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Inhibition of endoplasmic reticulum glucosidases is required for in vitro and in vivo dengue antiviral activity by the iminosugar UV-4

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Abstract

The antiviral activity of UV-4 was previously demonstrated against dengue virus serotype 2 (DENV2) in multiple mouse models. Herein, step-wise minimal effective dose and therapeutic window of efficacy studies of UV-4B (UV-4 hydrochloride salt) were conducted in an antibody-dependent enhancement (ADE) mouse model of severe DENV2 infection in AG129 mice lacking types I and II interferon receptors. Significant survival benefit was demonstrated with 10–20 mg/kg of UV-4B administered thrice daily (TID) for seven days with initiation of treatment up to 48 h after infection. UV-4B also reduced infectious virus production in in vitro antiviral activity assays against all four DENV serotypes, including clinical isolates. A set of purified enzyme, in vitro, and in vivo studies demonstrated that inhibition of endoplasmic reticulum (ER) α-glucosidases and not the glycosphingolipid pathway appears to be responsible for the antiviral activity of UV-4B against DENV. Along with a comprehensive safety package, these and
1. Main text

Dengue virus (DENV) can cause a range of disease manifestations from asymptomatic infection to severe dengue disease, which can result in death (Halstead, 2007). DENV is estimated to infect up to 390 million people worldwide annually (Bhatt et al., 2013). The resultant disease is a global health burden that strains medical systems in tropical and subtropical regions, where the virus circulates in Aedes mosquito populations. Currently no approved vaccine or antiviral therapy for DENV exists (Coller et al., 2010; Gubler, 1998; Julander et al., 2011). Four distinct serotypes of DENV (designated DENV1-4) infect humans, and epidemiological studies indicate that severe disease occurs most often during secondary infection with a heterologous serotype. A leading hypothesis to explain this phenomenon, the antibody-dependent enhancement (ADE) hypothesis, states that the presence of cross-reactive, non-neutralizing antibodies generated during primary infection or acquired passively at birth contributes to severe disease upon infection by another serotype (Halstead, 2007).

Iminosugars have been explored as antiviral agents against enveloped viruses because they demonstrate selective inhibition of viral assembly and secretion, presumably through the inhibition of the host endoplasmic reticulum (ER)-resident glycosylation pathway, leading to misfolding of viral glycoproteins (Chang et al., 2015). The antiviral activity of UV-4 against the mouse challenge virus DENV-2 S221 was found to be 38.98 μM.

In the in vivo studies described here, mice were dosed orally with varying concentrations (100, 40, 20 or 10 mg/kg) of UV-4 TID as aqueous UV-4B solution, starting 1 h before or 24 or 48 h after lethal DENV2 challenge (Plummer and Shresta, 2014; Zellweger and Shresta, 2014), and every 8 h thereafter for a total of seven days of treatment. Weight loss and health were monitored daily throughout infection and dosing, and continued for three days following the dosing period to quantitate changes in disease course. Health was determined on the basis of clinical scores ranging from 1 (completely healthy) to 7 (dead), based on a detailed rubric that includes evaluation of hunched posture, ruffling of fur, inset of eyes, and lethargy as previously described (Stavale et al., 2015). Based on extensive studies of this mouse model, animals that lost >20% of their original weight or had a clinical score >5 were considered to have succumbed to dengue disease and were euthanized immediately. Mice dosed with 100 mg/kg TID starting at −1, +24, or +48 h relative to infection exhibited survival rates of 90, 90, and 100%, respectively (all p-values <0.01 compared to vehicle treatment, Fig. 1). Similarly, animals dosed with 40 mg/kg TID starting at −1, +24, or +48 h relative to infection had survival rates of 100, 100, and 90%, respectively (all p-values <0.01 compared to vehicle treatment, Fig. 1). Animals dosed with 20 mg/kg TID starting at −1, +24, or +48 h relative to infection had survival rates of 85, 100, and 70%, respectively (all p-values <0.01 compared to vehicle treatment, Fig. 1). Animals dosed with 10 mg/kg TID showed statistically significant differences in survival compared to the vehicle group (Fig. 1). Animals dosed with 10 mg/kg TID showed statistically significant differences in survival compared to the vehicle group (Fig. 1).

Table 1

| Serotype Isolate | IC50 UV-4B μM | Replicate | Average | SD |
|------------------|---------------|-----------|---------|----|
|                  |               | 1         | 2       | 3 | 4 |
| DENV-1           | 779,172       | 0.47      | 3.52    | 8.52 | 8.08 | 5.15 | 3.85 |
|                  | SH29177       | 3.86      | 0.33    | –    | –    | 2.10 | 2.50 |
|                  | PRS41393      | 29.95     | 45.43   | –    | –    | 37.69 | 10.95 |
| DENV-2           | SL 5-17-04    | 7.42      | 9.91    | 41.09 | 30.96 | 22.34 | 16.36 |
|                  | NCG           | 5.41      | 5.84    | 5.75 | 8.95 | 6.49 | 1.65 |
|                  | H77           | 27.94     | 12.48   | 13.46 | 20.86 | 18.09 | 7.21 |
| DENV-3           | SL 5-29-04    | 2.33      | 2.73    | 5.38 | 4.11 | 3.64 | 1.39 |
|                  | HIS77         | 8.55      | 4.58    | 8.55 | 4.58 | 6.56 | 2.80 |
|                  | H87           | 87.60     | 85.37   | –    | –    | 86.49 | 1.58 |
| DENV-4           | 779,157       | 0.90      | 35.47   | –    | –    | 18.18 | 24.44 |
|                  | C258/97       | 9.83      | 8.06    | –    | –    | 8.95 | 1.25 |
|                  | H241          | 2.78      | 1.42    | –    | –    | 2.78 | 1.42 |

Not performed.

Table 1

Summary of antiviral activity based on a virus yield-reduction assay of UV-4B against multiple dengue strains in Vero cells. To determine the IC50, UV-4B was pre-incubated for 1 h, the cells were infected with the indicated dengue virus isolates for 1 h at MOI of 0.01 and the media replaced before a five day incubation period. The supernatants were then assayed for functional virus using an immunoplate assay. Shown are the results for the individual experiments, each as the average of 2–4 replicates along with the calculated average and standard deviation (SD).
significant survival when dosing started at -1 or +24 h but not +48 h relative to infection, with survival rates of 60 (p < 0.01), 56 (p < 0.05), and 36% (p > 0.05), respectively (Fig. 1). The control groups that were infected with the same lethal ADE DENV challenge but dosed TID with vehicle only (water) had 10–20% survival (Fig. 1). Additionally, the control groups receiving vehicle only lost significantly more weight and had significantly worse health scores as compared to animals dosed with UV-4B (data not shown). Under the conditions of this study, significant increases in survival were observed when mice were dosed at 10 mg/kg TID with treatment delayed for as long as 24 h post-infection; increased survival was observed at the 20 mg/kg TID dose when treatment was delayed as late as 48 h post-infection. In a separate experiment, reduction in viral titers in serum and various tissues was demonstrated in a slightly different ADE DENV2 model (Balsitis et al., 2010; Orozco et al., 2012; Perry et al., 2013; Rathore et al., 2011; Schul et al., 2007; Watanabe et al., 2012) when mice were treated with 100 mg/kg of UV-4 as UV-4B (Supplementary Figure 1).

The proposed antiviral mechanism of action of UV-4B is competitive inhibition of ER-resident α-glucosidases and glycosidases I and II. The potential utility of ER α-glucosidases as a pharmacological target capable of conferring broad-spectrum resistance to viral infectivity was demonstrated in a recent publication that characterized two children with a genetic defect resulting in absence of ER α-globulinemia, the children had no history of viral disease, were not hypogammaglobulinemia, the children had no history of viral disease, were not infected with UV-4B (data not shown). Under the conditions of this study, significant increases in survival were observed when mice were dosed at 10 mg/kg TID with treatment delayed for as long as 24 h post-infection; increased survival was observed at the 20 mg/kg TID dose when treatment was delayed as late as 48 h post-infection. In a separate experiment, reduction in viral titers in serum and various tissues was demonstrated in a slightly different ADE DENV2 model (Balsitis et al., 2010; Orozco et al., 2012; Perry et al., 2013; Rathore et al., 2011; Schul et al., 2007; Watanabe et al., 2012) when mice were treated with 100 mg/kg of UV-4 as UV-4B (Supplementary Figure 1).

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**Fig. 1.** Therapeutic window of various dose levels of UV-4B in lethal dengue ADE mouse model. Groups of AG129 mice (n = 10) received the first treatment dose of 100, 40, 20 or 10 mg/kg of UV-4 (dosed orally as UV-4B) or vehicle 1 h before or 24 or 48 h after infection with DENV2 S221 in the presence of DENV-specific antibody clone 2H2 at a dose of -1 LD90 (10^9 genomic equivalents); treatment continued every 8 h daily for seven days once initiated. (Top row) Survival data are plotted as percent survival against days post-infection. Asterisks denote statistical significance as determined by the Gehan–Breslow–Wilcoxon test (*, p = 0.05; **, p = 0.01; ***, p < 0.001). (Middle row) The mean percent weights for each group are plotted compared to their percent weight on Day -1 (baseline) against days post-infection. Error bars represent the standard error mean (SEM). (Bottom row) The mean health score, based on a standardized system with values from 1 to 7 given daily to each mouse, with the SEM for each group plotted against days post-infection. Error bars represent the standard error mean (SEM). (Bottom row) The mean health score, based on a standardized system with values from 1 to 7 given daily to each mouse, with the SEM for each group plotted against days post-infection. Error bars represent the standard error mean (SEM).
assayed for monosialodihexosylganglioside (GM3) levels as an indicator for modification of glycolipid processing (Platt et al., 1994a, 1994b). As shown in the inset graph in Fig. 2E, UV-4B and MON-6d-DGJ both reduced GM3 levels (normalized to total protein) by >90%. The dose-dependent relationship of UV-4B on GM3 levels was demonstrated in cells from three donors over a titration of 1e100 μM (Fig. 2E). The lowest dose tested (1 μM) reduced GM3 levels by an average of 73% and >90% inhibition was observed with 10 μM UV-4B. Generation of FOS is used as a marker of inhibition of ER-resident α-glucosidases and has been previously used in immortalized cell lines and rodents (Alonzi et al., 2008). The generation of FOS was assessed in the MDMFs after 48 h of treatment with 100 μM of UV-4B or 105 μM of MON-6d-DGJ. FOS that were detected in MON-6d-DGJ treated samples were identified as FOS generated by inhibition of lysosomal β-N-acetylgalosaminidases (Glawar et al., 2012), while monoglycosylated or triglycosylated

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### Table 2

| Class       | Enzyme                  | IC₅₀ (μM) |
|-------------|-------------------------|-----------|
| α-glucosidase | Mouse ER α-glucosidase I | 0.16      |
|             | Mouse ER α-glucosidase II | 1.8       |
|             | Rat intestinal maltase   | 0.28      |
|             | Rat intestinal isomaltase | 1.4       |
|             | Rat intestinal sucrase    | 0.5       |
|             | Human lysosome            | 0.39      |
|             | Human sucrase             | >1.05 mM  |
| Glucosyltransferase | HL60                  | 0.39      |
| α-galactosidase | Human lysosome           | >1 mM     |

α IC₅₀ is the concentration required to inhibit the enzyme to 50% activity. The drug did not reach an IC₅₀ for the given enzyme up to the maximal dose tested.

Glucosyltransferase in HL60 is a human promyelocytic cell line from which the glucosyltransferase has been isolated.

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Fig. 2. In vitro evaluation of iminosugar enzyme inhibition and antiviral activity using human monocyte-derived macrophages (MDMfs). Chemical structure of (A) N-(9'-methoxynonyl)-1-deoxynojirimycin hydrochloride (UV-4B, MON-DNJ) and (B) N-(9'-methoxynonyl)-1,6-dideoxygalactonojirimycin (MON-6d-DGJ). (C-D) Primary human MDMfs were infected with DENV2 strain 16681 at a multiplicity of infection of 1 and treated with a titration of iminosugar for 48 h. (C) Infectious virus titer was determined by plaque assay using LLC-MK2 (monkey kidney) cells. Cells from three donors were treated in triplicate and resulting samples quantitated using plaque assays in triplicate on each sample. Counts were normalized to 100% for untreated samples. Data are presented as mean ± SD. (D) Production of functional virus (quantitated using LLC-MK2 plaque assays) conducted on nine technical replicates was compared to total virus secreted (assessed using qRT-PCR conducted in technical duplicate) in untreated cells or cells treated with UV-4B. The values for both RNA and infectious virus are means normalized to untreated samples within a donor and a single representative donor is plotted as mean ± SD. (E) Uninfected human MDMfs were treated with iminosugars for 48 h and glycolipids were isolated from whole cell lysates. Production of monosialodihexosylganglioside (GM3) was normalized to total protein content for each sample and each treatment was normalized to untreated controls on a donor specific basis. Inhibition of GM3 production by 100 μM of UV-4B (black) or 105 μM of MON-6d-DGJ (red) was assessed (inset) and a range of UV-4B concentrations. Data are presented as mean ± SD from assay of three biological replicates assayed in single sample. (F) MDMfs were treated in technical duplicate for 48 h with iminosugar with a serial dilution of UV-4B or MON-6d-DGJ (not shown, results were negative) and inhibition of α-glucosidase I was measured by accumulation of Glc3Man5GlcNAc1 (white circles) while inhibition of α-glucosidase II was measured by accumulation of Glc1Man4GlcNAc1 (black circles). For each donor, the maximal concentration of each oligosaccharide species reached was normalized to 100%.
oligosaccharide species, which would be a product of inhibited glycoprotein processing, were not detected (data not shown). In contrast, addition of 100 μM of UV-4B led to accumulation of FOS representative of inhibition of both α-glucosidases, and the full dose-response relationship of treatment with UV-4B and production of FOS is shown in Fig. 2F.

Lastly, protection in the DENV ADE mouse model was compared after seven days of treatment with 100 mg/kg TID of UV-4 as UV-4B or the same dose and regimen of MON-6d-DGJ. The results clearly showed antiviral efficacy of UV-4B (p < 0.0001) but not MON-6d-DGJ (Fig. 3). Together, these findings suggest that antiviral activity of UV-4B is mediated by the inhibition of the ER α-glucosidases and not via inhibition of the glycosphingolipid pathway, which is inhibited by both UV-4B and MON-6d-DGJ. These data support and extend the ER α-glucosidases mechanism of action demonstrated for related glucose- and galactose-stereochemistry iminosugars (Sayce et al., 2015).

Our previous studies demonstrated the iminosugar UV-4 and its hydrochloride salt UV-4B are protective against lethal DENV2 challenge and reduce viremia and viral titers in multiple tissues (Perry et al., 2013). Additionally, we demonstrated there is no evidence of escape of DENV via resistance-conferring mutation after multiple passages in mice in the presence of UV-4B (Plummer et al., 2015), and that treatment with UV-4B does not alter the antibody response after DENV infection (Perry et al., 2013). The in vivo studies described herein were conducted to further define the minimum effective dose and the therapeutic window for oral dosing of UV-4B in the same ADE model of DENV infection, for the purpose of informing decisions about proposed dosing regimens for future human clinical trials. The effective oral dose of UV-4B given TID for seven days that resulted in statistically significant survival was considered to be 10 mg/kg and 20 mg/kg, when administration was initiated 24 or 48 h after infection, respectively. Additionally, we demonstrated efficacy against a diverse set of DENV1–4 clinical isolates in vitro. The UV-4B IC50 for the mouse challenge virus S221 is on the higher end and two-fold above the average in vitro IC50 (~20μM) of the other 12 isolates (Table 1); therefore, we predict the S221 mouse model is more, rather than less, stringent for assessing the antiviral activity of UV-4B in vivo. Further, the studies here confirm that ER α-glucosidase inhibition and not inhibition of glycolipid processing is likely responsible for the antiviral activity of the iminosugar UV-4B. These studies support the clinical development of UV-4B in ongoing Phase 1 (https://clinicaltrials.gov/ct2/show/NCT02061358) and planned Phase 2 clinical trials.

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The animal studies described herein were conducted in accordance with the approval of the Institutional Animal Care and Use Committee of La Jolla Institute for Allergy and Immunology (IACUC Protocol # AP028-S51-0615) or UC Berkeley Animal Care and Use Committee (MAUP #R252-10128). The studies were performed according to the regulations established by each local Animal Care and Use Committee, the United States Public Health Service, and the USDA Animal Welfare Act and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2010 Edition). The use of human blood was approved by the NHS National Research Ethics Service (09/H0606/3).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.03.001.

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