

# Liposome-Mediated Delivery of Iminosugars Enhances Efficacy against Dengue Virus *In Vivo*

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**A key challenge faced by promising antiviral drugs, such as iminosugars, is *in vivo* delivery to achieve effective levels of drug without toxicity. Four iminosugars, all deoxynojirimycin (DNJ) derivatives—*N*-butyl DNJ (NB-DNJ), *N*-nonyl DNJ, *N*-(9-methoxynonyl) DNJ, and *N*-(6'-[4"-azido-2"-nitrophenylamino]hexyl)-1-DNJ (NAP-DNJ)—potently inhibited both the percentage of cells infected with dengue virus and release of infectious virus from primary human monocyte-derived macrophages, demonstrating their efficacy in primary cells. In a lethal antibody-dependent enhancement mouse model of dengue pathogenesis, free NB-DNJ significantly enhanced survival and lowered viral load in organs and serum. Liposome-mediated delivery of NB-DNJ, in comparison with free NB-DNJ, resulted in a 3-log<sub>10</sub> reduction in the dose of drug sufficient to enhance animal survival. The optimizing of the effective dose in this way could liberate the therapeutic potential of many cytotoxic antivirals against both dengue virus and a wide array of other viruses.**

Dengue is the most prevalent arthropod-borne viral infection in the world and a growing public health concern. Over the past 50 years, the increase in international travel, rapid urbanization, difficulties in vector control, and possibly climate change have promoted a 30-fold increase in reported dengue virus (DENV) infection (22). As such, the financial, social, and individual costs of dengue are significant, although underappreciated (41, 46). As an arthropod-borne disease, the prevention of transmission is difficult to achieve, although several genetic strategies targeting the mosquito vector have recently shown promise in pilot studies (19). Despite concerted effort for over 70 years, and more recently an increase in funding for vaccine research, there is still no licensed prophylactic vaccine for dengue. With no specific antiviral treatment available, management is presently supportive and focused on symptom control primarily through fluid replacement. Thus, there is significant need for a postexposure (i.e., therapeutic) antiviral.

Iminosugars, such as deoxynojirimycin (DNJ) and its *N*-alkylated derivatives, demonstrate antiviral activity against DENV by targeting host cellular factors required for viral morphogenesis (reviewed in reference 36). These molecules are analogues of glucose that inhibit host cell glucosidases required for folding and maturation of glycoproteins. Inhibition of these enzymes prevents correct glycoprotein folding and oligomerization and thereby inhibits assembly of infectious virions, as viral envelope glycoproteins cannot interact with endoplasmic reticulum (ER) chaperones (e.g., calnexin and calreticulin). As they target a host process, iminosugars are of use against a wide range of viral infections. Furthermore, they are effective against all 4 serotypes of DENV (45), exhibit low toxicity, and may prevent emergence of viral escape mutants.

The iminosugar *N*-butyl DNJ (NB-DNJ) has been licensed for use in humans for over 10 years for treatment of Gaucher disease; thus, its potential clinical use against dengue is highly feasible. In trials as an anti-HIV therapy, administration of free drug did not sustain sufficiently high serum concentrations to achieve an anti-

viral effect (14). Since that time, iminosugars with improved *in vitro* potency (but concurrently greater cellular toxicity) have been developed (1, 7, 9, 16, 24, 33). As a parallel approach to enhance NB-DNJ potency, liposome-mediated intracellular delivery of iminosugars shows great promise (30). Liposomes have been used for many years as drug delivery vehicles (39), and recently, phosphoethanolamine and cholesteryl hemisuccinate liposome formulations encapsulating NB-DNJ inhibited HIV-1 with a 100,000-fold enhancement at the 50% inhibitory concentration (IC<sub>50</sub>) relative to free NB-DNJ (30). Subsequently, ER-targeting liposomes have been developed which traffic to the ER following cellular uptake (29). In addition to the benefits of targeted drug delivery, similar liposomes have inherent antiviral properties mediated by their cholesterol-lowering effects in treated cells (31).

The presence of DENV nonstructural proteins in activated monocytes in the blood (12, 21) and in phagocytes in the lymph node and spleen (2) indicates that monocytes and macrophages are key targets of human DENV infection. These findings support the hypothesis that these cells play a central role in severe dengue disease as sources of both new virus and immune mediators that shape the course of the subsequent immune response (4, 5, 13, 17). In addition to direct infection, macrophages are susceptible to targeted DENV infection via Fc-mediated opsonization in a process known as antibody-dependent enhancement (ADE) (11, 27, 28, 37). In spite of the relevance of the system, antiviral activity of iminosugars against DENV has only once been tested in a human

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cell line (N-nonyl DNJ [NN-DNJ] in Huh-7 cells [45]), and iminosugars have not been tested against DENV infection in primary human monocyte-derived macrophages (MDMφs).

Here we report the antiviral activity of iminosugars against DENV using an *in vitro* human macrophage model of infection and an ADE mouse model of dengue pathogenesis. NB-DNJ was chosen as a model iminosugar for our experiments examining the ability of polyunsaturated ER-targeting liposomes (PERLs) to enhance iminosugar efficacy against DENV because NB-DNJ is clinically licensed, has relatively low cytotoxicity, and allows easy comparison to previous results obtained for HIV-1. Although this drug is a prototype for other iminosugar derivatives that have antiviral activity against DENV (36), to our knowledge, there are currently no published data on the antiviral activity of NB-DNJ against DENV *in vitro* or *in vivo*. We show that NB-DNJ delivered as either a free drug or encapsulated in PERLs enhanced survival in the ADE mouse model and that liposome-mediated delivery increased NB-DNJ potency 2,660-fold.

## MATERIALS AND METHODS

**Viruses and cells.** DENV2 strain D2S10 was derived from the parental DENV2 PL046 Taiwanese isolate, isolated by 10 cycles of peripheral passage in mice and C6/36 mosquito cells, as previously described (40). In comparison with PL046, at the consensus sequence level, D2S10 varies by 8 nucleotides, with only one amino acid change in domain II of the E protein (K128E) and one nucleotide substitution in the 3' untranslated region (3' UTR). None of the N-linked glycosylation sites on prM, E, and NS1 are altered in this virus, in comparison to PL046 (40). DENV2 strain 16681 (a gift from E. Gould, Centre for Ecology and Hydrology, Oxford, United Kingdom) was propagated in the C6/36 cell line (a gift from the Armed Forces Research Institute of Medical Sciences, Thailand). Human monocytic cell lines U937 and HL60 (ATCC) were maintained in RPMI 1640 (Invitrogen) supplemented with 2 mM glutamine, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated fetal calf serum (Gibco). Primary human MDMφs were generated from human monocytes isolated from buffy coats (NHS Blood and Transplant, surplus to clinical requirements) as described previously (27) and incubated with 25 ng/ml recombinant human interleukin-4 (IL-4) (Peprotech) for 3 days prior to DENV infection to enhance their susceptibility to DENV infection. The use of human blood was approved by the NHS National Research Ethics Service (09/H0606/3).

**Iminosugar derivatives.** The iminosugar compounds tested in this study include NB-DNJ (solubilized in phosphate-buffered saline [PBS]), NN-DNJ (solubilized in 83% dimethyl sulfoxide [DMSO]), N-(9-methoxynonyl) DNJ (MON-DNJ), and N-(6'-[4"-azido-2"-nitrophenylamino]hexyl)-1-DNJ (NAP-DNJ) (34) (solubilized in DMSO) (all gifts from United Therapeutics, Inc.).

**Liposomes.** PERLs are composed of 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt), 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine, 1- $\alpha$ -phosphatidylinositol, and 1- $\alpha$ -phosphatidylserine, with a molar ratio of 1.5:1.5:1:1. All lipids were purchased from Avanti Polar Lipids. Fresh liposomes were prepared at 10 mM and diluted to 4 mM in PBS for injection for each experiment as described previously (30), and the distribution of liposome diameter (100 to 120 nm) was measured by dynamic light scattering. Inclusion of PEGylated phosphoethanolamine increases liposome circulation times *in vivo* (20). Calculation of the doses of iminosugar received in mice treated with 5 mM NB-DNJ PERLs was based on (i) an NB-DNJ encapsulation efficiency of 4.19% (95% confidence interval of  $\pm 1.49$ ,  $n = 19$ ) as established by measuring encapsulation of [ $^{14}$ C]NB-DNJ (data not shown) and (ii) factoring in average lipid volume changes during purification of 1.31% (95% confidence interval of  $\pm 2.11$ ,  $n = 72$ ) as established using the Stewart assay for phospholipids (42; data not shown). The estimated dose was calculated using the average values above,

while the "worst-case scenario" dose (with regard to fold enhancement calculations, i.e., if more drug was encapsulated and more lipid administered) was calculated using the average + 95% confidence intervals.

**Cytotoxicity assay.** Cell viability was measured using the Promega CellTiter 96 Aqueous One solution cell proliferation MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay according to the manufacturer's instructions. Cell viability was calculated as a percentage of the untreated cells. The values representing the concentration of iminosugar causing a 50% reduction in cell viability as a percentage of control, untreated cells ( $CC_{50}$  values) were calculated using the line of best fit on graphs of drug concentration versus percentage of cell viability.

**Inhibition of DENV infection of human macrophages.** MDMφs were infected with DENV2 strain 16681 at a multiplicity of infection of 1 for 90 min, and then virus containing supernatant was replaced with X-VIVO medium (Cambrex) supplemented with 1% autologous human plasma and serial dilutions of iminosugar. Cells were incubated for 2 days, and then the cell culture supernatant was removed, centrifuged for 5 min at  $400 \times g$  to pellet any cells, and stored at  $-80^{\circ}\text{C}$ . Cells were fixed with 4% paraformaldehyde. Infectious viral titers were obtained by viral plaque assay (27) on LLC-MK<sub>2</sub> monkey kidney cells (limit of plaque detection of 33 PFU/ml), and neither the presence of NB-DNJ nor that of liposomes in cell supernatants affected DENV titers (see Table S1 in the supplemental material). The percentage of infected cells was measured by immunofluorescence (27) using an anti-DENV2 E protein monoclonal antibody, with the average percentage of cells infected being  $20.9\% \pm 13.5\%$  ( $n = 22$ ).  $IC_{50}$ s, averaged for multiple donors, were expressed as means  $\pm$  standard deviations (SDs). Selectivity indices (SIs) were calculated as follows:  $SI1 = CC_{50}/IC_{50}$  and the more stringent  $SI2 = CC_{10}/IC_{90}$ .

***In vivo* ADE mouse model of DENV infection.** All experimental procedures were preapproved by the UC Berkeley Animal Care and Use Committee and were performed according to the guidelines of the UC Berkeley Animal Care and Use Committee and of the United State Public Health Service and the USDA Animal Welfare Act. AG129 mice (lacking alpha/beta and gamma interferon receptors) were primed with an enhancing concentration of anti-DENV monoclonal antibody prior to infection with DENV in an *in vivo* model of ADE as described previously (3). Specifically, mice were injected intraperitoneally with 2  $\mu\text{g}$  of 4G2, a pan-flavivirus monoclonal antibody against E protein, 20 to 24 h prior to intravenous infection with  $10^5$  PFU DENV D2S10. Beginning on the same day as infection, mice were treated intraperitoneally with 100  $\mu\text{l}$  PBS (no-drug controls) (twice a day), 100  $\mu\text{l}$  100 mg/ml NB-DNJ (twice a day), 100  $\mu\text{l}$  4 mM PERLs (once a day), 100  $\mu\text{l}$  4 mM PERLs encapsulating 5 mM NB-DNJ (once a day) (calculated to contain an equivalent daily dose of NB-DNJ as 200  $\mu\text{l}$  8.8- $\mu\text{g}/\text{ml}$  NB-DNJ), 100  $\mu\text{l}$  8.8- $\mu\text{g}/\text{ml}$  NB-DNJ (twice a day), or 100  $\mu\text{l}$  25-mg/ml NB-DNJ (twice a day). The group of mice administered free NB-DNJ received injections twice daily to increase the stability of drug levels due to efficient clearance times *in vivo* (half-life of 0.68 h in mice following intravenous injection [P. Laing, unpublished data]). For survival experiments, treatment continued up to 7 days, and mice were monitored four times daily for signs of morbidity and were euthanized if moribund, as approved through the animal protocol. Mice surviving at day 7 were monitored regularly for an additional 38 days (total of 45 days postinfection). Twelve of 28 animals (43%) showed no signs of disease or illness at day 45. Sixteen of 28 mice developed paralysis between days 11 and 43 postinfection and were euthanized. For viral load experiments, mice were euthanized at day 3.5, and virus was quantified in the liver, small intestine, and spleen by plaque assay and in serum by quantitative reverse transcription-PCR (qRT-PCR) (3). Viremia is expressed as PFU equivalents/ml, which was calculated by dividing the genomic RNA copy number in each sample by the genome/PFU ratio of C6/36-derived virus as determined by plaque assay and qRT-PCR. No mouse lost more than 18% of its starting body weight, including those euthanized when moribund.

TABLE 1 Anti-DENV activity and cytotoxicity of iminosugars in primary human macrophages<sup>a</sup>

Drug	CC, $\mu\text{M}$ ( <i>n</i> ) <sup>b</sup>		IC, $\mu\text{M}$ ( <i>n</i> ) <sup>c</sup>		SI <sup>d</sup>	
	50%	10%	50%	90%	1	2
NB-DNJ	24,903 $\pm$ 10,506 <sup>e</sup> (3)	837 $\pm$ 428	6.00 $\pm$ 7.31 (5)	62.1 $\pm$ 60.7 (4)	4,151	13.5
NN-DNJ	317 (1)	42	0.91 $\pm$ 0.40 (4)	8.02 $\pm$ 4.14 (3)	348	5.2
MON-DNJ	3,150 $\pm$ 1,211 (7)	837 $\pm$ 178 (4)	3.09 $\pm$ 3.93 (5)	7.74 $\pm$ 3.63 (5)	1,019	108.1
NAP-DNJ	300 $\pm$ 123 (2)	10	0.04 $\pm$ 0.01 (3)	0.28 $\pm$ 0.14 (3)	7,500	35.7

<sup>a</sup> Throughout, *n* represents the number of monocyte donors tested.<sup>b</sup> 50% and 10%, CC<sub>50</sub> and CC<sub>10</sub>, respectively. Human monocyte-derived macrophages from multiple donors were incubated for 2 days with serial dilutions of iminosugars, alongside controls containing equivalent medium and DMSO concentrations, as appropriate. Cell viability was measured as described in Materials and Methods and calculated as a percentage of that of the untreated cells. CC<sub>50</sub> values were calculated using the line of best fit on graphs of drug concentration versus the percentage of cell viability as a percentage of the control. Means and standard deviations are shown.<sup>c</sup> 50% and 90%, IC<sub>50</sub> and IC<sub>90</sub>, respectively. Data were calculated for inhibition of release of infectious virus.<sup>d</sup> Selectivity indices (SIs) were calculated as follows: SI1 = CC<sub>50</sub>/IC<sub>50</sub>, and the more stringent SI2 = CC<sub>10</sub>/IC<sub>90</sub>.<sup>e</sup> Cells from 5/8 donors showed no toxicity of NB-DNJ when tested at up to 31.6 mM and are not included in the calculation of this value.

**Statistical analysis.** Data from *in vitro* assays were analyzed using Kaleidagraph 4.0.3 (Synergy Software). Briefly, several logarithmic sigmoidal curve types were fitted to each data set with weighting applied based on precision of measurements (coefficient of variance). The curve providing the best description of the data (i.e., lowest  $\chi^2$ ) was used to calculate relevant values (IC<sub>50</sub>, IC<sub>90</sub>, etc.). Error bars represent standard deviations (SDs) unless otherwise noted. For a detailed description of methods used to analyze *in vitro* data, see the experimental procedures in the supplemental material. The Mantel-Cox log rank test was used to analyze the animal survival study.

## RESULTS

**NB-DNJ has low cytotoxicity for human cells.** For improved drug selectivity, DNJ has been derivatized (particularly by addition of variable-length *N*-linked alkyl chains), leading to changes in bioavailability, pharmacokinetics, and toxicity. We initially assessed the *in vitro* cytotoxicity of NB-DNJ, NN-DNJ, MON-DNJ, and NAP-DNJ in primary human macrophages by determining the concentration of iminosugar causing a 50% reduction in cell viability in comparison to control, untreated cells (CC<sub>50</sub>) (Table 1). Notably, no effect on cell viability was observed for NB-DNJ concentrations up to 31.6 mM for 5 donors tested. Further, NB-DNJ in human monocytic cell lines (U937 and HL60) and primary human monocytes displayed no detectable cytotoxicity following 2 days of treatment at drug concentrations up to 5 mM (see Table S2 in the supplemental material).

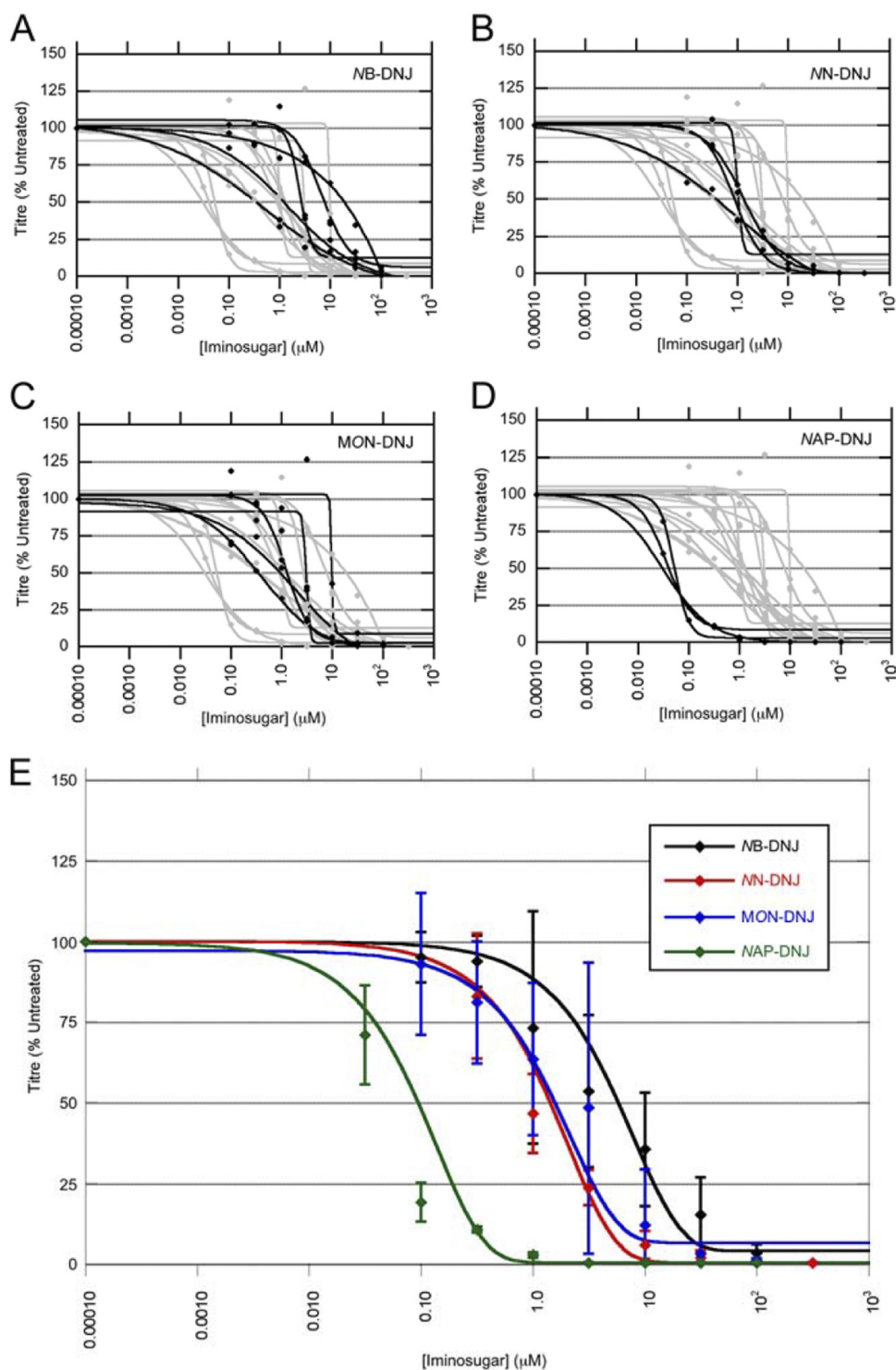
**Iminosugars inhibit multiple aspects of DENV infection of human macrophages.** We next tested the ability of NB-DNJ, NN-DNJ, MON-DNJ, and NAP-DNJ to inhibit the release of infectious DENV from primary human macrophages as a measure of antiviral activity. Untreated cells released 10<sup>3</sup> to 10<sup>5</sup> PFU/ml (data not shown). The antiviral effect was normalized to untreated cells, and curves of best fit (for a full description of the methodology, see the experimental procedures in the supplemental material) are shown for results from cells of individual donors (Fig. 1). All iminosugars tested reduced both the release of infectious virus from the infected macrophages (Fig. 1) and the percentage of cells infected (Fig. 2). Curves for individual donors demonstrate the variation between donors in potency of antiviral activity of the iminosugars, particularly for NB-DNJ (Fig. 1A to D). The concentration of drug required to inhibit the titer of virus released to 50% of that of the untreated control (IC<sub>50</sub>) varied for the iminosugars tested, ranging from 6.0  $\mu\text{M}$  for NB-DNJ to 0.04  $\mu\text{M}$  for NAP-DNJ (Table 1).

**PERLs demonstrate low cytotoxicity for human monocytic cell lines and primary human macrophages.** ER-targeting liposomes deliver NB-DNJ intracellularly, which leads to enhanced antiviral activity against HIV (29). To assess the possibility of using polyunsaturated ER-targeting liposomes (PERLs) to enhance the anti-DENV activity of iminosugars, we first investigated their cytotoxicity for uninfected cells. PERLs encapsulating either PBS or 5 mM NB-DNJ showed equivalent cytotoxicity at day 2 for primary human macrophages (CC<sub>50</sub> values around 500  $\mu\text{M}$ ) (see Table S1 in the supplemental material), similar to results with Huh-7.5 cells (S. Pollock, unpublished data). At concentrations up to 100  $\mu\text{M}$ , PBS-containing PERLs enhanced primary human macrophage viability to more than 200% of that of untreated controls (data not shown), possibly by acting as a source of lipid. This enhancing effect was not observed with primary human monocytes, although cytotoxicity remained low (see Table S1). The inclusion of 10 to 50% autologous human plasma in the cytotoxicity assay further reduced PERL cytotoxicity 2- to 4-fold (data not shown).

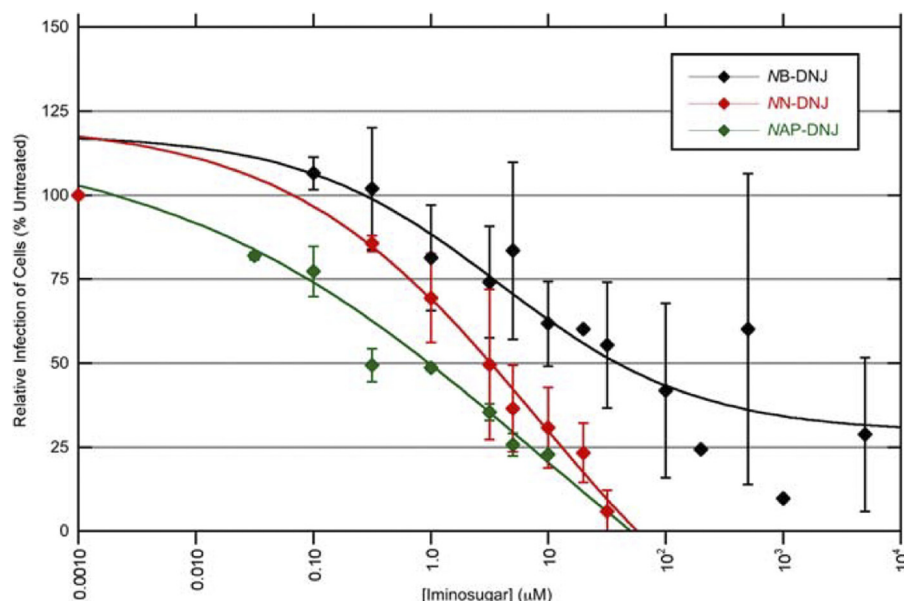
While NB-DNJ-containing PERLs are antiviral *in vitro* (antiviral effect observed in cells from 5 out of 5 human donors), tests using cells from 10 donors showed that PERLs alone are only antiviral against dengue virus in cells from half the donors. The interesting donor-linked factors influencing dengue virus susceptibility to inhibition by PERLs are now under investigation in our lab using human macrophages. Testing of the antiviral effects of PERLs  $\pm$  NB-DNJ *in vitro* never resulted in enhanced infectious virus release (data not shown).

**PERLs enhance the antiviral activity of NB-DNJ *in vivo*.** A recently developed ADE mouse model of dengue disease (3, 47) was used to compare the abilities of free NB-DNJ and PERL-encapsulated NB-DNJ to protect mice from a lethal DENV infection using daily treatment. The maximum PERL dose tested in toxicity studies caused no adverse effects or toxicity in mice (U. Ramstedt, unpublished data), and hence this dose (100  $\mu\text{l}$  of 4 mM PERLs) was used in our studies. All mice treated with either PBS or PBS-containing PERLs died between days 4 and 5 (Fig. 3). Free NB-DNJ administered at 1,000 mg/kg of body weight/day significantly ( $P < 0.0001$ , Mantel-Cox log rank test) enhanced survival (Fig. 3) and reduced morbidity (data not shown). When the dose of free NB-DNJ administered was reduced 4-fold (250 mg/kg body weight/day), only 20% of mice survived ( $P = 0.043$  compared to PBS-treated mice, Mantel-Cox log rank test). Mice treated with





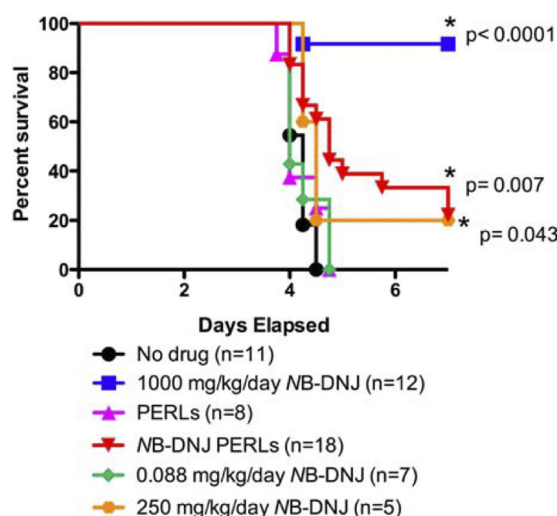
**FIG 1** Iminosugars inhibit release of infectious virus from DENV-infected macrophages. Primary human macrophages were infected with DENV and then incubated with medium containing NB-DNJ (A), NN-DNJ (B), MON-DNJ (C), or NAP-DNJ (D) for 2 days, with each drug concentration measured in triplicate. (A to D) Release of infectious virus from DENV-infected macrophages was measured by plaquing supernatants of cells treated with a drug. Plaques were counted and averaged for each drug concentration, and the percentage of infectious virus released was calculated as a percentage of the PFU/ml calculated from untreated cells. Curves of best fit are shown for cells of individual donors (black), with inhibition curves for other iminosugars shown in gray for comparison. (E) Iminosugar inhibition of release of infectious virus, with curves representing averages for multiple donors. Each line represents the results from cells of multiple donors. For detailed descriptions of how curves were derived, see the experimental procedures in the supplemental material. Error bars represent the SDs.



**FIG 2** Iminosugars inhibit infection of DENV-infected macrophages. Primary human macrophages were infected with DENV and then incubated with medium containing NB-DNJ, NN-DNJ, or NAP-DNJ for 2 days, with each drug concentration measured in triplicate. Each line represents average immunofluorescent results from cells of multiple donors. For detailed descriptions of how curves were derived, see the experimental procedures in the supplemental material. Error bars represent the SDs.

NB-DNJ-containing PERLs (labeled as NB-DNJ PERLs) also showed significantly enhanced survival ( $P = 0.007$ , Mantel-Cox log rank test) in comparison with PBS-treated mice.

We could not directly address what dose of PERL-encapsulated NB-DNJ would give equivalent protection to 1,000 mg/kg/day free NB-DNJ, as the required dose of PERLs would exceed the maximum doses tested and shown to have no toxicity (U. Ramstedt, unpublished data), so alternately we treated mice with an



**FIG 3** PERLs enhance the antiviral effects of NB-DNJ *in vivo*. Mice were administered 2  $\mu$ g anti-DENV MAb (4G2) intraperitoneally 24 h prior to infection. On day 0, mice were challenged intravenously with  $10^5$  PFU of DENV2 strain D2S10, followed by daily treatment with PBS (no drug), 1,000 mg/kg/day NB-DNJ, PERLs, or NB-DNJ PERLs or 0.088 mg/kg/day NB-DNJ and were monitored for survival. Kaplan-Meier survival curves and the numbers of mice per group are shown.

equivalent dose of NB-DNJ to that encapsulated in the PERLs. Initially assuming an encapsulation efficiency of 4% (based on measuring entrapment of self-quenching calcein [S. Pollock, unpublished data]), we conservatively calculated that daily treatment of mice with NB-DNJ PERLs (100  $\mu$ l of 4 mM PERLs encapsulating 5 mM NB-DNJ) was equivalent to 0.088 mg/kg/day NB-DNJ. Subsequently, we measured the encapsulation efficiency of NB-DNJ itself using [ $^{14}$ C]NB-DNJ. The measured value of 4.19% encapsulation efficiency was close to that of calcein and allowed us to calculate that mice treated daily with NB-DNJ PERLs received an equivalent dose of 0.094 mg/kg/day NB-DNJ (see Materials and Methods). Mice treated with a dose of free NB-DNJ (0.088 mg/kg/day) closely equivalent to that administered encapsulated in PERLs (0.094 mg/kg/day) did not show any enhanced survival in comparison with those in either the PBS- or empty-PERL-treated groups. These results indicate that when delivered via PERLs, 2,660-fold less NB-DNJ (250 mg/kg/day versus 0.094 mg/kg/day) provided a survival benefit against a lethal challenge dose of DENV in mice. Using a more conservative estimate, where the 95% confidence intervals for average percentages of encapsulation and losses/gains in lipid concentration are taken into account, PERL encapsulation provided a  $>1,900$ -fold reduction in the dose of drug able to enhance animal survival.

As an additional measure of antiviral effect, we compared the viral load in the same treatment groups as assessed above by euthanizing the mice at day 3.5 postinfection, just prior to commencement of severe illness (except for the 250-mg/kg/day treatment, for which these data were not available). Serum and tissues from various organs were assessed for viral load by plaque assay or qRT-PCR. As shown in Fig. 4, treatment with 1,000 mg/kg/day NB-DNJ resulted in a significant decrease in the viral load in liver, small intestine, sera, and spleen. However, treatment with NB-DNJ PERLs resulted in a small (not significant) decrease in viral

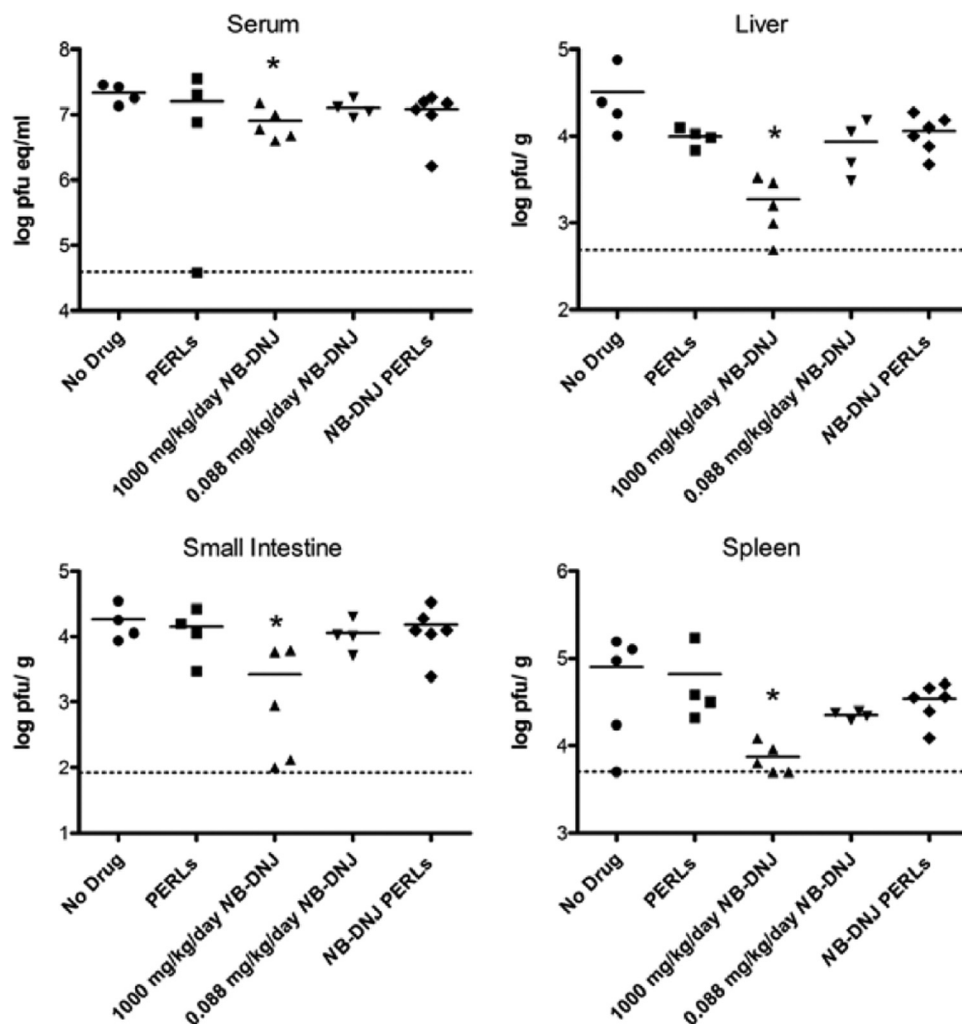


FIG 4 Effect of PERLs and NB-DNJ on viral load. Mouse treatment groups were as described in the legend to Fig. 3. Viral burden was measured in the indicated tissues at day 3.5 postinfection by qRT-PCR (serum) or plaque assay (all other tissues) as described by Balsitis et al. (2). Symbols indicate values in individual mice. The limit of detection is represented by dashed lines. eq, equivalents. Pairwise comparisons were performed with Mann-Whitney two-tailed  $P$  values, and asterisks show groups in which the average viral titer is significantly ( $P \leq 0.05$ ) different from that of the no-drug-treatment group.

load (in liver and spleen) in comparison with PBS-treated mice (Fig. 4), despite improved survival (Fig. 3). Treatment of the mice with 0.088 mg/kg/day NB-DNJ or administration of empty PERLs also resulted in a slight (although not significant) decrease in virus titers in liver and spleen, in comparison with those from PBS-treated (“no-drug”) mice.

## DISCUSSION

This study advances the field on a number of fronts. This is the first description of PERL-mediated delivery of iminosugars *in vivo*, demonstrating that this system results in a  $>3$ -log<sub>10</sub> reduction in the dose of drug sufficient to enhance animal survival. Furthermore, the lowering of the effective antiviral dose achieved by PERL-mediated drug delivery provides a potentially effective mechanism to reduce cytotoxicity of drugs with greater *in vitro* potency against DENV than those tested in this study. The use of a mouse ADE model of DENV infection to test antivirals extends previous data (36, 38, 45) to demonstrate an anti-DENV effect of NB-DNJ *in vivo*. Although previous studies evaluated DNJ (45)

and NN-DNJ (38), we demonstrate here for the first time the antiviral activity of NB-DNJ against DENV in cell lines, primary cells, and an *in vivo* model.

NB-DNJ has high *in vitro* selectivity (SI = 4,151). This impressive SI is largely due to the low cytotoxicity observed in this assay—a result consistent with the current safe use of NB-DNJ in humans. Other DNJ derivatives with more potent antiviral activity than NB-DNJ (shown in studies by ourselves and others) (7, 9, 36) generally show greater toxic effects on cells *in vitro*. In support of this trend, the cytotoxicity of the four iminosugars we tested (NB-DNJ, NN-DNJ, N9-MON-DNJ, and NAP-DNJ) was correlated with antiviral activity. However, due to the acute nature of DENV disease, short treatment periods (<5 days) are likely, and therefore, slightly higher host-associated toxicity of the more potently antiviral DNJ derivatives (if it occurs) may be more acceptable than in the context of chronic diseases, such as those caused by hepatitis C virus (HCV) or HIV/AIDS, for example, where much longer treatment periods are necessary. A shorter treatment period also reduces the probability of the development of DNJ-resistant mutants.

A  $>2\text{-log}_{10}$ -fold reduction of infectious DENV released from primary human macrophages was achieved by free NB-DNJ, NN-DNJ, MON-DNJ, and NAP-DNJ. The 1- to 2-log range in inhibition curves for individual donors indicates biological variation in humans (e.g., variation in expression or function of DENV receptors, ER  $\alpha$ -glucosidases, and/or molecules involved in antiviral defense, etc.). Because severe dengue disease in humans has been associated with 10- to 100-fold-higher viremia than dengue fever (25, 44), reduction of viral replication may be instrumental to limit the risk of developing severe disease. Supporting this hypothesis, mice treated with 1000 mg/kg/day NB-DNJ showed 92% survival in the mouse model and had significantly decreased viral loads in the liver, small intestine, serum, and spleen. In contrast, mice administered NB-DNJ PERLs, which also exhibited increased survival, showed a maximally 3-fold drop in viral titer in the organs at day 3.5 following treatment. Similar reductions in virus levels were seen in the mice treated with 0.088 mg/kg/day NB-DNJ, which was not protective here. Thus, the enhanced survival of mice treated with NB-DNJ PERLs in comparison with untreated mice and mice treated with 0.088 mg/kg/day NB-DNJ suggests that the PERLs are delivering the drug in such a way as to enhance its antiviral potential, although the mechanism remains unclear. This may be via improved retention in cells, more efficient delivery to the ER, and/or increased *in vivo* circulation times (due to PEGylation of liposomes), which will be studied in the future. No *in vivo* toxicity or adverse effects were seen at the highest liposome doses tested (U. Ramstedt, unpublished data)—the same doses as those used in the present study. In ongoing work, we are quantifying the effects of higher doses of liposomes in mice in order to determine the dose of PERL-encapsulated NB-DNJ that provides 90% survival in the DENV ADE mouse model. This will further our understanding of the potential for liposomes to enhance drug delivery *in vivo*.

In humans, however, the immunopathogenic reaction, rather than the viral load directly (15), is hypothesized to be responsible for the clinical manifestations of severe dengue. Thus, differential triggering of the inflammatory cascade (in particular tumor necrosis factor  $\alpha$  [TNF- $\alpha$ ] in the ADE mouse model) may explain increased survival in NB-DNJ PERL-treated mice. Of note, the kinetics of the entire infection and disease period is compacted in the mice, in comparison to humans; thus, it is possible that if viral load were measured earlier, then correlations between viral load and survival among all groups may have been apparent. Alternatively, our data could be explained by an indirect mechanism of action of iminosugars on factors that could influence disease outcome, other than viral load or TNF- $\alpha$ . This intriguing possibility is under investigation in our group.

NB-DNJ has high oral bioavailability (10), is already clinically licensed, and has been used to treat Gaucher's disease patients for over 10 years without any serious adverse effects reported other than those listed in the original labeling. It was also approved for treatment in Niemann-Pick disease in 2009. Chang et al. observed 100% protection of DENV-infected AG129 mice, when treated orally with 150 mg/kg/day DNJ derivative iminosugar CM-10-18 (8). As iminosugars target host-based pathways, emergence of treatment-resistant viral strains is predicted to be delayed, if not abolished. These make iminosugars strong candidates for clinical development as anti-DENV drugs. A safe and efficacious anti-DENV drug could potentially be used to reduce progression to more severe dengue disease (dengue hemorrhagic fever [DHF]

and dengue shock syndrome [DSS]) in patients living in regions of endemicity and presenting symptoms of DENV infection, as well as in hospitalized patients with laboratory-diagnosed DENV infection. Our and others' results demonstrate that iminosugars have considerable potential to meet these needs; however, as viral load generally peaks at or prior to hospitalization (18, 26, 43), a therapeutic drug targeted at reducing viral replication has only a short time (24 to 48 h) within which to act. Studies testing the window following exposure, during which iminosugars are successful as postexposure therapeutics, should be performed in the future. The infection and disease period in the ADE mouse model is shorter than that in humans; in light of the narrow window for effectiveness, the protection we observed from lethality is promising for its potential efficacy in humans.

Although much work will be necessary on the stability of the liposomes, as well as cargo loading and retention, these liposomes indicate that this area is worth pursuing. PERL-mediated delivery of iminosugars may significantly improve drug delivery (and therefore activity) while decreasing the necessary concentration (and therefore toxicity). For hepatitis B virus (HBV), HCV, and HIV-1, the cholesterol-lowering effects of PERLs *in vitro* provide antiviral activity (31). As recent reports have implicated cholesterol as an important molecule for DENV infection (6, 23, 32, 35), enhancement of iminosugar anti-DENV activity via PERL delivery may be a function of lowering cholesterol levels. Whereas NB-DNJ-containing PERLs administered to mice mediated enhancement of iminosugar efficacy, PBS-containing-PERLs themselves had no direct anti-DENV effects (apart from statistically insignificant lowering of viral load in tissues). Taken together, these results indicate that PERL-mediated enhancement of anti-DENV activity is likely an outcome of more complex processes than simply lowering cholesterol levels. To advance this work, we intend to perform further toxicity studies in mice to elucidate the dose of liposomes that manifests adverse effects *in vivo*. This will enable us to test higher liposome doses than those examined in the present study, to look more specifically at a correlation between PERL treatment and cholesterol lowering, and to assess the mechanism of antiviral enhancement.

The need for antiviral drugs effective against dengue virus is clear and ever increasing. Despite unanswered mechanistic questions, the *in vivo* mouse and *in vitro* human cell data presented here provide a strong indication that PERL-encapsulated iminosugars possess considerable potential as anti-DENV therapeutics. As such, this dual approach could be pursued for development of DENV antivirals for clinical use. Future studies on liposomes will aim to retain all of the necessary characteristics of liposomes tested here while incorporating both (i) alternate drug loading protocols to enhance loading capacity and (ii) lipid compositions with sufficient affordability of production, stability (shelf life and *in vivo*), and drug retention compatible with development for clinical use.

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