Antiviral Research 95 (2012) 216–223

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Resistance analysis of an antibody that selectively inhibits dengue virus serotype-1

Gang Zou [a,1], Petra Kukkaro [b,c], Shee-Mei Lok [b,c], Jowin K.W. Ng [d], Grace K. Tan [d], Brendon J. Hanson [e], Sylvie Alonso [d], Paul A. MacAry [d], Pei-Yong Shi [a,*]

[a] Novartis Institute for Tropical Diseases, Singapore
[b] Duke-NUS Graduate Medical School, Singapore
[c] Center for BioImaging Sciences, National University of Singapore, Singapore
[d] Department of Microbiology, Immunology Programme, National University of Singapore, Singapore
[e] Defense Medical and Environmental Research Institute, DSO National Laboratories, Singapore

ARTICLE INFO

Article history:
Received 22 May 2012
Revised 19 June 2012
Accepted 26 June 2012
Available online 4 July 2012

Keywords:
Dengue virus
Neutralizing antibody
Escape mutant
Virus entry
Flavivirus envelope protein

ABSTRACT

The four serotypes of dengue virus (DENV) are the causative agents of the most prevalent mosquito-borne viral disease in human. No clinically approved antiviral therapy is currently available. Therapeutic antibodies represent a viable approach for potential treatment of DENV infection. We recently isolated a human monoclonal antibody (HM14c10) that selectively neutralizes DENV serotype 1 (DENV-1), but not serotypes 2, 3, and 4. Here we report the resistance profile of DENV-1 against HM14c10 in cell culture. Escape mutant viruses readily emerged by culturing wild-type DENV-1 in the presence of the HM14c10 antibody. Sequencing of resistant viruses revealed a single T51K substitution in the domain I/II hinge region of the viral envelope protein. Residue T51 is located within the HM14c10 epitope and is highly conserved among various DENV-1 isolates. Recombinant DENV-1 containing the T51K mutation could not be neutralized by HM14c10 *in vitro* or *in vivo*. Biochemical assay revealed that the T51K mutation completely abolished the antibody binding to the DENV-1 virion. Collectively, the results demonstrate that a single amino acid change in DENV envelope protein can confer resistance to a potent antibody through abolishing the antibody-virus interaction.

1. Introduction

Dengue virus (DENV) is a mosquito-borne plus-sense RNA virus from the *Flavivirus* genus within the *Flaviviridae* family (Chambers et al., 1990). Approximately 2.5 billion people are at risk of DENV infection worldwide (Gubler et al., 2007; Guzman et al., 2010). Infection with DENV causes symptoms ranging from mild dengue fever [DF] to life-threatening dengue hemorrhagic fever [DHF] and dengue shock syndrome [DSS] (Gubler et al., 2007). There is currently no clinically approved vaccine or antiviral therapy for DENV infection (Whitehead et al., 2007). A number of approaches have been pursued to develop anti-DENV therapy, among which therapeutic antibody warrants further investment (Noble et al., 2010).

Similar to DENV, other flaviviruses such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV) contain genomic RNA that encodes a single open-reading-frame (ORF) that is translated into a polyprotein which is cleaved by viral and cellular proteases into ten mature proteins: three structural proteins (capsid [C], premembrane or membrane [M], and envelope [E] proteins) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Lindenbach et al., 2007). Structural analysis of mature DENV virion by cryoelectron microscopy (cryoEM) reveals a 50 nm virion with a smooth outer surface. The virion is covered by 90 anti-parallel E protein homodimers, arranged relatively flat along the virus surface with quasi-icosahedral $T = 3$ symmetry (Kuhn et al., 2002). The structure of E protein ectodomain has been solved by X-ray crystallography (Modis et al., 2004, 2005). The E protein consists of three distinct domains: domain I (DI) is a ten-stranded β-barrel and forms the central architecture of the protein; domain II (DII) is a long, finger-like protrusion from DI, with the putative fusion loop (residues 98–110) at its distal end, which participates in a type II fusion event (Allison et al., 2001). Domain III (DIII) is located on the opposite end of DI, forms a seven-stranded immunoglobulin-like fold, and has been suggested to be involved in receptor binding (Crill and Roehrig, 2001). Following exposure to low pH in the endosome, the E proteins rearrange from homodimers to homotrimers, exposing the fusion peptide. The fusion peptide inserts into the endosomal membrane, leading to fusion, uncoating, and nucleocapsid release into the cytoplasm (Bressanelli et al., 2004; Zhang et al., 2004).

* Corresponding author. Address: 10 Biopolis Road, #05-01 Chromos, Singapore 138670, Singapore. Tel.: +65 6722 2909; fax: +65 6722 2916.
E-mail address: pei_yong.shi@novartis.com (P.-Y. Shi).
[1] Present address: Institute Pasteur Shanghai, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025, China.

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http://dx.doi.org/10.1016/j.antiviral.2012.06.010

Humoral immunity is an essential component of the host response to flavivirus infection (Pierson et al., 2008; Roehrig et al., 2001). Upon flavivirus infection, most neutralizing antibodies are directed against viral E protein (Valdes et al., 2000). Neutralizing antibodies can inhibit virus infection by diverse mechanisms including inhibition of virus attachment to the cell surface, blockage of fusion to endosomal membranes, or suppression of release of progeny viruses (Lok et al., 2008; Rajamanonmani et al., 2009). We recently reported a potent neutralizing, serotype-specific monoclonal antibody (HM14c10) that could protect the AG129 mice (defective in interferon α/β/γ receptors) from DENV-1 infection at picomolar concentrations (Teoh et al., 2012). The antibody was derived from human B cells of an individual previously infected with DENV-1. Mechanistic analysis showed that the antibody specifically neutralizes DENV-1 mainly through inhibition of virus attachment to cell surface and, to a less extent, through suppression of a post-attachment step. The results indicate that the HM14c10 antibody is a promising therapeutic candidate for DENV-1 infection. However, the emergence of resistant virus against the antibody has not been studied.

The goal of this study is to examine the resistance profile of DENV-1 against HM14c10. We identified a single amino acid mutation (T51K), located in the DI/DII hinge region of viral E protein, that could confer complete resistance to the antibody neutralization both in cell culture and in the AG129 mouse model. Biochemical binding experiments showed that the T51K mutation abolished virus-antibody interaction. These results have important implications for development of antibody-based therapy for DENV infection.

2. Materials and methods

2.1. Cells, viruses, and antibodies

Baby hamster kidney cells (BHK21) and African green monkey kidney cells (Vero) were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) in 5% CO_2 at 37 °C. Mosquito C6/36 cells were cultured in RPMI medium with 10% FBS at 28 °C. DENV-1 virus (strain Westpac; GenBank accession No. U88535) was derived from an infectious cDNA clone (Dong et al., 2010). The generation and characterization of a human monoclonal antibody HM14c10 was recently reported (Teoh et al., 2012). Dengue specific anti-E monoclonal antibody 4G2 was prepared from hybridoma cell lines purchased from American Type Culture Collection (ATCC) (Rajamanonmani et al., 2009).

2.2. In vitro neutralization assays

Plaque reduction neutralization titer (PRNT) assays were performed on confluent monolayer of BHK21 cells. Briefly, serially diluted antibody (50 μl) were mixed with 100 PFU of DENV-1 in RPMI medium with 2% FBS (50 μl). The mixture (100 μl) was incubated for 1 h at 37 °C to allow the formation of virus-antibody complexes. The mixture was then added to individual wells of a 24-well plate in duplicates. Viral adsorption proceeded at 37 °C for 1 h, followed by the addition of 0.6 ml of RPMI-1640 plus 0.8% methylcellulose (Aquacide II, Calbiochem) and 2% FBS. After incubation at 37 °C with 5% CO_2 for 4 days, the cells were fixed with 3.7% formalin and stained with 1% crystal violet. The number of plaques were counted and normalized to the number of plaques derived from control wells in which virus was mixed with medium without antibody. The 50% effective dose (EC_{50}) of antibody was calculated using nonlinear regression analysis by Prism software (GraphPad, San Diego, CA).

2.3. Generation and characterization of neutralization escape mutants

DENV-1 was incubated with 1 μg/ml of HM14c10 at 37 °C for 1 h in DMEM. One hundred microliter of mixture was added to 3×10^5 Vero cells in a 12-well plate at a multiplicity of infection (MOI) of 0.3. After infection at 37 °C for 1 h, the cells were washed thrice with DMEM; fresh media containing 1 μg/ml of antibody were added. Virus growth under antibody selection proceeded at 37 °C for 72 h. At each passage, 50 μl of the culture fluid was mixed with 50 μl of fresh medium containing 2 μg/ml of antibody for 1 h; the mixture was added to Vero cells to establish next round of infection. After three passages under antibody selection, virus-containing cultures fluids were tested for resistance using PRNT. After the resistance phenotype was confirmed, viral RNAs were extracted from culture fluids using RNeasy kits (Qiagen). The extracted RNAs were amplified by RT-PCR using SuperScript III one-step RT-PCR kits (Invitrogen). The entire E gene was then directly sequenced from the gel-purified PCR products. As a negative control, DENV-1 was cultured in the absence of antibody for three rounds; the resulting virus was subjected to sequencing.

2.4. Plasmid construction, in vitro transcription, RNA transfection, and immunofluorescence assay (IFA)

An infectious cDNA clone of DENV-1 (pACYC-DENV-1; strain WestPac) and a subclone pACYC-DENV-1A were used to engineer mutations in the E gene in the context of DENV-1 genome-length RNA (Dong et al., 2010). The subclone pACYC-DENV-1A contains the *AscI-XhoI* fragment from the pACYC-DENV-1 (spanning from T7 promoter [for the RNA transcription of genome-length RNA] to nucleotide position 2,606 of the viral genome; GenBank No. U88535). To introduce E mutations into DENV-2, we used an infectious cDNA clone of DENV-2 (pACYC-DENV-2; strain NGC) and a subclone pACYC-DENV-2A (Zou et al., 2011). The subclone pACYC-DENV-2A contains the *SacII-XhoI* fragment from the pACYC-DENV-2 (spanning from T7 promoter to nucleotide position 5,426 of the viral genome; GenBank No. AF038403). For mutagenesis of both DENV-1 and DENV-2, a QuikChange II XL site-directed mutagenesis Kit (Stratagene) was used to engineer E mutations into the subclone. The mutated DNA fragment was cut and pasted back into the pACYC-DENV-1 plasmid with *AscI* and *XhoI*, and pACYC-DENV-2 plasmid with *SacII* and *XhoI*, respectively. All the constructs were verified by DNA sequencing.

The genome-length RNA of DENV-1 and DENV-2 were *in vitro* transcribed from corresponding cDNA plasmids that were pre-linearized with *SacII* and *XbaI*, respectively. A T7 mMESSAGE mMACHINE kit (Ambion) was used for RNA synthesis as described previous (Zhou et al., 2007). The RNAs were electroporated into BHK21 cells as previously described (Shi et al., 2002). After transfection of genome-length RNA, the cells were cultured at 37 °C for the first 24 h and then transferred to 30 °C for further incubation. Culture medium containing viruses was collected on day 4 post-transfection, aliquoted, and stored at −80 °C. IFA were performed as previously described (Hsieh et al., 2011).

2.5. ELISA-based virus-antibody binding assay

Wild-type (WT) and mutant T51 K DENV-1 were used as antigens for the ELISA-based virion-antibody binding assay. Culture fluids (collected from C6/36 cells on day 5 post-infection) were centrifuged at 4,000 *g* at 4 °C for 30 min to remove cell debris; the supernatants were centrifuged at 45,000 rpm (Type 70 Ti Rotor) at 4 °C for 2 h. The supernatant was carefully removed and the virus pellets were resuspended into TNE buffer (12 mM Tris–HCl, 120 mM NaCl, and 1 mM EDTA [pH 8.0]). The virus was diluted into PBS and coated onto 96-well flat bottom plates (Nunc Maxi-

Sorp) at 4 °C overnight. The amount of coated virus was set by determining a dilution of antigen that gave an equal high level signal when detected by dengue specific human polyclonal antibody that binds to all four serotypes of DENV (data not shown). The wells were blocked with 5% (w/v) milk/PBS at room temperature for 2 h. Serial dilutions of antibodies were prepared in 5% (w/v) milk/PBS, added to the antigen-coated plates, and incubated at room temperature for 1.5 h. The plates were washed four times with 0.1% Tween-20 in PBS and HRP conjugated anti-human IgG (Invitrogen, diluted 1,000-fold in 5% milk/PBS) was added to the plates. After incubation at room temperature for 1.5 h, the plates were washed again as described above. TMB substrate (Invitrogen) was then added, and the reaction was stopped by addition of 1 M HCl. Absorbance at 450 nm was quantified with Tecan Infinite M200 microplate reader (Tecan Group Ltd., Switzerland).

2.6. In vivo mouse efficacy assay

AG129 mice, deficient in IFN-α/β/γ receptors (van den Broek et al., 1995), were used for DENV-1 *in vivo* efficacy study (Schul et al., 2007). All mouse studies were approved and performed according to the guidelines of the Institutional Animal Care and Use Committee recommendations. Eight-week old AG129 mice were infected by intraperitoneal (i.p.) injection with 2×10^6 PFU of WT and T51K mutant viruses. A single dose of 20 μg of HM14c10 antibody or PBS control was administrated by i.p. injection at 24 h after infection. Mice were bled on day 3 and day 4 post-infection and viremia was quantified by plaque assay. The plaque assay was performed as described previously (Zou et al., 2011).

3. Results

3.1. Selection of escape mutant virus

We generated resistance virus by culturing WT DENV-1 in the presence of HM14c10 antibody (Fig. 1A). The $PRNT_{50}$ values (plaque reduction neutralization test) of HM14c10 antibody range from 5 ng/ml to 1.5 μg/ml against various DENV-1 genotypes; HM14c10 showed an $PRNT_{50}$ of 0.455 μg/ml against strain WestPac, the DENV-1 isolate used in the current resistance study (Teoh et al., 2012). After three rounds of culturing of DENV-1 on Vero cells under HM14c10 (1 μg/ml) selection, two out of four independent selections yielded viruses that could no longer be neutralized by HM14c10 in plaque reduction assay (Fig. 1B); viruses from the other two selections were still sensitive to HM14c10 neutralization (data not shown). We sequenced the complete E gene of the escape viruses without plaque purification. Both mutants exhibited a C → A change at nucleotide position 1,086 (GenBank accession No. U88535), leading to a Thr → Lys change at amino acid position 51 (T51K) in E protein; no other mutation was detected in the E gene. To analyze the kinetics of T51K emergence during selection, we sequenced viruses from each passage (P1–P3). As shown in Fig. 1C, the C1086A mutation appeared as a minor species in the P1 virus population; almost half of the P2 population contained the C1086A mutation; the C1086A species became dominant in the P3 population. The results demonstrate that DENV-1 gradually enriched a T51K mutation in E protein upon selection of HM14c10.

3.2. A single mutation of T51K in E protein confers resistance to antibody neutralization

Since we did not sequence the entire genome of the resistant viruses, it is possible that mutation(s) outside the E gene contributes to the escape phenotype. To exclude this possibility, we used an infectious cDNA clone of DENV-1 to prepare a recombinant T51K virus. The WT and mutant T51K recombinant viruses were analyzed for HM14c10 neutralization. As shown in Fig. 2A, the WT virus was neutralized by the antibody at an EC_{50} value of 0.43 μg/ml. In contrast, the T51K recombinant virus was not neutralized, even at the highest tested concentration of 810 μg/ml of antibody. These data clearly demonstrate that the E T51K mutation is responsible for the resistance to HM14c10 neutralization.

3.3. The T51K mutation in E protein abolishes antibody-virus binding

We performed an ELISA-based binding assay to examine the effect of E T51K mutation on virus-antibody interaction. A polyclonal antibody recognized both WT and mutant T51K recombinant viruses with an equivalent efficiency (Fig. 2B). In contrast, HM14c10 antibody recognized the WT virus, but failed to bind to the mutant virus. The results demonstrate that the T51K substitution in E protein confers resistance to HM14c10 through abolishing the binding between antibody and virus. On the crystal structure of E protein, amino acid T51 is located in a "hinge" region between Domains I and II (Fig. 3A). Such "hinge" region was suggested to be critical for membrane fusion in the endosome (Rey et al., 1995).

3.4. Amino acids outside the "epitope-forming" residues contribute to the specific binding of HM14c10 to DENV-1

Sequence analysis showed that DENV-1 and DENV-2 E protein contains a conserved Thr and Lys at amino acid 51, respectively (Teoh et al., 2012). Our results described above showed that a T51K mutation confers DENV-1 resistance to HM14c10. These data prompted us to ask whether engineering a K51T substitution in DENV-2 would make the serotype-2 virus susceptible to HM14c10 neutralization. To expand this hypothesis, cryoEM structure of DENV-1 in complex with HM14c10 suggested a number of amino acids that form the epitope of HM14c10 (Teoh et al., 2012). Among the "epitope-forming" residues, DENV-1 and DENV-2 showed seven amino acid differences at positions 51, 136, 160, 274, 275, 384, and 385; these residues are located on two adjacent dimeric E protein complexes (Fig. 3A, B). We then tested whether the substitution of these seven positions with DENV-1 residues into the backbone of DENV-2 could confer neutralization susceptibility of this chimeric virus to HM14c10. Using an infectious cDNA clone of DENV-2, we generated a panel of chimeric DENV-2 in which a single or multiple amino acids were substituted with their counterparts from DENV-1 (Fig. 3B). Among the nine chimeric viruses, E136K, PG384EK, and K51T + E136K + K160T + SG274GT + PG384EK viruses were defective in replication (though not lethal); their viral titers were too low to perform neutralization assay (Fig. 3C and data not shown). The titers of other six recombinant viruses were high enough for neutralization assay; however, none of them could be neutralized by HM14c10 (Fig. 3D). These results suggest that swapping some of the amino acids in the observed "epitope-forming" residues from DENV-1 to DENV-2 is not sufficient to confer DENV-2 susceptible to HM14c10 neutralization.

3.5. Effect of T51K mutation on viral replication in cell culture

To analyze the effect of T51K mutation on virus fitness, we compared viral replication of WT and mutant viruses in cell culture. As shown in Fig. 4A, after electroporation of BHK21 cells with equal amounts of genome-length RNA, both the WT and mutant RNA generated similar number of IFA-positive cells (detecting expression of viral E protein). The transfected cells produced WT and mutant viruses that exhibited similar plaque morphology (Fig. 4A) as well as growth kinetics in both mammalian Vero and mosquito C6/36 cells (Fig. 4B). Sequencing analysis confirmed that the engineered T51K substitution was retained in the mutant virus. These

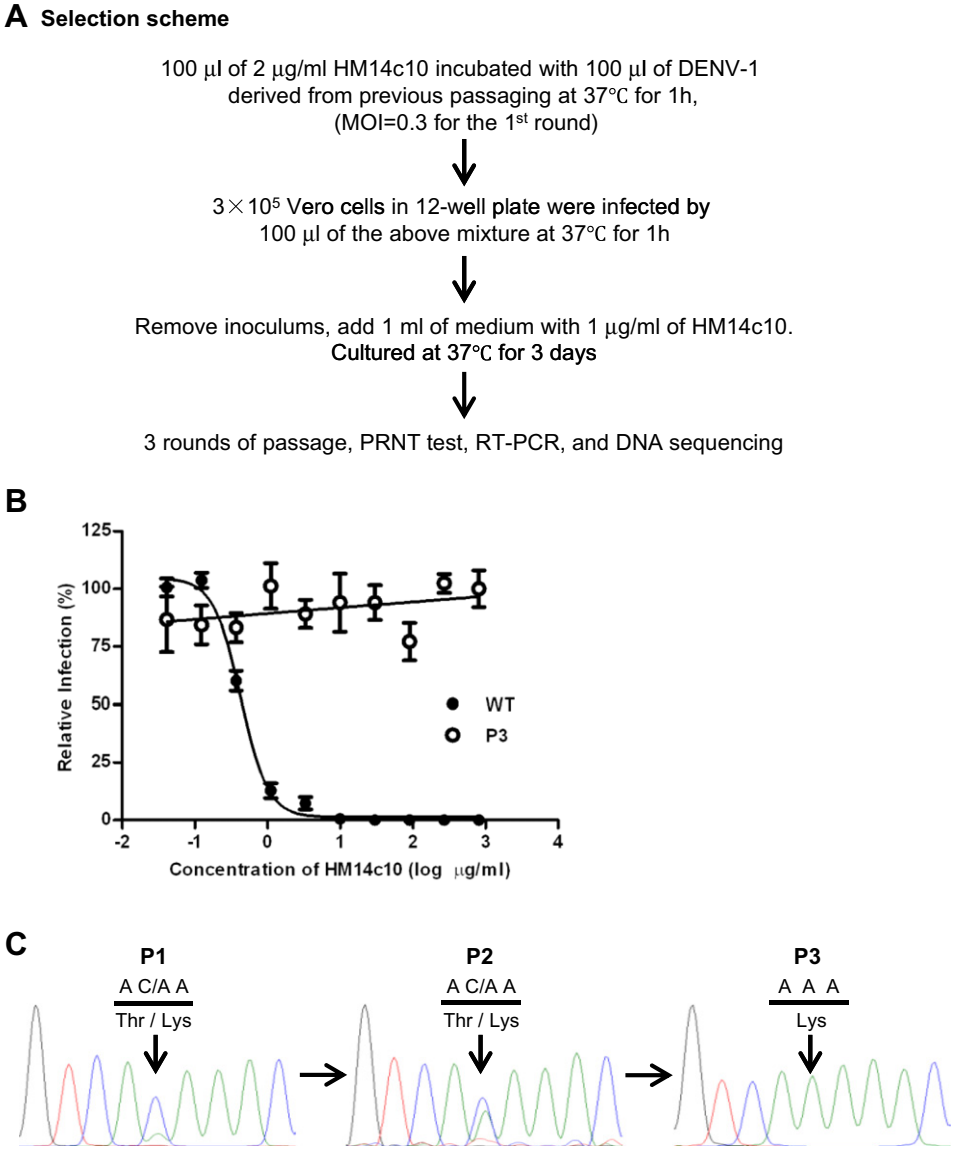


Fig. 1. Selection and characterization of HM14c10 escape mutants. (A) Scheme for selection of HM14c10 escape mutants. Four independent selections were selected at 1 µg/ml of HM14c10. (B) PRNT analysis of P3 virus to assess resistance phenotype. (C) Sequencing chromatograms of escape mutants from P1 to P3. Nucleoside and amino acid changes are indicated.

results suggest that the T51K mutation does not affect viral fitness in cell culture.

3.6. In vivo efficacy and viral fitness

To assess the effects of T51K mutation on viral fitness and efficacy *in vivo*, we infected AG129 mice with WT and mutant T51K DENV-1 (2×10^6 PFU) through intraperitoneal route. The DENV AG129 mice is a viremia model; the infected mice exhibited peak viremia at days 3 and 4 after infection (Fig. 5 and data not shown). The infected mice were treated with a single dose of HM14c10 (20 µg) or PBS at 24 h post-infection. For the WT virus-infected mice, treatment with HM14c10 suppressed viremia to an undetectable level on day 3 and day 4 post-infection, whereas viremia was readily detected in the PBS-treated group (Fig. 5). These data confirmed the *in vivo* efficacy of HM14c10 against the WT DENV-1. For the mutant virus-infected mice, treatment with HM14c10 or PBS showed similar levels of viremia on day 3 post-infection, indicating that the T51K mutation abolishes the *in vivo* efficacy of HM14c10. Notably, the viremia of mutant virus on day 3 was more than 10-fold lower than that of the WT virus. No viremia was detected on day 4 post-infection in the mutant virus-infected mice (treated with either HM14c10 or PBS) (Fig. 5). These results suggest that the T51K mutant virus is attenuated *in vivo*.

4. Discussion

Therapeutic antibody is a viable approach for the development of flavivirus treatment (Shadman and Wald, 2011). A number of protective antibodies with therapeutic potential have been reported for DENV (Brien et al., 2010; Shrestha et al., 2010; Sukupolvi-Petty et al., 2010). We recently reported a human antibody HM14c10 that selectively neutralizes DENV-1 in both cell culture and AG129 mouse (Teoh et al., 2012). In this study, we showed that HM14c10 neutralization escape mutants emerged rapidly in cell culture. After culturing WT virus for three rounds with antibody, we recovered two escape mutants from four independent selections. Both escape mutants accumulated a T51K

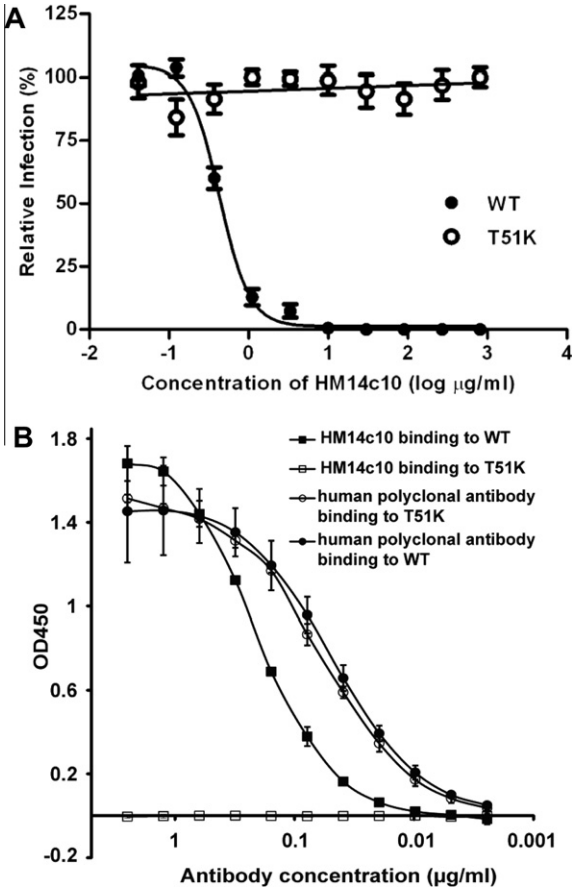


Fig. 2. T51K mutation confers resistance to HM14c10 through abolishment of binding to DENV-1. (A) PRNT analysis of DENV-1 T51K recombinant virus to confirm the escape phenotype in cell culture. The data are representative of two independent experiments performed in duplicate on BHK21 cells. (B) ELISA binding assay of WT and T51K mutant viruses. Binding of HM14c10 to WT (closed squares) and T51K mutant (open squares) was detected by ELISA. Human polyclonal antibody binding to WT (closed circles) and T51K mutant (open circles) was tested as control. Error bars represent standard deviation from three independent experiments.

mutation in E protein. The importance of residue T51 for neutralization was confirmed by introducing the T51K mutation into an infectious cDNA clone of DENV-1. Recombinant T51K virus was completely resistant to HM14c10, even when the culture was treated with high concentrations of antibody (810 μg/ml) (Fig. 2A). In line with the neutralization results, biochemical binding experiment showed that mutation T51K completely abolished the interaction between DENV-1 and HM14c10 (Fig. 2B). In crystal structure of E protein, DI and DII are linearly discontinuous structures linked by four peptide strands that function as a "molecular hinge" (designated H-1 to H-4). During fusion in endosome, the four hinge regions provide the flexibility that is required for the low-pH mediated conformational changes of E protein (Hurrelbrink and Mcminn, 2001; Rey et al., 1995). Residue T51 is located at the flexible DI/DII H-1 region. The angle between DI and DII of E protein rotates 27° during the transition from immature-to-mature state after the furin-mediated cleavage of prM (Zhang et al., 2004); the hinge then rotates back 30° during the prefusion-to-postfusion rearrangement (Bressanelli et al., 2004; Modis et al., 2004). The binding of HM14c10 to virion could block DENV-1 fusion by preventing this movement. However, it should be noted that although HM14c10 inhibits viral infection at a post-attachment step, the antibody exerts its antiviral activity mainly through blockage of virus attachment to cell (Teoh et al., 2012).

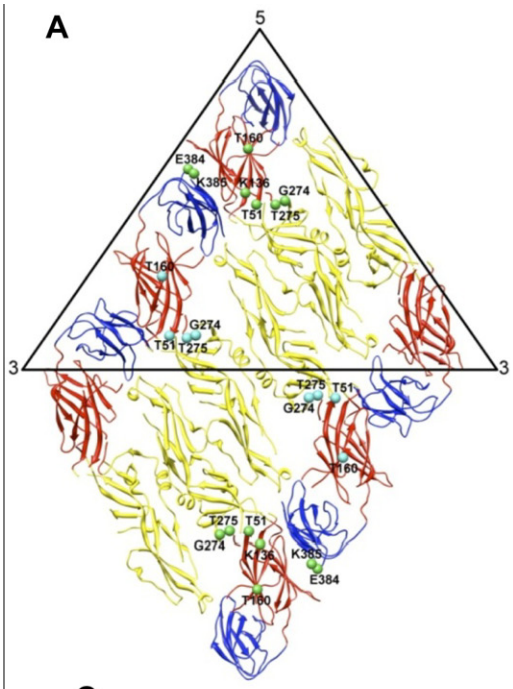
The resistance phenotype of T51K in E protein was demonstrated both *in vitro* (Figs. 1 and 2) and *in vivo* (Fig. 5). The *in vivo* experiment showed that the T51K mutant virus was attenuated in AG129 mouse. This is in contrast with the *in vitro* results indicating that WT and mutant viruses had similar replication kinetics in both mosquito and mammalian cells (Fig. 4). One possible explanation for the discrepancy (between the *in vitro* and *in vivo* viral fitness) is that the T51K change increases the positive charge of E protein, leading to an enhanced virion clearance in blood stream through binding to cell surface glycosaminoglycans (Lee et al., 2004).

Since a single mutation T51K in E protein confers resistance in DENV-1, only DENV-1 isolates containing residue T51 would be sensitive to HM14c10 inhibition. Sequence analysis indicates that 887 out of 896 DENV-1 isolates contained the conserved T51 residue, whereas 9 DENV-1 isolates have an Asp at this position (D51) (Table 1). It remains to be determined whether DENV-1 isolates containing the D51 variation could be inhibited by HM14c10. For other three serotypes, residue T51 was completely conserved in DENV-3, while K51 was conserved in both DENV-2 and DENV-4 (Table 1).

We attempted to explore the determinant(s) of serotype specificity of HM14c10. CryoEM structure of HM14c10 in complex with DENV-1 suggested a set of amino acids of E protein that directly interact with the antibody. These "epitope-forming" amino acids from DENV-1 were engineered into DENV-2. The resulting chimeric viruses were tested for their neutralization by HM14c10. None of the tested chimeric DENV-2 was susceptible to HM14c10 inhibition. Since the chimeric DENV-2 containing the complete "epitope-forming" amino acids (seven residues) from DENV-1 was replication defective, we were not be able to test this virus in neutralization assay. Based on these results, we could only conclude that swapping some of the "epitope-forming" residues is not sufficient to confer DENV-2 susceptible to HM14c10 neutralization.

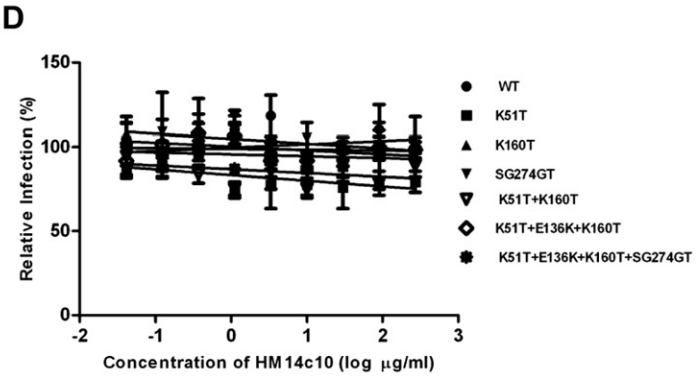
Although resistance viruses against HM14c10 developed quickly in cell culture, cautions should be taken when extrapolating the *in vitro* to the *in vivo* results. Similar to our observation, a previous study using a DENV-4 specific antibody 5H2 showed that resistance viruses emerged after three rounds of neutralization and propagation in cell culture; the same resistant virus was detected on day 6 after infected monkeys were treated with the antibody (Lai et al., 2007). Important sites for binding of 5H2 antibody were mapped to amino acids 174 and 176 in subdomain I of DENV-4 E protein (Lai et al., 2007). However, another study using WNV indicated that the emergence of escape mutants *in vitro* may not accurately reflect the *in vivo* situation; specifically, resistance to therapeutic antibody hE16 *in vivo* was less common than expected (Zhang et al., 2009). Antibody hE16 binds to subdomain III of E protein and possibly inhibits virus-endosome fusion (Zhang et al., 2009). The difference in resistance profile among these studies could be determined by the distinct mechanisms/epitopes of these antibodies. Flavivirus E protein is highly plastic due to their broad host range of mosquito/tick and bird/mammalian. Therefore, the use of combination of distinct antibodies (or combination of antibody with small molecule inhibitor) is required to overcome the emergence of resistance.

Two major hurdles must be considered for development of therapeutic antibodies for DENV. One hurdle is to prevent potential antibody-mediated enhancement (ADE) of virus infection (Dejnirattisai et al., 2010). Since ADE is mediated through the interaction of cellular surface Fc receptor with antibody, it could be mitigated through modifications of antibody residues to abolish the antibody-Fc receptor binding (Balsitis et al., 2010). The other hurdle is to achieve an antibody that could potently neutralize all four serotypes of DENV. The feasibility to obtain such antibody remains to be demonstrated. Given the economic status of most of



B

Amino acid position	51	136	160	274/275	384/385
DENV-1	T	K	T	GT	EK
DENV-2	K	E	K	SG	PG



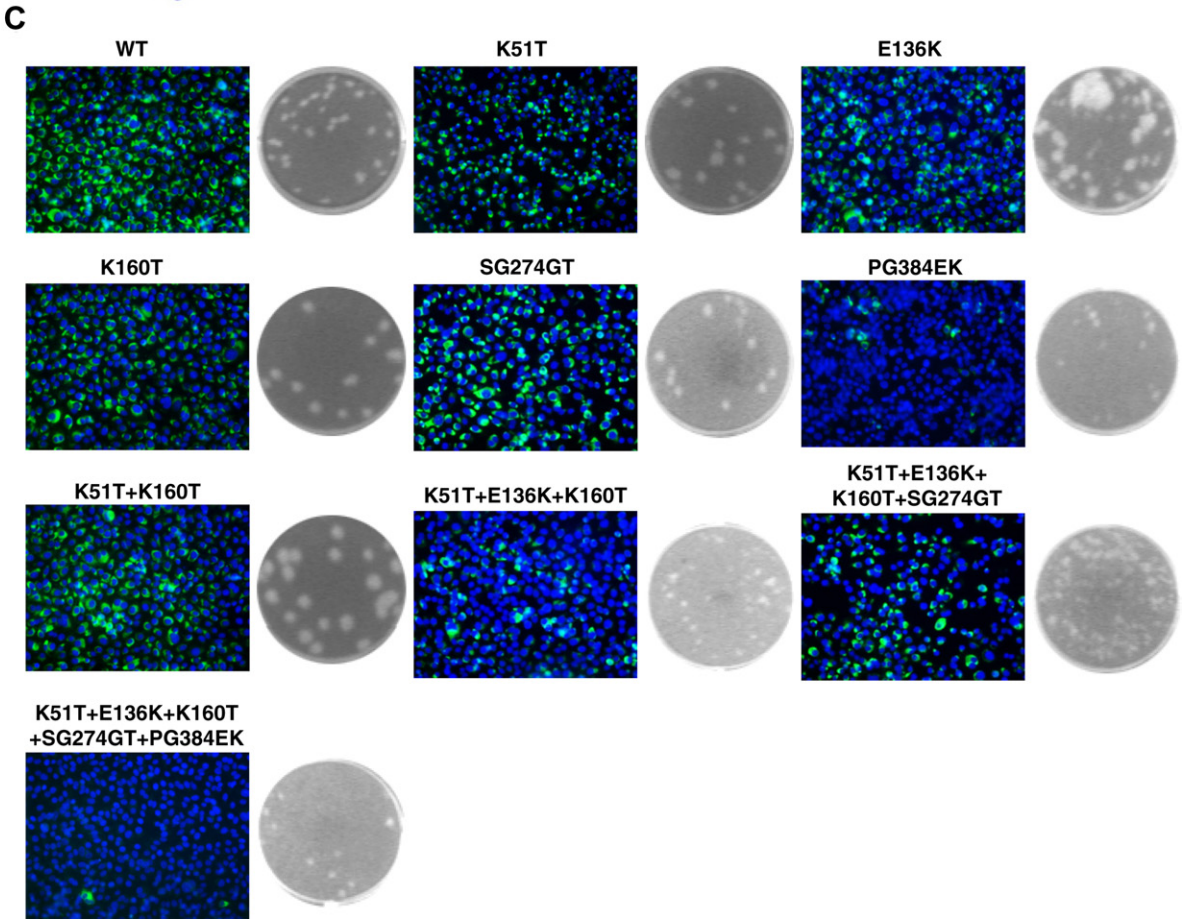


Fig. 3. Amino acids outside the "epitope-forming" residues contribute to the specific binding of HM14c10 to DENV-1. (A) Residues within HM14c10 epitope with variations between serotypes 1 and 2 are labeled and marked by sphere. CryoEM structure of DENV E protein ectodomain (Protein Data Bank entry 1P58) was used as a model. E ectodomains I, II and III are red, yellow and blue, respectively. The images were produced using Chimera. (B) Comparison of residues within HM14c10 epitope between DENV-1 and DENV-2. (C) Analysis of viral E protein synthesis and plaque morphology of chimeric DENV-2 viruses. Mutation(s) in each construct are indicated. Viral E protein expression was detected by IFA at 72 h post-transfection (left). Plaque morphologies of chimeric viruses were shown (right). (D) PRNT analysis of DENV-2 recombinant viruses. The data are representative of two independent experiments performed in duplicate on BHK21 cells.

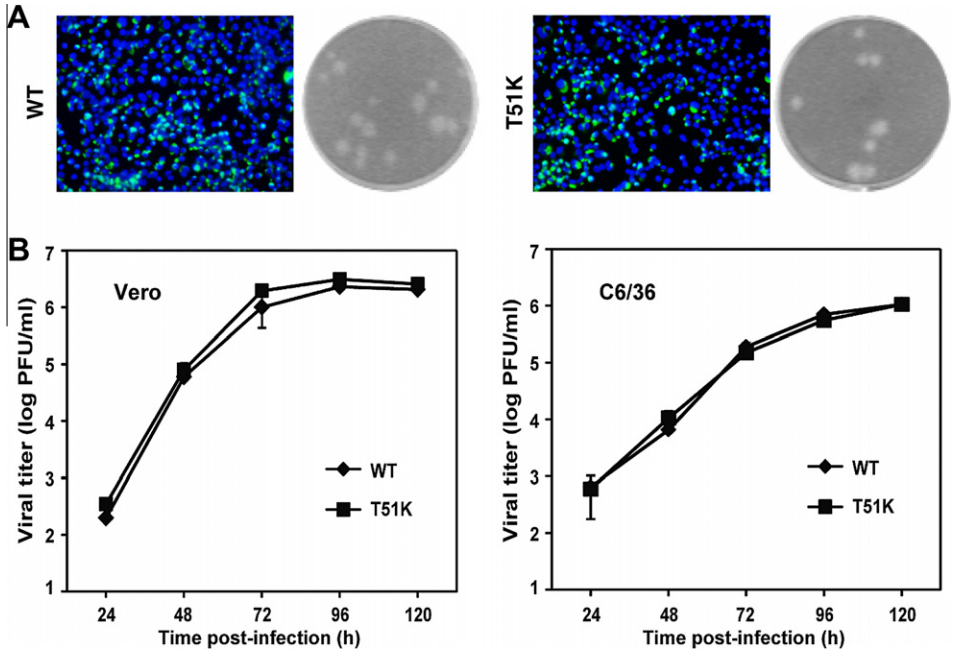


Fig. 4. Comparison of replication in cell culture between WT and T51K mutant DENV-1. (A) Comparison of viral E protein synthesis and plaque morphology between WT and T51K mutant viruses. BHK21 cells were transfected with WT and mutant genome-length RNAs (10 μg), and analyzed for viral E protein expression by IFA at 72 h post-transfection (left). Plaque morphologies of WT and T51K mutant viruses are shown (right). (B) Growth kinetics of WT and T51K mutant virus on Vero and C6/36 cells. Vero (3×10^5) and C6/36 (8×10^5) cells were seeded into each well of a 12-well plate. After incubation overnight, the cells were infected with WT and T51K mutant virus at an MOI of 0.1 for 1 h; the culture fluids were removed; the cells were washed three times by medium; 1 ml of fresh medium was added to the cells; viruses from culture fluids were collected every 24 h. Viral titers were determined by plaque assay on BHK21 cells. Error bars indicate the standard deviations from two independent experiments.

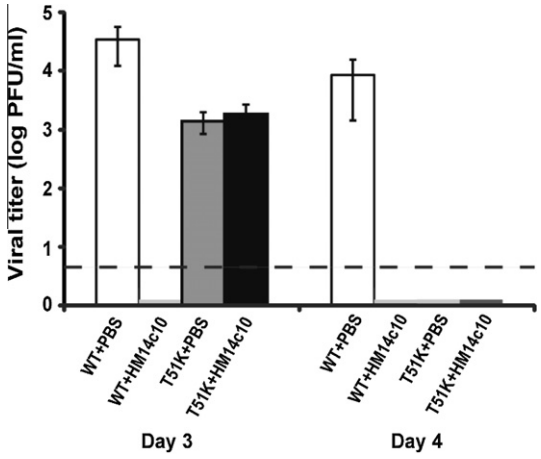


Fig. 5. *In vivo* efficacy analysis. Eight-week old AG129 mice were intraperitoneally (i.p.) infected with 2×10^6 PFU of WT and mutant T51K DENV-1. The infected mice were i.p. injected with a single dose of 20 μg of HM14c10 or PBS control at 24 h post-infection. Mice were bled on day 3 and day 4 post-infection and viremia were quantified by plaque assay. The dotted line represents the limit of detection. The data are presented as averages with error bars representing the standard error of the mean of the PFU/ml obtained from the 5 serum samples from each group.

Table 1
Amino acid alignment of DI/DII hinge region 1 of DENV E protein*.

Virus	DI/DII hinge region 1(E51–54)			
DENV-1 (n = 905)	T 896 D 9	N	P	V 830 I 75
DENV-2 (n = 353)	K 351 R 2	Q 301 H 44 E 8	P 351 S 2	A 352 V 1
DENV-3 (n = 169)	T	Q 168 R I	L	A
DENV-4 (n = 216)	K	E	V	A

* Four amino acids forming the DI/DII hinge region 1 are indicated for all four serotypes of DENV. For each serotype, variant amino acids are indicated below the consensus residues. The total number of virus isolates (n) for each serotype and the number of variant isolates are indicated.

the DENV endemic areas, transfusion of antibody for treatment is almost unrealistic. As discussed above, at least two distinct antibodies are needed to minimize emergence of resistance. Therefore, when using serotype specific antibodies, at least eight distinct antibodies should be developed (two for each of the four serotypes). Nevertheless, the available serotype-specific antibodies could be used to prove the concept whether direct antiviral agent can effectively impact on the disease progression and severity for DENV in clinics. The current rationale for dengue antiviral is that reduction of viremia at early stage of infection would prevent infected individuals from developing into severe forms of diseases – DHF and DSS.

Acknowledgement

We thank our colleagues at Novartis Institute for Tropical Diseases for technical support and helpful discussions.

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