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3′,5′Di-*O*-trityluridine inhibits *in vitro* flavivirus replication

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ABSTRACT

The dengue fever virus (DENV) and the yellow fever virus (YFV) are members of the genus flavivirus in the family *Flaviviridae*. An estimated 50–100 million cases of DENV infections occur each year and approximately half a million patients require hospitalization. There is no vaccine or effective antiviral treatment available. There is an urgent need for potent and safe inhibitors of DENV replication; ideally such compounds should have broad-spectrum activity against flaviviruses. We here report on the *in vitro* activity of 3′,5′di-*O*-trityluridine on flavivirus replication. The compound results in a dose-dependent inhibition of (i) DENV- and YFV-induced cytopathic effect (CPE) (EC_{50} values in the low micromolar range for the 4 DENV serotypes), (ii) RNA replication (DENV-2 EC_{50} = 1.5 μM; YFV-17D EC_{50} = 0.83 μM) and (iii) viral antigen production. Antiviral activity was also demonstrated in DENV subgenomic replicons (which do not encode the structural viral proteins) (EC_{50} = 2.3 μM), indicating that the compound inhibits intracellular events of the viral replication cycle. Preliminary data indicate that the molecule may inhibit the viral RNA-dependent RNA polymerase.

1. Introduction

The genus flavivirus (family *Flaviviridae)* comprises several pathogens, including the dengue virus (DENV) and the yellow fever virus (YFV). Flaviviruses that are pathogenic to man are transmitted to humans by bites of infected mosquitoes or ticks (Gould and Solomon, 2008). The incidence and geographical distribution of the four distinct DENV serotypes and its vector are increasing dramatically. DENV causes more than 50 million infections annually (mainly in South-East Asia and Latin America) and infections with this virus may develop into dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Halstead et al., 1980; World Health Organisation, 2009). There is, as is the case for most of the other flaviviruses, neither a vaccine nor a specific antiviral therapy available for the prophylaxis and/or treatment of DENV infections (Whitehead et al., 2007). Although a highly efficacious vaccine is available against the YFV, this virus is still a leading cause of hemorrhagic fever and therefore inhibitors against this virus are also urgently needed (Barnett, 2007). Vector-control strategies that were once successful in controlling YF cases have faltered, thereby leading to a re-emergence of the disease. The World Health Organization currently estimates that there are 200,000 cases of yellow fever annually of which over 90% occur in Africa, resulting in about 30,000 deaths per year (World Health Organization, 2001).

The flavivirus genome consists of a single-stranded RNA molecule of positive polarity (Perera and Kuhn, 2008; van Dijk et al., 2004). The viral genome encodes 3 structural and 7 non-structural proteins. Among them, the NS5 protein exerts methyltransferase as well as RNA-dependent RNA polymerase (RdRp) activities. The flavivirus RdRp is considered one of the most interesting targets for antiflaviviral drugs since (i) polymerase activity is essential for viral replication, (ii) the protein is conserved among all 4 DENV serotypes, (iii) human host cells are devoid of such RdRp activity and (iv) several highly potent and selective inhibitors of the HCV RdRp have recently successfully been evaluated in clinical trials (Malet et al., 2008). For flaviviruses, a nucleoside analogue (7-deaza-2′-C-methyl-adenosine), originally developed for hepatitis C virus (HCV), showed anti-DENV activity in cell culture (EC_{50} = 15 μM; SI > 21) and significantly reduced the viremia in a DENV mouse model (Olsen et al., 2004; Schul et al., 2007). Another adenosine analogue (7-deaza-2′-C-acetylene-adenosine) potently inhibited DENV replication both in cell culture and in mice; however, this compound showed serious side effects during *in vivo* toxicity studies in both rats and dogs (Yin et al., 2009b).

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β-D-2′-ethynyl-7-deaza-adenosine was able to inhibit all DENV serotype replication with EC_{50} values of about 1 μM. However, the catalytic efficiency of incorporating this molecule was 10-fold lower than that of ATP (Latour et al., 2010). T-705, a substituted pyrazine compound, which is used in clinical trials for the treatment of human influenza virus infection, significantly improved survival and disease parameters in YFV-infected hamsters despite the lack of good *in vitro* antiviral activity (Julander et al., 2009). An *in silico*-based search for non-nucleoside inhibitors (NNIs) resulted in the identification of *N*-sulfonylanthranilic acid derivatives, which inhibited the DENV polymerase activity with an IC_{50} of 0.7 μM (Yin et al., 2009a). These studies prove the concept that nucleoside analogues and NNIs could potentially be developed for flavivirus therapy although more potent compounds are needed and toxic effects on the host cells cannot be excluded.

Here, we report on the *in vitro* antiviral activity of 3′,5′di-*O*-trityluridine on flavivirus replication. The compound inhibits both DENV and YFV replication in the low μM range, and was shown to target intracellular events of the viral replication cycle, potentially inhibiting the viral RNA-dependent RNA polymerase.

2. Materials and methods

2.1. Cells and viruses

DENV serotype 2 New Guinea C [DENV-2 NGC (kindly provided by Dr. V. Deubel; formerly at Institute Pasteur, Paris, France)], 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 cultured on C6/36 mosquito cells (from *Aedes albopictus*; American Type Culture Collection (ATCC) CCL-1660) in Minimum essential medium (MEM; Gibco, Belgium) with 1% L-glutamine (Gibco), 1% penicillin (100 U/ml)/streptomycin (100 μg/ml) solution (Gibco), 1% non-essential amino acids (Gibco), 1% HEPES and 8% foetal bovine serum (FBS; Integro, The Netherlands) at 28 °C. DENV-1, DENV-3 and DENV-4 were kindly provided by Dr. X. de Lamballerie (Université de la Méditerranée, Marseille, France). Green monkey kidney cells [Vero-B cells (ECACC for DENV assays and ATCC CCL-81 for YFV assays)] were grown in MEM Rega-3 (Gibco) supplemented with 10% FBS, 1% L-glutamine and 1% sodium bicarbonate (Gibco). Antiviral assays were performed in medium with 2% FBS. Baby hamster kidney cells (BHK-21; ATCC CCL-10) were grown in DMEM supplemented with 10% FBS (culture medium) or 2% FBS (assay medium). BHK-21 cells harboring the subgenomic dengue replicon dCprMEPAC2NS3lucNS3 (derived from the dengue replicon construct pDENΔCprME-PAC2A) in which an antibiotic selection cassette encoding the puromycin *N*-acetyltransferase (PAC) together with the *Firefly* luciferase expression cassette was inserted upstream of the non-structural (NS) genes, will be referred to as BHK-Rep-Pac-LUC cells (Jones et al., 2005). 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, Belgium). Puromycin was omitted from the culture medium in antiviral assays. Yellow fever virus (YFV) 17D vaccine strain (Stamaril®) [Aventis Pasteur (MSD, Belgium)] was passaged once in Vero-B cells to prepare a working virus stock and stored at −80 °C until further use.

2.2. Antiviral molecules

Ribavirin [1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (Virazole; RBV)] was purchased from ICN Pharmaceuticals (Costa Mesa, CA). 5′-*O*-trityluridine (Fig. 1; molecule 2), 3′,5′di-*O*-trityluridine (Fig. 1; molecule 3) 2′,5′di-*O*-trityluridine (Fig. 1; molecule 4) were prepared as follows: Uridine (Fig. 1; molecule 1) (1.1 g, 4.4 mmol) was co-evaporated twice with dry pyridine and dissolved in dry pyridine (4.5 mL/mmol). Triphenylmethyl chloride (2.8 eq) was added and the solution was heated to 80 °C overnight under argon. The reaction was quenched by adding methanol (3 mL) at room temperature for 30 min. The solution was then concentrated and diluted in dichloromethane. The organic solution was washed with a solution of saturated $NaHCO_3$ (three times) and the combined aqueous layers were extracted with dichloromethane. Combined organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by column chromatography on silica (first 20-10% hexane/dichloromethane containing 0.5% triethylamine, then 0-2.5% MeOH/dichloromethane, 0.5% triethylamine) and afforded the tritylated compounds **2** (1184 mg, 2.43 mmol, 56% yield), **3** (262 mg, 0.36 mmol, 8% yield) and **4** (517 mg, 0.71 mmol, 16% yield) as white foams.

2: ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.20 (m, 1H, 5a′-H); 3.26 (m, 1H, 5b′-H); 3.96 (m, 1H, 4′-H); 4.09 (m, 2H, 2′-H and 3′-H); 4.16 (d, 1H, *J* = 5.1 Hz, OH); 5.32 (d, 1H, *J* = 5.7 Hz, 5-H); 5.50 (d, 1H, *J* = 4.4 Hz, OH); 5.75 (d, 1H, *J* = 4.0 Hz, 1′-H); 7.28–7.39 (m, 15H, h,i,j,k,l-H); 7.71 (d, 1H, *J* = 8.1 Hz, 6-H); 11.35 (br s, 1H, 3-NH). ^{13}C (DMSO-d_6, 75 MHz): 63.23 (C5′); 59.51 (C3′); 73.33 (C2′); 82.30 (C4′); 88.91 (Ph_3-C and C1′); 101.43 (C5); 127.15, 127.97, 128.27, 143.40 (arom-C); 140.58 (C6); 150.46(C2); 162.98(C4). HRMS (thgly) calcd. for $C_{28}H_{26}N_2O_6Na^+$ (MNa+) 509.1683; found 509.1708.

3: *3′,5′Di-O-trityluridine:* ^{1}H ($CDCl_3$, 500 Mhz): δ = 2.90 (d, *J* = 6.9 Hz, 1H, 2′-OH), 3.04 (dd, *J* = 2.6 and 10.9 Hz, 1H, 5′-H), 3.36 (dd, *J* = 2.1 and 10.9 Hz, 1H, 5′-H), 3.65–3.69 (m, 1H, 2′-H), 3.75–3.78 (m, 1H, 4′-H), 4.32 (dd, *J* = 4.0 and 5.0 Hz, 1H, 3′-H), 5.25 (dd, *J* = 8.2 and 2.2 Hz, 1H, 5-H), 5.99 (d, *J* = 5.1 Hz, 1H, 1′-H), 7.18–7.44 (m, 30H, Tr), 7.59 (d, *J* = 8.2 Hz, 1H, 6-H), 8.54 (s, 1H, 3-NH) ppm. ^{13}C ($CDCl_3$, 500 MHz): δ = 63.08 (C5′), 73.38 (C3′), 74.80 (C2′), 82.82 (C4′), 87.77 (4 °C of Tr), 88.19 (4 °C of Tr), 89.69 (C1′), 102.21 (C5), 127.40, 127.73, 127.94, 128.19, 128.72, 143.11, 143.38 (Tr), 140.35 (C6), 150.38 (C2), 162.78 (C4) ppm. A HMBC-spectrum showed a correlation between 3′-H and the quaternary carbon of the trityl-group, it also showed the lack of correlation between 2′-H and the quaternary carbon of the trityl-group. Full details for assignments of alkylated compounds can be found in the accompanying paper [Chatelain, personal communication]. HRMS (thgly) calcd. for $C_{47}H_{40}N_2O_6Na^+$ (MNa^+): 125 751.2779; found 751.2776.

4: *2′,5′Di-O-trityluridine:* ^{1}H ($CDCl_3$, 500 Mhz): δ = 2.79 (d, *J* = 4.5 Hz, 1H, 3′-H), 3.11 (d, *J* = 1.5 Hz, 2H, 5′-H), 3.98 (s, 1H, 4′-H), 4.51 (dd, *J* = 4.5 and 7.5 Hz, 1H, 2′-H), 5.11 (dd, *J* = 2.1 and 8.1 Hz, 1H, 5-H), 6.57 (d, *J* = 7.5 Hz, 1H, 1′-H), 7.05–7.31 (m, 30H, Tr), 7.70 (d, *J* = 8.1 Hz, 1H, 6-H), 8.85 (s, 1H, 3-NH) ppm. ^{13}C ($CDCl_3$, 500 MHz): δ = 64.37 (C5′), 70.87 (C3′), 77.77 (C2′), 84.32 (C4′), 86.22 (C1′), 87.84 (4 °C of Tr), 87.98 (4 °C of Tr), 102.68 (C5), 127.50, 127.81, 127.98, 128.28, 128.50, 128.55, 142.84, 143.14 (Tr), 140.98 (C6), 150.72 (C2), 163.00 (C4) ppm. A HMBC-spectrum showed a correlation between 2′-H and the quaternary carbon of the trityl-group, it also showed the lack of correlation between 3′-H and the quaternary carbon of the trityl-group. HRMS (thgly) calcd. for $C_{47}H_{40}N_2O_6Na^+$ (MNa^+): 751.2779, found 751.2772.

2.3. CPE-reduction assays

Vero-B cells were seeded in 96-well plates (Becton Dikinson Labware, Franklin Lakes, NJ) at a density of 7×10^3 (ECACC) or 2×10^4 (ATCC CCL-81) cells/well in 100 μl assay medium and were allowed to adhere overnight. Subsequently, a compound dilution series was added after which cultures were infected with 100

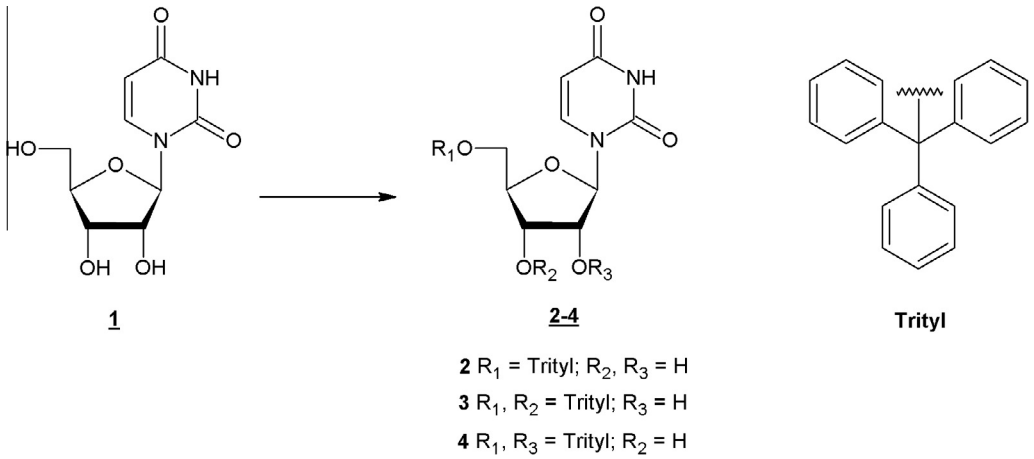

Fig. 1. Synthesis of 3′,5′di-*O*-trityluridine (compound 3). Uridine (**1**) was tritylated using triphenylmethyl chloride in pyridine at 80 °C as described before (Blank and Pfleiderer 1967; Yung and Fox 1961; Zemlicka, 1964). The amounts and ratios obtained of the different tritylated products **2–4**, depend on the excess of reagent added and the heating time. A typical procedure is given in the Section 2. The position of attachment of the trityl moiety at either the 2′ or 3′ oxygen was determined by 2D NMR spectroscopy.

$CCID_{50}$ (i.e., 50% cell culture infectious dose) DENV-2 NGC or YFV 17D in 100 μl assay medium. Plates were incubated at 37 °C [95–99% relative humidity and 5% CO_2]. On day 8 post infection (p.i.) the cultures were fixed with 70% ethanol and stained with 1% methylene blue. Ribavirin was included in the assay as a reference compound. Antiviral assays with Coxsackie B3 virus and HCV were performed as described before (De Palma et al., 2007; Paeshuyse et al., 2006).

Table 1
Primer and probe sequences of DENV-1, 2, 3 and 4.

Primer/probe	Sequence (5′–3′)
DENV-1-2-3-4-forward	GGA TAG ACC AGA GAT CCT GCT GT
DENV-1-2-3-reverse	CAT TCC ATT TTC TGG CGT TC
DENV-1-2-3-probe	FAM-CAG CAT CAT TCC AGG CAC AG-MGB
DENV-4-reverse	CAA TCC ATC TTG CGG CGC TC
DENV-4-probe	FAM-CAA CAT CAA TCC AGG CAC AG-MGB

2.4. Virus yield reduction assays

Vero-B cells (5×10^4) were seeded in 96-well plates. One day later, culture medium was replaced with 100 μl assay medium containing a 2× serial dilution of the compound and 100 μl of virus inoculum [either DENV-2 NGC, YFV-17D, DENV-1 Djibouti, DENV-3 H87 or DENV-4 Dak HD 34 460; 50 $CCID_{50}$/well] that was pre-incubated for 2 h on 37 °C. Following a 2 h incubation period, the cell monolayer was washed 3 times with assay medium to remove non-adsorbed virus and cultures were further incubated for 4 days (for DENV-2 and YFV) or 7 days (DENV-1, DENV-3 and DENV-4) in the presence of the inhibitor. Supernatant was harvested and viral RNA load was determined by real-time quantitative RT-PCR. The 50% effective concentration (EC_{50}), which is defined as the compound concentration that is required to inhibit viral RNA replication by 50%, was determined using logarithmic interpolation. Ribavirin was included as a reference compound. Potential cytotoxic/cytostatic effects of the compound were evaluated in uninfected cells by means of the MTS/PMS method as described earlier (Kaptein et al., 2010). The 50% cytotoxic concentration (CC_{50}; i.e., the concentration that reduces the total cell number by 50%) was calculated using logarithmic interpolation.

2.5. Quantitative reverse transcriptase-PCR (qRT-PCR)

RNA was isolated from 150 μl supernatant with the NucleoSpin RNA virus kit (Filter Services, Germany) as described by the manufacturer. Primers and probe sequences are described earlier (Kaptein et al., 2010) or are described in Table 1. The TaqMan probe was fluorescently labeled with 6-carboxyfluorescein (FAM) at the 5′ end as the reporter dye and with minor groove binder (MGB) at the 3′ end as the quencher. One-step, quantitative RT-PCR was performed in a total volume of 25 μl, containing 13.9375 μl H_2O, 6.25 μl master mix (Eurogentec, Belgium), 0.375 μl forward primer, 0.375 μl reverse primer, 1 μl probe, 0.0625 μl reverse transcriptase (Eurogentec) and 3 μl sample. RT-PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Branchburg, NJ) using the following conditions: 30 min at 48 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The data was analyzed using the ABI PRISM 7500 SDS software (version 1.3.1; Applied Biosystems). For absolute quantification, standard curves were generated using 10-fold dilutions of template preparations of known concentrations.

2.6. Immunofluorescence assays

Vero-B cells were seeded in a 8-well chamber slide (Lab-tek, II, Nunc, Germany) at a density of 2×10^4 cells/well; 24 h later, cells were infected with 50 $CCID_{50}$ DENV-2 NGC or YFV-17D in the presence or absence of 5 μM 3′,5′di-*O*-trityluridine. The virus inoculum was removed after 1 h; cells were washed and further incubated in the presence of the compound for 72 h. Cells were stained with the anti-dengue E protein antibody (Ab) clone 3H5 (Millipore, Billerica, MA) or the anti-YFV NS1 Ab 1A5, and the secondary Ab Alexa Fluor 488 (Millipore) (Charlier et al., 2004). Following DAPI staining, the cultures were visualized using a confocal laser scanning microscope (LCSM, Leica Microsystems, Germany).

2.7. DENV-2 subgenomic replicon

BHK-Rep-Pac-LUC cells were seeded at a density of 1×10^4 -cells/well in a tissue culture-treated white view 96-well plate (Perkin–Elmer, Boston, MA). The next day, a 2-fold serial dilution of 3′,5′di-*O*-trityluridine or 2′,5′di-*O*-trityluridine was added. After 72 h, luciferase activity was measured using the Luciferase Assay System according to the manufacturer's protocol (Promega, Leiden, The Netherlands). Luciferase activity was compared to that of untreated replicon cells. The cytotoxic effect of the compounds on

BHK-Rep-Pac-LUC cells was evaluated in parallel cultures (Kaptein et al., 2010). Ribavirin was included, for comparative reasons, as a replication inhibitor. The inhibitory effect of the compounds on luciferase activity was adjusted for inhibitory effects on cell proliferation.

3. Results

3.1. Inhibition of DENV-2- and YFV-induced CPE formation by 3′,5′di-O-trityluridine

The starting point of the present study was the identification of 2′,5′di-*O*-trityluridine in a large cell-based antiviral screening effort as an *in vitro* inhibitor of DENV and YFV infection in Vero cells. The EC_{50} values for inhibition of DENV- and YFV-induced cytopathic effect (CPE) were respectively 30 and 1.2 μM. This finding prompted us to evaluate the antiviral activity of a selection of tritylated (triphenylmethyl) nucleoside analogues (Fig. 1) [personal communications]. The evaluation was based on the inhibition of DENV-2- and YFV-induced CPE. 3′,5′Di-*O*-trityluridine (compound 3 in Fig. 1) was identified as being 15 and 3-fold more effective than the parent compound in inhibiting respectively DENV-2 and YFV-17D replication ($EC_{50\ DENV}$ = 2 μM and $EC_{50\ YFV} \leqslant 0.4$ μM). At a concentration of 3 μM, DENV- and YFV-induced CPE formation was inhibited completely (Fig. 2). Concentrations up to 100 μM did not result in adverse effects on the morphology of growing Vero cell cultures used for YFV, although 3′,5′di-*O*-trityluridine inhibited the proliferation of another clone of Vero cells that were selected to proliferate rapidly (data not shown) used for DENV assays (CC_{50} = 10 μM). The 2′,5′ tritylated parent compound had no cytotoxic effect on this particular clone of Vero cells. 3′,5′Di-*O*-trityluridine proved selective against flaviviruses, it did not inhibit CPE induced by Coxsackie virus B3 (CVB3; EC_{50} > 100 μM), but antiviral activity was observed in an HCV replicon assay (EC_{50} = 4.6 ± 3.6 μM).

3.2. Flaviviral RNA replication and viral antigen expression is inhibited by 3′,5′di-O-trityluridine

RNA levels in the supernatant of DENV- and YFV-infected cultures were quantified using RT-qPCR. 3′,5′Di-*O*-trityluridine inhibited DENV-2 (EC_{50} = 1.5 ± 1.2 μM) and YFV-17D (EC_{50} = 0.83 ± 0.34 μM) RNA formation in a dose-dependent manner (Fig. 3). The antiviral activity of 3′,5′di-*O*-trityluridine against respectively DENV and YFV was 34- and 6-fold more pronounced than that of the reference replication inhibitor ribavirin ($EC_{50\ DENV}$ = 51 ± 10 - μM; $EC_{50\ YFV}$ = 4.8 ± 0.6 μM). The antiviral effect was further corroborated by the fact that at a concentration of 12.5 μM, the formation of infectious DENV-2 was reduced by 3.3 $\log_{10}$ and for YFV-17D by 4.5 $\log_{10}$ (data not shown). In addition, 3′,5′di-*O*-trityluridine was found to inhibit the progeny formation of DENV-1 (EC_{50} = 0.92 ± 0.18 μM), DENV-3 (EC_{50} = 1.54 ± 0.88 μM) and DENV-4 (EC_{50} = 1.1 ± 0.06 μM) in a dose-dependent manner as was assessed in virus yield reduction assays. Moreover 3′,5′di-*O*-trityluridine was, at a concentration of 5 μM, able to inhibit the expression of DENV-2 E and YFV-17D NS1 antigen (Fig. 4).

3.3. Antiviral activity of 3′,5′di-O-trityluridine in the DENV subgenomic replicon system

In order to have an indication at which stage/process of the viral replication cycle (i.e. level of replication, binding or release) the molecules act, it was studied whether 3′,5′di-*O*-trityluridine and 2′,5′di-*O*-trityluridine inhibit DENV subgenomic replicon replication. These subgenomic replicons only harbor the non-structural genes of DENV-2 and the structural genes are replaced by a luciferase cassette. BHK cells carrying DENV subgenomic replicons were cultured in the presence of various concentrations of 3′,5′di-*O*-trityluridine, 2′,5′di-*O*-trityluridine or the reference molecule ribavirin. The replication of the subgenomic replicons was shown to be inhibited by 3′,5′di-*O*-trityluridine and 2′,5′di-*O*-trityluridine in a dose-dependent manner (EC_{50} = 2.3 ± 0.06 μM and 9.74 ± 0.72 μM) (Fig. 5). Ribavirin was able to inhibit the replicon replication with an EC_{50} value of 1.2 ± 0.8 μM (data not shown).

3.4. Long term compound pressure does not result in the generation of resistant virus

To identify the molecular target of 3′,5′di-*O*-trityluridine, an attempt was made to generate drug-resistant variants. So far, even following 30 passages (30 weeks) we have not been able to select for drug-resistant variants. We continue our efforts to select for drug-resistant variants.

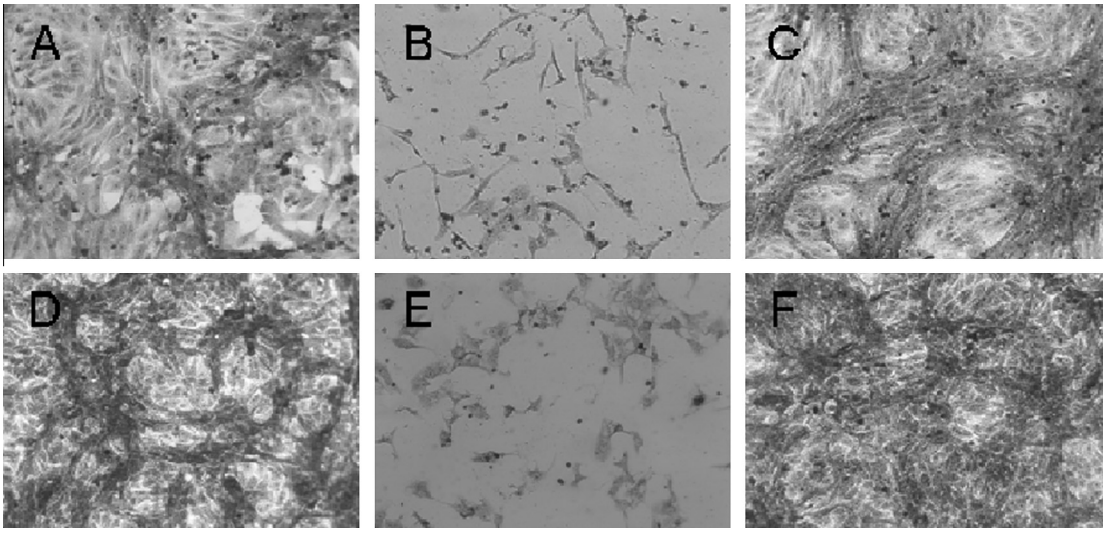

Fig. 2. Inhibition of DENV-2- and YFV-induced CPE by 3′,5′di-*O*-trityluridine. Vero cells (uninfected cell controls: panels C and F) were infected with DENV-2 (panels A and B) or YFV-17D (panels D and E) and were left untreated (panels B and E) or were treated with 3 μM 3′,5′di-*O*-trityluridine (panels A and D). Inhibition of CPE formation was monitored on day 8 p.i.

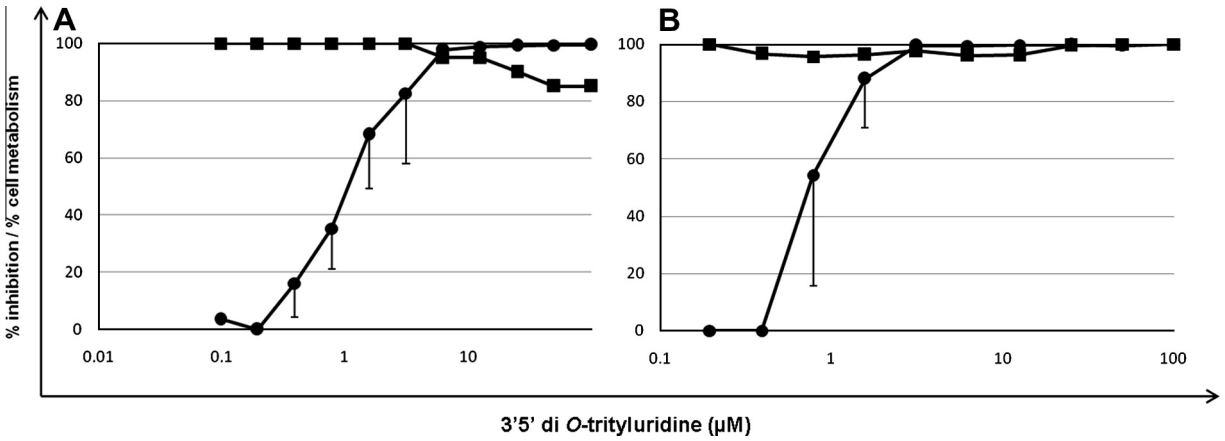

Fig. 3. Dose-dependent inhibition of flavivirus RNA replication by 3′,5′di-*O*-trityluridine. Vero-B cell cultures infected with DENV-2 (panel A) or YFV-17D (panel B) were treated with different concentrations of 3′,5′di-*O*-trityluridine. Viral RNA levels were quantified on day 4 p.i. by means of RNA RT-qPCR (black circles). Mock-infected cells were treated with the same dilution series of 3′,5′di-*O*-trityluridine. Cell viability was determined by means of the MTS/PMS method (black squares). Data represent mean values ± standard deviations (SD) for three independent experiments.

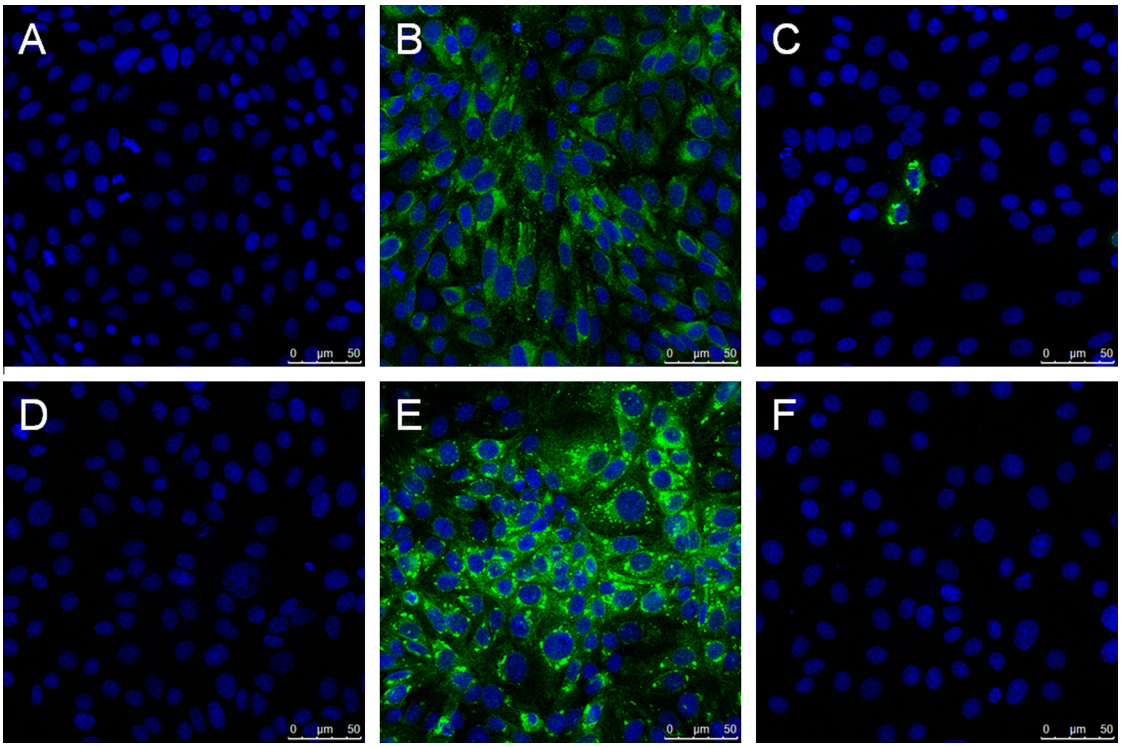

Fig. 4. Inhibition of viral antigen expression by 3′,5′di-*O*-trityluridine. Vero cells (panels A and D: cell control) were infected with DENV-2 (panels B and C) or YFV-17D (panels E and F) and were treated with 5 μM 3′,5′di-*O*-trityluridine (panels C and F). DENV-2 E protein and YFV-17D NS1 protein expression was visualized on day 3 p.i.

4. Discussion

There is an urgent need for safe, efficient and pan-serotype DENV drugs. Such drugs should allow reduction of the total number of people developing dengue fever, dengue hemorrhagic fever, and dengue shock syndrome, in particular during epidemic situations in areas where DENV is endemic. Moreover, people travelling to and through regions where DENV is endemic may use such drugs prophylactically. Ideally, a DENV inhibitor should in addition to exerting DENV pan-serotype activity also be active against other flaviviruses, such as the YFV, the Japanese encephalitis and the West Nile virus. Promising targets for antiviral drug development include key processes/activities of the viral life cycle such as viral entry, RNA capping, protease cleavage, RNA-dependent RNA polymerase (RdRp) and helicase activity (Bollati et al., 2010).

In a large scale cell-based screening campaign we identified 2′,5′di-*O*-trityluridine as an inhibitor of *in vitro* YFV and DENV replication and subsequently observed that the 3′,5′ tritylated analogue is more potent than the parent compound. No activity was observed against unrelated viruses such as the Coxsackie B3 virus (*Picornaviridae*). 3′,5′Di-*O*-trityluridine inhibits DENV and YFV-induced CPE formation, RNA replication and viral antigen expression with comparable efficacies. Moreover, the molecule inhibits the replication of DENV subgenomic replicons, which demonstrates that the mechanism of action is based on inhibition of intracellular viral replication events rather than on early or late processes in the replication cycle such as virus entry, assembly or egress.

The effect of 3′,5′di-*O*-trityluridine on the activity of the DENV RNA-dependent RNA polymerase (RdRp) activity was studied using a homopolymeric polyC template (Supplemental data).

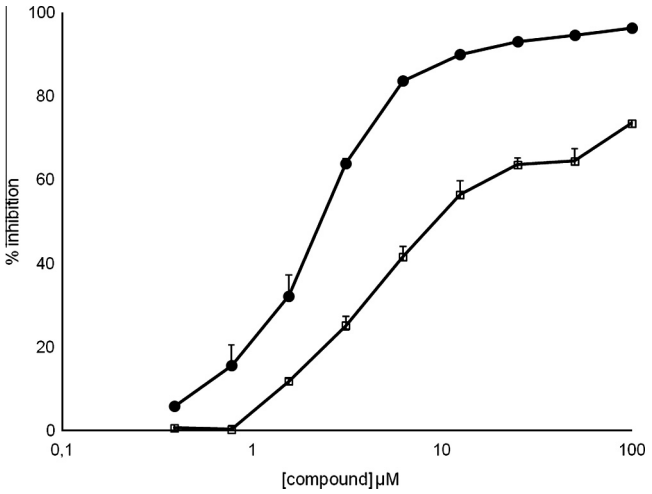

Fig. 5. Evaluation of the antiviral activity of 3′,5′di-*O*-trityluridine and 2′,5′di-*O*-trityluridine on subgenomic DENV replicon replication. BHK cells containing the DENV subgenomic replicon were cultured in the presence of different concentrations of 3′,5′di-*O*-trityluridine (rounds) or 2′,5′di-*O*-trityluridine (open squares). Luciferase activity was measured 72 h later and is expressed as percentage of untreated controls. Data represent mean values ± SD for three independent experiments.

3′,5′Di-*O*-trityluridine inhibited DENV polymerase with IC_{50} values ranging from 3.1 to 6.9 μM (Fig. S1 in the supplemental material) and it did not inhibit the polymerase activity of the RdRp of the CVB3 (data not shown), a virus that is not susceptible to inhibition by the molecule in cell-based assays, at a concentration up to 100 μM and using the same amount of enzyme. Furthermore, our results indicate that the compound inhibits DENV RNA elongation rather than initiation on a DENV-specific "minigenomic" RNA template (Fig. S2 in the supplemental material).

A confirmation of the viral polymerase as the only molecular target of 3′,5′di-*O*-trityluridine would be obtained when demonstrating that drug-resistant variants select for mutations in this region. To this end we tried to select for drug-resistant variants. However, even following 30 passages in the presence of suboptimal concentrations of the molecule, no resistant virus was obtained, either indicating that the compound has a very high barrier to resistance or that resistant variants are so unfit that we were unable to amplify them under our culture conditions. We currently continue our efforts to select for drug-resistant variants.

Due to the highly hydrophobic nature of the compound, we cannot rule out any nonspecific inhibition of the polymerase activity. However, the fact that the activity of an RdRp from another +ssRNA virus (CVB3) is not inhibited under similar conditions might argue against a nonspecific inhibitory effect. Co-crystallization or soaking studies of the polymerase and the inhibitor will be needed to reveal the molecular details of its inhibitory effect and to exclude an additional mechanism of action. 3′,5′Di-*O*-trityluridine may then serve as an interesting tool for the design of more selective and potent inhibitors. The molecule itself, given the tritylgroups, may not be ideally suitable for further development. Its chemical nature, however, may provide valuable information for the development of related pharmacophores. In conclusion, we here report on the anti-flavivirus activity of 3′,5′di-*O*-trityluridine. Further studies are needed to confirm or exclude that the molecule targets the flavivirus polymerase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.01.011.

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