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Optimizing celgosivir therapy in mouse models of dengue virus infection of serotypes 1 and 2: The search for a window for potential therapeutic efficacy

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ABSTRACT

Although the antiviral drug celgosivir, an α-glucosidase I inhibitor, is highly protective when given twice daily to AG129 mice infected with dengue virus, a similar regimen of twice daily dosing did not significantly reduce serum viral loads in patients in a recent clinical trial. This failure presumably might reflect the initiation of treatment when patients were already viremic. To better mimic the clinical setting, we used viruses isolated from patients to develop new mouse models of DENV1 and DENV2 infection and employed the models to test the twice daily treatment, begun either on the day of infection or on the third day post-infection, when the mice had peak of viremia. We found that, although the treatment started on day 0 was effective on viral load reduction, it provided no benefit when begun on day 3, indicating that *in vivo* antiviral efficacy becomes less prominent once viremia reaches the peak level. To determine if the therapeutic regimen in humans could be improved, we tested regimen of four-times daily treatment and found that the treatment significantly reduced viremia, suggesting that a similar regimen may be effective in a human clinical trial. A new clinical trial to investigate an altered dosing regimen has been approved (NCT02569827).

1. Introduction

Dengue virus (DENV) infection with any of the 4 related viral serotypes (DENV1–4) causes a variety of clinical manifestations ranging from self-limiting febrile illness, known as dengue fever (DF), to the life-threatening severe diseases, such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), characterized by vascular leakage, thrombocytopenia, bleeding and elevated levels of cytokines (Gubler, 2006; Halstead, 2007; Simmons et al., 2012). Dengue is emerging as a global public health threat with an estimated 400 million human infections and several hundred thousand cases of severe dengue occurring yearly (Bhatt et al., 2013). At present, however, there are no approved antiviral drugs or preventive vaccines. Antiviral strategy for dengue aims to inhibit viral replication and promote faster viremia clearance in patients, so that the febrile period can be shortened and further progression to more severe dengue diseases (DHF/DSS) can be prevented (Keller et al., 2006).

Celgosivir, also known as 6-O-butanoyl castanospermine (Bu-Cast), exerts antiviral activity by inhibiting the ER-resident alpha-glucosidase I enzyme (Mehta et al., 1998). Recently, celgosivir was tested in a Phase 1b randomized, double-blind, placebo-controlled trial in 50 adult dengue patients in Singapore (the CELgosivir as a treatment Against DENgue; CELADEN) (Low et al., 2014). In the pre-clinical study, we showed that celgosivir had the half maximal effective concentration (EC_{50}) of below micromolar against all 4 DENV serotypes in *in vitro* cell-based assay (Rathore et al., 2011). In a lethal AG129 mouse model of DENV infection, it was demonstrated that the compound had 100% protective efficacy against lethal infection when the mice were treated twice daily at 50 mg/kg (Rathore et al., 2011; Watanabe et al., 2012a), which is comparable to a clinical dose (initial 400 mg loading dose followed by 200 mg twice daily) (Low et al., 2014) when translated to human equivalent dose (HED) (Reagan-Shaw et al., 2008). In addition, we

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demonstrated that celgosivir improved mouse survival even when treatment was delayed for 48 h post-infection (Rathore et al., 2011). Thus, CELADEN became the first clinical trial for dengue where the antiviral effect of the drug was pre-clinically proven in an animal model. Unexpectedly, however, the trial failed to show statistically significant levels of lowering of viremia or fever in patients unstratified by prior exposure status (Low et al., 2014). Understanding the reason for the discrepancy between the pre-clinical experimental data and clinical outcome in humans is important for dengue antiviral research and also for the exploration of any potential for further development of celgosivir.

In past studies using mouse models of dengue, the levels of "peak viremia reduction" have been taken into account to assess the antiviral effect of the drugs (Chang et al., 2011; Chen et al., 2014; Fraser et al., 2014; Perry et al., 2013; Rathore et al., 2011; Schul et al., 2007; Watanabe et al., 2012a; Yin et al., 2009). In the present study, since human dengue patients are viremic at the initiation of treatment, we measured drug efficacy when therapy was begun either at the time of infection or at the time of peak viremia using new mouse models of clinical isolates DENV1 and DENV2, and also compared the benefit provided by twice-daily or four-times-daily treatment. Here we show that, although twice-daily treatment from the time of infection was beneficial, it had poor effect when begun during the peak viremia. However, four-times-daily treatment was beneficial even when the mice were viremic, suggesting that such a dosing regimen should be evaluated in further clinical trials.

2. Material and methods

2.1. Cells, virus and antibodies

BHK-21 (baby hamster kidney fibroblast cells, ATCC), THP-1 (human monocytic cells, ATCC), Vero (kidney epithelial cells derived from an African green monkey, ATCC) and C6/36 (*Aedes albopictus* cells, ATCC) were maintained as described in Supplementary methods. A mouse-adapted DENV2 strain S221 (Yauch et al., 2009) was a gift from Dr. Sujan Shresta (La Jolla Institute for Allergy and Immunology, CA). DENV1-2402 (EDEN1, GenBank accession EU081230.1) and DENV2-3295 (EDEN2, GenBank accession EU081177.1) were obtained from the Early Dengue infection and outcome (EDEN) study in Singapore (Low et al., 2006). DENV strains were grown in C6/36 cells and the supernatants were stored at −80 °C after filtration through a 0.45 μm membrane. DENV strains obtained from CELADEN (Low et al., 2014) were amplified from the patient's serum (pre-treatment) in C6/36 cells, and the supernatants after 2 passages in C6/36 cells were used for the experiments. Virus titer was determined by standard plaque assay on BHK-21 cells. 4G2 (mouse IgG2a, anti-E) hybridoma was purchased from ATCC. Purified 4G2 Abs was prepared as described previously (Watanabe et al., 2012b).

Table 1
Antiviral effect of celgosivir and NITD008 against DENV strains in BHK-21, Huh-7, Vero and THP-1 cells.

Cell line	Virus	MOI	EC_{50} (SD)	
			Celgosivir	NITD008
BHK-21	EDEN1	0.3	0.105 (0.059)	0.865 (0.172)
		0.01	0.066 (0.019)	0.608 (0.139)
	EDEN2	0.3	0.061 (0.003)	0.354 (0.157)
	S221	0.3	0.119 (0.000)	0.509 (0.285)
HuH-7	EDEN1	0.3	17.430 (4.921)	0.684 (0.019)
		0.01	5.961 (1.258)	0.356 (0.058)
	EDEN2	0.3	0.824 (0.109)	0.098 (0.058)
	S221	0.3	5.093 (1.036)	0.074 (0.008)
Vero	EDEN1	0.3	51.035 (14.47)	2.815 (0.069)
		0.01	13.805 (1.902)	0.910 (0.223)
	EDEN2	0.3	2.434 (0.773)	1.174 (0.066)
	S221	0.3	8.336 (1.041)	1.477 (0.047)
THP-1	EDEN1	2	3.236 (–)	0.358 (–)
	EDEN2	50	0.756 (–)	0.679 (–)
	S221	2	2.135 (–)	0.390 (–)

Experiment was conducted in duplicate and the results show the average EC_{50} values with standard deviations (SD). Data on THP-1 cells were obtained from a single experiment and shown without SD.

2.2. In vitro cell-based antiviral assay

Celgosivir was purchased from Dalton Pharma Services, Canada. NITD008 was a generous gift from Novartis Institute for Tropical Diseases, Singapore. BHK-21, HuH-7 and Vero cells (1×10^5 cells) were seeded on 24-well plates and incubated overnight to allow the cells to adhere. Cells were infected with 200 μl of DENV strains at a different multiplicity of infection (MOI) as indicated in the result section and incubated at 37 °C for 1 h. For antibody-dependent enhancement (ADE) infection, virus and 0.05 μg of 4G2 were mixed on ice for 1 h, and infected on THP-1 cells (1×10^5 cells) for 1.5 h at 37 °C. After removal of virus, cells were washed once with media and replaced with 500 μl culture media containing celgosivir or NITD008 with a serial 4-fold dilution starting from 200 μM or 50 μM. Cells were incubated for an additional 48 h at 37 °C, and the supernatants were collected and subjected to plaque assay using BHK-21 cells to determine virus titer. EC_{50} was determined using the GraphPad Prism software.

2.3. In vivo experiments

Sv/129 mice deficient in type I/II IFN receptors (AG129), purchased from B&K Universal (UK), were housed in the BSL-2 animal facility at Duke-NUS, Singapore and all animal experiments (protocol 2012/SHS/713) were approved by the Institutional Animal Care And Use Committee at Singapore Health Services. Seven to eleven week-old mice were inoculated intravenously with DENV including clinical isolates of DENV1 and DENV2 as well as mouse-adapted DENV2 strain S221 with different virus titer indicated in the figure legends. For lethal infection under ADE condition, 50 μg (for EDEN1 and EDEN2) or 10 μg (for S221) of 4G2 Ab was administered intraperitoneally into mice one day before infection (Watanabe et al., 2012b). Celgosivir, stored at 100 mg/ml in PBS at −30 °C, and NITD008, stored at 100 mg/ml in 95% DMSO at −30 °C, were diluted with PBS before each dosing to obtain 1 mg/200 μl (50 mg/kg) or 0.2 mg/200 μl (10 mg/kg) for celgosivir and 0.5 mg/200 μl (25 mg/kg) for NITD008. For a twice-daily dosing, mice were administered orally with 200 μl of the diluted compounds at 8/16 h cycle (10 AM and 6 PM on each day). For a four-times daily dosing, mice were treated at 4/4/4/12 h cycle (9 AM, 1 PM, 5 PM and 9 PM on each day). Blood were collected on each day up to 8 days post-infection by submandibular bleeding and serum samples were used to measure viral copy number by reverse transcription quantitative real-time PCR (qRT-PCR).

2.4. Quantification of viral load in serum by qRT-PCR

Serum viral RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. qRT-PCR was carried out in Bio-Rad Real-time thermal cycler CFX96 by the use of qScript One-Step qRT-PCR kit (Quanta). Primers and TaqMan probes for DENV1 and DENV2 (Houng et al., 2000) are described in Supplementary methods. Plasmids containing whole genome sequences of DENV1-2402 (EDEN1) or DENV2-3295 (EDEN2) (Low et al., 2006) were used to generate a standard curve for the quantification of viral genome copy number.