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Antibody-dependent enhancement of dengue virus infection is inhibited by SA-17, a doxorubicin derivative

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ABSTRACT

Antibody-dependent enhancement (ADE) is thought to play a critical role in the exacerbation of dengue virus (DENV)-induced disease during a heterologous re-infection. Despite ADE's clinical impact, only a few antiviral compounds have been assessed for their anti-ADE activity. We reported earlier that SA-17, a doxorubicin derivative, efficiently inhibits the *in vitro* infection of DENV and yellow fever virus. Here we explored SA-17's mechanism of inhibition and investigated if the compound is active against ADE of DENV infection. Since enhanced infectivity stimulated by antibodies has been observed with standard and immature DENV, both types of virions were included in the study. We observed that SA-17 (i) inhibits DENV infection by preventing binding/entry to the cell and (ii) interferes with antibody-mediated infection of both standard and immature DENV2. SA-17 markedly reduced the infectivity of DENV2 in ADE conditions, with IC_{50}s ranging from 0.26 to 2.89 μM. The compound exerted its activity when added before, during, and after antibody-opsonization of standard and immature virus. Thus, molecules with the characteristics of SA-17 may be attractive antiviral agents since they can be used both to block DENV2 entry during primary and secondary infection and to inhibit ADE of standard and immature virus.

1. Introduction

Dengue is the most widespread vector-borne viral disease observed in humans, with an estimated 50–100 million dengue infections per year. Currently, an approximate of 2.5 billion people (over 40% of the world's population) live in dengue endemic countries, mainly in south and south-east Asia, the Caribbean, Central and South America, and Africa (World Health Organization,). There is neither a vaccine nor a specific antiviral therapy available, but early detection and access to proper medical care can lower the fatality rate to below 1% (World Health Organization, 2012a,b). Dengue is associated with explosive urban epidemics and has become a major public health problem, with large economic, political, and social impact (Simmons et al., 2012).

Dengue virus (DENV) is a member of the Flavivirus genus of the *Flaviviridae* family (Mukhopadhyay et al., 2005). All four DENV serotypes are transmitted to humans by *Aedes* mosquito bites, with *Aedes aegypti* being the main vector (Stephenson, 2005b). DENV is an enveloped single-stranded RNA virus with a plus-sense viral genome of ~10,700 nucleotides (Mukhopadhyay et al., 2005). DENV enters the host cell by receptor-mediated endocytosis and fuse from within acidic endosomes. Upon membrane fusion, the genome is released in the cytoplasm and viral replication is initiated. Virus assembly occurs on the surface of the endoplasmic reticulum (ER) (Mukhopadhyay et al., 2005). Newly formed immature virions contain heterodimers of the E and prM glycoproteins which protrude from the viral membrane. These immature particles are transported through the cellular secretory pathway, where furin-mediated cleavage of prM occurs to mature the virion (Li et al., 2008). DENV maturation is, however, not very efficient as infected cells secrete a heterogeneous mixture of mature, partially immature and fully immature virus particles (Junjhon et al., 2010).

Infection with DENV can result in dengue fever (DF), dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), a range of intermediate responses, or no clinical manifestations (Halstead, 2007). Severe dengue disease is characterized by severe plasma leakage, hemorrhage, and in some cases organ impairment (World Health Organization, 2012a,b). Increased disease severity has been associated with pre-existing heterologous dengue antibodies, a phenomenon described as antibody-dependent enhancement (ADE) of infection (Halstead, 2003). Cross-reactive antibodies are believed to enhance viral entry resulting in a higher infected cell mass and an increased viral burden. This is followed by an imbalanced secretion of inflammatory cytokines and mediators causing the capillary leakage characteristics seen in DHF/DSS cases (World Health Organization, 2012a,b; Cardier et al., 2005). *In vitro* experiments revealed that both anti-E and anti-prM antibodies can stimulate viral infectivity (Halstead and O'Rourke, 1977; Huang et al., 2006; Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010).

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Interestingly, anti-prM and anti-E antibodies were observed to render essentially non-infectious fully immature particles almost as infectious as standard virus particles, suggesting that these particles may also contribute to the development of DHF/DSS (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010; da Silva Voorham et al., 2012).

The role of ADE in DHF/DSS is considered as one of the principal challenges for the development of a vaccine (Stephenson, 2005a; Guzman and Vazquez, 2010). A tetravalent DENV vaccine is currently being tested in the field but, unfortunately, the first results were disappointing as DENV2 immunity was not achieved (Sabchareon et al., 2012). Prevention of dengue is still mainly focused on vector control and personal protection from mosquito bites (World Health Organization, 2012a,b). For the above reasons, there is a need for the development of anti-DENV agents that can be used for treatment and prevention of DENV dissemination.

Previously, we explored the antiviral properties of SA-17, a doxorubicin derivative which carries a squaric acid amide ester moiety at the carbohydrate (α-L-daunosaminyl) group (Kaptein et al., 2010). We defined that SA-17 is active against DENV and yellow fever virus at non-cytotoxic concentrations. Time-of-drug-addition studies revealed that SA-17 acts at the early stages of the viral replication cycle (i.e., virus attachment and/or virus entry), without being virucidal. By computer modeling, SA-17 was predicted to dock in the β-OG binding cleft of the dengue virus E protein (binding pocket between DI and DII) (Modis et al., 2003). However, the mechanism underlying the DENV-inhibitory activity of SA-17 was not elucidated.

The observation that SA-17 acts at an early stage of the viral cycle prompted us to further evaluate the mechanism of inhibition and to investigate if the compound inhibits ADE of DENV infection. To this end, we used a binding and fusion fluorescence microscopy-based assay and a panel of *in vitro* assays with enhancing monoclonal antibodies. The data from these experiments revealed that SA-17 markedly reduced the binding and fusion of standard DENV particles to cells in a dose-dependent manner. Furthermore, SA-17 efficiently blocked antibody-mediated infection of standard and immature DENV, thus potentially inhibiting ADE of infection.

2. Materials and methods

2.1. Virus and cells

Standard (std) DENV2 strain 16681, kindly provided by Dr. Claire Huang (Center for Disease Control and Prevention, USA), was propagated in C6/36 cells as described before (Zybert et al., 2008). Viral infectivity was determined with a standard plaque assay in Baby hamster kidney (BHK-15) cells (plaque forming units, PFU) (Diamond et al., 2000). The absolute number of viral particles in solution was determined by reverse transcriptase quantitative PCR (RT-qPCR), which detects the number of genome-containing particles (GCPs) (van der Schaar et al., 2007). Virus infection was performed on the basis of multiplicity of genome-containing particles per cell (MOG).

Immature DENV2 particles were produced in LoVo cells, as previously described (Diamond et al., 2000). In brief, LoVo cells were infected at MOI 5 with std DENV2 16681. After 3 h, the virus inoculum was removed, the cells were washed three times with PBS, and fresh 10% FCS medium was added. At 72 h post-infection, the medium containing the virus particles was harvested. When indicated, std and immature DENV virus preparations were concentrated and purified on tartrate gradients by ultracentrifugation, exactly as described before (van der Schaar et al., 2007).

Mouse macrophage P388D1 cells were maintained in DMEM (PAA) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), sodium bicarbonate (Invitrogen, 7,5% solution) and 1.0 mM sodium pyruvate (Gibco). BS-C-1 cells (African Green Monkey kidney cells) and BHK-15 cells were cultured in 1× high glucose, L-glutamine-enriched DMEM with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). *Aedes albopictus* C6/36 cells were maintained in MEM (Gibco) supplemented with 10% FBS, 25 mM HEPES, 7.5% sodium bicarbonate, penicillin (100 U/mL), streptomycin (100 μg/mL), 200 mM glutamine and 100 μM nonessential amino acids at 30 °C. Human adenocarcinoma LoVo cells were cultured in Ham's F-12 medium (Gibco) supplemented with 20% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells except for C6/36 were maintained at 37 °C and 5% CO_2.

2.2. Compounds and antibodies

The doxorubicin derivative SA-17 was synthesized as reported elsewhere (Sztaricskai et al., 2005). SA-17 was diluted in DMSO to a final concentration of 10 mM. The resulting solution (color red) was aliquoted and stored at 4 °C. Ammonium chloride and chlorpromazine were purchased from Sigma–Aldrich. Monoclonal murine anti-E antibody 4G2 was obtained from Millipore. Anti-E 104 monoclonal antibody was generously provided by M. Diamond (Washington University, St. Louis, USA) and human anti-prM antibody 2F5 was kindly provided by Gavin Screaton (Imperial College, London, UK).

2.3. Binding assay of SA-17 to DENV

We applied a filtration method to assess whether SA-17 directly binds to DENV. To this end, we used an Amicon Ultra 100 kDa centrifugal filter device (Millipore). Three conditions were tested. First, to confirm that SA-17 passes through the filter and does not stay in the retentate, SA-17 was mixed with HNE and subjected to filtration. The harvested filtrates and retentate (see below) were then challenged with std DENV2. Second, as a control for virus recovery, virus in HNE was subjected to filtration. Third, to assess whether SA-17 directly binds to DENV, tartrate purified virus was mixed with SA-17 (60 and 240 μM) and incubated at 37 °C for 30 min and subjected to filtration. In all conditions, 9×10^4 PFUs of tartrate-purified virus (equivalent to 7.5×10^7 GCPs) was used. Filtration was performed by centrifugation for 2 min at 9000*g*. Upon centrifugation, the filtrate was harvested and 400 μL HNE buffer was added to the filter device (retentate had a residual volume of 50 μL). This procedure was repeated 3 times. Viral titers of the retentate obtained after 3 washes with HNE and the collected filtrates were quantified by RT-qPCR and/or plaque assay, as previously described (van der Schaar et al., 2007).

2.4. Binding and fusion assays with DiD-labeled std DENV2 particles

The lipophilic fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) (Molecular Probes No. D307) was used to label the viral membrane of DENV2 virus particles, as previously described (van der Schaar et al., 2007). When incorporated in the viral membrane at a relatively high surface density, the emitted fluorescence level is largely quenched, but single DiD-labeled virus particles can still be clearly detected. Membrane fusion of virus particles labeled with a relatively high surface density of DiD can be observed as a fluorescence dequenching due to the dilution of the DiD probe from the viral membrane into the target cell membrane. DiD-labeling of DENV preparations was done as described before (Ayala-Nunez et al., 2011).

The extent of virus-cell binding and membrane fusion was determined as described before (Ayala-Nunez et al., 2011). In brief,

for the binding assay, BS-C-1 cells were seeded in a Lab-Tek II Chambered Coverglass (Nunc No. 155409). The following day, cells were incubated for 10 min at 4 °C and DiD-labeled std DENV2 (MOG 3000) was added in the presence or absence of twofold serial dilutions of SA-17 or a DMSO control. Subsequently, incubation was continued for 1 h at 4 °C. Unbound virus was removed by washing the cells three times with cold phenol red-free MEM. Then, cold phenol red-free MEM containing 1% glucose and a glucose oxidase solution (GLOX) was added to the cells and the microscopic analysis was done, as described below. For the fusion assay, the same experimental setup was used except that the cells were incubated 30 min at 37 °C to allow infection. Ammonium chloride (NH_4Cl, 50 mM) and chlorpromazine (60 μM) were used as negative controls of the fusion assay. Both compounds were added 1 h before infection. Microscopy analysis was done by taking 10 snapshots of randomly selected fields using both differential interference contrast (DIC) and DiD channels. DiD-labeled viruses were detected by epi-fluorescence microscopy in a Leica Biosystems 6000B instrument by using a 635-nm helium–neon laser.

The acquired images were processed and analyzed with ImageJ using an in-house macro. In the binding assay, the images were analyzed by counting the total number of bound DiD-particles. The extent of membrane fusion was analyzed by measuring the total fluorescent signal per field of view. Since the fusion assay quantifies total post-fusion fluorescence, it was used to provide an indirect measurement of how much fusion took place during a particular infection. The quantitative results of the negative controls in the fusion assay were taken as background; hence, they were subtracted from the total fluorescence intensity of the positive control and SA-17 treatments.

2.5. Binding assay measured by RT-qPCR

To further confirm the effect of SA-17 on binding of std DENV virions to the cell, a binding assay based on RT-qPCR was performed. In brief, BS-C-1 cells were seeded in 24-wells plates (7.5×10^4 cells/well) one day before infection. The cells were incubated for 10 min at 4 °C. Serial twofold dilutions of SA-17 were then mixed with std DENV2 (MOG 3000) and added to the cells. Incubation was continued for 1 h at 4 °C. Unbound virus was removed by washing the cells three times with cold PBS 1×. The number of GCPs that bound to the cell were determined by RT-qPCR, as described before (van der Schaar et al., 2007).

2.6. DENV infectivity inhibition assay

Serial twofold dilutions of SA-17 were mixed with std DENV2 (MOG 100 or ~MOI 1), incubated for 1 h at 37 °C, and added to 2×10^5 P388D1 cells seeded in 24-wells plates. The inoculum was removed at 1 h post-infection after which the cells were washed and new medium was added. The cell supernatant was harvested at 43 h post-infection and virus particle production was assessed by plaque assay (Diamond et al., 2000). The 50% inhibitory concentration (IC_{50}) was defined according to the percentage of infectivity inhibition relative to the positive non-treated control. In case of BS-C-1 cells, an MOG 3000 was used in 7.5×10^4 BS-C-1 cells seeded in 24-wells plates.

2.7. Cytotoxicity assay

To assess the toxic effect of SA-17 in the DENV infectivity assay, a cytotoxic assay was performed in P388D1 and BS-C-1 cells under the same experimental conditions. A stock solution of SA-17 was serially diluted in growth medium and added to 24-wells plates containing BS-C-1 (7.5×10^4 cells/well) or P388D1 cells (2×10^5 cells/well). Cells were washed with medium after 1 h to remove the compound, new medium was added and incubation was continued at 37 °C in a 5% CO_2 air humidified atmosphere for 2 days. Cell viability was assessed by a MTT assay, as described before (Twentyman and Luscombe, 1987). The 50% cytotoxic concentration (CC_{50}) was defined based on the percentage of cell survival relative to the positive control (non-treated).

2.8. ADE assay

For virus-antibody complex formation, virus particles (MOG 100 of std as well as immature) were incubated for 30 min at 37 °C with serial 10-fold dilutions of antibodies in cell culture medium containing 2% FBS prior to the addition to cells. The virus-antibody complexes were then added to 2×10^5 P388D1 cells/well, and incubated at 37 °C with 5% CO_2. At 43 h post-infection, the supernatant was collected and virus particle production was measured with a plaque assay. As a control, std DENV infection was performed in the absence of antibodies.

2.9. ADE-inhibition assays

To evaluate SA-17's efficacy against DENV2 ADE, P388D1 cells were infected with DENV2 (MOG 100 of std or immature) in the presence of enhancing concentrations of mAb 4G2 (120 ng/mL), 104 (40 ng/mL), or 2F5 (40 ng/mL) and increasing concentrations of the compound. The addition of SA-17 to the virus was done under three experimental conditions: (1) before, (2) during, and (3) after mAb-opsonization. In the first condition, the virus was pretreated with serial twofold dilutions of SA-17 for 1 h at 37 °C before the addition of the indicated antibody. Opsonization was then done in the presence of the compound, for 30 min at 37 °C. In the second experimental condition, the virus, the antibody, and a serial twofold dilution of SA-17 were simultaneously mixed and incubated for 1.5 h at 37 °C. In the third experiment, the virus-antibody complexes were first formed by a 30 min incubation at 37 °C, and then added to serial twofold dilutions of SA-17. Afterwards, the mixture was further incubated for 1 h at 37 °C. All compound-virus-antibody mixtures were finally added to 2×10^5 P388D1 cells/well in 24-wells plates and incubated at 37 °C with 5% CO_2. Assessment of DENV2 particle production was performed with a plaque assay at 43 hpi.

2.10. Statistical analysis

The CC_{50} and IC_{50} values were calculated with a nonlinear regression model (Sigmoidal dose–response curves) in GraphPad Prism 5 software. Statistical analysis on the potency of inhibition was done using a Mann–Whitney test (one-tailed). P values lower than 0.05 ($P < 0.05$) were considered significant.

3. Results

3.1. SA-17 directly binds to DENV2 and inhibits virus-cell binding

SA-17 was previously shown to inhibit DENV2 infection and was predicted to interfere with an early stage of the viral life cycle (Kaptein et al., 2010). Here, we extended our studies and unraveled the mode of action of SA-17. First, we assessed whether SA-17 directly binds to the virion using a filtration method. As seen in Fig. 1A, free SA-17 passes through the 100 kDa filter device as only the filtrate showed reduced infectivity upon virus challenge. It should be noted however that an unknown fraction of SA-17 stays behind in the filter, as upon centrifugation the filter membrane colored pink. SA-17 trapped in the filter could not be removed even after extensive washing. Furthermore, as a control for virus

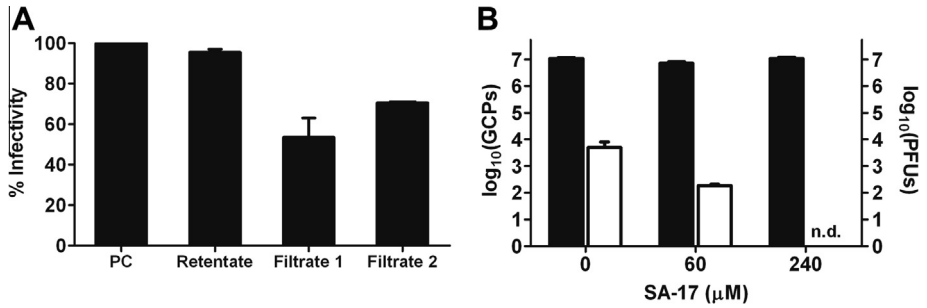

Fig. 1. SA-17 directly binds to DENV virions. SA-17-DENV binding was assessed by use of an Amicon Ultra 100 kDa centrifugal filter device. (A) SA-17 mixed with HNE was filtered to test if the compound passes through the filter in the absence of virus. Both the filtrates and the final retentate were challenged with std virus (9×10^4 PFUs of std DENV2) to evaluate the localization and activity of SA-17. The positive control (PC) represents a sample of non-filtered virus. (B) std DENV2 (9×10^4 total PFUs) was applied to the filter with and without prior treatment with SA-17 (60 and 240 μM). The viral titer of the final retentate was determined by qPCR (GCPs) and plaque assay (PFUs). The bars represent the average of three independent experiments ± standard error of the mean (SEM). N.d. represents "not detectable".

recovery, DENV alone was applied to the filter and the results show that the virus stayed in the retentate (Fig. 1B). Interestingly, when SA-17 was mixed with DENV before addition to the filter, a dose-dependent inhibition of viral infectivity was observed in the retentate. Importantly, the virus recovery yields were similar under all conditions tested (Fig. 1B). Taken together, the above results show that SA-17 directly binds to the virion

Next we aimed to evaluate the effect of SA-17 on virus binding to the cell surface and membrane fusion activity using a fluorescence microscopy-based assay with DiD-labeled particles. But before performing the analysis we first assessed the anti-DENV activity and cytotoxicity of SA-17 in BS-C-1 cells. The infectivity inhibition assay was performed at an MOG 3000 of std DENV, since this resembles the experimental condition needed for the binding and fusion microscopy assays (Ayala-Nunez et al., 2011). A dose-dependent inhibition of DENV2 infection was observed as a result of SA-17 treatment (Fig. 2A). Furthermore, no cytotoxic effect was observed at the concentration range where antiviral activity of SA-17 was found (Fig. 2A).

Subsequently, the binding and fusion microscopy assay was applied. As seen in Fig. 2B–D, SA-17 markedly reduced both the binding and fusion capacity of DiD-labeled std DENV2 particles to cells in a dose-dependent manner. These results indicate that SA-17 predominantly acts to prevent binding of the virus to the host cell receptor. The higher extent of inhibition observed in the fusion assay when compared to the binding assay suggests that there is an additive effect on membrane fusion, albeit not significant. Negative controls for the fusion assay were ammonium chloride and chlorpromazine as these compounds are known to interfere with viral infectivity in these cells (Fig. 2D, (van der Schaar et al., 2008)). The result that SA-17 acts at the level of virus binding to the cell surface was confirmed by RT-qPCR, which determines the number of GCPs bound per cell (Fig. 2C).

3.2. SA-17 inhibits ADE of std DENV2 infection in murine macrophages

The result that SA-17 blocks infection through virus binding inhibition prompted us to evaluate whether the compound also exerts an antiviral effect towards ADE of std DENV2. Given our previous prediction that SA-17 inserts in the β-OG binding pocket between E DI and DII cleft, we decided to use antibodies directed against epitopes in E DIII (mAb 104, (Sukupolvi-Petty et al., 2010)) and E DI/II (mAb 4G2, (Henchal et al., 1982)) for these studies. ADE is dependent on the interaction of the virus-antibody complex with Fcγ-receptors and therefore we used murine macrophage-like P388D1 cells for the following experiments. P388D1 cells express FcγRIII (CD16), FcγRII (CD32), and FcγRI (CD64) (Sung, 1985; Ochiai et al., 1988), and are known to support ADE of DENV (Halstead et al., 1983; Morens et al., 1987; Huang et al., 2006; da Silva Voorham et al., 2012).

We first determined the antiviral activity of SA-17 on these cells in the absence of antibodies. Fig. 3 shows that SA-17 inhibits the infection of std DENV2 (MOG 100) in a dose-dependent manner, with an IC_{50} of 0.52 ± 0.15 μM (Table 1). SA-17 completely inhibited the infection at 30 μM. These results are comparable to our previous findings in Vero cells using DENV NGC (Kaptein et al., 2010). No cytotoxic effect was observed within the tested concentration range, as measured by an MTT assay. The CC_{50} was 937 ± 240 μM and the Selectivity Index (SI) was 1802 (Table 1), indicating that SA-17 efficiently inhibits DENV infection within a wide non-cytotoxic range (Fig. 3, gray area).

In parallel, the enhancing profile of mAb 104 and mAb 4G2 was assessed. For mAb 4G2, the maximum enhancement (2 $\log_{10}$ increase in titers compared to the positive control) was obtained at a mAb concentration of 120 ng/mL (Fig. 4A). In case of mAb 104, optimal ADE was reached at 40 ng/mL (Fig. 4B).

We then investigated SA-17's activity against std DENV2 under optimal conditions of ADE. The addition of the compound to the virus was performed either (1) before, (2) during, or (3) after antibody-opsonization, to mimic the different scenarios in the DENV-infected patient. SA-17 treatment inhibited the infectivity of std DENV2 in a dose-dependent manner in the three experimental conditions and with both monoclonal antibodies (Fig. 4C and D). For mAb 4G2, the IC_{50}s ranged from 0.78 ± 0.24 to 2.89 ± 0.99 μM (Table 1). In the case of mAb 104, the measured IC_{50}s were in the range of 1.51 ± 0.24 to 2.32 ± 0.10 μM. For both antibodies, the lowest IC_{50}s were found when treating the virus with SA-17 before opsonization and the highest ones when the treatment was done after opsonization. Particularly, SA-17's treatment before Ab-opsonization significantly inhibited ($P < 0.05$) the viral infection as compared with the other two experimental conditions using the same antibody (Table 1).

Approximately four times more SA-17 was needed to inhibit the infection of std DENV2 in the presence of antibodies. Since the infectivity enhancement mediated by antibodies results in a 2-$\log_{10}$ increase of the viral titer, SA-17 could be considered as potential effective treatment against ADE. In addition, no cytotoxicity was observed (CC_{50} of 937 ± 240 μM), which resulted in an effective blockage of ADE of std DENV2 infection (high SIs).

3.3. SA-17 inhibits ADE of immature DENV2 infection

The possible role of antibody-opsonized immature particles in dengue disease (Rodenhuis-Zybert et al., 2010) encouraged us to further assess the extent of SA-17's antiviral activity against immature DENV2. Immature DENV2 16681 particles were produced in

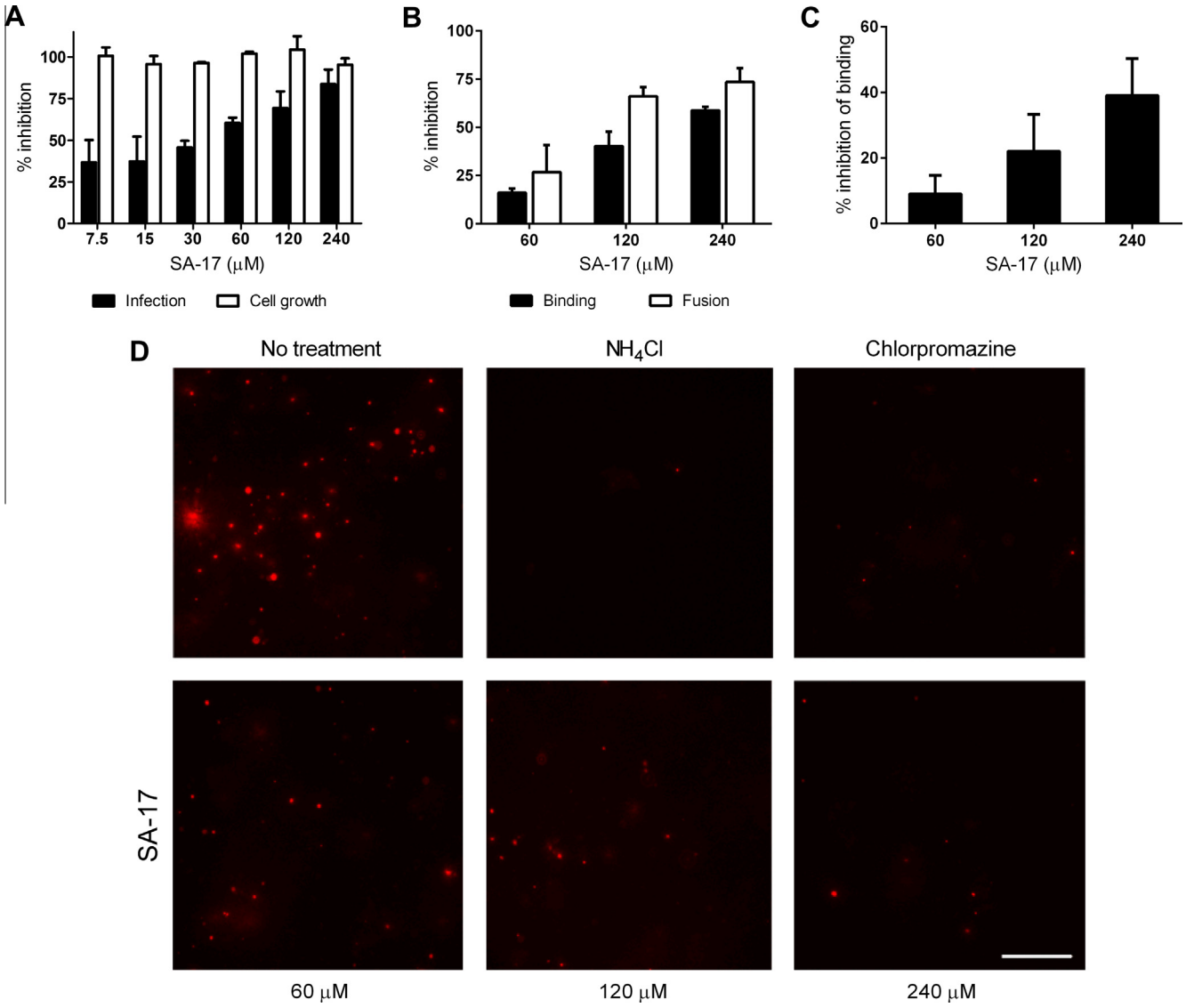

Fig. 2. SA-17 inhibits binding of DENV to the cell surface. (A) Virus particle production after SA-17 treatment was assessed in BS-C-1 cells by plaque assay. Cell viability was determined in the same cell line by the MTT method. The percentage of cell viability and infection inhibition was calculated with respect to the non-treated control. (B) Binding and membrane fusion of DiD-labeled virus was assessed in BS-C-1 cells in the presence or absence of SA-17 by a microscopy-based assay. The percentage of binding or fusion inhibition of SA-17 was calculated with respect to the positive non-treated control using ImageJ. The average of three independent experiments is shown ± SEM. (C) To complement these results, a binding assay measured by RT-qPCR was performed under the same experimental conditions. The number of viral particles bound per cell was quantified in the absence or presence of SA-17. The percentage of inhibition of binding with respect to the non-treated control is shown. The bars represent the average of two independent experiments ± SEM. (D) Snapshots of the controls of a fusion assay: no treatment, NH_4Cl (50 mM), chlorpromazine (60 μM), and three SA-17 treatments are shown. Fusion of DiD-labeled virus within endosomes is observed as highly fluorescent red puncta. Scale bar: 25 μm.

LoVo cells (which lack functional furin) as previously described (Zybert et al., 2008). The PFU-to-particle ratio of the obtained virus preparation was 115,000-fold lower than that of the C6/36-produced std virus preparation, confirming that the used immature particles were essentially non-infectious.

An ADE assay was then performed with serial ten-fold dilutions of 2F5, a cross-reactive human anti-prM mAb previously shown to enhance the infectivity of the four DENV serotypes (Dejnirattisai et al., 2010). Non-opsonized std and immature virus were used as controls. Enhancement of immature DENV2 infection ($4 \log_{10}$) was seen at both 4 and 40 ng/mL (Fig. 5A). Under optimal conditions for ADE, the infectivity of antibody-opsonized immature DENV was comparable to that of the std virus control (white bar, Fig. 5A).

SA-17 was also added to immature DENV2 before, during, and after opsonization with mAb 2F5. As seen in Fig. 5B, SA-17 inhibited antibody-mediated infection of immature virus in a dose-dependent manner. Inhibition of infection was comparable among the three experimental conditions, with an IC_{50} of 0.54 ± 0.2 μM when SA-17 was added before antibody-opsonization, an IC_{50} of 0.26 ± 0.04 μM when added during opsonization, and an IC_{50} of 0.68 ± 0.41 μM when the compound was added after mAb-opsonization (Table 1). All IC_{50}s were in the same range than those from the non-opsonized std DENV2 infection, with no significant difference among the mentioned conditions. Complete inhibition of infection was achieved at 15–30 μM SA-17. The relative low permissiveness and thickness of P388D1 cells compared to BS-C-1 cells limited us to further analyze the molecular mechanism by which SA-17 blocks infection of antibody-opsonized std and immature DENV.

4. Discussion

Our results reveal, for the first time, that SA-17 directly binds to DENV2 and efficiently inhibits infection in the absence and

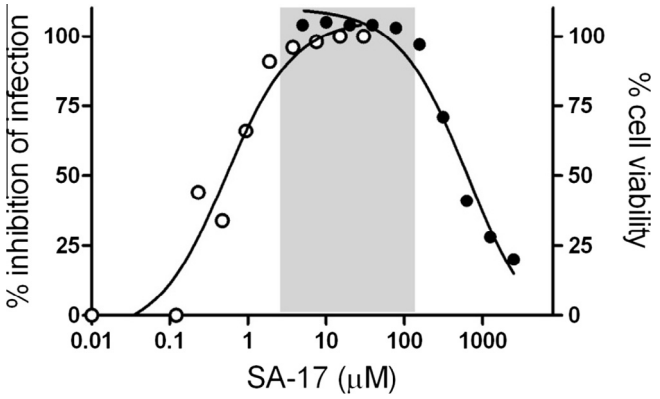

Fig. 3. Dose-dependent inhibition of DENV infection by SA-17 in P388D1 cells. Virus particle production after SA-17 treatment was assessed in P388D1 cells by plaque assay (white circles). Cell viability was determined in the same cell line by the MTT method (black circles). Each data point represents the average of three independent experiments carried out in duplo. Sigmoidal dose–response curves are shown. The gray area indicates the concentration range of SA-17 that inhibits the infection with no cytotoxic effect to P388D1 cells.

presence of enhancing antibodies, thus potentially inhibiting ADE of disease. We showed that SA-17 impedes DENV2 infection by targeting the early stage (binding/entry) of the viral cycle. Interestingly, SA-17 inhibits entry of antibody-opsonized DENV2 irrespective of its maturation state.

Despite the great therapeutic potential of DENV entry inhibitors only a limited number of molecules have been identified. These include, glycosidase inhibitors (Wu et al., 2002; Whitby et al., 2005), carbohydrate-binding agents (Alen et al., 2009), heparan mimetics (Ono et al., 2003; Lee et al., 2006), and other molecules (Wang et al., 2009; Costin et al., 2010). Here, we showed that SA-17 inhibits binding of DENV to target membranes. We demonstrated that SA-17 acts by direct binding to the virus in a manner that prevents virion binding, fusion and, as a consequence viral replication. Entry is an attractive therapeutic target in case of DENV as its inhibition will prevent both host cell infection and subsequent immune activation (Alen and Schols, 2012). DENV entry inhibitors therefore have great potential to be used alone or in combination therapy with viral replication inhibitors. The use of a combined antiviral approach is always preferable as it will decrease the chance of the emergence of resistant virus strains.

Upon a heterologous secondary DENV infection, individuals have an increased risk to develop severe disease due to ADE of infection (Halstead, 2007). Thus far, two compounds have been shown to be active against ADE of DENV (Nicholson et al., 2011; Rathore et al., 2011). Celgosivir, a compound that inhibits DENV replication by targeting the NS1 protein, was described to effectively enhance survival and reduced viremia in a lethal challenge mouse model of ADE DENV infection (Rathore et al., 2011). Furthermore, Nicholson et al. (2011) identified peptides that behave as inhibitors of both std DENV entry and ADE, but no details were given regarding their mode of action (Nicholson et al., 2011). We report that SA-17 interferes with DENV infection in the absence and presence of antibodies, irrespective of the maturation status of the particle. SA-17 is the first compound known to interfere with ADE of both std and immature particles. The ability of SA-17 to inhibit infectivity of immature DENV-immune complexes is important as these particles have been postulated to contribute to the development of disease (Rodenhuis-Zybert et al., 2010; Dejnirattisai et al., 2010; da Silva Voorham et al., 2012; Simmons et al., 2012). The combined action of SA-17 makes it an attractive therapeutic compound against DENV.

SA-17 inhibited std and immature DENV2 infection before, during and after antibody-opsonization. Interestingly, the addition of SA-17 treatment before opsonization of std DENV2 with mAbs 4G2 and 104 significantly increased the inhibitory activity of the compound with respect to the other two experimental conditions of the same group (during and after antibody-opsonization). Furthermore, the IC_{50} value of SA-17 against std DENV in the presence of mAb 4G2 is similar to that of std infection in the absence of antibody. Together, this indicates that the addition of SA-17 prior to antibody opsonization may allow more SA-17 molecules to bind to the virion thereby increasing its inhibitory action. On the other hand, for mAb 104, a higher IC_{50} value was observed when compared to std infection without antibodies, suggesting that E DIII antibodies compete with binding of SA-17. Indeed, when SA-17 is added to the virus in competing conditions (during antibody-opsonization) or after mAb opsonization, a lower antiviral effect was measured. Anti-prM antibodies do not seem to compete with SA-17 binding as in all three tested conditions the IC_{50} value was similar to std virus in the absence of antibody. Since an anti-prM antibody was used, the binding of SA-17 would not interfere with the opsonization, supporting the notion that SA-17 directly binds to the virion. Importantly, despite the lower antiviral effect observed, SA-17 was found active against antibody-opsonized std and immature virus. This suggests that the compound was able to reach and bind to unoccupied residues of E. We hypothesize that ADE of infection is blocked at the level of membrane fusion as the antibodies bound to the virion are likely to facilitate entry of the virus-immune complex through interaction with the Fc receptor.

Table 1
Antiviral effect of SA-17 against Ab-opsonized std and immature DENV2.

Virus	Antibody	Time of SA-17 addition with respect to Ab-opsonization	IC_{50} (μM)	SI[b]
std	None		0.52 ± 0.15	1802
std	4G2 (anti-E DI/II)	Before	0.78 ± 0.24[a]	1203
		During	2.32 ± 0.47	404
		After	2.89 ± 0.99	324
std	104 (anti-E DIII)	Before	1.51 ± 0.24[a]	621
		During	2.15 ± 0.04	436
		After	2.32 ± 0.10	404
Immature	2F5 (anti-prM)	Before	0.54 ± 0.2	1729
		During	0.26 ± 0.04	3591
		After	0.68 ± 0.41	1374

[a] Significantly different ($P < 0.05$) compared with the other two experimental conditions of the same group ("During" and "After"). Calculated using a Mann Whitney test (one-tailed).
[b] Selectivity Index (SI) calculated with a CC_{50} of 937 ± 240 μM.

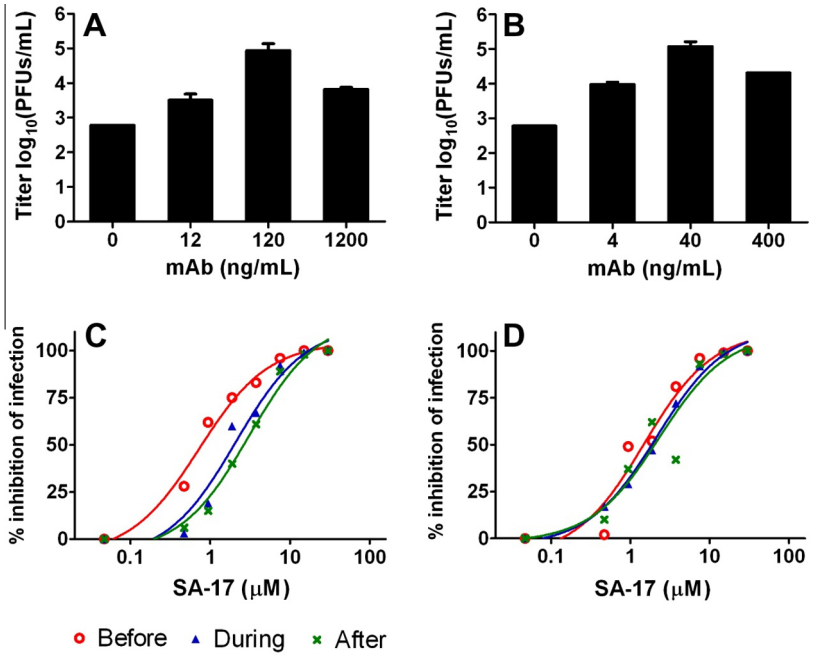

Fig. 4. SA-17 inhibits antibody-dependent enhancement of DENV infection. The enhancing profile of anti-E mAb (A) 4G2 and (B) 104 was assessed with std DENV2 in P388D1 cells. To evaluate SA-17's efficacy against DENV2 ADE, the cells were infected with std DENV2 in the presence of SA-17 and enhancing concentrations of (C) mAb 4G2 and (D) mAb 104. The addition of SA-17 to the virus was done under three experimental conditions: (1) before, (2) during, and (3) after mAb-opsonization. The percentage of inhibition of infection of SA-17 treatment was calculated with respect to the positive control of untreated opsonized virus. Each data point represents the average of three independent experiments carried out in duplo. Sigmoidal dose–response curves are shown.

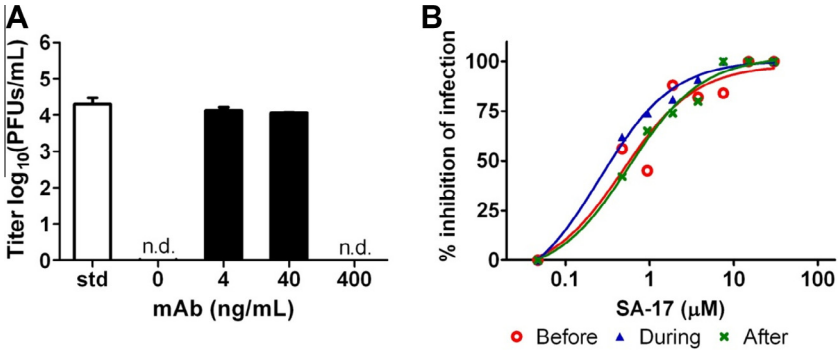

Fig. 5. Antiviral activity of SA-17 against ADE of immature DENV infection. (A) The enhancing activity of anti-prM mAb 2F5 was evaluated with immature DENV2 in P388D1. (B) The addition of SA-17 to the virus in the presence of mAb 2F5 was done under the same experimental setup as described in the legend to Fig. 4, except that here immature DENV is used. Each data point represents the average of three independent experiments carried out in duplo. Sigmoidal dose–response curves are shown.

However, further mechanistic analyses should be done in order to clearly understand how the compound interacts with the immune complex.

In conclusion, the doxorubicin derivative SA-17 is a promising antiviral agent against DENV2, as it is active against DENV2 infection and effectively inhibits ADE of std and immature virus. The data presented here contribute to a new and still largely unexplored area, the use of antiviral compounds against ADE of viral infection.

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