

## A Derivate of the Antibiotic Doxorubicin Is a Selective Inhibitor of Dengue and Yellow Fever Virus Replication *In Vitro*<sup>∇†</sup>

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**A doxorubicin derivate, SA-17, that carries a squaric acid amide ester moiety at the carbohydrate ( $\alpha$ -L-daunosaminy) group was identified as a selective inhibitor of *in vitro* dengue virus (DENV) serotype 2 replication (50% effective concentration [EC<sub>50</sub>] = 0.34 ± 0.20 μg/ml [0.52 ± 0.31 μM]). SA-17 is markedly less cytostatic than the parent compound, resulting in a selectivity index value of ~100. SA-17 also inhibits yellow fever virus 17D (YFV-17D) replication (EC<sub>50</sub> = 3.1 ± 1.0 μg/ml [4.8 ± 1.5 μM]), although less efficiently than DENV replication, but proved inactive against a variety of enveloped and nonenveloped viruses. SA-17 inhibits *in vitro* flavivirus replication in a dose-dependent manner, as was assessed by virus yield reduction assays and quantification of viral RNA by means of real-time quantitative reverse transcriptase PCR (RT-qPCR) (~2 to 3 log reduction). The anti-DENV activity was confirmed using a *Renilla* luciferase-expressing dengue reporter virus. Time-of-drug-addition studies revealed that SA-17 acts at the very early stages of the viral replication cycle (i.e., virus attachment and/or virus entry). This observation was corroborated by the observation that SA-17, unlike the nucleoside analogue ribavirin, does not inhibit the replication of DENV subgenomic replicons. Preincubation of high-titer stocks of DENV or YFV-17D with ≥5 μg/ml SA-17 resulted in 100% inhibition of viral infectivity (≥3 log reduction). SA-17, however, did not prove virucidal.**

Dengue virus (DENV), of which four serotypes (DENV-1, -2, -3, and -4) are known, and yellow fever virus (YFV) belong to the mosquito-borne cluster of the genus *Flavivirus* (family *Flaviviridae*) (25). According to the World Health Organization (WHO), 2.5 billion people, of whom 1 billion are children, are at risk of DENV infection (72). An estimated 50 to 100 million cases of dengue fever, half a million cases of severe dengue disease (i.e., dengue hemorrhagic fever [DHF] and dengue shock syndrome [DSS]), and more than 20,000 deaths occur worldwide each year (69, 72). Every year, increasing numbers of dengue outbreaks/cases are reported. Travelers visiting areas where DENV is endemic (a steadily increasing number) are also at risk of exposure to dengue (70). Dengue fever has been diagnosed in increasing numbers of febrile travelers returning from the tropics, ranging from 2% in the early 1990s to 16% or more recently (4, 5, 33, 59). Due to the nonspecific and self-limiting nature of the milder infections, these data very likely represent an underestimation of the true incidence. In addition, dengue fever represents an emerging problem for troops as well as personnel of nongovernmental organizations (NGOs) deployed in tropical countries where

dengue is endemic (49). In Africa, 33 countries, comprising a total population of nearly 510 million people, are at risk of YFV infection (71). Yellow fever is also endemic in South American countries and in several Caribbean islands. Annually, 200,000 estimated cases of yellow fever, with 30,000 deaths, occur (8, 71). The number of yellow fever epidemics is rising, and more countries are reporting cases. Besides vector control measures, vaccination with the attenuated 17D strain is the most important weapon in the fight against yellow fever. This vaccine has an outstanding safety profile and is highly effective. Mass vaccination campaigns between 1933 and 1961 resulted in the gradual disappearance of the disease. However, these programs have lapsed over the years, leading to a resurgence of yellow fever epidemics.

To prevent and/or control dengue disease, the only currently available methods are mosquito control strategies. The development of effective and safe dengue virus vaccines has proven to be a true challenge, mostly because of the link between secondary dengue virus infection (i.e., reinfection with a different serotype) and severe clinical disease (DHF) (15, 35, 60). Recovery from an infection by one serotype provides long-lasting immunity against that particular serotype but confers only partial and transient protection against a subsequent infection by any of the other three serotypes. In secondary infections, antibodies against the first serotype appear to enhance infection with the second serotype (a phenomenon known as antibody-dependent enhancement of infection), resulting in higher peak viral titers. Higher viral loads have been

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TABLE 1. Structural formulae and effect of doxorubicin and analogues on DENV-2 NGC and YFV-17D replication<sup>a</sup>

Name	Structure	Mol wt	EC <sub>50</sub> (DENV)	EC <sub>50</sub> (YFV)	CC <sub>50</sub>
Doxorubicin		544	0.67 ± 0.37 (1.2 ± 0.68)	0.32 ± 0.01 (0.59 ± 0.02)	13 ± 0.7 (24 ± 1.3)
SA-3		637	>100 (>157)	>100 (>157)	22 ± 1.6 (35 ± 2.5)
SA-11		623	>100 (>161)	>100 (>161)	<0.2 (<0.32)
SA-17		653	0.34 ± 0.20 (0.52 ± 0.31)	3.1 ± 1.0 (4.8 ± 1.5)	28 ± 2.7 (43 ± 4.1)

<sup>a</sup> EC<sub>50</sub> and CC<sub>50</sub> data are expressed in μg/ml (μM in parentheses) and are mean values for at least two independent experiments.

correlated with more severe dengue disease (32, 64, 67). It is of utmost importance to have better means (other than mosquito control efforts and, hopefully within a few years, vaccines) at hand to control dengue (and yellow fever) outbreaks. Moreover, it would also be important to have potent and selective inhibitors of YFV replication and other flaviviruses (such as the Japanese encephalitis virus [JEV], the tick-borne encephalitis virus [TBEVR], and the West Nile virus [WNV]) at hand for the treatment of life-threatening infections caused by these viruses. Recently, a number of inhibitors of DENV and/or other flaviviruses have been reported. These include nucleoside/nucleotide analogues targeting either the polymerase (10, 29, 37, 53, 74) or the protease of the virus (63). A few molecules have been shown to inhibit the early stages of the dengue virus replication cycle (51, 66). Here, we report on the activity

against DENV and some other flaviviruses of SA-17, a derivate of the anthracycline antibiotic doxorubicin. The parent compound doxorubicin was earlier shown to be active against a variety of other viruses, such as HIV and herpes simplex virus (3, 21, 22, 48). SA-17 inhibits virus replication by targeting the very early stages of the viral replication cycle.

#### MATERIALS AND METHODS

**Compounds.** Ribavirin or 1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (Virazole; RBV) was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Doxorubicin or (8S,10S)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (trade name Adriamycin) was obtained from Sigma-Aldrich (Bornem, Belgium). The doxorubicin derivatives SA-3, SA-11, and SA-17 (Table 1) were synthesized as reported elsewhere (61, 62).

**Cells and replicons.** African green monkey kidney cells (Vero-B cells; ATCC CCL-81) were grown in minimum essential medium (MEM; Invitrogen, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS; Integro, Zaandam, Netherlands), 0.075% sodium bicarbonate (Invitrogen), and 2 mM L-glutamine (Invitrogen) at 37°C and in a 5% CO<sub>2</sub> incubator. Antiviral assays using Vero-B cells were performed in assay medium (MEM supplemented with 2% FCS, 0.075% sodium bicarbonate, and 2 mM L-glutamine). C6/36 mosquito cells (from *Aedes albopictus*; ATCC CRL-1660) were grown in MEM supplemented with 8% FCS, 2 mM L-glutamine, 0.01 M HEPES buffer (Invitrogen), 1× nonessential amino acids (Invitrogen), and penicillin (100 U/ml)-streptomycin (100 µg/ml) solution (Invitrogen) at 28°C in the absence of 5% CO<sub>2</sub>. Baby hamster kidney (BHK-21; ATCC CCL10) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% FCS and 1 mM sodium pyruvate (Invitrogen) at 37°C and 5% CO<sub>2</sub>, and assays using these cells were performed in DMEM supplemented with 2% FCS and 1 mM sodium pyruvate. BHK-21 cells harboring the subgenomic dengue virus replicon dCprMEPAC2NS3lucNS3 (derived from the dengue virus replicon construct pDENACprME-PAC2A [23]), in which an antibiotic selection cassette encoding the puromycin *N*-acetyltransferase (PAC) together with the firefly luciferase expression cassette was inserted upstream of the nonstructural (NS) genes (see Fig. 5A), will be referred to as BHK-Rep-Pac-LUC cells. BHK-Rep-Pac-LUC cells were cultured as the parental BHK-21 cells, with the exception that 3.3 µg/ml of puromycin was added to the culture medium (Sigma-Aldrich, Bornem, Belgium). Puromycin was omitted from the culture medium in antiviral assays.

**Viruses.** Modoc virus (MODV) strain M544 (ATCC VR415) was propagated in BHK-21 cells. The YFV-17D vaccine strain (Stamaril) (Aventis Pasteur MSD, Brussels, Belgium) was passaged once in Vero-B cells to prepare a working virus stock and stored at -80°C until further use. DENV serotype 2 New Guinea C (NGC) strain (kindly provided by V. Deubel [formerly at Institut Pasteur, Lyon, France]) was cultivated in either Vero-B or C6/36 mosquito cells. Dengue virus serotype 1 Djibouti strain D1/H/IMTSSA/98/606 (GenBank accession number AF298808), dengue virus serotype 3 strain H87 prototype (c93130), and dengue virus serotype 4 strain Dak HD 34 460 (only partial, unpublished sequences available) were propagated in C6/36 mosquito cells. All four DENV serotypes that were passaged in C6/36 mosquito cells were titrated by means of a flow cytometry-based assay to calculate the number of infectious units (26) prior to use. The construction of an infectious, full-length dengue virus (DENV-2 16681), in which a *Renilla* luciferase expression cassette under the translational control of the encephalomyocarditis virus internal ribosome entry site (EMCV IRES) was inserted upstream of the 3' untranslated (UTR) region (see Fig. 3A), was reported earlier (42). In the current study, this virus is referred to as "dengue reporter virus." All work using infectious Japanese encephalitis virus (JEV) strain SA-14, tick-borne encephalitis virus (TBEV) strain Oshima, and West Nile virus (WNV) strain NY99 was carried out in a biosafety level 3 laboratory. The batches of viral inoculum (JEV, TBEV, and WNV) were prepared by culturing virus twice on Vero cells in MEM supplemented with 7% FCS, 2 mM L-glutamine, and penicillin-streptomycin. Finally, cell culture supernatants were collected and frozen at -80°C in 50 mM HEPES. JEV, TBEV, and WNV were titrated by performing a plaque assay on Vero or BHK-21 cells.

**Cytotoxic and cytostatic assay in Vero-B cells.** Potential cytotoxic effects of the compounds were evaluated in uninfected quiescent Vero-B cells. Cells were seeded at 1 × 10<sup>4</sup> cells/well in a 96-well plate in the presence of 2-fold serial dilutions (ranging from 0.20 to 100 µg/ml) of either SA-17 (0.30 to 153 µM) or doxorubicin (0.36 to 184 µM) and incubated for 1 week. After 1 week, culture medium was discarded, and 100 µl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazinemetosulfate (MTS/PMS; Promega, Leiden, Netherlands) in phosphate-buffered saline (PBS) was added to each well. Following a 2-hour incubation period at 37°C, the optical density (OD) was determined at 498 nm. Cytotoxic activity was calculated using the following formula: % CPE = 100 × (OD<sub>Compound</sub>/OD<sub>CC</sub>), where CPE is the cytopathic effect and OD<sub>Compound</sub> and OD<sub>CC</sub> correspond to the optical density at 498 nm of the uninfected cell cultures treated with compound and that of uninfected, untreated cell cultures, respectively. The 50% cytotoxic concentration (CC<sub>50</sub>; i.e., the concentration that reduces the total cell number by 50%) was calculated using linear interpolation. The cytostatic effect was assessed on exponentially growing Vero-B cells that were cultured for 20 consecutive passages. During each passage, cells were seeded at a fixed number (3 × 10<sup>5</sup> cells in 25-cm<sup>2</sup> tissue culture flasks) in the absence/presence of either 1 or 10 µg/ml of compound. Cells were allowed to proliferate for 7 days after which they were trypsinized and the cell number was determined using a Coulter Counter. Subsequently, 3 × 10<sup>5</sup> cells were seeded per flask and were further cultured in the presence of the drug until reaching passage 20.

**Antiviral assay.** To each well of a 96-well plate, 100 µl of culture medium containing 100 50% cell culture infectious doses (i.e., CCID<sub>50</sub>) of virus was added to each well, after which 2-fold serial dilutions of the compounds and 100 µl cell suspension (2.5 × 10<sup>4</sup> Vero-B or BHK-21) were added. After a 1-week incubation, culture medium was discarded, and 100 µl MTS/PMS (Promega) in PBS was added to each well. Following a 2-hour incubation period at 37°C, the optical density was determined at 498 nm (Safire; Tecan, Mechelen, Belgium), and the percentage of the CPE was calculated as follows: % CPE = 100 × [(OD<sub>Virus+Compound</sub> - OD<sub>VC</sub>)/(OD<sub>CC</sub> - OD<sub>VC</sub>)]. In this formula, OD<sub>CC</sub> represents the optical density of the uninfected untreated cells, whereas OD<sub>VC</sub> and OD<sub>Virus+Compound</sub> represent the optical densities of infected, untreated cells and virus-infected cells that were treated with a given concentration of compound, respectively. The 50% effective concentration (EC<sub>50</sub>), which is defined as the compound concentration that is required to inhibit the virus-induced CPE by 50%, was determined using logarithmic interpolation. Ribavirin was included as a reference compound. Each experiment was carried out at least in quadruplicate, and two independent experiments were carried out.

Antiviral assays for coxsackievirus B3 (CVB3; Nancy strain), herpes simplex virus type 1 (KOS), HIV-1, and influenza A virus H2N2 (A2 Japan/305/57) were carried out as described earlier (6, 7, 12, 56, 76).

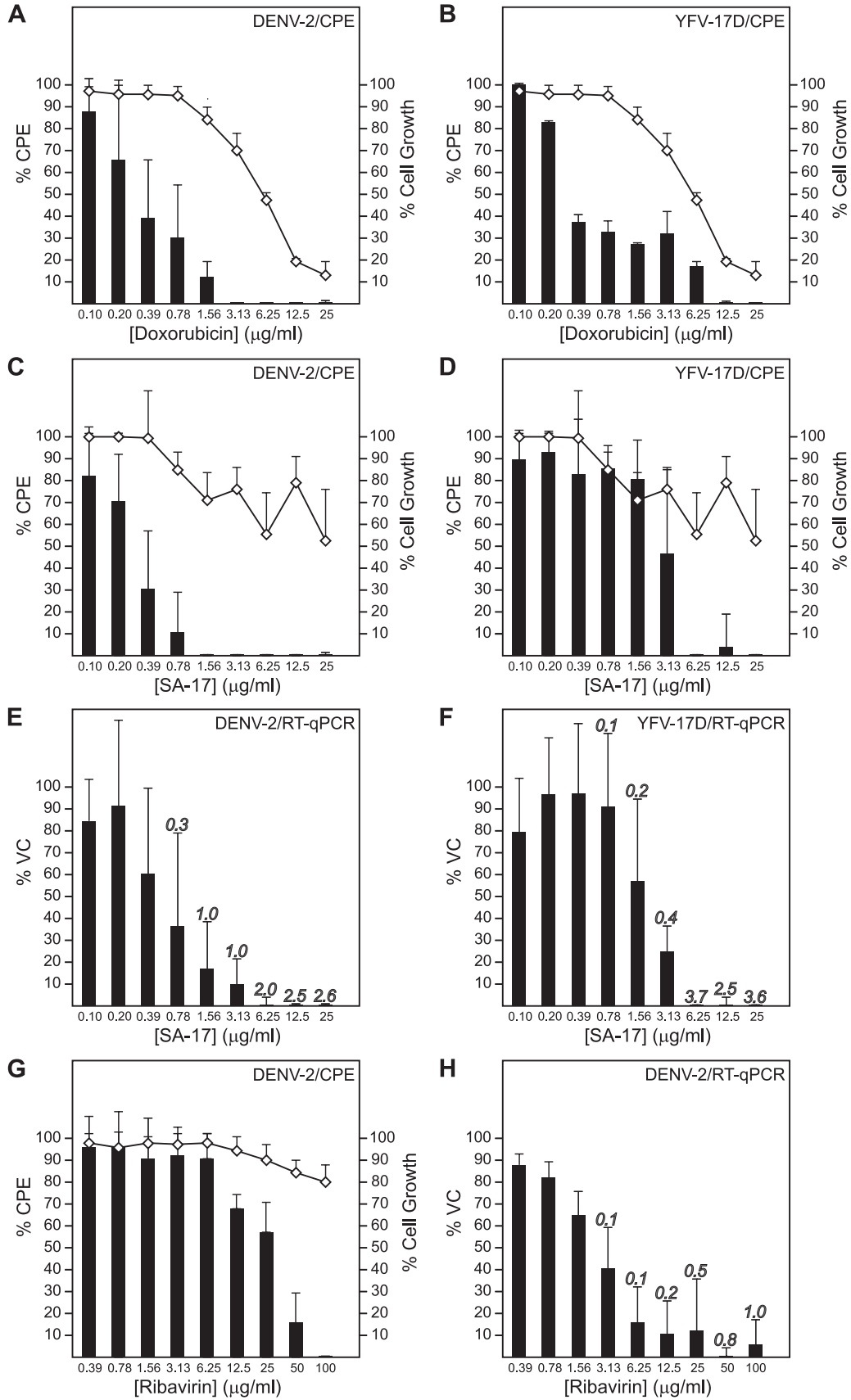
**Antiviral assay using dengue reporter virus.** One day prior to infection, 1 × 10<sup>4</sup> BHK-21 cells were seeded per well in a tissue culture-treated white-view 96-well plate (Perkin-Elmer, Boston, MA). The next day, cells were infected with 2 × 10<sup>5</sup> PFU/well of dengue reporter virus in the absence or presence of 0.1, 1, or 10 µg/ml (0.15, 1.5 or 15 µM) of SA-17. The viral inoculum was removed 1 h later, and cells were rinsed three times with PBS. Luciferase activity was measured at 10, 24, 48, and 72 h postinfection (p.i.) using the *Renilla* luciferase assay system according to the manufacturer's protocol (Promega). Counts were detected using a Safire plate reader (Tecan, Mechelen, Belgium).

**Virus yield assay.** DENV-2 NGC or YFV-17D (100 CCID<sub>50</sub>/well) was added to a 96-well plate, after which 2-fold serial dilutions of the compounds and 100-µl cell suspensions (5 × 10<sup>4</sup> Vero-B or BHK-21) were added. Four hours later, culture medium was carefully removed and the monolayers were washed three times with assay medium to remove residual, nonadsorbed virus. Cells were cultivated with fresh assay medium containing the respective 2-fold serial dilutions of the compounds for 7 days, after which the clarified supernatant of four replicate wells per plate was collected and pooled; 150 µl was stored at -80°C for later use in quantitative reverse transcriptase PCR (RT-PCR) to determine viral RNA load. Each treatment condition was carried out in quadruplicate, and two independent experiments were performed.

Antiviral assays for JEV, TBEV, and WNV were carried out in Vero-B cells in 24-well plates (5 × 10<sup>4</sup> cells/well). Briefly cells were cultured in MEM supplemented with 7% FCS, 2 mM L-glutamine, and penicillin-streptomycin. One day after seeding, cells were infected with 100 µl of the viral inoculum (at a multiplicity of infection of either 0.1 or 1) in the presence or absence of 10 µg/ml (15 µM) SA-17. Following incubation for 90 min (37°C; 5% CO<sub>2</sub>), cultures were washed twice with Hanks balanced salt solution (HBSS) after which 1 ml fresh medium either supplemented or not with 10 µg/ml (15 µM) SA-17 was added to the wells. Cells were further incubated at 37°C (medium with compound was refreshed at days 3 and 5 postinfection). At various time points p.i., supernatants were harvested and stored at -80°C for later use in quantitative RT-PCR.

**Time-of-drug-addition assay.** One day prior to infection, 2 × 10<sup>4</sup> BHK-21 cells were seeded in a tissue culture-treated white-view 96-well plate. The next day, cells were infected with 2 × 10<sup>5</sup> PFU/well of dengue reporter virus. After 1 h, the virus inoculum was replaced by assay medium. SA-17 at concentrations of 0.1, 1, or 10 µg/ml (0.15, 1.5 or 15 µM) was added to the assay medium either at 0 (i.e., together with the virus) or at 1, 2, 4, 6, 8, 10, or 12 h postinfection. Luciferase activity was measured at 24 h p.i. using the *Renilla* luciferase assay system. Luciferase activity of treated, infected cells was compared to that of untreated, infected cells.

**Real-time RT-qPCR.** Total RNA was extracted from 150 µl of cell culture supernatant using the NucleoSpin RNA virus kit according to the manufacturer's protocol (Macherey-Nagel, Düren, Germany) or using an EZ1 robot (Qiagen) in the case of JEV, TBEV, and WNV. The sequences of the TaqMan primers (DENV-For, DENV-Rev, MODV-For, MODV-Rev, YFV-For, YFV-Rev, JEV-For, JEV-Rev, TBEV-For, TBEV-Rev, WNV-For, and WNV-Rev) (see Table S1 in the supplemental material) and TaqMan probes (DENV-Probe, MODV-Probe, YFV-Probe, JEV-Probe, TBEV-Probe, and WNV-Probe) (see Table S1) were selected from nonstructural gene 3 (NS3), NS5, or the 3' UTR of the respective flaviviruses using Primer Express software (version 2.0; Applied Biosystems, Lennik, Belgium). The TaqMan probe was fluorescently labeled with 6-carboxyfluorescein (FAM) at the 5' end as the reporter dye and with minor groove binder (MGB) or tetramethylrhodamine (TAMRA) at the 3' end as the



quencher (see Table S1). One-step, quantitative RT-PCR (RT-qPCR) was performed essentially as described earlier (1). For absolute quantification, standard curves were generated using 10-fold dilutions of template preparations of known concentration (PCR product using primers DENV-For and DENV-Rev, cloned into a TOPO vector using the TOPO TA cloning kit [Invitrogen]). Data are expressed as the percentage of untreated virus control, and log reduction values are calculated. The detection limit of the RT-qPCR in this experimental setup was 111 RNA copies/ml for YFV (30), 200 copies for DENV, and 6,016 copies for MODV.

**Flow cytometric analysis.** C6/36 mosquito cells ( $4 \times 10^6$  cells) were seeded in 25-cm<sup>2</sup> tissue culture flasks and allowed to proliferate for 3 days. Prior to infection, all four serotypes of DENV ( $2 \times 10^6$  IU) were separately incubated with or without SA-17 (ranging from 0.5 to 10  $\mu\text{g/ml}$  [0.77 to 15  $\mu\text{M}$ ]) for 1 h at 37°C. C6/36 cells were then infected with the virus-compound mixtures for 2 h at 28°C, after which the infection medium was replaced with MEM containing 2% FCS. At day 3 p.i., flow cytometric analysis was performed essentially as described previously (1). In short, cells were fixed and permeabilized with Cytofix/Cytoperm fixation/permeabilization solution (Becton Dickinson, Erembodegem, Belgium) for 20 min at 4°C and rinsed with Perm/Wash buffer (Becton Dickinson). Next, cells were subsequently stained with anti-dengue virus complex directed against the prM protein of all dengue virus serotypes (clone D3-2H2-9-21; Chemicon International/Millipore, Billerica, MA) for 30 min at 4°C and secondary phycoerythrin (PE)-conjugated goat F(ab')<sub>2</sub> anti-mouse immunoglobulins (M30004-1; Caltag Invitrogen, Carlsbad, CA) for 30 min at 4°C. Cells were washed and analyzed using a FACSCalibur flow cytometer equipped with CellQuest software (BD, San Jose, CA). The percentage of DENV-infected cells was determined by subtracting the mean fluorescence of intensity (MFI) of the background staining from the MFI of each sample. EC<sub>50</sub>s were obtained as described above.

**Virucidal assay.** Virus stocks ( $5 \times 10^4$  PFU [DENV or YFV-17D];  $1 \times 10^6$  PFU [DENV or YFV-17D];  $5 \times 10^6$  PFU [YFV-17D]) were preincubated for various periods of time at 37°C in the presence of various concentrations of SA-17, doxorubicin, or an equivalent volume of the solvent (1% dimethyl sulfoxide [DMSO] in culture medium; negative control). Following this incubation, the virus-compound mixture was serially diluted (10- to 31,250-fold for DENV and 60- to 187,500-fold for YFV-17D) and used to infect Vero-B cells ( $5 \times 10^5$  cells/well in a 6-well plate). Cultures were incubated for 2 h, after which they were rinsed three times with assay medium and overlaid with a solution containing 0.5% Avicel (a codried blend of microcrystalline cellulose and sodium carboxymethylcellulose [FMC BioPolymer, Brussels, Belgium]) and assay medium at a ratio of 1:1 (36). One week later, the Avicel/MEM solution was removed, cells were washed (PBS), fixed, and stained with 1% methylene blue solution, and the number of plaques was counted.

In a separate experiment, YFV-17D ( $1 \times 10^6$  PFU) was incubated in the absence or presence of various concentrations of SA-17 (0.1 to 10  $\mu\text{g/ml}$  [0.15 to 15  $\mu\text{M}$ ]). To one-third of the samples, 0.5  $\mu\text{l}$  RNase A (4 mg/ml; Promega) was added to the reaction mixture, whereas in yet another third of the samples, 1  $\mu\text{l}$  RNase A was added. All samples were subjected to incubation for 1 h at 37°C, after which viral RNA was extracted (using the NucleoSpin RNA virus kit) in the presence of proteinase K (0.4 mg; Promega) and detected via RT-qPCR. As a positive control, YFV-17D was incubated with proteinase K for 1 h at 37°C. Subsequently, RNase A together with phenylmethanesulfonyl fluoride (PMSF; 5 mM; Sigma-Aldrich) was added to the mixture, which was incubated for another 30 min at 37°C. PMSF was added to the reaction to inhibit proteinase K activity. Viral RNA extraction and quantification were performed as described above.

**Dengue virus subgenomic replicon assay.** BHK-Rep-Pac-LUC cells were seeded at a density of  $1 \times 10^4$  cells/well in a tissue culture-treated white-view 96-well plate (Perkin-Elmer). The next day, medium was replaced by assay medium containing various concentrations of SA-17 or doxorubicin (concentrations ranged from 0.1 to 5  $\mu\text{g/ml}$  [0.15 to 7.7  $\mu\text{M}$  and 0.18 to 9.2  $\mu\text{M}$ , respectively]). After 72 h, luciferase activity was measured using the luciferase assay system according to the manufacturer's protocol (Promega). Luciferase activity

was compared to that of untreated replicon cells. The cytotoxic effect of the compounds on BHK-Rep-Pac-LUC cells was evaluated with parallel cultures. Ribavirin was included as a replication inhibitor for comparative reasons. The inhibitory effect of the compounds on luciferase activity was adjusted for inhibitory effects on cell proliferation.

**Docking experiments.** Docking of SA-17 in the prefusion form of the DENV-2 E glycoprotein (PDB code 1oke) (39) was performed using Autodock 4.0 (43). A docking zone of a 50-angstrom cube situated around Ile-270 in the  $\beta$ -OG (*n*-octyl- $\beta$ -D-glucoside) (40) binding cleft of the dengue virus E glycoprotein dimer was defined. The SA-17 conformation was obtained from the Dundee PRODRG2 server (55). Gasteiger charges were added to ligand and protein; only polar H was kept for the docking experiments. All default docking parameters were used, except the number of dockings was increased to at least 50, and docking runs were repeated three times. The final selection was based on the low score and as many possible interactions with the surrounding amino acids (hydrogen bonds and hydrophobic interactions).

## RESULTS

**SA-17 is a selective inhibitor of flavivirus replication.** Doxorubicin was identified as an *in vitro* inhibitor of DENV-2 NGC (EC<sub>50</sub> =  $0.67 \pm 0.37$   $\mu\text{g/ml}$  [ $1.2 \pm 0.68$   $\mu\text{M}$ ]) and YFV-17D (EC<sub>50</sub> =  $0.32 \pm 0.01$   $\mu\text{g/ml}$  [ $0.59 \pm 0.02$   $\mu\text{M}$ ]) induced CPE formation (Table 1; Fig. 1A and B). However, doxorubicin also exhibited rather pronounced cytotoxic activity (CC<sub>50</sub> =  $13 \pm 0.7$   $\mu\text{g/ml}$  [ $24 \pm 1.3$   $\mu\text{M}$ ]) in the host (Vero-B) cell (Fig. 1A and B). This finding prompted the evaluation of the antiviral activity of a selection of doxorubicin analogues. The squaric amide ester SA-17 was found to be roughly twice as effective as the parent compound in inhibiting DENV-2 NGC replication (EC<sub>50</sub> =  $0.34 \pm 0.20$   $\mu\text{g/ml}$  [ $0.52 \pm 0.31$   $\mu\text{M}$ ]) (Fig. 1C) but was less effective than the parent compound with YFV-17D replication (EC<sub>50</sub> =  $3.1 \pm 1.0$   $\mu\text{g/ml}$  [ $4.8 \pm 1.5$   $\mu\text{M}$ ]) (Fig. 1D). In addition, SA-17 proved markedly less cytostatic (CC<sub>50</sub> =  $28 \pm 2.7$   $\mu\text{g/ml}$  [ $43 \pm 4.1$   $\mu\text{M}$ ]) than the parent compound.

The antiviral activity ( $\geq 2$  log reduction) was confirmed in a virus yield assay using DENV-2 NGC (EC<sub>50</sub> DENV =  $0.76 \pm 0.60$   $\mu\text{g/ml}$  [ $1.2 \pm 0.92$   $\mu\text{M}$ ]) (Fig. 1E) and YFV-17D (EC<sub>50</sub> YFV =  $1.9 \pm 1.2$   $\mu\text{g/ml}$  [ $2.9 \pm 1.8$   $\mu\text{M}$ ]) (Fig. 1F) and by means of flow cytometry (EC<sub>50</sub> DENV-2 < 0.5  $\mu\text{g/ml}$  [ $< 0.77$   $\mu\text{M}$ ]; Table 2). SA-17 also inhibited the replication of an infectious, full-length dengue reporter virus expressing *Renilla* luciferase in a dose- and time-dependent manner (EC<sub>50</sub> =  $3.6 \pm 1.6$   $\mu\text{g/ml}$  [ $5.5 \pm 2.5$   $\mu\text{M}$ ] at  $t = 48$  h p.i.) (data not shown). In addition, SA-17 was found to inhibit the replication of DENV-1 (EC<sub>50</sub> =  $8.3 \pm 1.4$   $\mu\text{g/ml}$  [ $12 \pm 2.1$   $\mu\text{M}$ ]) and DENV-3 (EC<sub>50</sub> =  $1.1 \pm 0.5$   $\mu\text{g/ml}$  [ $1.7 \pm 0.77$   $\mu\text{M}$ ]) in insect cells, but not that of DENV-4 (EC<sub>50</sub> > 10  $\mu\text{g/ml}$  [ $> 15$   $\mu\text{M}$ ]) (Table 2). SA-17 also inhibited the replication of the murine flavivirus Modoc virus (EC<sub>50</sub> =  $1.9 \pm 0.3$   $\mu\text{g/ml}$  [ $2.9 \pm 0.46$   $\mu\text{M}$ ]) (Table 2) but proved inactive against other flaviviruses (i.e., JEV, TBEV, WNV [EC<sub>50</sub> > 10  $\mu\text{g/ml}$  ( $> 15$   $\mu\text{M}$ )]) (data not shown) or a selection of other viruses (with either a DNA

FIG. 1. Dose-response curves for doxorubicin and SA-17 against DENV and YFV. Cytotoxic (white diamonds) and antiviral effect (black bars) of doxorubicin against DENV-2 NGC (A) and YFV-17D (B). Cytotoxic and antiviral effect of SA-17 against DENV-2 NGC (C and E) and YFV-17D (D and F). Cytotoxic and antiviral effect of the positive-control compound ribavirin against DENV-2 NGC (G and H). Antiviral activity was determined by means of a CPE reduction assay using MTS/PMS (A, B, C, D, and G) and RT-qPCR (E, F, and H). Antiviral data are expressed as percentage of the cytopathic effect (CPE) of untreated virus control (VC). Mean log reduction values ( $> 0$ ) are depicted in italics at the top of each bar (E, F, and H). Cytotoxic data are expressed as percentage of cell growth of untreated control. Data are mean values  $\pm$  standard deviation (SD) for at least three independent experiments.

TABLE 2. Effect of SA-17 on the replication of selected flaviviruses

Virus	EC <sub>50</sub> (μg/ml) <sup>a,b</sup>		
	CPE-based	RT-qPCR	Antigen expression
Dengue 1 Djibouti D1/H/IMTSSA/98/606	NT	NT	8.3 ± 1.4 (12 ± 2.1)
Dengue 2 New Guinea C	0.34 ± 0.20 (0.52 ± 0.31)	0.76 ± 0.60 (1.2 ± 0.92)	<0.5 (<0.77)
Dengue 3 H87	NT	NT	1.1 ± 0.5 (1.7 ± 0.77)
Dengue 4 Dak HD 34 460	NT	NT	>10 (>15)
Yellow fever virus 17D	3.1 ± 1.0 (4.8 ± 1.5)	1.9 ± 1.2 (2.9 ± 1.8)	NT
Modoc	NT	1.9 ± 0.3 (2.9 ± 0.46)	NT

<sup>a</sup> Data are expressed in μg/ml (μM in parentheses) and are mean values ± standard deviations from at least two independent experiments. NT, not tested.

<sup>b</sup> EC<sub>50</sub> of SA-17 for inhibition of DENV-2 NGC and YFV-17D replication was determined with Vero-B cells. EC<sub>50</sub> of SA-17 for inhibition of MODV replication was determined with BHK-21 cells by means of RT-qPCR. The effect of SA-17 on DENV-1, DENV-2, DENV-3, and DENV-4 antigen expression was determined with C6/36 mosquito cells by means of flow cytometry.

or RNA genome and either enveloped or not (i.e., coxsackievirus B3, HIV-1, influenza A virus, and HSV-1 [EC<sub>50</sub> > 100 μg/ml (>153 μM)]) (data not shown).

The effect of SA-17 and doxorubicin was evaluated on the proliferation of exponentially growing Vero-B cells during 20 consecutive passages. Doxorubicin at concentrations of 1 and 10 μg/ml resulted in complete destruction of Vero-B cells from the culture following, respectively, three and two consecutive passages. At the same concentrations, cells that had been cultured with SA-17 proliferated efficiently after 20 passages of drug pressure (Fig. 2).

**Time-of-drug-addition studies.** To examine which stage of the viral replication cycle (i.e., the complete process from virus binding to release) is affected by SA-17, a time-of(-drug)-addition experiment was carried out. Various concentrations of SA-17 were added to dengue reporter virus-infected cells either at the time of infection or at several time points postinfection. At 24 h postinfection, luciferase activity was quantified and compared to that of untreated, infected cells. Infection in the presence of 1 or 10 μg/ml (1.5 or 15 μM) SA-17 resulted in, respectively, 41% or 98% inhibition of virus replication (Fig. 3B). When the compound was added at later stages postinfection, SA-17 failed to efficiently inhibit viral replication. This provides evidence that SA-17 either interferes with the earliest

stages of the viral replication cycle (i.e., virus attachment and/or virus entry) or, alternatively, exerts a virucidal action.

#### SA-17 inhibits the infectivity of DENV and YFV particles.

High-titer stocks of DENV-2 NGC or YFV-17D were preincubated at 37°C with either 1 or 10 μg/ml (1.5 or 15 μM) SA-17 for various periods of time, after which serial dilutions of the virus-compound mixture were used to infect cell cultures. Preincubation of a virus preparation of 5 × 10<sup>4</sup> PFU with 1 μg/ml (1.5 μM) SA-17 inhibited subsequent DENV- and YFV-induced plaque formation by ~90% (≥1 log reduction) (Fig. 4D and E). Complete inhibition of plaque formation was observed when the virus stocks were incubated with SA-17 at concentrations of 5 μg/ml (7.7 μM) or higher (≥3 log reduction) (Fig. 4D and E). EC<sub>50</sub>s for inhibition of virus infectivity by SA-17 following preincubation were calculated to be 0.17 ± 0.01 μg/ml (0.26 ± 0.02 μM) for DENV-2 and 0.15 ± 0.06 μg/ml (0.23 ± 0.09 μM) for YFV-17D. The inhibitory activity of SA-17 was not attributable to a carryover effect of the compound, since the sixth well shown in Fig. 4C (containing 0.32 ng/ml SA-17 [0.49 nM]) did not show any plaques, whereas the first well shown in Fig. 4B (containing 0.1 μg/ml SA-17 [0.15 μM]) contained numerous plaques. When the titer of the viral inoculum was 20-fold higher (1 × 10<sup>6</sup> PFU), SA-17 retained about the same efficacy in this assay (EC<sub>50</sub>s of 0.25 ± 0.04 μg/ml [0.38 ± 0.06 μM] and 0.21 ± 0.01 μg/ml [0.21 ± 0.02 μM] for DENV-2 and YFV, respectively) (Fig. 4D and E). Preincubation of 5 × 10<sup>6</sup> PFU of a YFV-17D stock (100-fold-higher titer) with 10 μg/ml (15 μM) SA-17 resulted in 98% inhibition of plaque formation (1.7 log reduction) (Fig. 4E). Doxorubicin proved markedly less potent than SA-17 in this assay. When 5 × 10<sup>4</sup> PFU of DENV-2 stock was preincubated with 10 μg/ml (18 μM) doxorubicin, a maximum of 73% reduction in plaque formation was obtained (0.6 log reduction) (Fig. 4F).

Subsequently, the time dependency of the virus-inactivating activity of SA-17 was studied (Fig. 4G). A 50% inhibition of plaque formation (0.4 log reduction) was observed when YFV-17D stock was preincubated with 10 μg/ml (15 μM) SA-17 for 5 min. Complete inhibition of YFV infection of the cells (≥2.4 log reduction) was obtained following preincubation with 5 μg/ml (7.7 μM) SA-17 for periods of 45 min and longer.

To study whether the inhibitory effect of SA-17 was the result of a direct virucidal activity, YFV-17D was incubated for 1 h with various concentrations of SA-17 in the absence or presence of RNase A. Next, viral RNA was extracted and

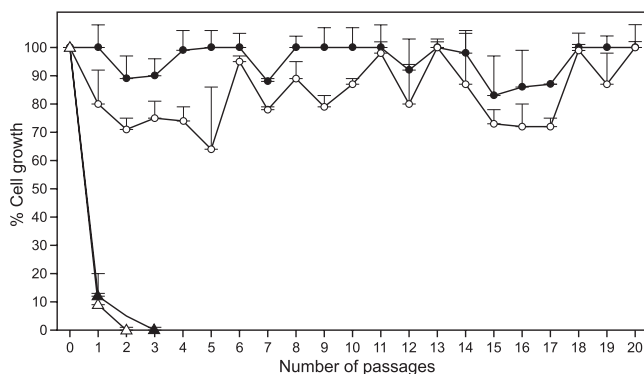


FIG. 2. The effect of SA-17 and doxorubicin on exponentially growing Vero-B cells. Cells were seeded in the absence or presence of either 1 (●) or 10 (○) μg/ml (1.5 or 15 μM) SA-17 or 1 (▲) or 10 (△) μg/ml (1.8 or 18 μM) doxorubicin for 20 passages. The cell number was determined at 7 days postseeding (after every passage). Data are expressed as percentage of cell growth of untreated cultures and represent mean values ± SD for at least three independent experiments.

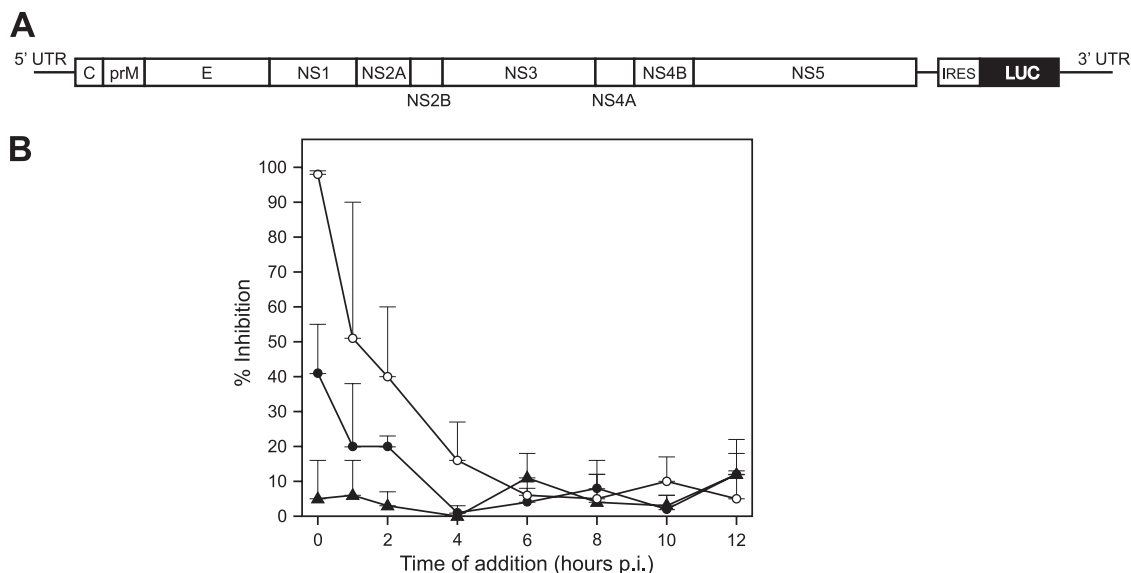


FIG. 3. Time-of-addition study using dengue reporter virus. (A) Genome organization of an infectious, full-length dengue reporter virus expressing *Renilla* luciferase. White boxes represent the proteins encoded by DENV-2 16681, both structural (C, prM, and E) and nonstructural proteins (NS1 to NS5). The *Renilla* luciferase coding sequence (LUC; black box) is fused to the encephalomyocarditis virus internal ribosome entry site (IRES) (42). UTR, untranslated region. (B) The effect of time-of-(drug-)addition on the antiviral activity of SA-17 using an infectious, full-length dengue reporter virus. Infected BHK-21 cells were treated with 0.1 (▲), 1 (●), or 10 (○)  $\mu\text{g/ml}$  (0.15, 1.5 or 15  $\mu\text{M}$ ) SA-17 at various time points p.i., starting at 0 h p.i. Luciferase activity was measured at 24 h p.i. Luciferase activity of treated, infected cells was compared to that of untreated, infected cells and expressed as percentage of inhibition. Data are mean values  $\pm$  SD for at least three independent experiments.

quantified by means of RT-qPCR. Viral RNA levels were identical for both RNase-treated and RNase-untreated samples (data not shown). In contrast, the positive-control sample (i.e., YFV treated with proteinase K and RNase A in the presence of PMSF) showed a 95% reduction of viral RNA load (data not shown). Together, these data and the central experiment demonstrate that SA-17 does not disrupt virion integrity.

**SA-17 does not inhibit DENV subgenomic replicon replication.** If SA-17 exerts its activity at a very early step, the compound may be expected not to inhibit the replication of a DENV subgenomic replicon. To test this hypothesis, DENV subgenomic replicon-containing cells (BHK-Rep-Pac-LUC) were cultured in the presence of various concentrations (0.1 to 5  $\mu\text{g/ml}$ ) of SA-17 (0.15 to 7.7  $\mu\text{M}$ ) or doxorubicin (0.18 to 9.2  $\mu\text{M}$ ). Replication of the subgenomic replicons was not significantly impaired in the presence of 0.1 to 5  $\mu\text{g/ml}$  (0.18 to 7.7  $\mu\text{M}$ ) SA-17 (Fig. 5B). In contrast to SA-17, ribavirin, which was included as a replication inhibitor, clearly reduced the replication of the dengue virus replicons (Fig. 5C) (79% and 68% inhibition of luciferase activity at a concentration of 1 and 5  $\mu\text{g/ml}$  [4.1 and 21  $\mu\text{M}$ ], respectively). Thus, SA-17 does not inhibit DENV RNA replication.

**Long-term antiviral pressure with SA-17 does not result in the selection of drug-resistant variants.** It was studied whether DENV-2 or YFV-17D drug-resistant variants could be generated following long-term selective pressure with various concentrations of SA-17. Even following 30 passages in the presence of various concentrations of the compound (0.1 to 20  $\mu\text{g/ml}$  [0.15 to 31  $\mu\text{M}$ ]), no shift in sensitivity/the  $\text{EC}_{50}$  was noted.

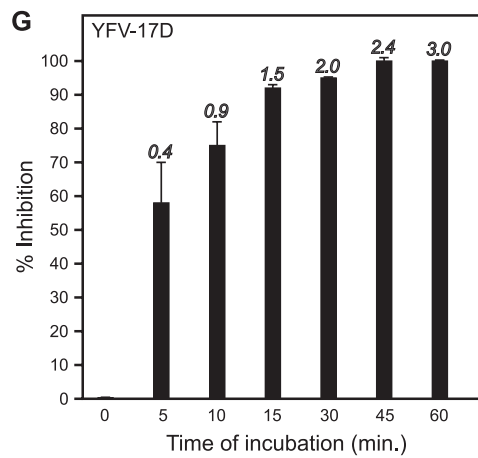
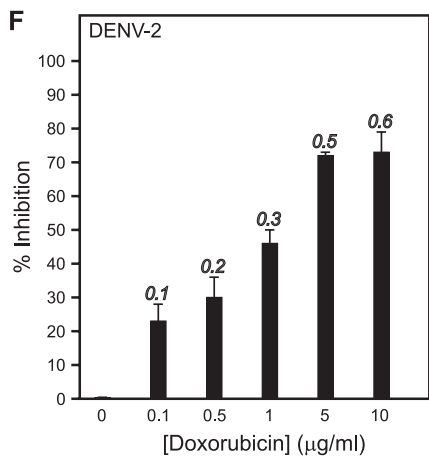
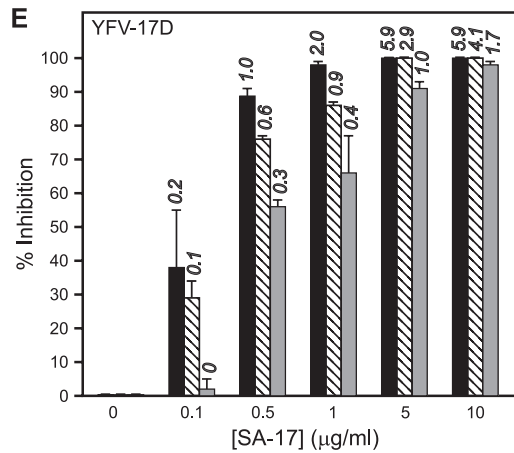
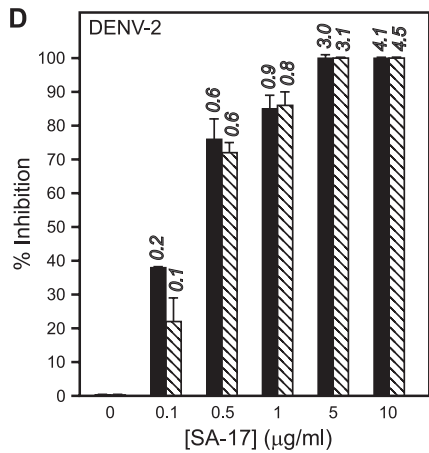
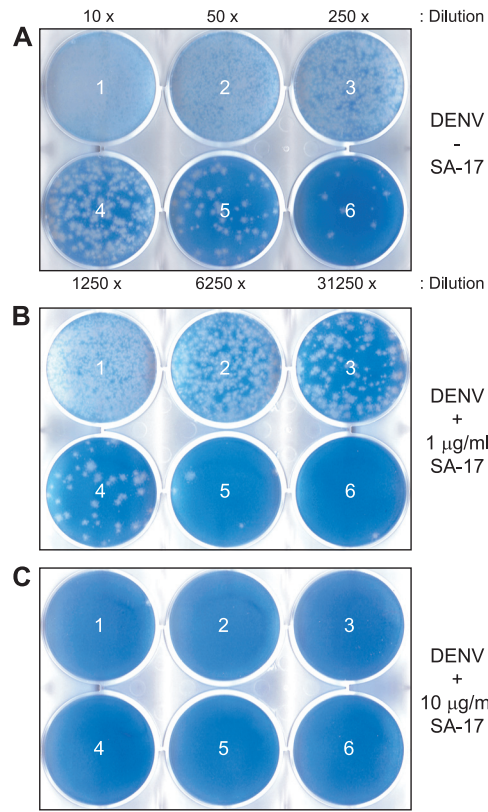
**Docking of SA-17.** Since SA-17 was shown to decrease viral infectivity when preincubated with the virus without being viru-

cidal, whether the compound has the potential to interact with the prefusion form of the DENV E glycoprotein was studied by means of a docking algorithm. As depicted in Fig. 6A and B, SA-17 is predicted to dock in the  $\beta$ -OG binding cleft of the dengue virus E protein. SA-17 may interact predominantly with amino acid residues Ala-50, Tyr-137, and Gln-200 through hydrogen bonds (Fig. 6A). Other nonligand residues that might be involved in hydrophobic interactions are Thr-48, Pro-53, Lys-128, Leu-135, Phe-193, Leu-198, Ala-205, Ile-270, Gln-271, Thr-280, and Gly-281 (Fig. 6A).

## DISCUSSION

A safe and efficient anti-dengue virus drug (or combination of drugs) may have the potential to (i) reduce the total number of people developing dengue fever, dengue hemorrhagic fever, and dengue shock syndrome, in particular during epidemic situations in areas where dengue virus is endemic, and (ii) provide protection for people who travel to and through regions where dengue virus is endemic.

The anthracycline doxorubicin is an antineoplastic antibiotic from *Streptomyces peuceetius* (34). The drug exhibits broad-spectrum antitumor activity and is used for the treatment of cancer, in particular solid tumors (2, 13, 44, 46, 50). Doxorubicin was reported to elicit antiviral activity *in vitro*, including against HIV, herpes simplex virus, Rauscher leukemia virus, and avian myeloblastosis virus (3, 21, 22, 48). On the other hand, however, doxorubicin was reported to cause reactivation of latent viruses, including herpes simplex virus and the Epstein-Barr virus (17, 18, 54). Our aim was to study whether doxorubicin inhibits the *in vitro* replication of flaviviruses and, if so, whether analogues can be developed that are more po-



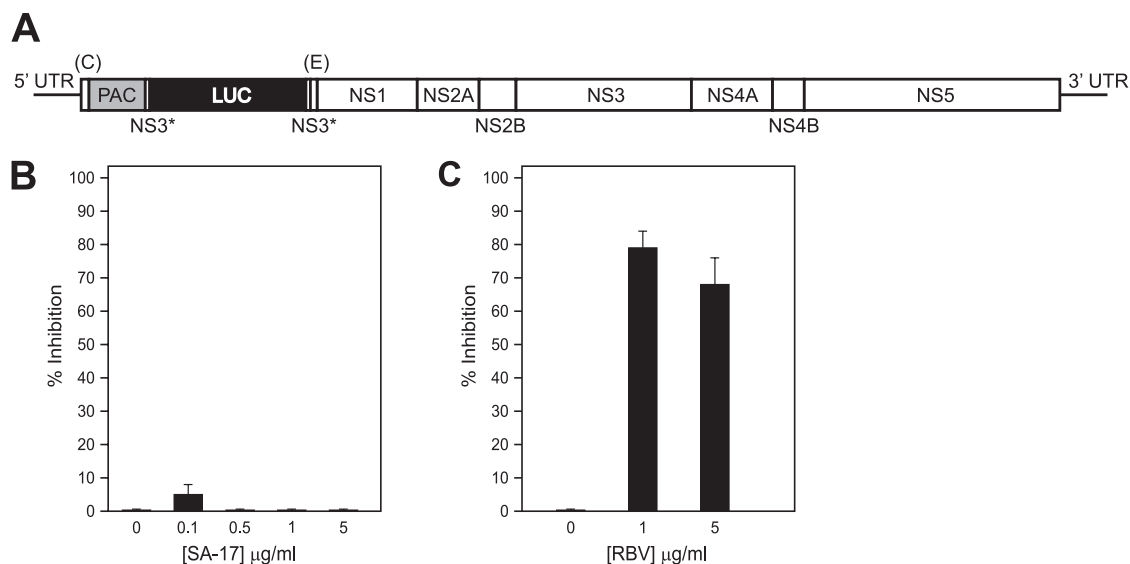


FIG. 5. Effect of SA-17 on dengue virus subgenomic replicon replication. (A) Genome organization of dengue virus subgenomic replicons that harbor only the NS genes of dengue virus 2 NGC (23). The puromycin *N*-acetyltransferase (PAC) gene together with the firefly luciferase (LUC) expression cassette was inserted upstream of the NS genes. Effect of SA-17 (B) and the reference compound ribavirin (RBV) (C) on dengue virus subgenomic replicon replication. Various concentrations of SA-17 or RBV were added to the culture medium of replicon cells (BHK-Rep-Pac-LUC), and luciferase activity was measured after 72 h. Luciferase activity was compared to that of untreated replicon cells and expressed as percentage of inhibition. The inhibitory effect of SA-17 and RBV on luciferase activity was adjusted for inhibitory effects on cell proliferation. Data are mean values  $\pm$  SD for at least three independent experiments.

tent/selective. We indeed observed that doxorubicin exhibits *in vitro* antiviral activity against the flaviviruses DENV-2 and YFV-17D. However, and as expected, doxorubicin exerts pronounced cytotoxic and cytostatic activity. To study whether the antiviral activity extended to analogues and, if so, if the antiviral activity could be dissected from the cytotoxicity, the anti-flavivirus activity and effect on the uninfected host cell of a number of analogues of doxorubicin (62) were evaluated. In particular, the SA-17 derivative, which carries a squaric acid amide ester moiety at the carbohydrate ( $\alpha$ -L-daunosaminy) group, was found to efficiently inhibit DENV-2 replication as well as replication of DENV-1, DENV-3, YFV-17D, and the murine flavivirus Modoc virus. The compound proved inactive against DENV-4, JEV, WNV, and TBEV as well as against a number of viruses belonging to other families. SA-17 proved markedly less cytotoxic and cytostatic than the parent compound doxorubicin. Time-of-drug-addition studies revealed that the compound acts at a stage that coincides with the early events of the flavivirus replication cycle. Preincubation of the virus with SA-17 (e.g., 5  $\mu$ g/ml [7.7  $\mu$ M]) for 45 min was able to completely abolish viral infectivity. In the case that SA-17 acts at the level of the viral particle or at a very early stage of the viral replication cycle, it may be assumed that the com-

ound will have no effect on intracellular RNA replication. Indeed, SA-17 did not inhibit the replication of a subgenomic DENV replicon, whereas the replication inhibitor ribavirin did. The question remains as to precisely how SA-17 interacts with the flavivirus particle. It is rather unlikely that the compound targets the lipid bilayer of the virus, since other unrelated enveloped viruses (i.e., HIV-1 and influenza virus) are not sensitive to SA-17. A direct virucidal effect was also excluded since incubation of the virus with SA-17 did not result in degradation of viral RNA by subsequent RNase treatment. An alternative explanation may be that SA-17 interacts in a specific manner with the envelope glycoprotein and thereby hinders proper functioning of this glycoprotein. This was recently also suggested for two tetracycline derivatives, i.e., rolitetracycline and doxycycline, based on computational approaches (73). The flavivirus E glycoprotein is involved in virus attachment and entry, for which several conformational rearrangements are needed. Both tetracycline derivatives are believed to putatively block the conformational change of the E protein during membrane fusion and, thereby, viral entry (73). Likewise, and given the structural relationship between tetracyclines and the anthracycline doxorubicin (i.e., anthracyclines consist of a tetracycline moiety), SA-17 may interfere with the

FIG. 4. Effect of SA-17 on viral infectivity. DENV-2 NGC ( $5 \times 10^4$  PFU) was preincubated in the absence (–) (A) or presence of 1 (1.5  $\mu$ M) (B) or 10  $\mu$ g/ml (15  $\mu$ M) SA-17 (C). Cells in 6-well plates were infected with serial dilutions (ranging from a  $10\times$  to a  $31,250\times$  dilution corresponding to wells 1 to 6) of preincubated virus with or without compound. Effect of SA-17 on DENV-2 NGC (D) and YFV-17D (E and G) using an inoculum of  $5 \times 10^4$  PFU (black bars),  $1 \times 10^6$  PFU (hatched bars), or  $5 \times 10^6$  PFU (gray bars). Effect of doxorubicin on DENV-2 NGC (F). Virus with or without compound was preincubated for either 1 h (D, E, and F) or various periods of time (G) at 37°C. Data are expressed as percentage of inhibition of plaque formation and represent mean values  $\pm$  SD for at least three independent experiments. Mean log reduction values ( $>0$ ) are depicted in italics at the top of each bar.

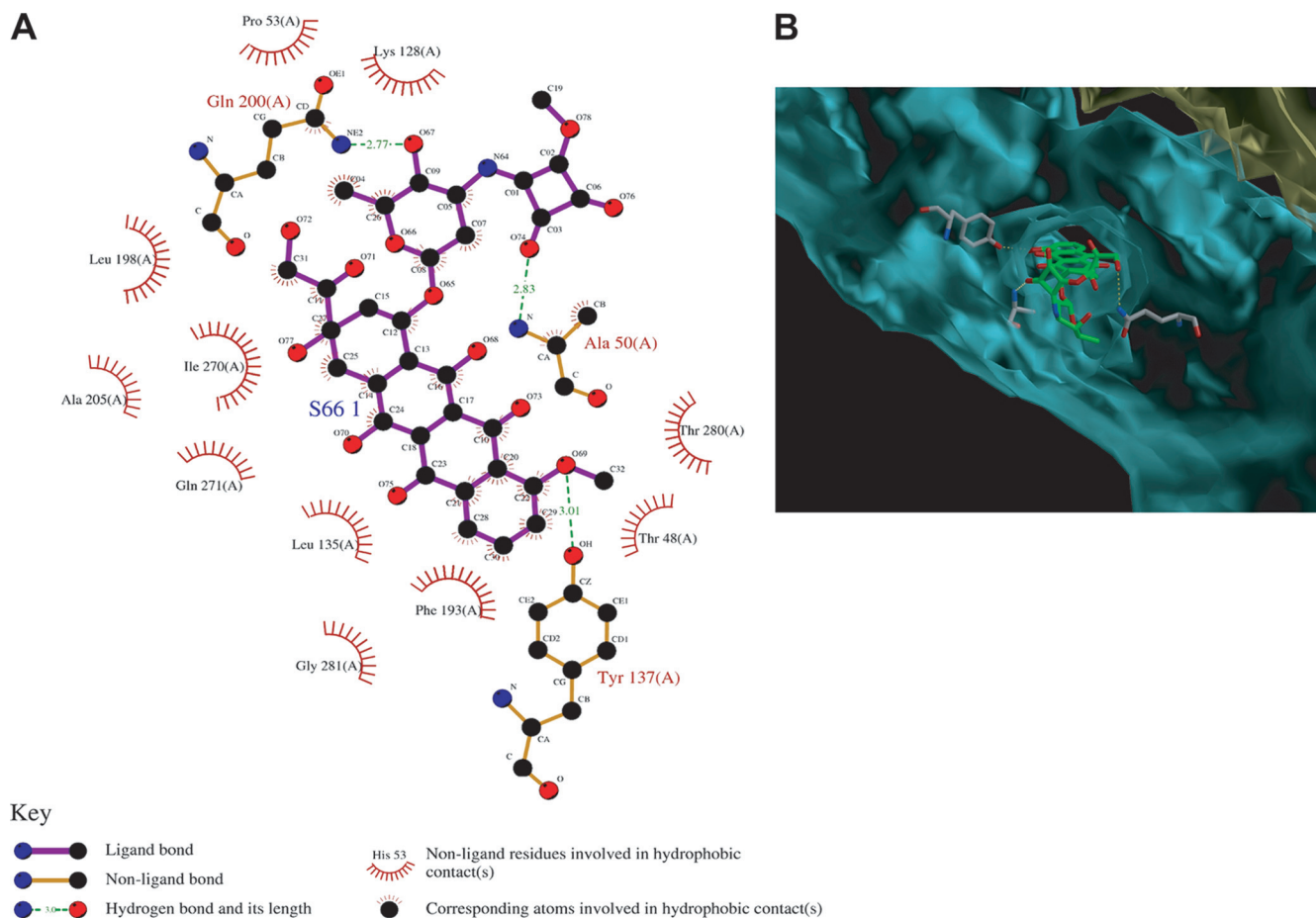


FIG. 6. Results of docking calculations. (A) A ligplot interaction map of docked SA-17 in the  $\beta$ -OG binding pocket in one molecule ([indicated by "(A)"] of the dengue virus envelope dimer. (B) Representation of the SA-17 molecule located in the  $\beta$ -OG binding site. One molecule of the E glycoprotein dimer is represented by a blue surface; the other one is represented by a yellow surface. The SA-17 molecule is displayed in green; nonligand residues are exhibited in gray. The formation of hydrogen bonds is shown by a green dashed line. Docking results were generated using Ligplot software (A) or bobscrip, molscript, and raster3D (B) (14, 24, 38, 65).

conformational change of the E protein that is essential for DENV entry and, eventually, infection of the host cell. Docking experiments revealed that SA-17, like rolitetracycline and doxycycline, fits into the hydrophobic detergent-binding pocket of the E protein. Interestingly, among the residues possibly involved in binding SA-17 are those reported to be critical in membrane fusion during virus entry, namely, Thr-48, Glu-49, Ala-50, Lys-51, and Gln-52 (39, 40). Even following 20 passages of culturing in the presence of suboptimal concentrations of SA-17, we were unable to select for drug-resistant variants. Identification of mutations responsible for the drug-resistant phenotype should have allowed the identification of a viral factor as the antiviral target, such as the E protein. The reason for this failure might be that 20 passages of antiviral pressure are too short to select for resistant virus against this particular compound and, thus, that the barrier to resistance is high. Even antiviral molecules that target a cellular factor, such as the cyclophilin-binding compound Debio 025, which is a potent inhibitor of HCV replication (11, 47), may result in mutations in the viral genome that explain the antiviral resistance (L. Coelmont, X. Hanouille, U. Chatterji, C. Berger, J. Snoeck, M. Bobardt, P. Lim, I. Vliegen, J. Paeshuyse, G. Vuagniaux, R.

Bartenschlager, P. Gally, G. Lippens, and J. Neyts, submitted for publication). We continue our effort to select for drug-resistant variants.

It is intriguing that SA-17 seems to lack activity against dengue virus serotype 4. We have at present no explanation for this observation. Sequence alignment and phylogenetic tree analysis of the dengue virus E proteins, on which DENV serotype classification is based, demonstrate that DENV-4 is the most divergent dengue virus serotype (16, 27, 68). Likewise, we can as yet not explain the lack of activity against JEV, TBEV, and WNV. However, as is the case for DENV-4, sequence divergence at the position where the inhibitor interacts with the virus likely explains this difference.

Challenges for future studies will be to (i) confirm that SA-17 indeed interacts with the viral E glycoprotein, (ii) evaluate the antiviral activity of SA-17 in DENV/mouse models (19, 53, 57, 58), and (iii) improve antiviral potency against flaviviruses on the one hand and reduce or even eliminate any residual cytotoxic/cytostatic activity on the other hand (i.e., increase activity and selectivity) through lead optimization procedures. Drugs with properties like those of SA-17 may be effective in lowering circulating infectious virus titers during

viremia. DENV viral load positively correlates with the severity of clinical disease (32, 64, 67). Safe drugs that prevent infection of cells may, either alone or in combination with, for example, replication inhibitors, reduce the number of patients who develop significant clinical disease. Reducing viral loads may not only prevent clinically significant disease in individuals but may also contribute to epidemic control. Mosquitoes are remarkably resistant to dengue virus infection by the oral route and require high titers ( $>10^5$  to  $10^7$  particles/ml) in human blood to become infected (41). Any reduction in viral load brought about by drug treatment will therefore reduce the chance of transmission of DENV between humans, which may play a crucial role in the future control of dengue epidemics.

#### ACKNOWLEDGMENTS

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