iminosugar for controlling DENV infection and disease in this mouse model. Specifically, administration of UV-4 reduced mortality, as well as viremia and viral RNA in key tissues, and cytokine storm. In addition, UV-4 treatment can be delayed, and it does not alter the anti-DENV antibody response. These results have set the foundation for development of UV-4 as a DENV-specific antiviral in phase I human clinical trials.

1. Introduction

With rapid increase in geographic spread and lack of vector control, dengue virus (DENV) has re-emerged as an important infectious disease pathogen. Transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes, the virus causes a wide variety of symptoms in people in a complicated interplay with the host immune system (Halstead, 2007). Clinical disease ranges from dengue fever (DF), an acute, self-limiting febrile illness, to the more severe and life-threatening dengue hemorrhagic fever and shock syndrome (DHF/DSS) (Halstead, 2007). Despite an annual number of infections approaching 50 million worldwide and a rise in disease severity, no vaccines or antiviral therapies are currently available (Coller et al., 2010; Gubler, 1998; Julander et al., 2011).

Although vaccines have been successfully created against other flaviviruses, such as yellow fever virus (YFV) and Japanese encephalitis virus (JEV), an incomplete understanding of dengue immunopathogenesis has impeded the development of a safe and effective vaccine or antiviral. DENV exists as four antigenically distinct serotypes (DENV1-4), each containing multiple genotypes that cause infection in humans (Gubler, 1998). Epidemiological studies of dengue endemic areas have revealed an increased risk for severe disease during a secondary infection. One of the proposed mechanisms of this phenomenon is known as antibody-dependent enhancement (ADE) of disease. In brief, cross-reactive, non-neutralizing antibodies generated during a primary infection by one DENV serotype or acquired passively at birth, contribute to the development of severe disease upon infection by one of the other three serotypes (Balsitis et al., 2010; Zellweger et al., 2010). Another leading theory, T cell original antigenic sin, involves cross-reactive T cells from the original infecting serotype contributing to the immunopathogenesis of a secondary heterologous infection due to their low-affinity (Rothman, 2011). In light of these theories, a DENV vaccine that induces robust, long-term protection across all serotypes and a therapeutic that has broad-spectrum antiviral activity would be most effective.

An antiviral that targets a common host pathway could provide an advantage to the issues faced by therapies targeting viral enzymes or mechanisms, such as viral heterogeneity and the

Abbreviations: DENV, dengue virus; DF, dengue fever; DHF/DSS, dengue hemorrhagic fever/dengue shock syndrome; YFV, yellow fever virus; JEV, Japanese encephalitis virus; ADE, antibody dependent enhancement; ER, endoplasmic reticulum; DNJ, deoxynojirimycin; NGC, New Guinea C; GE, genomic equivalents; PFU, plaque forming units; PrM, pre-membrane; E, envelope; LD_{90}, 90% lethal dose.

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emergence of drug resistant mutants. Many mammalian viruses, including DENV, rely on host glycosylation machinery to modify envelope glycoproteins. In particular, as flaviviruses assemble in the endoplasmic reticulum (ER), the oligosaccharide $Glc_3Man_9GlcNAc_2$ is added to specific asparagine residues on the structural proteins prM (membrane) and E (envelope) that form the immature viral particle (Whitby et al., 2005). ER resident enzymes α-glucosidases I and II sequentially modify the attached carbohydrate for additional processing with protein folding chaperones calnexin and calreticulin (Block and Jordan, 2001; Chang et al., 2011a; Zitzmann et al., 1999). Inhibiting host glycosylation pathways can lead to the misfolding of viral glycoproteins resulting in the reduced assembly, secretion, and infectivity of viral particles (Block and Jordan, 2001; Chapel et al., 2007).

Iminosugars are monosaccharide mimics that competitively inhibit glycoprotein processing enzymes α-glucosidases I and II (Chang et al., 2011a; Qu et al., 2011). Iminosugars have been widely explored as antivirals against many enveloped viruses, including DENV, and demonstrate selective inhibition of viral assembly and secretion (Chang et al., 2009). Recent studies with iminosugars, including celgosivir, indicate that these compounds may be effective *in vivo*, although the impact of iminosugars on levels of viral RNA in tissues and cytokines needs to be determined (Watanabe et al., 2012). Unlike published studies to date, we present a thorough characterization of an iminosugar compound and its effect on DENV infection *in vivo*. An *in vivo* screen of iminosugars derived from the molecule deoxynojirimycin (DNJ) identified one compound, UV-4, to be effective at protecting mice from lethal DENV challenge in a dose dependent manner. Subsequent studies indicated that UV-4 reduced both viral titer in tissues and various cytokine levels in circulation without impacting DENV-specific antibody response, and that UV-4 treatment can be delayed. This comprehensive analysis has set the foundation to evaluate UV-4 as an effective therapeutic against DENV infection.

2. Materials and methods

2.1. Compounds

Iminosugar compounds tested in this study include *N*-butyldeoxynojirimycin (*N*B-DNJ; UV-1), *N*-nonyl-DNJ (UV-2), *N*-7-oxadecyl-DNJ (UV-3), *N*-9-methoxynonyl-DNJ (UV-4), and *N*-*N*-4′-azido-2′-nitrophenyl-6-aminohexyl-DNJ (UV-5). All were solubilized in H_2O at a concentration of 20–40 mg/mL by sonication in water bath (Branson 200, Ultrasonic Cleaner) with addition of HCl to a final pH of 6.2–6.5. Ribavirin (SIGMA) was diluted in PBS.

2.2. Cells and viruses

For *in vitro* studies, DENV2 New Guinea C (NGC) was used for determining antiviral effects of UV-1 through UV-5 on Vero cells. For *in vivo* studies, DENV2 strain S221 was used (Perry et al., 2009; Prestwood et al., 2012; Zellweger et al., 2010). Viral stocks were grown in C6/36 cells, concentrated by ultracentrifugation, purified over a sucrose gradient, and quantified by real-time reverse transcription-PCR (RT-PCR) expressed as genomic equivalents (GEs) (Prestwood et al., 2008). Infectious titer was determined by plaque assay on BHK-21 cells described below, and a ratio of approximately 5×10^4 GE per plaque forming unit (PFU) was determined (Prestwood et al., 2008).

2.3. Plaque and $TCID_{50}$ assay for in vitro DENV inhibition

All incubations were performed at 37 °C and 5% CO_2. For plaque assay, Vero cells were seeded at 2×10^5 cells per well into 6-well plates and incubated overnight. Cells were infected with DENV2 NGC with various dilutions of compounds UV-1, UV-2, UV-3, or UV-5 for 1 h before infection with virus. After 5 days the supernatents were harvested and the virus concentration determined using plaque assay. Vero cells in 6 well plates were infected with serial dilutions of the supernatents. The infection medium was aspirated and replaced with 1% low-melt agarose (Lonza) and 1X MEM supplemented with 5% heat-inactivated FBS. After incubation of the plates for 5–7 days, 5% glutaraldehyde in PBS was added, incubated for 1 h at room temperature, and aspirated. Cells were stained with 0.1% crystal violet/5% methanol and scored visually for plaques. IC_{50} values were calculated using a 4PL curve-fit in Microsoft Excel add-in XLFit (IDBS). For compound UV-4, $TCID_{50}$ assay was performed. Vero cells were seeded in 96-well plates in 1X MEM supplemented with 1% FBS at 1×10^4 cells per well and incubated overnight. Cells were infected with DENV2 NGC at nine wells per virus dilution and incubated for 7 days at 37 °C. Cells were fixed with 5% glutaraldehyde and stained with 0.1% crystal violet/5% methanol. Cytopathic effect was scored visually and $TCID_{50}$ calculated using the Spearman–Karber method. All compounds were prepared at Integrated Biotherapeutics, Inc., in 100 mM DMSO.

2.4. Plaque assay for measuring infectious DENV titers in mice

Infectious titer of DENV2 strain S221 in animal tissues was determined by plaque assay as previously described (Shresta et al., 2004). Briefly, 2×10^5 BHK-21 cells were seeded in 24-well plates in 1X MEM (GIBCO) supplemented with 10% FBS, penicillin, streptomycin, and HEPES. Confluent monolayers were infected with 100 μL of diluted infected serum or whole wet tissue homogenates. After one-hour incubation, BHK-21 cells were covered with 1 mL 1% carboxymethyl cellulose in 1X MEM (2% FBS, penicillin, streptomycin, HEPES). Four days later, cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet diluted in 20% ethanol. Plaques were scored visually and expressed as number of PFU per mL or per gram of wet tissue.

2.5. Pharmacokinetics study

SRI International (Menlo Park, CA, USA) performed pharmacokinetic (PK) analysis of compound UV-4 using Balb/C mice 6–8 weeks of age. UV-4 was dissolved in water for oral administration (p.o.) and dissolved in PBS for intravenous (i.v.) administration. A total of three mice per time point were bled with no more than two time point samples collected from each mouse. A maximum of 300 μL of blood was collected from the retro-orbital sinus under isoflurane anesthesia into tubes containing K_3EDTA and processed to plasma. Plasma samples were kept at $\leqslant -60$ °C until analysis. Blood collections were done 5, 15, and 30 min and 1, 2, 4, 8, 24, and 48 h post-dosing. Quantification of UV-4 entailed the addition of 200 μL of a solution of 100 ng/mL UV-4 (internal standard) in 100% methyl alcohol to precipitate plasma proteins. Samples were vortexed for 10 min, centrifuged, followed by transfer of supernatants to be dried by vacuum centrifugation for 2 h. The samples were reconstituted with Milli-Q water and ethyl acetate, vortexed, and centrifuged. The upper organic layer was transferred, dried, and reconstituted with 90% acetonitrile in Milli-Q-Water with 0.1% formic acid. These mixtures were vortexed for 10 min, clarified by centrifugation, and transferred for LC–MS/MS analysis. Study samples were quantitated using a set of calibration standards prepared in blank matrix that were processed in parallel. Plasma drug level data were analyzed using WinNonlin version 5.2 Professional by noncompartmental modeling.

2.6. Mice

AG129 mice (129/Sv mice deficient in type I and II IFN receptors) were bred and housed under SPF conditions at the La Jolla

Institute for Allergy & Immunology (LIAI). All experimental procedures were preapproved and performed according to guidelines set by the LIAI Animal Care and Use Committee. Mice sex-matched at 5–6 weeks of age were infected i.v. via tail vein with S221 diluted into 200 μL PBS with 5% FBS. 5 μg of monoclonal antibody 2H2 (IgG2a anti-DENV1-4 prM/M) was administered intraperitoneally (i.p.) in 200 μL PBS 30 min prior to infection when indicated. For survival studies, mice were sacrificed when moribund or at the first signs of paralysis.

2.7. Viral RNA harvest and quantification

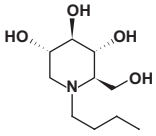
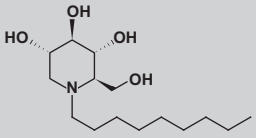
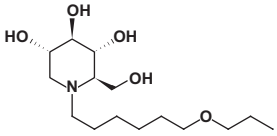
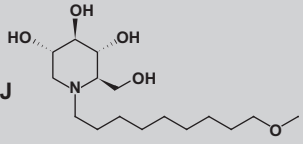
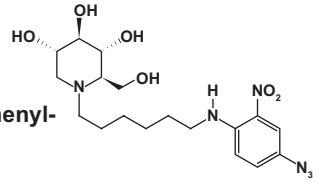
Mice were euthanized via isoflurane inhalation and blood was collected by cardiac puncture. Mice were perfused with 60 mL of PBS and tissues collected into RNALater (QIAGEN). Whole tissues were homogenized for 3 min at 4 °C in 1 mL of tissue lysis buffer (QIAGEN RLT Buffer) using the QIAGEN TissueLyser (Retsch). Liver and small intestine homogenates were diluted 5-fold and homogenized for three additional minutes. RNA was isolated from 30 μL serum using the QIAmp viral RNA minikit (QIAGEN) and from tissue homogenates using the RNeasy minikit (QIAGEN) according to the manufacturer's instructions, eluted, and stored at −80 °C until analysis. Quantitative RT-PCR to detect DENV RNA and 18S rRNA was performed as previously described (Prestwood et al., 2008). Viral load is expressed as GE per mL in serum or GE normalized to copies of relative 18S in tissues.

2.8. Cytokines

Serum from naïve and infected animals was analyzed by the Duke Human Vaccine Institute, Duke University, Durham, NC USA, using the Mouse 20-Plex kit (Invitrogen). TNF in the serum was measured using the mouse TNF-α ELISA Ready-Set-Go kit (eBioscience) according to the manufacturer's instructions.

2.9. DENV ELISA for IgM and IgG

Sucrose gradient-purified DENV2 strain S221 (10^9 GE per well) was used to coat 96-well plates. Next, virus on plates was UV inactivated and plates were washed of unbound virus using 0.05% (v/v) Tween 20 (Sigma) in PBS (GIBCO). After blocking with Blocker Casein in PBS (Thermo Scientific) for 1 h at room temperature, naïve and infected serum samples were titrated on the plate. After 1.5 h at room temperature, plates were washed with 0.05% (v/v) Tween 20 in PBS to remove unbound antibody. Bound antibody was detected using either HRP-conjugated goat anti-mouse IgM antibodies or goat anti-mouse IgG antibodies (Sigma) and TMB (eBioscience).

Compound	Structure	Molecular Formula & Weight	Toxicity (CC_{50} μM)	Potency (IC_{50} μM)	SI
UV-1 N-butyl-DNJ		$C_{10}H_{21}NO_4$ 219.28 g/mol	>500	162	>3
UV-2 N-nonyl-DNJ		$C_{15}H_{31}NO_4$ 289.42 g/mol	125	9	13.8
UV-3 N-7-oxadecyl-DNJ		$C_{15}H_{31}NO_5$ 305.41 g/mol	>500	41	>12
UV-4 N-9-methoxy-nonyl-DNJ		$C_{16}H_{33}NO_5$ 319.44 g/mol	>500	17	>29
UV-5 N-N-4'-azido-2'-nitrophenyl-6-aminohexyl-DNJ		$C_{18}H_{28}N_6O_6$ 424.46 g/mol	350	2	175

DNJ, deoxynojirimycin; CC_{50}, 50% cytotoxic concentration; IC_{50}, 50% DENV inhibition concentration; SI, Selective Index (CC_{50}/IC_{50})

Fig. 1. Antiviral profiles of deoxynojirimycin iminosugar compounds against DENV. Toxicity and antiviral profiles of compounds UV-1 through UV-5 and their corresponding structures and molecular characteristics. CC_{50} and IC_{50} values were calculated using Vero cells that were infected with DENV2 NGC in the presence of compound.

3. Results

3.1. Antiviral activity of selected iminosugars in vitro

Many preclinical studies have pointed to the potential of iminosugars as antiviral agents against enveloped viruses (Chang et al., 2011a,b, 2009; Durantel et al., 2001; Fischer et al., 1995; Gu et al., 2007; Gubler, 1998; Mehta et al., 2002; Nash et al., 2011; Qu et al., 2011; Wu et al., 2002; Zitzmann et al., 1999). A panel of five iminosugar derivatives based on the natural product 1-deoxynojirimycin (DNJ) (referred to as UV-1, UV-2, UV-3, UV-4, and UV-5), were selected for evaluation as possible antiviral therapies against DENV (Fig. 1). DNJ analogues characteristically have a DNJ head group, various N-alkyl side chains, and are the only iminosugars that have been marketed for clinical use (Block and Jordan, 2001). As one of the first derivatives of DNJ developed, UV-1 has been extensively studied for its antiviral properties (Chang et al., 2009; Durantel et al., 2001; Fischer et al., 1995; Zitzmann et al., 1999). However, it has reached clinical application only against Gaucher's disease (Chang et al., 2011a). UV-2, UV-3, and UV-4 possess longer alkyl side chains, which have been previously shown to increase cellular uptake and enhance antiviral potency against hepatitis B virus and bovine viral diarrhea virus (Durantel et al., 2001; Mehta et al., 2002; Zitzmann et al., 1999). UV-3 and UV-4 also have an oxygen atom introduced into their side chains that is reported to improve glucosidase inhibition, while reducing toxicity (Durantel et al., 2001; Gu et al., 2007; Mehta et al., 2002). UV-5 is a DNJ derivative that has an aminohexyl group introduced at the end of its carbon tail.

In vitro potency against DENV was determined by plaque assay or $TCID_{50}$ in Vero cells. All five compounds possessed low μM anti-DENV activity with IC_{50} values ranging from 2 to 162 μM. However, UV-2 and UV-5 exhibited measurable cytotoxicity in Vero cells, while UV-1, 3 and 4 had CC_{50} values greater than 500 μM. This toxicity was observed in multiple cell lines including human primary monocytes and macrophages (data not shown).

3.2. UV-2 and UV-4 promote survival of AG129 mice following lethal DENV infection

Ribavirin is a classic antiviral that is effective against several RNA and DNA viruses. While ribavirin potently inhibits DENV replication *in vitro*, it is ineffective against DENV as an *in vivo* treatment (Chang et al., 2011b). As the efficacy of an antiviral *in vitro* does not always translate into efficacy *in vivo*, compounds UV-1 through UV-5 were immediately evaluated in a murine primary infection model of severe dengue disease. Mice lacking both the type I and type II IFN receptors on the 129/Sv genetic background (AG129) were infected with 10^{11} GE ($\approx 2 \times 10^6$ PFU) of DENV2 strain S221 intravenously (i.v.) via tail vein injection. Animals were then administered UV-1, UV-2, UV-3, UV-4, or UV-5 via oral gavage (i.g.), at 100 mg/kg/dose every 12 h (BID) for 7 days, and monitored for weight loss and survival (Fig. 2). As previously described, early death with DHF/DSS features occurs in this model during the first 4–6 days following infection (Shresta et al., 2006). Mice that survive beyond this early window suffer from paralysis starting at 10–12 days post-infection. As paralysis is not representative of human dengue pathogenesis, and is a side effect of the absence of the IFNγR pathway in AG129 mice (Prestwood et al., 2012), survival was not monitored beyond day 9 post-infection. Animals were administered either water or compound via intragastric (i.g.) oral gavage twice daily for 7 days starting at the time of infection. Ribavirin was included as a control and was given daily by subcutaneous (s.c.) injection. Animals receiving only water or ribavirin became moribund between days 4–6 post-infection (median survival: 4.0 days) and were euthanized (Fig. 2A). Despite their potency against DENV *in vitro*, UV-1, UV-3, and UV-5 had minimal effects upon the survival times of animals receiving these compounds. Only groups receiving UV-2 (median survival: 8.0 days) or UV-4 (median survival: 7.5 days) had statistically longer survival times than the control groups, indicating UV-2 and UV-4 were both effective agents against DENV in this lethal model of direct infection.

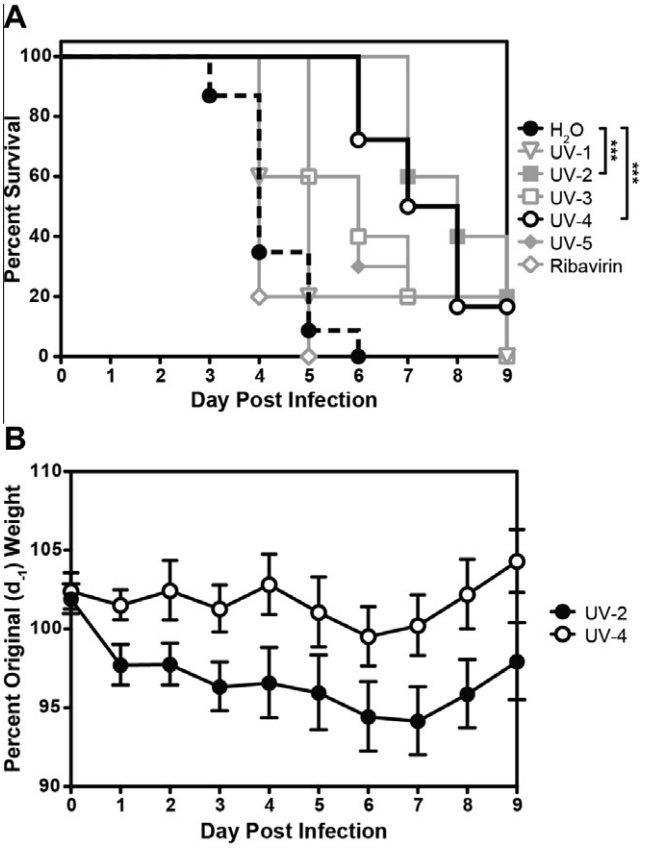


Fig. 2. Evaluation of UV-1 through UV-5 against DENV infection in mice. AG129 mice were infected with 10^{11} GE ($\approx 2 \times 10^6$ PFU) of DENV2 S221 i.v. (A) Survival of mice after treatment with H_2O ($n = 23$), UV-1 ($n = 10$), UV-2 ($n = 13$), UV-3 ($n = 5$), UV-4 ($n = 18$), or UV-5 ($n = 10$) intragastric (i.g.) BID (100 mg/kg/dose) or Ribavrin ($n = 5$) s.c. daily (100 mg/kg/dose) for 7 days starting at the time of viral challenge. (B) Percent original body weight of mice treated with UV-2 or UV-4. Asterisks denote significant differences between groups ($^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$). Error bars denote the SEM.

Weight of the infected animals was also monitored daily during the course of the experiment. Animals receiving UV-2 gradually decreased in mean group weight, and this trend continued until 36 h following the last dose of UV-2 (day 8) (Fig. 2B). In contrast, the mean weight of animals receiving UV-4 was significantly higher than the control group of mice throughout the course of the experiment ($^*p < 0.05$), and a weight increase was seen immediately following the end of dosing. This is consistent with our data in which UV-2 exhibited antiviral potency but also higher toxicity than other DNJ derivatives *in vitro*. Other studies have reported similar findings (Durantel et al., 2001; Gu et al., 2007; Mehta et al., 2002; Zitzmann et al., 1999). Because UV-4 was better tolerated than UV-2 in the present study, we chose UV-4 as the most suitable candidate to pursue for further study.

3.3. Pharmacokinetics of antiviral candidate UV-4 in mice

To guide the dosing regimen in additional *in vivo* studies, PK analysis of compound UV-4 was performed. Balb/c mice were

given a single dose of UV-4 orally at 200 mg/kg or via the intravenous route at 5 mg/kg (Fig. 3). After oral administration, UV-4 was rapidly absorbed, reaching a peak plasma concentration of 90.99 μg/mL at 15 min. The drug also demonstrated high bioavailability of approximately 84%. The elimination half-life for UV-4 was 5.65 h and 5.14 h for intravenous and oral dosing respectively, suggesting that dosing mice twice daily (12 h intervals) is suboptimal.

3.4. UV-4 promotes survival of mice in an ADE of model of DENV infection

In further evaluation of UV-4 *in vivo*, several experimental parameters were adjusted to accommodate the results obtained from the PK studies and to incorporate significant developments in DENV disease mouse models. Animals were previously dosed twice per day (BID) every 12 h (Fig. 2). However, because the half-life of the compound was short, we implemented dosing three times per day (100 mg/kg/dose, TID) every 8 h. Recent publications have described the ADE model of DENV infection in mice as a more representative model of human severe disease pathogenesis than the direct infection model (Balsitis et al., 2010; Zellweger et al., 2010). Therefore, we changed from the primary infection model to the ADE model system of infection, and both virus and anti-DENV antibody doses were modified to achieve a 90% lethal dose (LD_{90}) response in control animals. These doses were empirically determined (data not shown) and implemented to improve the resolution of the experimental system.

AG129 mice were administered 5 μg of anti-DENV prM antibody (clone 2H2), infected with 10^9 GE ($\approx 2 \times 10^4$ PFU) of DENV2 strain S221, and given compound UV-4 (100 mg/kg/dose) every 8 h for 7 days commencing at the time of infection (Fig. 4). By day 4 post-infection, 60% of control animals given only water had died with prior visible signs of distress (ruffled fur, hunched posture, lethargy) and by day 9 post-infection, only 10% of the group remained (median survival: 4.0 days). In contrast, 24 out of 27 UV-4 treated mice survived without developing any outward symptoms of disease throughout the course of infection, demonstrating the efficacy of UV-4 in the ADE model of DENV infection.

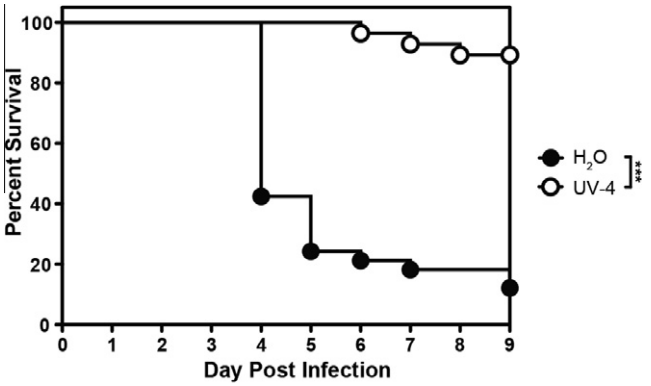
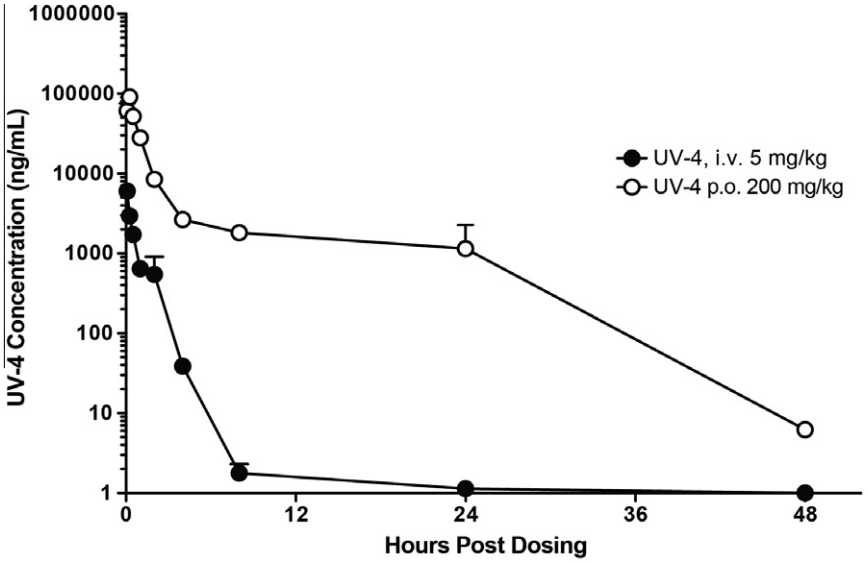


Fig. 4. Evaluation of UV-4 against lethal ADE DENV infection in mice. AG129 mice were administered anti-DENV prM antibody (clone 2H2) i.p., infected with 10^9 GE ($\approx 2 \times 10^4$ PFU) of DENV2 S221 i.v., and monitored for survival after treatment with H_2O ($n = 33$) or UV-4 ($n = 29$) i.g. TID (100 mg/kg/dose) for 7 days starting at the time of viral challenge. Asterisks denote significant differences in survival ($^{***}p < 0.0001$).

3.5. Viral loads are decreased in UV-4 treated animals

To evaluate the impact of UV-4 on DENV infection *in vivo*, viral load in the infected animals was examined (Fig. 5). Tissues were harvested at 24, 48, 72, and 96 h post-infection and analyzed for



Dose (mg/kg)	$T_{1/2}$ (hr)	T_{max} (hr)	C_{max} (ng/mL) Mean	SE	C_0 (ng/mL)	AUC_{last} (hr ng/mL) Mean	SE	AUC_{inf} (hr ng/mL)	F
5 i.v.	5.65	NA	6,021	737	8,567	3,819	595	3,831	NA
200 p.o.	5.14	0.25	90,993	2,757	NA	128,971	21,712	129,017	84.2

Fig. 3. Pharmacokinetics of UV-4 in mice. Balb/c mice were given UV-4 i.v. (5 mg/kg) or p.o. (200 mg/kg) and plasma drug concentrations were measured from blood collected at 5, 15, 30 min, 1, 2, 4, 8, 24, and 48 h post-dose ($n = 3$ per timepoint). Error bars represent the SEM. Pharmacokinetic analysis is indicated below the graph. Terminal elimination half-life ($T_{1/2}$), time to maximum plasma concentration (T_{max}), observed maximal plasma concentration (C_{max}), extrapolated maximum plasma concentration after i.v. administration (C_0), area under the plasma concentration time curve (AUC), AUC calculated to the last time point (AUC_{last}), AUC extrapolated to infinity (AUC_{inf}). Bioavailability (F) may be estimated for the test article in each formulation using the formula: $F = AUC_{po} \times Dose_{iv}/AUC_{iv} \times Dose_{po}$.

amount of viral genomes via qRT-PCR and infectious viral particles by plaque assay.

Viral RNA levels in the serum increased for both treated and untreated animals during the first 72 h post-infection (Fig 5A). Although UV-4 treated animals had 4-fold lower viral load than untreated mice at 48 h ($p = 0.0003$), viral load was roughly equivalent between the two groups by 72 h. However, at 96 h post-infection, serum viral RNA levels in the control animals remained above 10^9 GE/mL while those in UV-4 treated animals had dropped 100-fold, demonstrating a direct effect of UV-4 in decreasing viremia. To confirm that virus being replicated in the tissues was infectious, plaque assay experiments were performed. The titers observed in the serum (Fig. 5B) corresponded to the qRT-PCR results.

In the ADE mouse model, increased viral load in the liver is a key indicator of ADE-mediated pathogenesis. This is followed by replication in other non-lymphoid tissues such as the intestine and kidney (Zellweger et al., 2010). Therefore, viral RNA levels were examined in the liver, small intestine, and kidney of infected mice between 24 and 96 h post-infection (Fig. 5C–E). In these tissues, viral RNA levels remained 1–2 logs lower in UV-4 treated animals compared to control animals. Although the differences between UV-4 treated and untreated control mice were less striking when infectious titers were measured by plaque assay, the trends remained significant for both the liver and kidney when compared at 72 h post-infection (Fig. 5F and G). Taken together, these results indicate that UV-4 has a significant effect in reducing both viremia and liver titer on day 3 post infection, which is a good indicator of disease severity in the ADE mouse model (Zellweger et al., 2010).

3.6. Inflammatory cytokine induction is reduced in UV-4 treated animals

Cytokine storm is a key feature of DHF/DSS and we have previously reported that increased TNF activity predicts the severity of disease in this mouse model and neutralization of TNF prevents lethal DHF/DSS-like disease (Perry et al., 2011; Shresta et al., 2006; Zellweger et al., 2010). Thus, serum of UV-4 treated and control animals was evaluated by ELISA for the presence of various cytokines, including TNF, IP-10, IFN-γ, IL-6, IL-12, MCP-1, and KC (CXCL1). As a negative control, serum from naïve mice treated with water or UV-4 was also included. As expected, TNF levels of control mice increased over the first 96 h post-infection (Fig. 6A). In contrast, TNF in UV-4 treated animals was not significantly higher than levels observed in naïve mice until 72 h post-infection, and remained below 150 pg/mL throughout the first 96 h of infection. These results demonstrate that UV-4 treatment leads to reduction in TNF levels, leading to survival of the infected mice.

Levels of other inflammatory cytokines induced in the circulation of UV-4 treated mice were also significantly lower than in control mice (Fig. 6) at either 48 h (IL-12 and MCP-1) or 72 h post-infection (IP-10, IFN-γ, IL-6, KC). However, serum concentrations of some of these cytokines were roughly equivalent between the

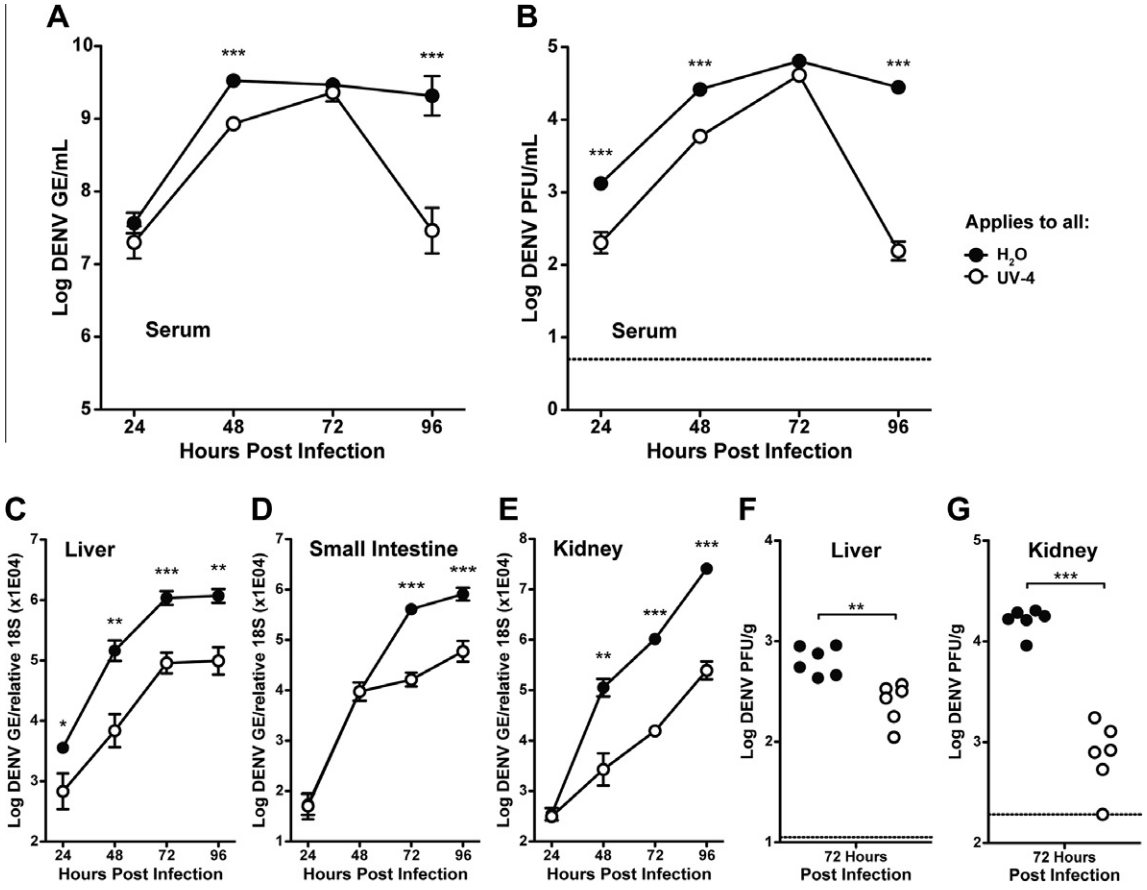


Fig. 5. Viral load and infectious titer analysis of UV-4 treated mice during DENV infection. AG129 mice were administered anti-DENV prM antibody (clone 2H2) i.p., infected with 10^9 GE (≈2×10^4 PFU) of DENV2 S221 i.v., and treated with H_2O or UV-4 i.g. TID (100 mg/kg/dose) until tissue harvest. Quantification of DENV RNA in the (A) serum, (C) liver, (D) small intestine, and (E) kidney at 24, 48, 72, and 96 h post-infection as determined by qRT-PCR (24 h $n = 6$–12; 48 h $n = 6$; 72 h $n = 6$–16; 96 h $n = 6$–10). Data are shown as GE per mL of serum or GE per copy of 18S RNA for tissues. Infectious DENV titers in the (B) serum at 24 ($n = 6$), 48 ($n = 6$), 72 ($n = 4$), and 96 ($n = 4$) hours post-infection, (F) liver, and (G) kidney at 72 h-post infection as determined by plaque assay. Data are shown as PFU per mL of serum or PFU per gram tissue weight. The dotted line represents the limit of detection of the assay for each tissue. Error bars represent the SEM and asterisks denote statistically significant differences ($^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$).

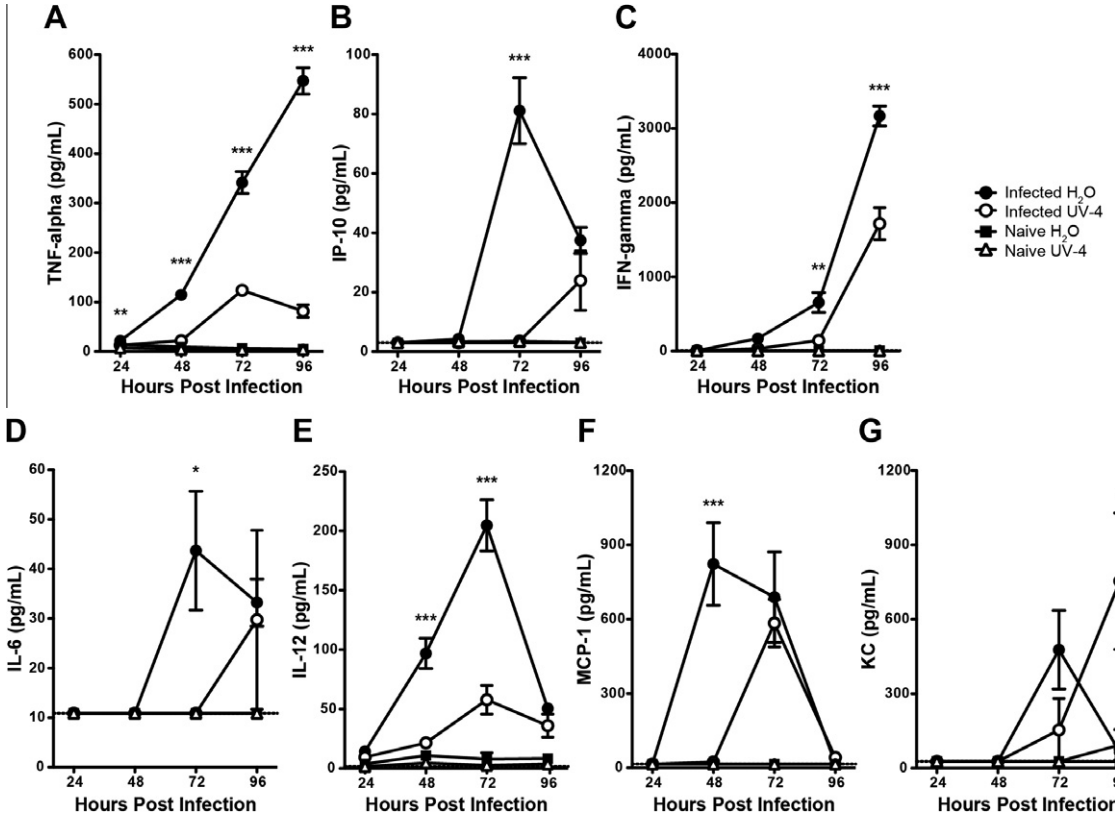


Fig. 6. Cytokine levels in serum of UV-4 treated mice during DENV infection. Serum from non-infected (n = 3 mice per group) and AG129 mice infected (n = 6 mice per group) with 10^9 GE ($\approx 2 \times 10^4$ PFU) of DENV2 S221 i.v., both administered anti-DENV prM antibody (clone 2H2) i.p., and treated with H_2O or UV-4 i.g. TID (100 mg/kg/dose) were evaluated for cytokine production. Levels of (A) TNF were determined by ELISA. (B) IP-10, (C) IFN-γ, (D) IL-6, (E) IL-12, (F) MCP-1, and (G) KC levels were measured using multi-plex ELISA (Invitrogen). Error bars represent the SEM. Asterisks denote statistically significant differences ($^*p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$).

UV-4 treated and untreated animals by either 72 h (MCP-1) or 96 h post infection (IP-10, IL-6, and IL-12). IFN-γ levels increased at 96 h post-infection in both animal groups, but the levels observed in UV-4 treated animals remained significantly lower than in control mice. Levels of one cytokine, KC, were higher in UV-4 treated animals than in control animals. By 96 h post-treatment, KC levels also increased slightly in naïve UV-4 treated mice, suggesting the increase in KC may be a result of UV-4 treatment alone. These results indicate that UV-4 reduces cytokine release in DENV-infected mice. However, it remains unclear whether the reduced cytokine levels is due to a direct effect of UV-4 upon cytokine production, or as a response to the decreased viral load upon UV-4 treatment.

3.7. Levels of DENV-specific IgM and IgG are unaltered by administration of UV-4

As sub-protective levels of antibodies enhance infection and disease severity in this mouse model, it is important to monitor any changes the administration of the compound has on antibody response. Thus, we analyzed levels of DENV-specific IgM at 4 days post-infection and IgG at 9 days post-infection in UV-4 treated and untreated control mice. The administration of UV-4 had no significant effect on the induction of DENV-specific IgM or IgG (Fig. 7).

3.8. Dose response in survival of UV-4 treated animals

In order to determine a minimal effective dose, we tested doses of UV-4 ranging from 100 mg/kg to 2.5 mg/kg (Fig. 8A). Doses of 100 and 10 mg/kg UV-4 were most effective, resulting in 100% and 90% survival respectively, while lower doses resulted in increased mortality. These results show that efficacy of UV-4 is dose dependent.

3.9. Efficacy of UV-4 therapeutic dosing

Data presented thus far have demonstrated that UV-4 affects both viral load and cytokine expression, leading to increased survival rates in animals treated with UV-4 as compared to control animals. In these experiments, virus and UV-4 were administered nearly simultaneously. In a clinical setting, humans would seek medical attention only after infection with DENV, highlighting the need for a therapeutic that has efficacy after DENV infection has occurred. To test the potential of UV-4 as a therapeutic treatment, mice were infected with DENV as previously described, and UV-4 administration was begun at time of infection, 24 h, 48 h, or 72 h post-infection (Fig. 8B). As in previous experiments, animals received UV-4 for a total of 7 days from the start of administration and were monitored for 3 days after cessation of treatment. Animals receiving UV-4 starting 24 h following infection had an equivalent survival rate as those treated at the time of infection. Mice receiving the first dose of UV-4 at 48 h post-infection had a slightly lower survival rate (median survival: 11 days), but survival remained significantly higher than in control mice. By 72 h post-infection, the survival rate had dropped to what was observed in control mice, suggesting that UV-4 has a therapeutic window that extends into the first 48 h of infection.

4. Discussion

In the present study, we identified iminosugar compound UV-4 to be effective at protecting mice from lethal DENV2 challenge.

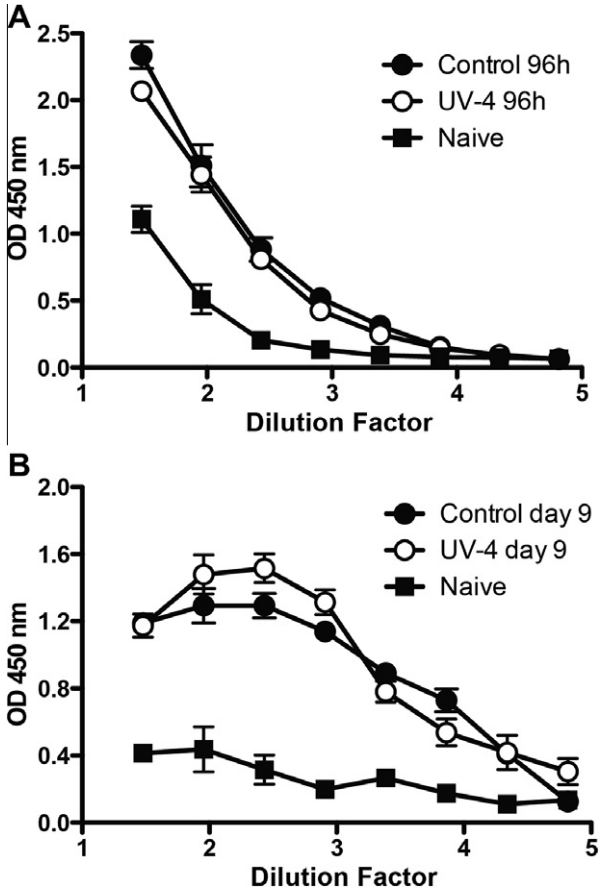


Fig. 7. Antibody response to DENV infection during UV-4 administration. AG129 mice were administered anti-DENV prM antibody (clone 2H2) i.p., infected with 10^9 GE (≈2×10^4 PFU) of DENV2 S221 i.v., and serum was collected at day 4 for IgM (A) or day 9 for IgG (B) analysis. Serum samples were titrated on an S221-coated plate and bound antibody was detected using IgM (A) or IgG (B) antibodies.

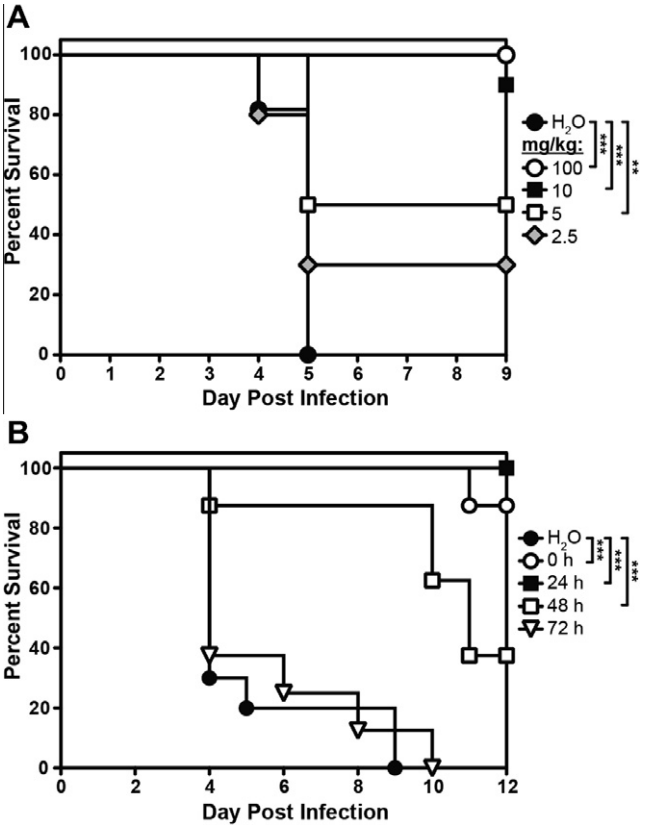


Fig. 8. Dose titration and therapeutic effect of UV-4 against lethal DENV infection in mice. AG129 mice were administered anti-DENV prM antibody (clone 2H2) i.p., infected with 10^9 GE (≈2×10^4 PFU) of DENV2 S221 i.v., and monitored for survival after dosing with H_2O or UV-4 i.g. TID for 7 days. (A) Dose titration of UV-4 treatment (H_2O $n = 11$; 100 mg/kg/dose, 10 mg/kg/dose, 5 mg/kg/dose, 2.5 mg/kg/dose $n = 10$ per group). (B) Time delay of treatment with H_2O ($n = 10$) at time of infection or UV-4 at 0, 24, 48, and 72 h post-infection ($n = 8$ per group). Asterisks denote significant differences in survival ($^{**}p = 0.0057$; $^{***}p < 0.0005$).

Kinetic analysis of DENV replication and dissemination into various tissues demonstrated a significant reduction in viremia and viral titers in tissues of mice administered UV-4. UV-4 treatment decreased cytokine levels and can be delayed up to 48 h post-infection. In addition, UV-4 showed antiviral efficacy *in vitro* against all four dengue serotypes (Supplemental Table 1), suggesting UV-4 may have similar antiviral effects *in vivo*. These results support UV-4 as a potential candidate for a phase 1 clinical trial in humans.

Several dengue antiviral studies have targeted host glycosylation pathway by utilizing iminosugars to inhibit α-glucosidases (Chang et al., 2011a,b, 2009; Rathore et al., 2011; Schul et al., 2007; Yu et al., 2012). Long-term success is more likely for treatments that target host pathways to control viral infection because the virus is less likely to develop mutations that select for drug resistance. Various iminosugars have been tested *in vivo* for their potential as a DENV treatment, but the effect on viremia with other compounds is modest. The iminosugar known as Compound 7 (CM-10-18) mildly reduced viremia in a mouse model of direct DENV infection (2-fold reduction compared to control) (Chang et al., 2009). A derivative called 3 l showed improved efficacy *in vitro* compared to compound 7, but performance *in vivo* is unknown at this point (Yu et al., 2012). Another example of an iminosugar that has been tested against various viral infections, celgosivir, reduced DENV viral titers *in vivo*. In a mouse model of DENV infection similar to the one used in our study, treatment with celgosivir reduced viremia 10-fold on day 1 and 5-fold on day 3 compared to untreated controls (Rathore et al., 2011). In our study, treatment with UV-4 modestly reduced viremia at early timepoints (4-fold reduction on day 2 post-infection). However, by day 4 post-infection, viremia had reduced 100-fold compared to untreated mice. The effect of UV-4 treatment on viremia supports its potential as a DENV therapy, as reduction of viremia is believed to be sufficient to prevent the progression of DF to DHF/DSS, based on studies demonstrating a 10-fold higher viremia in DHF/DSS patients compared to DF patients (sLibraty et al., 2002; Vaughn et al., 2000).

While viremia is an important feature when studying control of DENV infection, a limitation of published studies to date is their focus only on survival and viremia. Our previous findings have demonstrated the importance of high viral titer in the liver in severity of DENV disease (Zellweger et al., 2010). Therefore, we performed a thorough analysis of the early kinetics of DENV infection in the liver, as well as small intestine and kidney. This allowed us to conclude that UV-4 drastically reduces viral RNA levels in vital organs. Results of our previous work have also emphasized the importance of cytokines, particularly TNF, in severity of DENV disease. DENV-infected animals treated with anti-TNF antibodies do not succumb to early lethal disease, identifying TNF as a critical component of DENV pathogenesis (Zellweger et al., 2010). In the present study, by day 4 post-infection, mice treated with UV-4 have over 5-fold lower levels of TNF in the serum when compared to animals administered water only, indicating the reduction of this key inflammatory cytokine by treatment with UV-4 likely plays an important role in protecting mice from lethal DENV challenge. This

study provides an understanding of the modulation of many inflammatory cytokines during DENV infection under UV-4 treatment.

Several characteristics make UV-4 a good candidate for development as a DENV-specific antiviral. Considering the geographic range of DENV-transmitting mosquito populations, it is critical to consider ease of administration, especially in resource-poor regions. UV-4 can be prepared in the form of a salt, and this solid is stable and easy to transport and dissolves readily in water. Its high bioavailability makes it a viable option for oral administration, which is easier to distribute in resource-poor areas. Additionally, of particular relevance to DENV infected patients who seek treatment after exhibiting viremia, UV-4 treatment can be delayed up to 48 h in this mouse model, when mice have significant levels of viremia.

In this study, we have thoroughly characterized the effect of UV-4 treatment on many aspects of DENV infection, including viremia, viral RNA levels in tissues, and cytokine storm. As a result, we present UV-4 as a potent iminosugar for controlling DENV infection. Similar to the recent study that served as the basis for starting a Phase 1b human clinical trial with celgosivir (Watanabe et al., 2012), the results of this study have set the foundation for development of UV-4 as a DENV-specific antiviral in phase I human clinical trials.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.01.004.

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