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Celgosivir treatment misfolds dengue virus NS1 protein, induces cellular pro-survival genes and protects against lethal challenge mouse model ☆

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ABSTRACT

Dengue virus (DENV) infections continue to spread aggressively around the world. Here we demonstrate that celgosivir (6-*O*-butanoyl castanospermine), strongly inhibits all four DENV serotypes. We show by fluorescence microscopy that the antiviral mechanism of celgosivir, is in part, due to misfolding and accumulation of DENV non-structural protein 1 (NS1) in the endoplasmic reticulum. Moreover, celgosivir modulates the host's unfolded protein response (UPR) for its antiviral action. Significantly, celgosivir is effective in lethal challenge mouse models that recapitulate primary or secondary antibody-dependent enhanced DENV infection. Celgosivir treated mice showed enhanced survival, reduced viremia and robust immune response, as reflected by serum cytokine analysis. Importantly, survival increased even after treatment was delayed till 2 days post-infection. Together the present study suggests that celgosivir, which has been clinically determined to be safe in humans, may be a valuable candidate for clinical testing in dengue patients.

1. Introduction

Dengue fever (DF) is the most prevalent mosquito-borne viral infection in humans and is caused by DENV, a member of the *Flaviviridae* family. Infection of humans by any of the four DENV serotypes leads to an incubation period ranging from 3 to 14 days (average 4–7 days) followed by the rapid onset of clinical manifestations of DF (Guzman et al., 2010; Halstead, 2007). While DF is usually self-limiting, some patients progress to life-threatening severe diseases, such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). The phenomenon that non-neutralizing heterotypic antibodies can cause antibody-dependent enhancement (ADE), together with the increasing prevalence of DENV infections around the world, raises the probability of increasing cases of severe DENV disease (Sabin, 1952; Thein et al., 1997; Whitehorn and Farrar, 2010). Despite the observed association between DHF/DSS and higher viremia compared to DF (Vaughn et al., 2000), no antiviral treatment exists. There is, thus, a global urgency to develop drugs that can reduce the burden of DENV morbidity and mortality (Keller et al., 2006).

The plus-strand RNA genome of DENV is translated as a single polyprotein precursor which is processed by host and virus-encoded proteinases to produce three structural proteins (Capsid, prM, Envelop) and seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Chambers et al., 1990). The virus assembly occurs in the endoplasmic reticulum (ER), where heterodimers of pre-membrane protein (prM) and envelope (E) localize to the luminal side of the ER to form an immature particle (Chambers et al., 1990; Zhang et al., 2003). Previous studies have shown that N-linked glycosylation of prM and E proteins are required for the proper assembly and release of infectious DENV particles (Courageot et al., 2000).

Celgosivir is a water soluble oral pro-drug of the natural alkaloid castanospermine (Cast), derived from the Moreton Bay chestnut tree (*Castanospermum australae*) (Molyneux et al., 1986). It readily crosses cell membranes and is rapidly converted to Cast (Kang, 1996). The compound inhibits the catalytic activity of ER resident enzymes, α-glucosidase I and II, which play a critical role in the proper folding of *N*-glycosylated glycoproteins. Treatment with Cast may affect the folding of some viral proteins by preventing the removal of the terminal glucose residue from N-linked glycans (Taylor et al., 1991). Lack of modification of the high mannose

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sugar on viral proteins may result in misfolding and protein aggregation that leads to cellular ER stress. ER stress is marked by activation of unfolded protein response (UPR) machinery, and the expression of UPR genes which, in turn, induces various signaling pathways associated with cell survival/death (Schröder and Kaufman, 2005). It has been shown that DENV proteins prM and E are glycosylated and undergo high mannose modification for proper folding and function (Courageot et al., 2000). However, NS1, a viral protein whose function is currently being intensively studied, is also glycosylated at positions N130 and N207 (Somnuke et al., 2011), and secreted from infected cells (Gutsche et al., 2011; Libraty et al., 2002). Due to its direct and systemic effects on the host, NS1 could be a potential target for an antiviral drug (Somnuke et al., 2011). Indeed, our studies reveal that DENV NS1 is a target of the action of celgosivir.

AG129 mice lacking the interferon (IFN)-α/β and -γ receptors have been shown to be useful for testing antiviral compounds. Infection of these mice with DENV2 TSV01 strain (non-mouse adapted clinical strain) produced viremia that peaked at 3 days post-infection. The peak viremia and the serum concentration of several cytokines and NS1 were reduced when infected animals were treated with several antiviral inhibitors including celgosivir (Schul et al., 2007). More recently, the AG129 model has been used with non-mouse-adapted DENV2 (D2Y98P) and mouse-adapted DENV2 (S221), which presents with severe disease, including high viremia and death (Balsitis et al., 2010; Tan et al., 2010; Zellweger et al., 2010). Furthermore, it has been shown that ADE infection of AG129 mice can be achieved by prior passive immunization using a mouse monoclonal antibody against DENV E protein (4G2), which leads to an increased plasma leakage and mortality (Zellweger et al., 2010). Together, these studies support the use of AG129 mice to test antiviral compounds in a setting where mice experience prolonged viremia and even death.

In this study, we address the lack of a suitable antiviral for DENV infection by detailed examination of the mechanism of celgosivir and show that it is an effective antiviral *in vitro* against all four DENV serotypes and *in vivo* against DENV2.

2. Materials and methods

2.1. Cells and virus strains

THP-1 (human monocytic cells, ATCC) and BHK-21 (baby hamster kidney fibroblast cells, ATCC) cells were grown in RPMI1640 medium (Gibco). A549 cells (human lung epithelial cells, ATCC) were maintained in Ham's F-12K (Gibco) medium. A549 replicon cells (containing DENV2 NS genes) were grown as described elsewhere (Ng et al., 2007). All growth media were supplemented with 10% FBS and 1% penicillin–streptomycin and cells were cultured at 37 °C in 5% CO_2. C6/36, an *Aedes albopictus* cell line (ATCC), was maintained in RPMI1640 medium with 10% FBS, 25 mM HEPES and 1% penicillin–streptomycin, at 28 °C in the absence of CO_2.

The four DENV serotypes (DENV1-2402, DENV2-3295, DENV3-863 and DENV4-2270) used in the study were obtained from the Early Dengue infection and outcome (EDEN) study in Singapore (Low et al., 2006) and the mouse adapted strain of DENV2 (S221) was a gift from Dr. Sujan Shresta (La Jolla Institute for Allergy and Immunology, CA). All virus strains were grown in C6/36 cells. Virus stocks were tittered by plaque assay and stored at −80 °C.

2.2. Therapeutic compounds

Celgosivir was purchased from Dalton Pharma Services, Canada. Castanospermine was purchased from Sigma (C3784).

2.3. Antibodies

Humanized monoclonal antibody against DENV E protein (clone 4G2) was previously described (Paradkar et al., 2011). Mouse monoclonal antibody against DENV NS1 was provided by Dr. Christiane Ruedl (Division of Molecular & Cell Biology, School of Biological Sciences, NTU, Singapore). Mouse monoclonal antibody 4G2 was produced in-house from hybridoma cells.

2.4. Cell viability and infection assays

The cytotoxicity of each test compound was measured by the Cell titer-Glo Luminescent cell viability assay, according to the Manufacturer's protocol (G7570; Promega). The luminescence signals for cells treated with the test compounds were compared to those for cells treated with the maximum tolerated dimethyl sulfoxide (DMSO) to determine the 50% cytotoxic concentration (CC_{50}). Further detailed methods for virus quantification, cell based infection assays, RT PCR, immunofluorescence microscopy and Western blotting can be found in the supplemental materials section.

2.5. In vivo infection

Sv/129 mice deficient in type I and II IFN receptors (AG129), purchased from B&K Universal (UK), were housed in the BSL-2 animal facility at Duke-NUS, Singapore. The Animal Care Committee at Singapore General Hospital/NUS approved all animal experiments. For infection, mice ($n = 8$) were inoculated intraperitoneally (i.p.) with 2×10^5 pfu of S221. To model ADE, mice were injected i.p. with 20 μg/mouse of mouse monoclonal antibody against DENV E protein (4G2 clone) one day prior to infection. For treatment during infection, celgosivir (50 mg/kg) was injected i.p. twice daily for 5 days, starting from day 0, 1 or 2. Blood was collected at days 1, 3 and 7 by submandibular bleeding. Survival of mice was followed until day 10 and survival curves were plotted in GraphPad Prism-5 software with survival significance evaluated using log-rank Test.

3. Results

3.1. Celgosivir is effective in vitro at nM concentrations against DENV1-4

To examine the antiviral effect of celgosivir *in vitro*, we used the cell-based flavivirus immune detection (CFI) assay, which comprises the complete replication cycle of DENV. BHK-21 cells were infected with a clinical isolate of DENV2 at an MOI of 0.3 in the presence of different concentrations of celgosivir, and virus E protein was quantified 48 h later using 4G2 antibody. The result showed an inhibitory effect of celgosivir on DENV2 replication with a half maximal effective concentration (EC_{50}) value of ~0.2 μM (Fig. 1A). Similarly, EC_{50} values against DENV1, 3 and 4 were obtained (Table 1) and found to be below 0.7 μM. Furthermore, immunofluorescence microscopy confirmed the effect of celgosivir on DENV2, since NS1 was hardly detected in the drug-treated cells (Fig. 1B). Interestingly, celgosivir is about 100 times more effective against DENV2 than the parent compound castanospermine (Fig. S1). These results suggest that celgosivir has a robust inhibitory effect against all four DENV serotypes (DENV1-4), with greater potency than we and others have observed for castanospermine (Schul et al., 2007; Whitby et al., 2005).

Since monocytes are subject to dramatically increased DENV infection during severe secondary infection (Halstead and O'Rourke, 1977), we recapitulated this scenario with *in vitro* ADE infection (in the presence of either sub-neutralizing or enhancing

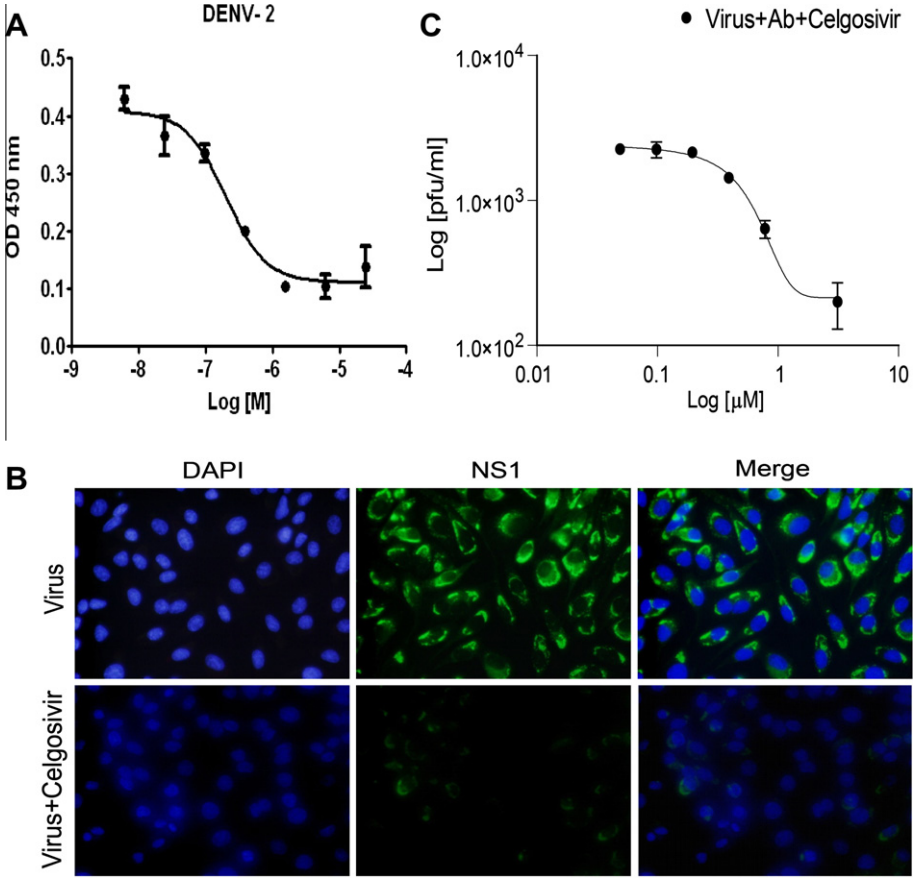


Fig. 1. Inhibition of DENV replication by celgosivir (A) Dose response curve of celgosivir against DENV2 infection on BHK-21 cells at 48 h is shown. (B) Immunofluorescence microscopy of DENV2-infected BHK21 cells following mock or celgosivir (20 μM) treatment. Virus replication was detected at 48 h using monoclonal anti-NS1 antibody (green). (C) ADE-infected THP-1 cells were treated with various concentrations of celgosivir and the EC_{50} (0.496 μM) value was determined by plaque assay using media supernatants at 48 h.

concentrations of 4G2) of THP-1 monocytic cells and examined whether celgosivir would have the same inhibitory action as observed for direct infection of BHK-21 cells (Fig. 1). Our results showed that the supernatant from the ADE infected cells had 10-fold more infectious virus particles compared with direct virus infection after 48 h (Fig. S2). Celgosivir significantly reduced DENV2 ADE infection by plaque assay (Fig. 1C), with an EC_{50} of ~0.5 μM. The excellent potency of celgosivir in these *in vitro* models supported its further evaluation as an antiviral compound in a murine DENV infection model that recapitulates ADE.

3.2. Mechanism of celgosivir action: viral and host targets

3.2.1. DENV NS1 as novel target of celgosivir action

It has been previously shown that α-glucosidase inhibitors disrupt the folding of prM and E and prevent the maturation and release of viable DENV particles (Courageot et al., 2000). First we confirmed by immunofluorescence microscopy that the transport of DENV E protein from the ER to the Golgi is blocked in the presence of celgosivir (Fig. S3). Since the impact of celgosivir on NS1 has not been reported, we addressed this question using the sub-genomic replicon assay, which, rather than measuring the complete viral life cycle, determines the effect of celgosivir on the replication of DENV NS genes. This assay relies on the use of replicon cells, which are stably transfected with a DENV2 sub-genomic replicon comprising of viral NS genes, with a luciferase reporter (Ng et al., 2007). Interestingly, incubation of these cells with different concentrations of celgosivir showed a decrease in luciferase reporter activity. As a control, cells were transfected with a luciferase plasmid to rule-out the possibility that celgosivir directly inhibited the luciferase activity. The EC_{50} of celgosivir against the DENV replicon was thus determined to be around 2.2 μM (Fig. 2A). This result suggests that glycosylated DENV NS1 protein plays a significant role in the viral genome replication cycle.

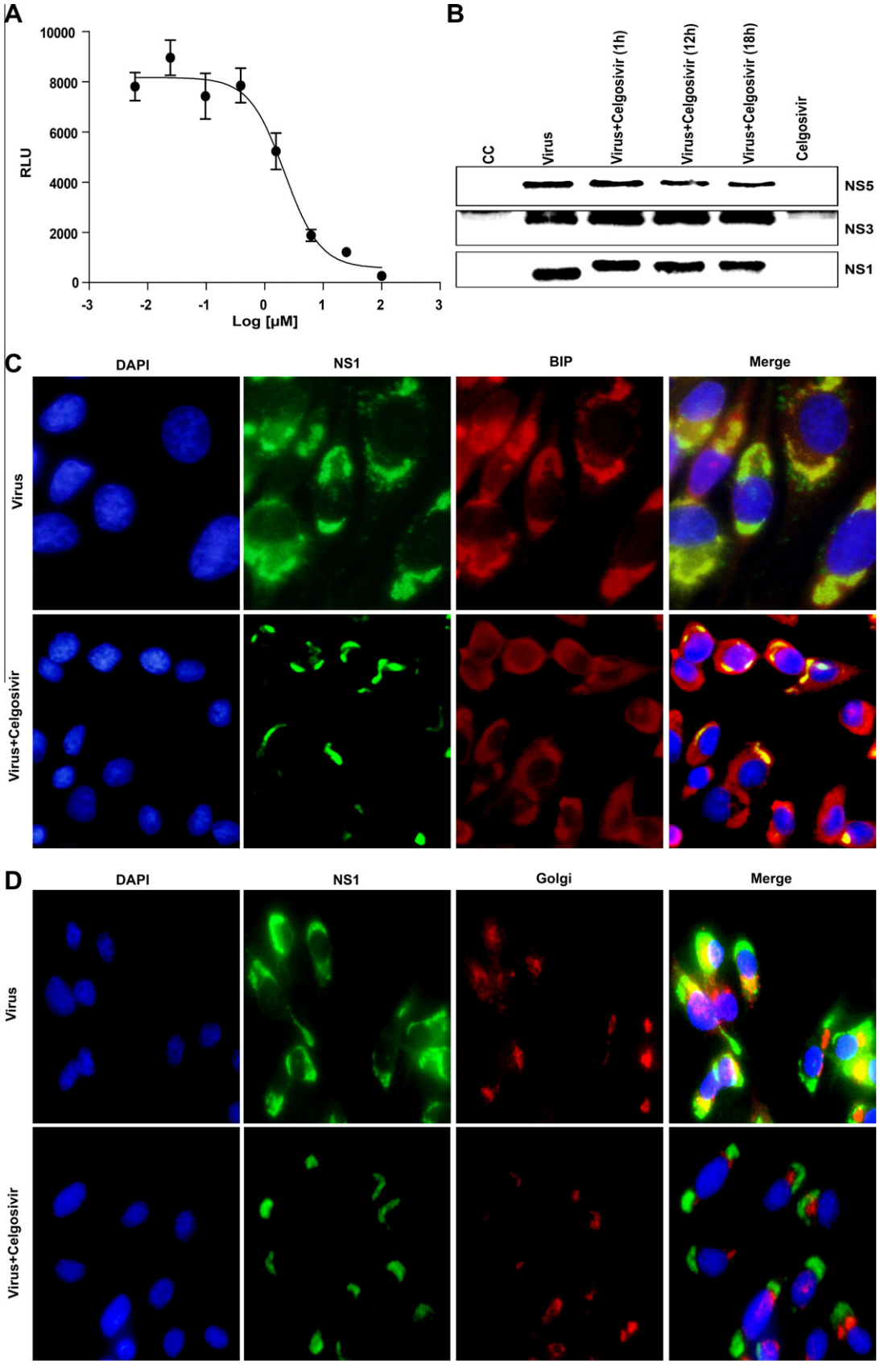
To examine the effect of celgosivir on DENV NS proteins, DENV2 infected BHK-21 cells were treated with celgosivir (20 μM) starting at 1, 12 or 18 h post-infection and cells were harvested at 36 h post-infection. Samples were separated by SDS–PAGE and detected by Western blotting using antibodies against DENV NS1, NS3 or NS5 (Fig. 2B). As the mode of action of celgosivir is through the inhibition of glycosylation, the difference in band migration between the treated and untreated DENV NS1 protein suggests a defect in N-linked glycosylation of this protein. At the same time the other NS proteins such as NS3 and NS5, which are not glycosylated, remained unaffected (Fig. 2B). Furthermore, using immunofluores-

Table 1
Efficacy of celgosivir in DENV serotypes.

DENV	CFI (EC_{50})*
DENV-1	0.65 + 0.16 μM
DENV-2	0.22 + 0.01 μM
DENV-3	0.68 + 0.02 μM
DENV-4	0.31 + 0.12 μM

* The average EC_{50} values and SDS ($n = 3$) for celgosivir determined by CFI assay (see Fig. 1A).

cence microscopy, we observed that celgosivir treatment resulted in accumulation of NS1 in the ER and blocked the transport of NS1 from the ER to the Golgi (Fig. 2C and D). Together, these data showed that celgosivir inhibited the replication of DENV by affecting the folding and transport of DENV NS1 between cellular compartments, which is an essential part of its secretion process.



3.2.2. Celgosivir activates the UPR pathway

Celgosivir treatment triggers the accumulation of misfolded proteins in the ER lumen which activates the UPR pathway (Schröder and Kaufman, 2005). We showed previously that DENV infection caused the activation of UPR machinery (Paradkar et al., 2011; Umareddy et al., 2006). To examine the impact of celgosivir treatment on cellular UPR pathway, we infected THP-1 monocytes-directly or via ADE with DENV2 and monitored several UPR marker genes using real-time RT-PCR or relevant proteins by western blotting. Celgosivir treatment resulted in significant reduction in the level of NS1 mRNA during direct or ADE infection (Fig. 3A). BIP (Grp78), the ER-resident chaperone and master regulator of UPR machinery, was activated as seen by real-time RT-PCR and western blotting analysis (Fig. 3C and B). BIP levels were found to be slightly increased in the cells treated with celgosivir during direct and ADE infection (Fig. 3B and C). Other proximal sensors of UPR pathway activation, such as PERK and IRE-1, were activated in the presence of celgosivir alone, as shown by measuring the activation of downstream factors in each pathway, i.e. phosphorylation of eIF2α (Fig. 3B) and transcription of XBP-1 (Fig. 3C) respectively, which were both increased over the untreated cells. However, celgosivir treatment of direct or ADE-infected cells showed a significant reduction in the phosphorylation of eIF2α compared to the corresponding infected cells without treatment (Fig. 3B). This suggests that while celgosivir promotes the moderate activation of the UPR pathway on its own, it acts to limit the heightened activation that occurs in DENV-infected cells. Interestingly, we observed that EDEM-1, an ER-resident chaperone that facilitates and hastens the sorting of unfolded proteins to the degradation pathway (Hosokawa et al., 2010), is strongly transcribed in the presence of celgosivir (Fig. 3C). EDEM-1 is a pro-survival protein that promotes the recovery of cells from ER stress by clearing the calnexin cycle and facilitating terminal degradation of misfolded proteins (Molinari et al., 2003; Oda et al., 2003). The level of EDEM-1 transcripts were lower in THP-1 cells infected with virus directly or via ADE compared with infected cells treated with celgosivir. In fact for the ADE-infected cells with treatment, the EDEM trascript level was more than 100-fold higher than the cell control. Conversely, the level of CHOP (an apoptotic marker) transcripts was found to be 16-fold higher in ADE infected THP-1 cells, suggesting that many of these cells were infected and likely to undergo apoptosis. Remarkably the level of CHOP-transcripts was significantly reduced ($P < 0.05$) in celgosivir-treated ADE infected cells (Fig. 3C). Collectively, our data suggest that celgosivir treatment during DENV infection modulates the UPR machinery and promotes cell survival as depicted in the model (Fig. 3D).

3.3. Celgosivir is effective in both primary and lethal ADE mouse models of DENV infection

Previously we showed that celgosivir significantly reduced viremia in AG129 mice infected with a clinical strain of DENV2 (TSV01) (Schul et al., 2007). Now we have used the recently developed lethal DENV infection model (Zellweger et al., 2010) to test the efficacy of celgosivir. During primary infection with a mouse-adapted DENV strain S221, mice showed increased viremia on day 3, yet 80% survived day 10 (Fig. 4A and B) with virus completely cleared by day 8 (data not shown). On the other hand, mice infected with the same virus dose 24 h after passive immunization with 4G2 antibody (ADE infection model) displayed very high viremia on day 3 (Fig. 4B), with severe morbidity and 100% mortality by day 5 (Fig. 4A). To test the antiviral effect of celgosivir in both primary and severe ADE infection models, mice were treated with 50 mg/kg of celgosivir, twice-a-day, for 5 days starting from the day of infection. For delayed treatment studies, the twice a day dose celgosivir treatment was administered 1 or 2 days post-infections. Remarkably, mice treated from the day of infection showed 100% survival and those treated from day 1 or day 2-post-infection showed 75% and 50% survival, respectively (Fig. 4A). Viremia data corresponding to the survival curve showed significant reduction in the viral burden of celgosivir-treated groups (Fig. 4B). We also sought to determine the immune fitness of the surviving mice by kinetic serum cytokine analysis. In all cases virally challenged mice developed a strong Th1 polarized signature by day 3, which in the case of virus treatment alone progressed into a systemic inflammatory signature by day 7. Interestingly, our data show that celgosivir-treated mice were capable of maintaining significantly higher levels of cytokines such as IFN-γ, IL-9, RANTES, which are associated with immune clearance of viral pathogens (St. John et al., 2011) and lower levels of proinflammatory cytokines including TNF-α, MCP-1 and IL-6, which are associated with lethal immune pathology during DENV infection (Priyadarshini et al., 2010) (Fig. 4C). Cumulatively, these data suggest that celgosivir is a robust antiviral compound, effective in an *in vivo* model of both primary and severe ADE DENV infection.

4. Discussion

Despite the significant disease burden caused by DENV, no specific antiviral therapy is currently licensed for treatment. Promising clinical candidates developed by the leading dengue drug discovery groups have been abandoned because of adverse toxicology effects in animal models (Yin et al., 2009). The use of clinical candidates that have undergone extensive safety studies in humans for other diseases as potential treatment in diseases like DF is a viable strategy, if efficacy can be demonstrated in suitable *in vitro* and *in vivo* settings. In this respect, a prior study reported that the plant alkaloid, castanospermine, an ER α-glucosidase inhibitor, blocked DENV1 infection (Courageot et al., 2000) and that this inhibition was due to the misfolding of structural glycoproteins E and prM. The prodrug of castanospermine, celgosivir, has been evaluated in humans as a potential treatment against human immunodeficiency virus (HIV) and hepatitis C virus (HCV) and tested for the safety and tolerability in over 600 subjects at doses between 10 and 600 mg (Kaita et al., 2007). It was dosed in some HCV patients for between 24 and 48 weeks at 400 mg daily (Durantel, 2009). Furthermore, we had previously tested celgosivir, along with other well-known nucleoside inhibitors to establish the AG129 mouse model as vehicle to obtain *in vivo* efficacies of novel antiviral compounds against DENV that were in the drug discovery pipeline (Schul et al., 2007; Yin et al., 2009). The study by Schul and colleagues showed that the viral load of DENV2 TSV01 strain could be reduced by high-dose (75 mg/kg) treatment with celgosivir and efficacy was also maintained following treatment of up to 1 day post-infection.

Fig. 2. NS1 post-translational modification is affected by celgosivir. (A) Dose response curve showing inhibitory activity of celgosivir in DENV2 sub-genomic replicon cells at 48 h with an EC_{50} value of 2.2 μM. (B) Western blot analysis on DENV2-infected BHK-21 cells, after treatment with celgosivir (20 μM) at 1, 12 and 18 h post-infection and harvested at 36 h, using antibodies against DENV NS1, NS3 and NS5 proteins. The difference in protein migration shows the effect of celgosivir on the glycosylation of NS1; however, NS3 and NS5 remain unaffected. Uninfected BHK-21 cells (CC) were used as western blotting negative control. (C) Immunofluorescence microscopy performed on DENV2-infected BHK-21 cells at 36 h in the presence or absence of 20 μM celgosivir added 12 h post-infection using antibodies against host protein BIP and viral protein NS1. In the presence of celgosivir DENV NS1 (green) accumulates in the ER and co localize with BIP (red). (D) Mock-treated DENV2-infected BHK21 cells show co-localization of NS1 (green) with gigantin (red) and no such co-localization was observed in the presence of celgosivir.

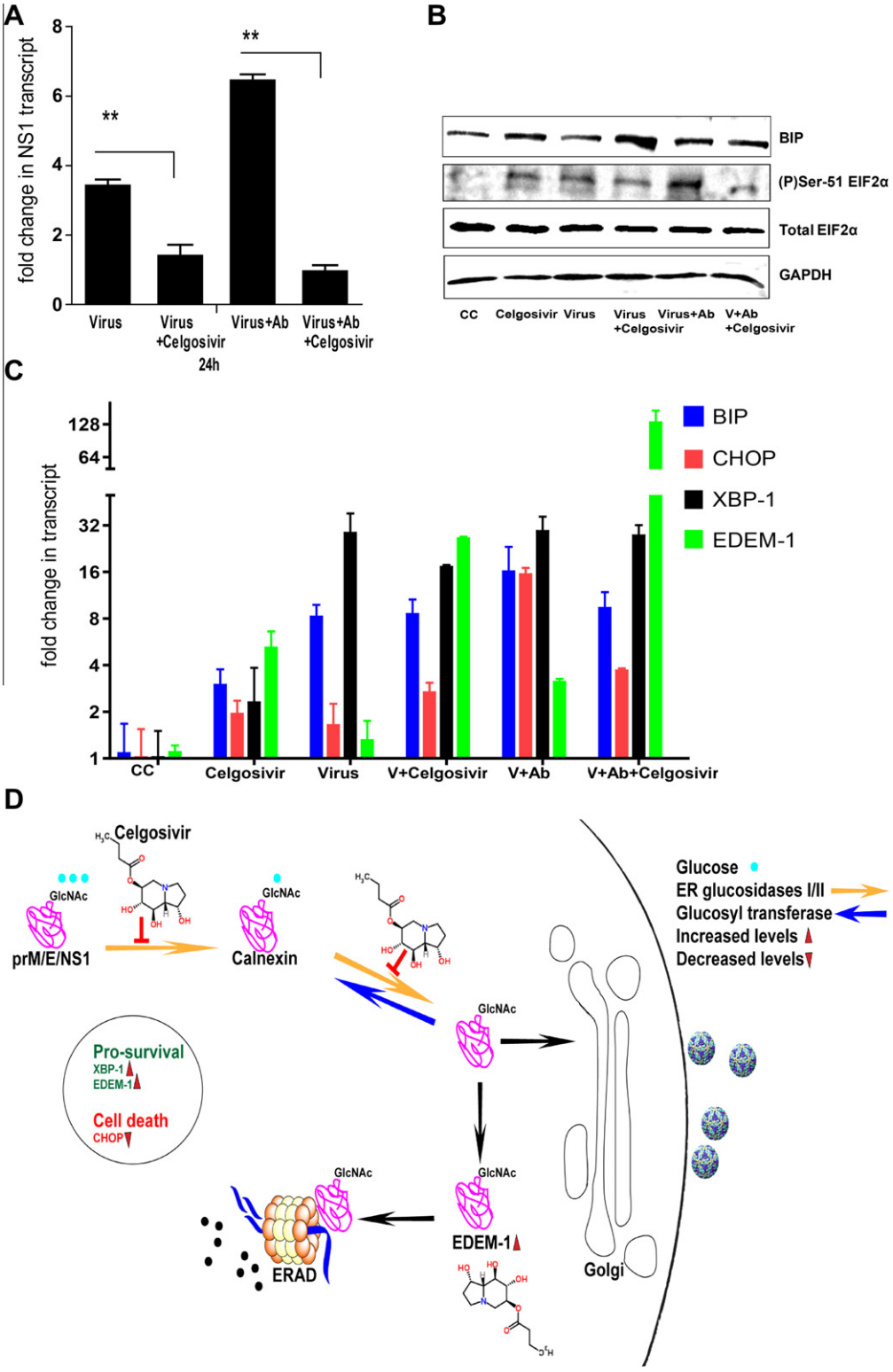


Fig. 3. Celgosivir modulates host cellular UPR machinery. (A) ADE-infected THP-1 cells were mock or celgosivir (20 μM) treated for 48 h followed by real time PCR detection of viral/host UPR gene transcripts or western analysis. As shown, celgosivir treatment significantly reduced the levels of NS1 RNA compared to the mock treated cells. (B) Western blot showing increased protein levels of BIP and eIF2-α phosphorylation upon celgosivir treatment. Uninfected/untreated BHK-21 cells (CC) were used as cell control. (C) Real time PCR analysis shows celgosivir treatment leads to transcription induction of UPR genes such as BIP and XBP-1 and reduction in the levels of CHOP. Significance was determined by 1-way ANOVA. (D) Schematic model showing the celgosivir driven modulation of UPR machinery in a DENV infected cell.

In this background more detailed studies were carried out with celgosivir in the present study. We showed that celgosivir is effective against all four serotypes of DENV and that it also inhibited DENV-2 ADE infectionof human monocytic cells. Since, in our previous mouse model, the viral load in serum reduced without treatment after peaking at day 3 post-infection, we tested the efficacy of celgosivir in the newly established lethal dengue model (Balsitis et al., 2010; Tan et al., 2010; Zellweger et al., 2010). We established a window of infection where a selected dose of the mouse adapted virus alone resulted in ~80% survival. However passive immuniza-

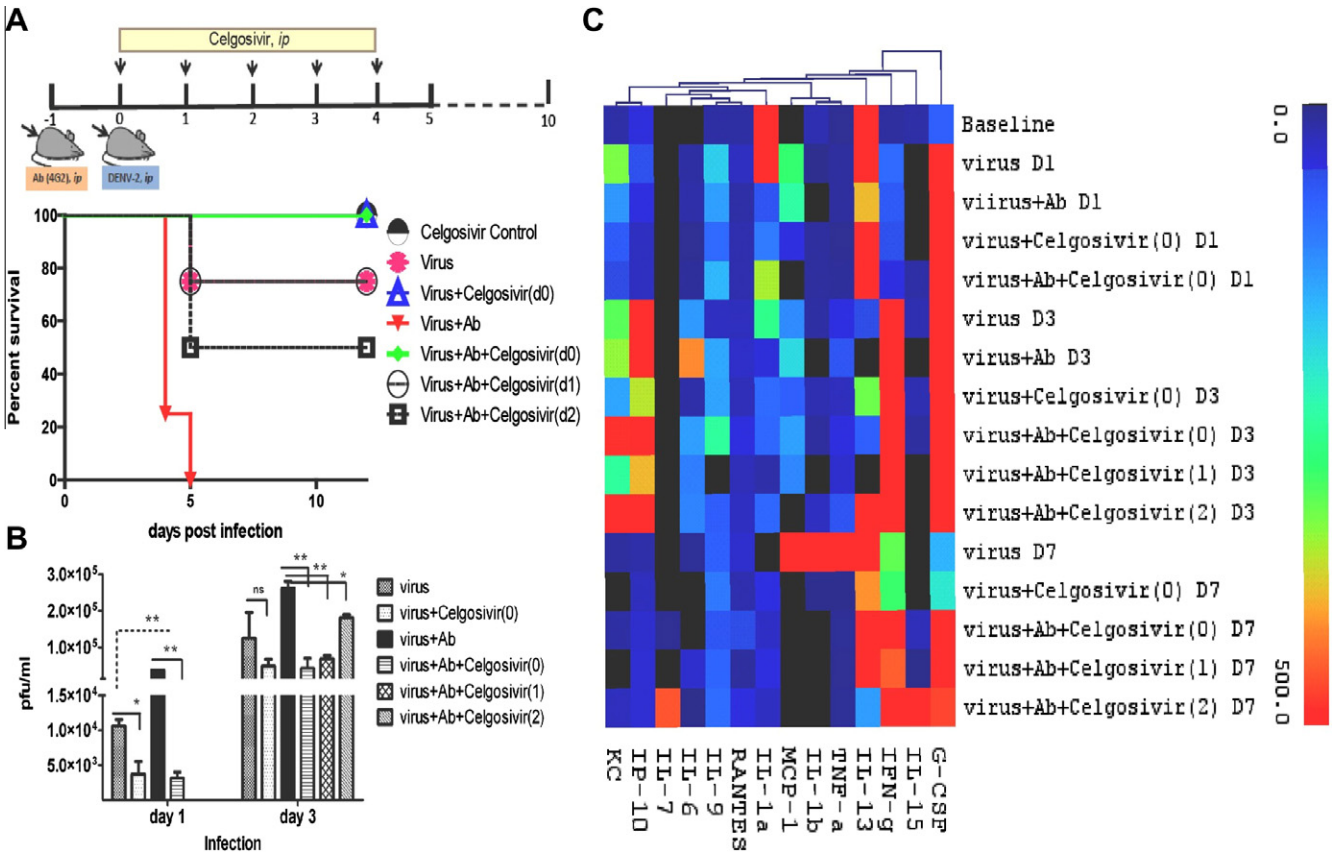


Fig. 4. Celgosivir is effective in a lethal challenge mouse model of DENV infection. (A) Direct and ADE DENV infection was modeled in AG129 mice and treatment was done with vehicle control (1% DMSO in PBS) or celgosivir (50 mg/kg/mouse) twice daily for 5 days starting from day 0, 1 or 2 post-infection. Celgosivir treatment significantly enhanced mouse survival ($P < 0.0001$). (B) Celgosivir-treated mice showed reduced viremia over the vehicle control as determined by plaque assay. (A) Serum cytokine analysis was performed on the mock- or celgosivir-treated mice. Heat map data displayed from 0 to 500 pg/ml shows elevated levels of cytokines such as IFN-γ, IL-9, RANTES and reduced levels, TNF-α and IL-6. The day celgosivir treatment was initiated is in parentheses group's label. Samples from days 1 (D1), 3 (D3) and 7 (D7) were analyzed. For significant data analysis the bioplex assay was repeated 2 times with the respective serum samples.

tion with E protein cross-reactive monoclonal antibody (4G2) 24 h prior to infection with the same virus dose resulted in 100% mortality at day 5. In this condition we found that the celgosivir dose that was chosen on the basis of our previous work was 100% protective when administered twice a day starting from the day of infection. Strikingly, in this lethal infection model we observed that even with treatment delayed by two days post-infection, 50% of mice survived the ADE challenge. These findings together with the data from the kinetic immunomonitoring, which showed improved immune status of the drug, treated animals raise the confidence on the efficacy of celgosivir and its potential use in the clinic against dengue fever, an acute viral infection that only requires a short course of treatment.

To further strengthen the case we demonstrated that in addition to the prM and E proteins (Courageot et al., 2000), celgosivir strongly inhibited viral genome replication by causing the NS1 protein to misfold and accumulate in the ER. That NS1 is also a target of celgosivir and a component of the overall effect of DENV replication is a finding that potentially has clinical significance due to NS1's association with severe DENV pathogenesis (Avirutnan et al., 2006, 2007;Falconar, 1997; Falconar and Martinez, 2011; Libraty et al., 2002).

The UPR machinery that maintains the cell's protein production homeostasis is activated when newly synthesized proteins in the ER, misfold and accumulate in the lumen. Since celgosivir treatment without infection creates general ER stress in a cell, the transcription level of several UPR genes and some of the proteins were monitored to understand the "yin and yang" of the drug treatment on infected THP-1 cells. The increase in pro-apoptotic transcripts, BIP and CHOP, and the enhanced phosphorylation of eIF2α in celgosivir-treated uninfected cells appears to be balanced by a significant increase in the transcription levels of EDEM-1, which encodes the pro-survival ER-resident chaperone that helps in terminal degradation of misfolded proteins to mitigate ER stress. In the direct or ADE-infected THP-1 cells without drug treatment the pro-apoptotic genes are up-regulated and the level of EDEM-1 transcript is lower than CHOP. This is consistent with DENV infection promoting ER stress and causing cell-death by increased apoptotic activity. In contrast, DENV infection in the presence of celgosivir is marked by the modulation of the UPR machinery, where ER stress is reduced and survival is enhanced likely due to celgosivir-induced increased levels of pro-survival proteins, EDEM-1 and XBP-1, and significantly reduced levels of the apoptotic marker, CHOP. The caveat in the present interpretation, however, is that the increased transcription levels need not necessarily imply higher protein production of the pro-survival factors.

In conclusion, the ER α-glucosidase inhibitor celgosivir is a promising antiviral agent against DENV infection, with clear evidence of *in vitro* and *in vivo* activity. As its precursor, Cast, is derived readily from trees that can be grown in tropical/sub-tropical climates, it is interesting to consider celgosivir as a possible affordable therapeutic agent in DENV endemic regions. Based on the detailed pre-clinical data presented here, we believe that further development of celgosivir is warranted as a possible treatment for DENV infection.

Conflict of interest

The authors ascertain that they do not have any commercial or other association that might pose a conflict of interest in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.10.002.

References

Avirutnan, P., Punyadee, N., Noisakran, S., Komoltri, C., Thiemmeca, S., Auethavornanan, K., Jairungsri, A., Kanlaya, R., Tangthawornchaikul, N., Puttikhunt, C., Pattanakitsakul, S.N., Yenchitsomanus, P.T., Mongkolsapaya, J., Kasinrerk, W., Sittisombut, N., Husmann, M., Blettner, M., Vasanawathana, S., Bhakdi, S., Malasit, P., 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J. Infect. Dis. 193, 1078–1088.

Avirutnan, P., Zhang, L., Punyadee, N., Manuyakorn, A., Puttikhunt, C., Kasinrerk, W., Malasit, P., Atkinson, J.P., Diamond, M.S., 2007. Secreted NS1 of dengue virus attaches to the surface of cells via interactions with heparan sulfate and chondroitin sulfate E. PLoS Pathog. 3, e183.

Balsitis, S.J., Williams, K.L., Lachica, R., Flores, D., Kyle, J.L., Mehlhop, E., Johnson, S., Diamond, M.S., Beatty, P.R., Harris, E., 2010. Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog. 6, e1000790.

Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. 44, 649–688.

Courageot, M.P., Frenkiel, M.P., Dos Santos, C.D., Deubel, V., Desprès, P., 2000. Alpha-glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. J. Virol. 74, 564–572.

Durantel, D., 2009. Celgosivir, an alpha-glucosidase I inhibitor for the potential treatment of HCV infection. Curr. Opin. Invest. Drugs 10, 860–870.

Falconar, A.K., 1997. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch. Virol. 142, 897–916.

Falconar, A.K., Martinez, F., 2011. The NS1 glycoprotein can generate dramatic antibody-enhanced dengue viral replication in normal out-bred mice resulting in lethal multi-organ disease. PLoS ONE 6, e21024.

Gutsche, I., Coulibaly, F., Voss, J.E., Salmon, J., d'Alayer, J., Ermonval, M., Larquet, E., Charneau, P., Krey, T., Mégret, F., Guittet, E., Rey, F.A., Flamand, M., 2011. Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. Proc. Natl. Acad. Sci. USA 108, 8003–8008.

Guzman, M.G., Halstead, S.B., Artsob, H., Buchy, P., Farrar, J., Gubler, D.J., Hunsperger, E., Kroeger, A., Margolis, H.S., Martinez, E., Nathan, M.B., Pelegrino, J.L., Simmons, C., Yoksan, S., Peeling, R.W., 2010. Dengue: a continuing global threat. Nat. Rev. Microbiol. 8, S7–16.

Halstead, S.B., 2007. Dengue. Lancet 370, 1644–1652.

Halstead, S.B., O'Rourke, E.J., 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J. Exp. Med. 146, 201–217.

Hosokawa, N., Tremblay, L.O., Sleno, B., Kamiya, Y., Wada, I., Nagata, K., Kato, K., Herscovics, A., 2010. EDEM1 accelerates the trimming of alpha1, 2-linked mannose on the C branch of *N*-glycans. Glycobiology 20, 567–575.

K. Kaita, E. Yoshida, D. Kunimoto, F. Anderson, M. Sherman, P. Marotta, L. Scully, K. Peltekian, R. Enns, F. Diaz-Mitoma, S. Lee, L. Worobetz, J. Pankovich, A. Petersen, Phase II proof of concept study of celgosivir in combination with peginterferon alfa-2b and ribavirin in chronic hepatitis C genotype-1 non-responder patients. Digestive Disease Weekly May, Abstr 442 (2007).

Kang, M.S., 1996. Uptake and metabolism of BuCast: a glycoprotein processing inhibitor and a potential anti-HIV drug. Glycobiology 6, 209–216.

Keller, T.H., Chen, Y.L., Knox, J.E., Lim, S.P., Ma, N.L., Patel, S.J., Sampath, A., Wang, Q.Y., Yin, Z., Vasudevan, S.G., 2006. Finding new medicines for flaviviral targets. Novartis Found Symp. 277, 102–114 (discussion 114–109, 251–103).

Libraty, D.H., Young, P.R., Pickering, D., Endy, T.P., Kalayanarooj, S., Green, S., Vaughn, D.W., Nisalak, A., Ennis, F.A., Rothman, A.L., 2002. High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J. Infect. Dis. 186, 1165–1168.

Low, J.G., Ooi, E.E., Tolfvenstam, T., Leo, Y.S., Hibberd, M.L., Ng, L.C., Lai, Y.L., Yap, G.S., Li, C.S., Vasudevan, S.G., Ong, A., 2006. Early Dengue infection and outcome study (EDEN) – Study design and preliminary findings. Ann. Acad. Med. Singapore 35, 783–789.

Molinari, M., Calanca, V., Galli, C., Lucca, P., Paganetti, P., 2003. Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. Science 299, 1397–1400.

Molyneux, R.J., Roitman, J.N., Dunnheim, G., Szumilo, T., Elbein, A.D., 1986. 6-Epicastanospermine, a novel indolizidine alkaloid that inhibits alpha-glucosidase. Arch. Biochem. Biophys. 251, 450–457.

Ng, C.Y., Gu, F., Phong, W.Y., Chen, Y.L., Lim, S.P., Davidson, A., Vasudevan, S.G., 2007. Construction and characterization of a stable subgenomic dengue virus type 2 replicon system for antiviral compound and siRNA testing. Antiviral Res. 76, 222–231.

Oda, Y., Hosokawa, N., Wada, I., Nagata, K., 2003. EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. Science 299, 1394–1397.

Paradkar, P.N., Ooi, E.E., Hanson, B.J., Gubler, D.J., Vasudevan, S.G., 2011. Unfolded protein response (UPR) gene expression during antibody-dependent enhanced infection of cultured monocytes correlates with dengue disease severity. Biosci. Rep. 31, 221–230.

Priyadarshini, D., Gadia, R.R., Tripathy, A., Gurukumar, K.R., Bhagat, A., Patwardhan, S., Mokashi, N., Vaidya, D., Shah, P.S., Cecilia, D., 2010. Clinical findings and pro-inflammatory cytokines in dengue patients in Western India: a facility-based study. PLoS ONE 5, e8709.

Sabin, A.B., 1952. Research on dengue during World War II. Am. J. Trop. Med. Hyg. 1, 30–50.

Schröder, M., Kaufman, R.J., 2005. The mammalian unfolded protein response. Annu. Rev. Biochem. 74, 739–789.

Schul, W., Liu, W., Xu, H.Y., Flamand, M., Vasudevan, S.G., 2007. A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J. Infect. Dis. 195, 665–674.

Somnuke, P., Hauhart, R.E., Atkinson, J.P., Diamond, M.S., Avirutnan, P., 2011. N-linked glycosylation of dengue virus NS1 protein modulates secretion, cell-surface expression, hexamer stability, and interactions with human complement. Virology 413, 253–264.

St. John, A.L., Rathore, A.P., Yap, H., Ng, M.L., Metcalfe, D.D., Vasudevan, S.G., Abraham, S.N., 2011. Immune surveillance by mast cells during dengue infection promotes natural killer (NK) and NKT-cell recruitment and viral clearance. Proc. Natl. Acad. Sci. USA 108, 9190–9195.

Tan, G.K., Ng, J.K., Trasti, S.L., Schul, W., Yip, G., Alonso, S., 2010. A non-mouse-adapted dengue virus strain as a new model of severe dengue infection in AG129 mice. PLoS Negl. Trop. Dis. 4, e672.

Taylor, D.L., Sunkara, P.S., Liu, P.S., Kang, M.S., Bowlin, T.L., Tyms, A.S., 1991. 6-*O*-Butanoylcastanospermine (MDL 28, 574) inhibits glycoprotein processing and the growth of HIVs. AIDS 5, 693–698.

Thein, S., Aung, M.M., Shwe, T.N., Aye, M., Zaw, A., Aye, K., Aye, K.M., Aaskov, J., 1997. Risk factors in dengue shock syndrome. Am. J. Trop. Med. Hyg. 56, 566–572.

Umareddy, I., Chao, A., Sampath, A., Gu, F., Vasudevan, S.G., 2006. Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. J. Gen. Virol. 87, 2605–2614.

Vaughn, D.W., Green, S., Kalayanarooj, S., Innis, B.L., Nimmannitya, S., Suntayakorn, S., Endy, T.P., Raengsakulrach, B., Rothman, A.L., Ennis, F.A., Nisalak, A., 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J. Infect. Dis. 181, 2–9.

Whitby, K., Pierson, T.C., Geiss, B., Lane, K., Engle, M., Zhou, Y., Doms, R.W., Diamond, M.S., 2005. Castanospermine, a potent inhibitor of dengue virus infection in vitro and in vivo. J. Virol. 79, 8698–8706.

Whitehorn, J., Farrar, J., 2010. Dengue. Br. Med. Bull. 95, 161–173.

Yin, Z., Chen, Y.L., Schul, W., Wang, Q.Y., Gu, F., Duraiswamy, J., Kondreddi, R.R., Niyomrattanakit, P., Lakshminarayana, S.B., Goh, A., Xu, H.Y., Liu, W., Liu, B., Lim, J.Y., Ng, C.Y., Qing, M., Lim, C.C., Yip, A., Wang, G., Chan, W.L., Tan, H.P., Lin, K., Zhang, B., Zou, G., Bernard, K.A., Garrett, C., Beltz, K., Dong, M., Weaver, M., He, H., Pichota, A., Dartois, V., Keller, T.H., Shi, P.Y., 2009. An adenosine nucleoside inhibitor of dengue virus. Proc. Natl. Acad. Sci. USA 106, 20435–20439.

Zellweger, R.M., Prestwood, T.R., Shresta, S., 2010. Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. Cell Host Microbe 7, 128–139.

Zhang, Y., Corver, J., Chipman, P.R., Zhang, W., Pletnev, S.V., Sedlak, D., Baker, T.S., Strauss, J.H., Kuhn, R.J., Rossmann, M.G., 2003. Structures of immature flavivirus particles. EMBO J. 22, 2604–2613.