Chapter 24

Evaluation of Dengue Antiviral Candidates In Vivo in Mouse Model

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Abstract

In vivo evaluation of antiviral compounds can serve as criteria in the drug discovery process for selection of compounds that are suitable to enter late preclinical studies and further development. Dengue virus serotypes 1–4 can infect and replicate in the interferon type I and type II receptor deficient mice (AG129). Here we describe the use of a mouse-adapted dengue 2 virus strain (S221) that has been used to develop a robust lethal model of infection. Treatment with small molecule inhibitors of DENV replication at the time of infection or delayed treatment up to 48 h post infection can result in measurable protection that reflects the efficacy of the tested compound.

Key words Dengue mouse model, Dengue antiviral testing, Virus quantification by plaque assay, Antibody-dependent enhanced infection, 4G2 antibody

1 Introduction

Dengue is a global public health threat caused by infection with any of the four related viral serotypes (DENV1–4). Clinical manifestations range 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) [1, 2]. Most cases of DHF/DSS are associated with secondary heterotypic infections, probably due to the phenomenon known as antibody-dependent enhanced (ADE) infection [2, 3]. More than two billion people are at risk of infection worldwide, and there are more than 400 million human infections and several hundred thousand cases of DHF/DSS per year [2, 4]. At present, however, there are no approved preventive vaccines or antiviral drugs against DENV infection. In vitro cell-based assays [5–7] are routinely used to determine concentration of drug that results in 50 % reduction in infection (EC50). The testing of compounds in suitable in vivo models provides an opportunity to optimize the potency of the compound in terms of pharmacokinetics, pharmacodynamics, and toxicity [6, 8, 9].

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1.1 Animal Models for DENV Infection

The lack of a suitable animal model has hampered the evaluation of novel antiviral candidates for DENV infection. The AG129 (Sv/129 mice deficient in type I and II IFN receptors) viremia model was used to show that animals infected with a clinical (non-lethal) isolate of DENV2 resulted in reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drug [10]. The infected animals showed a transient increase in serum viral levels that peaked 3 days post infection (dpi) and also showed an increase in virus-expressed antigen NS1, thus confirming that the unadapted virus was able to replicate in the AG129 mouse model. Similar to human infection, the viral load and NS1 antigen levels decreased over time. Furthermore, infected mice had significantly enlarged spleens as well as higher levels of pro-inflammatory cytokines. Directly acting antivirals which target the viral RNA-dependent RNA polymerase (RdRp) activity of NS5 protein, such as 7-deaza 2′-C-methyl adenosine, and host alpha-glucosidase inhibitors, such as *N*′-nonyl-deoxynojirimycin or 6-*O*-butanoyl castanospermine (celgosivir) reduced viral load in a dose-dependent manner in this murine model. Most importantly, levels of pro-inflammatory cytokines and the extent of splenomegaly were also reduced with the drug treatment thus demonstrating the value of antiviral treatment, albeit in a mouse infection model. More recently, AG129 mouse was used as a lethal dengue mouse model that recapitulated several of the major pathologies of human infection including high level of viremia, elevated levels of cytokines, vascular leakage, intestinal bleeding, thrombocytopenia, and death [11, 12], which further supports the use of this mouse model to select the suitable candidates and treatment strategy before the clinical study. In this chapter, we describe the method for the in vivo drug evaluation using the example of celgosivir treatment in mice [6, 9].

2 Materials

2.1 Generation of DENV Stocks

1. Cells of the *Aedes albopictus* C6/36 line (ATCC catalogue number: CRL-1660).
2. RPMI1640 (10 % FBS): RPMI1640 medium (GIBCO) supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, 100 U/mL penicillin and streptomycin (PenStrep; GIBCO), 25 mM HEPES.
3. RPMI1640 (2 % FBS): RPMI1640 medium supplemented with 2 % heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin, 25 mM HEPES.
4. Incubator: No CO_2 atmosphere at 28 °C.
5. 1× PBS: 137 mM NaCl, 6.25 mM Na_2HPO_4, 2.5 mM Na_2PO_4; sterilized by autoclave.

6. 0.25 % Trypsin–EDTA.
7. Dengue virus stock: DENV-2 mouse-adapted S221.
8. Sterile 2 ml microcentrifuge tubes (screw cap and safe for storage at –80 °C; Sarstedt or similar).
9. –80 °C freezer.

2.2 Determination of Viral Titer by Plaque Assay

1. Cells of the baby hamster kidney cell line, BHK-21 (ATCC catalogue number: CCL-10).
2. RPMI1640 (10 % FBS): RPMI1640 medium supplemented with 10 % heat-inactivated FBS; Gibco, 100 U/mL penicillin and streptomycin, 25 mM HEPES.
3. RPMI1640 (no serum): RPMI1640 medium supplemented with 2 mM L-glutamine.
4. Humidified incubator with 5 % CO_2 atmosphere at 37 °C.
5. 1× PBS.
6. 0.25 % Trypsin–EDTA.
7. Dengue virus samples to titer (collected from infected C6/36 cell supernatant).
8. 0.8 % Methyl-cellulose medium with 2 % FBS.
9. 3.7 % Formaldehyde.
10. 1 % Crystal violet.

2.3 Preparation of a-DENV E Protein Antibodies (4G2) for the Induction of Antibody-Dependent Enhanced (ADE) Infection

1. Hybridoma cells; 4G2 (ATCC catalogue number: HB-112).
2. RPMI1640 (10 % FBS): RPMI1640 medium supplemented with 10 % heat-inactivated FBS; Gibco, 100 U/mL penicillin and streptomycin, 25 mM HEPES.
3. PFHM-II: Protein-Free hybridoma Medium (GIBCO) supplemented with 2 mM L-glutamine, 100 U/mL penicillin and streptomycin.
4. Humidified incubator with 5 % CO_2 atmosphere at 37 °C.
5. 1× phosphate-buffered saline (PBS), filtered, pH 7.2.
6. 0.1 M glycine (pH 2.7).
7. 1 M Tris–HCl (pH 7.2).
8. 5 ml Protein G column (GE Healthcare).
9. AKTApurifier™ UPC 10 (GE Healthcare).

2.4 DENV Infection and Drug Treatment in AG129 Mice

1. Sv/129 mice deficient in type I and II IFN receptors (AG129 mice).
2. Purified 4G2 Abs.
3. S221 virus stock.

4. Test compounds.
5. Dimethyl sulfoxide, DMSO.
6. 29G insulin syringe (BD).
7. 27G needle (BD).

2.5 Determination of Serum Viral Load by Plaque Assay

1. BHK-21 cells.
2. RPMI1640 (10 % FBS).
3. RPMI1640 (no serum).
4. Humidified incubator with 5 % CO_2 atmosphere at 37 °C.
5. 1× PBS.
6. 0.25 % Trypsin–EDTA.
7. Mouse serum samples.
8. 0.8 % Methyl-cellulose medium with 2 % FBS.
9. 3.7 % Formaldehyde.
10. 1 % Crystal violet.

3 Methods

3.1 Generation of DENV Stocks

3.1.1 Preparation of C6/36 Insect Cells for Generation of Viral Stocks

1. Maintain C6/36 cells in RPMI1640 (10 % FBS) media in sterile tissue culture flasks (*see* **Note 1**).
2. Dislodge healthy cells in 0.25 % Trypsin–EDTA for less than 5 min. Add culture medium to resuspend cells and transfer into a centrifugal tube.
3. Centrifuge cells at 900 × *g* for 5 min at room temperature.
4. Discard medium and resuspend cells in fresh growth medium.
5. Seed cells into 175 cm^2 sterile tissue culture flasks in 25 ml of culture media and incubate at 28 °C under non-CO_2 atmosphere condition.

3.1.2 Generation of Viral Stocks

1. Once cells have reached 90 % confluency in 175 cm^2 tissue culture flasks, remove the existing media and infect virus with 0.1 MOI in 5 ml RPMI1640 (2 % FBS).
2. Incubate for 1 h at 28 °C under non-CO_2 atmosphere condition.
3. Remove virus and add 25 ml RPMI1640 (2 % FBS).
4. Incubate for 4–7 days at 28 °C under non-CO_2 atmosphere condition (*see* **Note 2**).
5. Scrape cells and transfer into centrifugal tubes, then centrifuge cells at 1,800 × *g* for 20 min at 4 °C.

6. Collect supernatant by syringe and transfer into fresh tubes by filtration with 0.45 mm filter.
7. Aliquot virus into 2 ml cryotubes and store in liquid nitrogen until use.

3.2 Determination of Viral Titer by Plaque Assay

3.2.1 Preparation of BHK-21 Cells for Plaque Assay

1. Maintain BHK-21 cells in RPMI1640 (10 % FBS) media in sterile tissue culture flasks.
2. Dislodge healthy cells in 0.25 % Trypsin–EDTA for less than 5 min. Add culture medium to resuspend cells and transfer into a centrifuge tube.
3. Centrifuge cells at 900 × *g* for 5 min at room temperature.
4. Discard medium and resuspend cells in fresh growth medium.
5. Count cell number and seed cells at 2×10^5 cells/well/500 ml in 24-well plate (*see* **Note 3**).
6. Incubate cells overnight at 37 °C in 5 % CO_2 incubator to allow cells to adhere plate.

3.2.2 Plaque Assay

1. Serially dilute virus tenfold in RPMI1640 (no serum).
2. Remove culture supernatant of BHK-21 cells and add 200 ml of diluted virus into each well (*see* **Note 4**).
3. Incubate plate for exactly 1 h at 37 °C in 5 % CO_2 incubator.
4. Remove virus and add 500 ml of 0.8 % Methyl-cellulose medium supplemented with 2 % FBS (*see* **Note 5**).
5. Incubate plate for 4–5 days at 37 °C in 5 % CO_2 incubator (*see* **Note 6**).
6. Fix cells with 3.7 % formaldehyde for 20 min.
7. Rinse plate with copious volume of water in a container. Shake plate robustly to remove Methyl-cellulose medium completely.
8. Add 500 ml of 1 % crystal violet into each well and stain for 1 min.
9. Rinse plate with copious volume of water in a container and shake plate to remove excess water.
10. Dry plate and count number of plaques to determine virus titer.

3.3 Preparation of a-DENV E Protein Antibodies (4G2) for the Induction of ADE Infection

3.3.1 Collection of 4G2 Hybridoma Cell Culture Supernatant

1. Culture 4G2 hybridoma cells in 50–75 ml of PFHM-II (Protein-Free hybridoma Medium) in sterile 175 cm^2 tissue culture flasks (*see* **Note 7**).
2. Once the color of culture media turns to orange or yellow, collect cell suspension into a centrifuge tube.
3. Centrifuge cells at 900 × *g* for 5 min at room temperature.
4. Collect supernatant (*see* **Note 8**).
5. If more supernatant is required, continue culture of cells and repeat **steps 1–4**.

3.3.2 Purification of Antibodies (See Note 9)

1. Filter combined supernatant through a 0.45 mm membrane.
2. Load the 4G2 supernatant onto a 5 ml Protein G column pre-equilibrated in pH 7.2 PBS.
3. Wash the column with PBS using 5× the column volume (i.e., 25 ml).
4. Prepare a 96-well block containing 60 ml 1 M Tris–HCl (*see* **Note 10**).
5. Elute using 100 % 0.1 M glycine and 1 ml fractions are collected into the wells of the block.
6. Check the quality of purified antibody by running a SDS PAGE.
7. Collect fractions of similar quality into a dialysis membrane and dialysed against PBS overnight.
8. Quantitate the concentration of the purified antibody using nanodrop.

3.4 DENV Infection and Drug Treatment in AG129 Mice

3.4.1 Administration of 4G2 to Induce ADE Infection

1. Prepare male AG129 mice aged 6–10 weeks (6–10 mice per group) (*see* **Note 11**).
2. Adjust purified 4G2 Abs at the concentration of 1–20 mg/200 ml in PBS (*see* **Note 12**).
3. Administer of 200 ml of Abs into AG129 mice intraperitoneally by 29G insulin syringe 1 day prior to virus infection.

3.4.2 Virus Infection and Drug Treatment

1. Dilute S221 virus stock with PBS into 2×10^5 pfu/200 ml.
2. Dissolve solid test compound in DMSO to 100 mg/ml stock (*see* **Note 13**).
3. Dilute test compounds with PBS (*see* **Note 14**).
4. Inoculate with 200 ml of virus intraperitoneally into mice by 29G insulin syringe.
5. Administer test compound intraperitoneally or orally into mice according to appropriate dosage regimen (*see* **Note 15**).
6. Observe mouse disease status and survival rate until day 10 post infection.
7. Collect blood samples at periodical time points to determine viremia level.

3.5 Determination of Serum Viral Load by Plaque Assay

3.5.1 Preparation of Mouse Serum Samples

1. Obtain blood samples from the facial vein of mice by using 27G needles.
2. Store blood samples overnight at 4 °C before separation of serum.
3. Centrifuge blood samples at 12,000 rpm for 5 min.
4. Collect serum and store at −80 °C until use.

3.5.2 Plaque Assay

1. Seed BHK-21 cells at 2×10^5 cells/well/500 ml in 24-well plate.
2. Incubate cells overnight at 37 °C in 5 % CO_2 incubator to allow cells to adhere plate.
3. Serially dilute serum samples (up to 10,000-fold) in RPMI1640 (no serum).
4. Remove culture supernatant of BHK-21 cells and add 200 ml of diluted serum samples into each well.
5. Incubate plate for exactly 1 h at 37 °C in 5 % CO_2 incubator.
6. Remove diluted serum and add 500 ml of 0.8 % Methyl-cellulose medium supplemented with 2 % FBS.
7. Incubate plate for 4–5 days at 37 °C in 5 % CO_2 incubator.
8. Fix cells and stain plate as indicated in Subheading 3.2.2.
9. Count plaque number and calculate viremia as *pfu/ml* (*see* **Note 16**).

4 Notes

1. Growth media supplemented with 20 % FBS improves cell growth if C6/36 cells do not proliferate desirably.
2. Keep incubation until the cytopatic effect (syncytium) can be clearly seen by microscopy. Some virus strains do not induce syncytium. In this case, check virus titer in the supernatant of different time point to determine the optimal day to harvest.
3. Cells become confluent on the following day by seeding 2×10^5 cells into each well. Two days incubation after seeding of 1×10^5 cells is also viable.
4. Virus should be added immediately after removing culture supernatant to avoid cells dried out.
5. Prepare 0.8 % methyl-cellulose medium as follows;
   Add 8 g of methyl-cellulose powder into 500 ml water followed by autoclave to dissolve powder. Prepare 500 ml of 2× RPMI1640 media by dissolving RPMI1640 powder in water followed by supplement with 2 % heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin and 25 mM HEPES. After filtration of 2× RPMI1640 media by 0.2 mm membrane, mix well with 500 ml of prepared methyl-cellulose and store at 4 °C.
6. The rate of plaque formation is affected by virus replication rate of the strain. Check the plaque size by visual observation before fixation.

7. Recommend the use of RPMI1640 (10 %) during the initial period of a couple of days after thawing cells. Once cells grow well, replace the media gradually with PFHM-II media.
8. The supernatant can be stored at 4 °C without filtration until the enough amount of supernatant can be obtained.
9. 4G2 antibody is purified using the AKTApurifer (GE). Please refer to the manufacturer's instruction guides regarding sample loading specifications.
10. The standard ratio of Tris–HCl to glycine (100:6) for neutralization is subjected to change depending on the concentration of buffers prepared. Volume of Tris–HCl to be added required for neutralization (pH 7) can be adjusted by pH paper testing.
11. AG129 mice were purchased from B&K Universal (UK) and maintained under specific pathogen-free (SPF) conditions in the animal facility. Maintenance of 20-breeding pairs produce more than 100 mice per month. Male mice are suitable for the drug evaluation since female mice are more sensitive to some kind of drugs.
12. Administration of 4G2 Abs ranged from 1 mg to 20 mg induce 100 % lethal infection by subsequent intraperitoneal inoculation with 2×10^5 pfu of S221 or intravenous inoculation with 2×10^4 pfu of S221.
13. Test compound should be dissolved in compatible solvent, generally DMSO, to high concentration stock.
14. If test compound is not soluble in PBS, appropriate solvent should be selected.
15. For celgosivir treatment, a twice-a-day regimen (BID) of 50 mg/kg achieved 100 % protective efficacy, while a single daily dose (QD) of 100 mg/kg for 5 days failed to protect mice from lethal infection (Fig. 1a).
16. Successful treatment will show clear reduction of viremia level compared with vehicle control (Fig. 1b).

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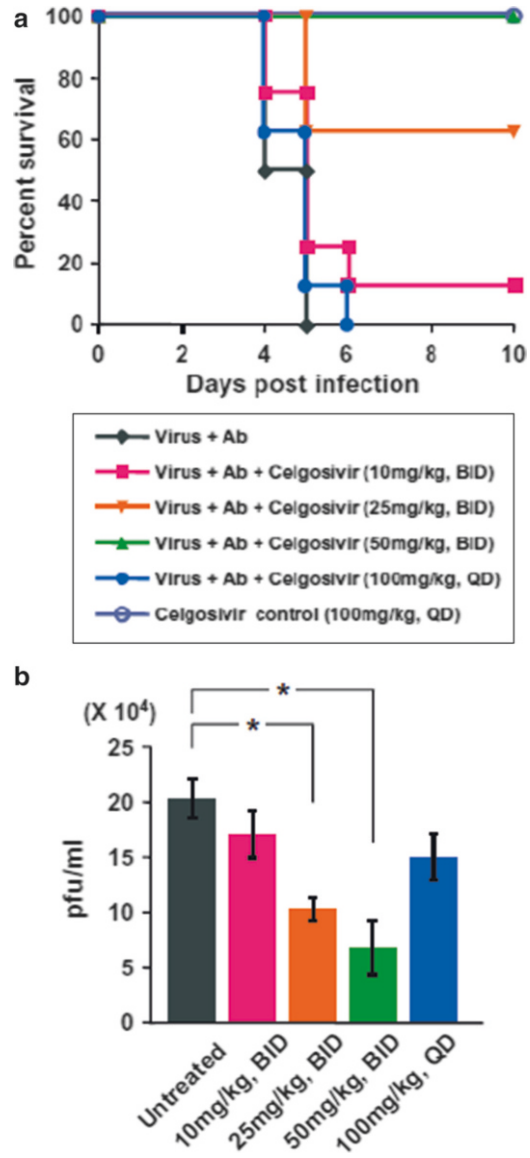

Fig. 1 Dose and schedule effects of celgosivir against lethal DENV infection in mice. AG129 mice were inoculated i.p. with 2×10^5 pfu of S221 in the presence of 20 mg of DENV anti-E Ab (4G2). Mice were treated with vehicle or different concentrations and doses of celgosivir at the time of infection and daily for 5 days. Mouse survival rates were monitored until day 10 post infection (**a**) and survival significance was evaluated using log-rank test. Viremia on day 3 post infection was measured by plaque assay (**b**). Significant differences between data groups were determined by two-tailed Student *t*-test analysis and *P* value less than 0.05 was considered significant ($^*P < 0.05$). A number of mice per group are 7–8

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