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Identification of a new dengue virus inhibitor that targets the viral NS4B protein and restricts genomic RNA replication

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ABSTRACT

Dengue virus (DENV) is an important human arthropod-borne virus with a major impact on public health. Nevertheless, a licensed vaccine or specific treatment is still lacking. We therefore screened the NIH Clinical Collection (NCC), a library of drug-like small molecules, for inhibitors of DENV replication using a cell line that contains a stably replicating DENV serotype 2 (DENV2) subgenomic replicon. The most potent DENV inhibitor in the NCC was δ opioid receptor antagonist SDM25N. This compound showed antiviral activity against wild-type DENV2 in both Hela and BHK-21 cells, but not in the C6/36 cell line derived from the mosquito *Aedes albopictus*. The structurally related compound naltrindole also inhibited DENV replication, albeit less potently. Using a transient subgenomic replicon, we demonstrate that SDM25N restricts genomic RNA replication rather than translation of the viral genome. We identified a single amino acid substitution (F164L) in the NS4B protein that confers resistance to SDM25N. Remarkably, an NS4B amino acid substitution (P104L), which was previously shown to confer resistance to the DENV inhibitor NITD-618, also provided resistance to SDM25N. In conclusion, we have identified a new DENV inhibitor, SDM25N, which restricts genomic RNA replication by – directly or indirectly – targeting the viral NS4B protein.

1. Introduction

Dengue virus (DENV) causes severe, sometimes fatal disease and is considered the most important human arthropod-borne virus by the World Health Organization (WHO, 2013). DENV is a member of the genus *Flavivirus* within the family *Flaviviridae* and is mainly found in tropical and subtropical areas in Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific (Guzman and Istúriz, 2010; Guzman et al., 2010; WHO, 2013). DENV is transmitted by *Aedes* mosquitoes and circulates as four distinct, but closely related serotypes (DENV1–4). Each year, an estimated 50–100 million people are infected with DENV (Guzman and Istúriz, 2010; WHO, 2013). Most of these infections resolve without clinical symptoms or result in dengue fever, a relatively mild and self-limited flu-like illness. However, 500,000 of these DENV-infected people present with dengue hemorrhagic fever (DHF) (Guzman and Istúriz, 2010; WHO, 2013), the more severe manifestation of infection that is characterized by plasma leakage. Plasma leakage in DHF can be so profound that it leads to circulatory compromise and shock, a life-threatening condition that is referred to as dengue shock syndrome. Annually, DENV is responsible for an estimated 22,000 deaths (Guzman and Istúriz, 2010; WHO, 2013).

The incidence of DENV infections has increased dramatically during the last decades and the virus is now endemic in more than 100 countries (Guzman and Istúriz, 2010; Guzman et al., 2010; WHO, 2013). Cases not associated with travel to endemic countries have recently emerged in Florida, France and Croatia (Franco et al., 2010; Gjenero-Margan et al., 2011; La Ruche et al., 2010). At

Abbreviations: DENV, dengue virus; DHF, dengue hemorrhagic fever; NCC, NIH Clinical Collection; NGC, strain New Guinea C; CPE, cytopathic effect; FMDV, foot-and-mouth disease virus; EC_{50}, 50% effective concentration; CCID50, cell culture infectious dose 50%; hpi, hours post-infection; hpt, hours post-transfection; IFN, interferon.

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present, up to 2.5 billion people are at risk of infection (Guzman and Istúriz, 2010; WHO, 2013). Despite the enormous disease burden and health care costs associated with DENV infections, there is currently no licensed vaccine or specific therapy available. Hence, there is an urgent need for compounds with anti-DENV activity. Potential targets for the development of antiviral therapy include both viral proteins and host factors that are required for viral replication (Julander et al., 2011; Noble et al., 2010). Viral enzymes are excellent therapeutic targets, since they are indispensable for viral replication and are not expressed by host cells. Indeed, most of the anti-DENV drugs that are currently under investigation target the NS3 and NS5 proteins, the only viral proteins with known enzymatic activities (Julander et al., 2011; Noble et al., 2010). However, non-enzymatic viral proteins may also provide viable targets for therapeutic intervention. Nevertheless, only a few flavivirus inhibitors have been identified that target non-enzymatic viral proteins (Botting and Kuhn, 2012).

In an unbiased replication-based screen of the NIH Clinical Collection (NCC), a library of small molecules that have been used in human clinical trials, we have identified a novel inhibitor of DENV. This inhibitor, the δ opioid receptor antagonist SDM25N, inhibits DENV at the level of genomic RNA replication. Moreover, single amino acid substitutions in the viral NS4B protein (F164L and P104L) were sufficient to confer resistance to SDM25N. These results indicate that NS4B is a promising target for specific anti-DENV drug development.

2. Materials and methods

2.1. Cells and virus

Hela and BHK-21 cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) Ready Mix (PAA Laboratories), which contains 10% fetal bovine serum (FBS). The medium was supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin (pen/strep; Life Technologies). BHK-21 clone 15 (BHK-15) cells were grown at 37 °C and 5% CO_2 in minimum essential medium (MEM; Life Technologies) supplemented with 10% heat-inactivated FBS (PAA Laboratories), 0.075% (w/v) sodium bicarbonate (Life Technologies), 25 mM HEPES (Life Technologies) and pen/strep. The pH of the medium was adjusted to 7.2 with NaOH. *Aedes albopictus* C6/36 cells were cultured at 28 °C without CO_2 in Leibovitz's L-15 medium (Life Technologies) supplemented with 10% heat-inactivated FBS, 2% tryptose phosphate broth (Sigma–Aldrich), 1× MEM non-essential amino acids (Life Technologies) and pen/strep.

DENV2 (strain New Guinea C [NGC]) stocks were prepared on C6/36 cells. The cells were infected one day after seeding. When the cells had reached advanced cytopathic effect (CPE), their culture supernatant was harvested, cleared from cell debris by low-speed centrifugation and divided into aliquots. The aliquots were flash-frozen in liquid nitrogen and stored at −80 °C. All DENV2 infections were done in complete growth medium of the corresponding cells.

DENV2 titers were determined by end-point dilution on BHK-15 cells. The cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well. The following day, serial 10-fold dilutions of virus suspension were added to the cells. Each sample was titered in quadruplicate. After 1 week, infection was scored based on CPE. Viral titers were calculated according to the method of Reed and Muench (Reed and Muench, 1938).

2.2. Compounds

The NCC was obtained from BioFocus (http://www.nihclinical-collection.com). SDM25N (CAS No. 342884-62-2) and naltrindole (CAS No. 111469-81-9) were purchased from Tocris Bioscience, and ribavirin (CAS No. 36791-04-5) from Sigma–Aldrich. All compounds were dissolved in DMSO.

2.3. DENV2 subgenomic replicons

All DENV2 replicons are generated by *in vitro* transcription of plasmid templates derived from DENV2 NGC cDNA clone pDVWS601 (Pryor et al., 2001). Replicon dCprMEPAC2NS3lucNS3 (here referred to as RepDVPacLuc) has been described previously (Kaptein et al., 2010).

Plasmid pRepDVRLuc was derived from plasmid pDENΔCprME-EGFP-PAC-1D2A (Massé et al., 2010). First, a cassette encoding a *Renilla* luciferase and a foot-and-mouth disease virus (FMDV) 2A sequence was constructed. The *Renilla* luciferase and FMDV 2A sequences were amplified by PCR. The *Renilla* luciferase sequence was amplified from plasmid pXpA-RenR (Belov et al., 2007) using primers KC13 (ACGTTGTACAAACCGGTATGGCTTCCAAGGTGTACGA) and KC21 (AAATTCAAAGTCTGAGATCTCTGCTCGTTCTTCAGCACGC). The FMDV 2A sequence was amplified from plasmid pDENΔCprME-EGFP-PAC-1D2A using primers KC22 (TGAAGAACGAGCAGAGATCTCAGACTTTGAATTTTGACCT) and KC16 (ACGTGC TAGCTTTGAAGGGGATTC). The *Renilla* luciferase and FMDV 2A sequences were then fused by overlap-extension PCR using primers KC13 and KC16. These primers introduce BsrGI and NheI restriction sites at the 5′ and 3′ ends of the amplified fragment, respectively. The resulting PCR fragment was digested with BsrGI and NheI and cloned into the corresponding restriction sites of plasmid pDENΔCprME-EGFP-PAC-1D2A, generating plasmid pRepDVRLuc.

Plasmids pRepDVRLuc-NS4B$_{F164L}$, pRepDVRLuc-NS4B$_{P104L}$ and pRepDVRLuc-NS4B$_{A119T}$ were made by the introduction of T7315C, C7136T and G7180A substitutions, respectively, into the pRepDVRLuc plasmid (nucleotide positions relative to the DENV2 NGC genome; GenBank accession No. AF038403.1 (Gualano et al., 1998)).

To generate *in vitro* transcribed replicon RNA, replicon plasmids were linearized with XbaI and used as templates in *in vitro* transcription reactions using the T7 RiboMAX Large Scale RNA Production System (Promega). *In vitro* transcription was done in the presence of Ribo m^7G Cap Analog (Promega) at a cap analog to GTP ratio of 2.5:1. *In vitro* transcribed replicon RNA was purified with the RNeasy Mini Kit (QIAGEN).

2.4. Antiviral assays with Hela DENV2 replicon cells and selection of SDM25N-resistant replicons

The Hela DENV2 replicon cell line was generated by transfection of Hela cells with *in vitro* transcribed RepDVPacLuc RNA using Effectene Transfection Reagent (QIAGEN). Two days after transfection, 0.375 μg/ml puromycin (Sigma–Aldrich) was added to the culture supernatant to select for cells with stably replicating replicons. The Hela DENV2 replicon cells were purified by two rounds of single-colony purification and maintained in culture medium with 0.375 μg/ml puromycin.

For antiviral assays, Hela DENV2 replicon cells were seeded in either 24-well (1×10^5 cells/well) or 96-well (1.5×10^4 cells/well) plates in culture medium without puromycin. Compounds or equal volumes of DMSO were added to the culture supernatant the following day. Luciferase activity and cell viability assays as well as quantitative reverse transcription PCRs (qRT-PCRs) were performed two days after the addition of compounds. The 50% effective concentration (EC_{50}) was defined as the concentration of compound that reduced luciferase activity of the Hela DENV2 replicon cells by 50%. EC_{50}s with 95% confidence intervals (CIs) were calculated by non-linear regression analysis using GraphPad Prism software (version 5.03).

SDM25N-resistant replicons were selected by culturing Hela DENV2 replicon cells for 15 passages in the presence of both puromycin and SDM25N. During these passages, the puromycin concentration was kept constant at 10 μg/ml, whereas the concentration of SDM25N was gradually increased from 2.5 μM to a final concentration of 10 μM. The replicons in the three resulting SDM25N-selected as well as in the parental Hela DENV2 replicon cell lines were sequenced in order to identify mutations that may account for SDM25N resistance.

2.5. Viral titer reduction assays

Hela, BHK-21 and C6/36 cells were seeded in 24-well plates at densities of 1×10^5, 1×10^5 and 5×10^5 cells/well, respectively. The next day, the cells were inoculated with DENV2 at 1 cell culture infectious dose 50% (CCID50)/cell during a 2-h infection period. The culture supernatant was then replaced by fresh culture medium supplemented with compounds or equal volumes of DMSO. At different time-points post-infection, the viral titers in

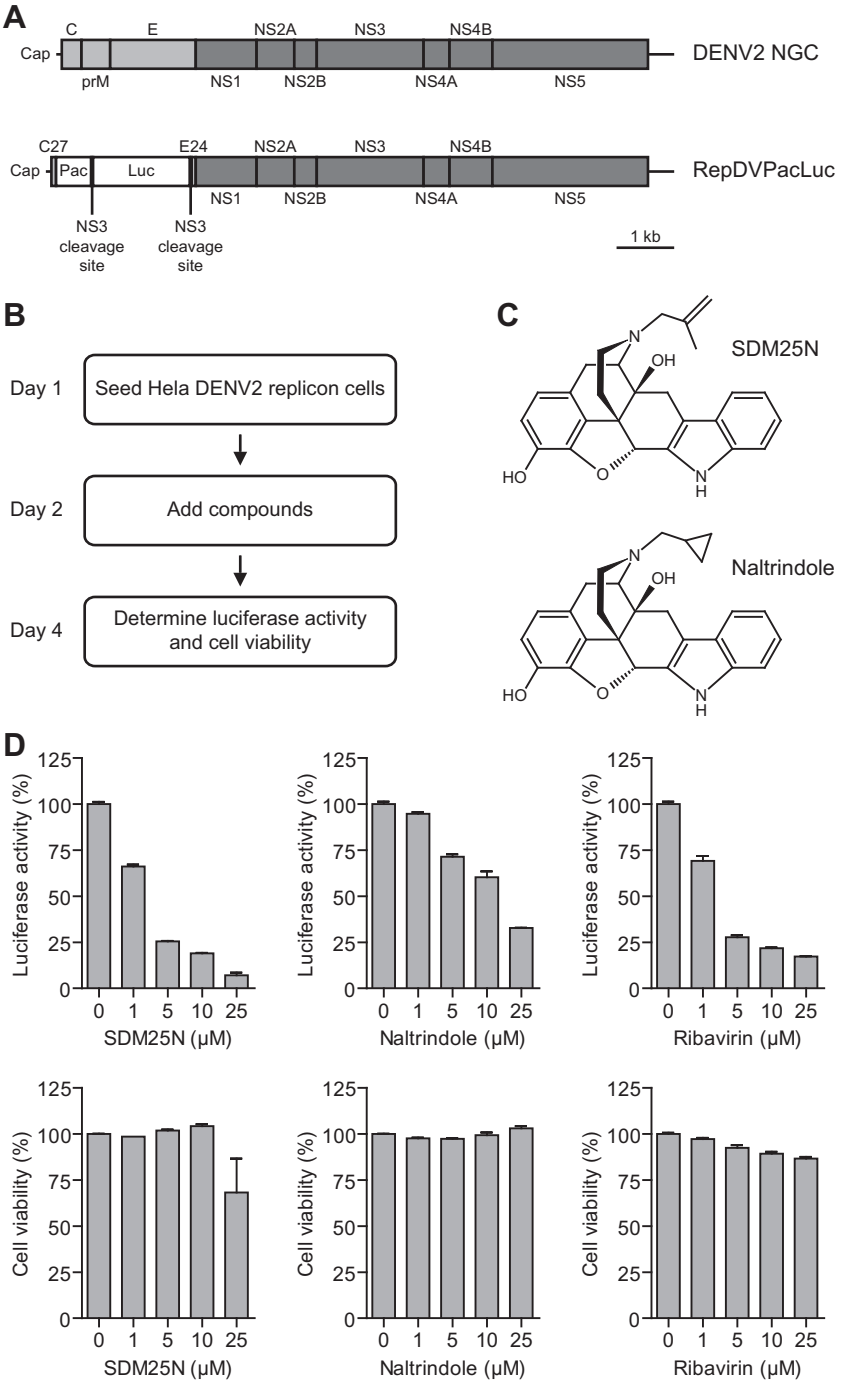

Fig. 1. Identification of a compound with anti-DENV activity in the NIH Clinical Collection. (A) Schematic representations of the DENV2 NGC genome and the subgenomic replicon RepDVPacLuc. The structural and non-structural regions are shown in light and dark grey, respectively. Pac, puromycin-*N*-acetyl-transferase; Luc, firefly luciferase. (B) Flow chart of the screen. (C) Molecular structures of SDM25N and naltrindole. (D) Luciferase activity (upper panels) and cell viability (lower panels) of Hela DENV2 replicon cells after 48 h of culture in the presence of increasing concentrations of SDM25N (left panels), naltrindole (middle panels) or ribavirin (right panels). All data were normalized to the DMSO control (0 μM). Bars and error bars represent mean and standard error of the mean of three independent samples.

the culture supernatant were determined by end-point dilution on BHK-15 cells. Viral genome copies in the cells were quantified by qRT-PCRs. Cell viability assays were run in parallel.

2.6. Transient translation/replication assays

Hela cells were seeded in 24-well plates at a density of 1×10^5 cells/well. The following day, the culture supernatant was supplemented with compounds or equal volumes of DMSO. One day after the addition of compounds, 300 ng of *in vitro* transcribed replicon RNA was transfected into the cells using Effectene Transfection Reagent. Luciferase activity assays were performed at different time-points post-transfection.

2.7. qRT-PCRs

Total RNA was isolated with either Isol-RNA Lysis Reagent (5 PRIME) or the GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich), treated with DNase I (Life Technologies) and reverse transcribed into cDNA using the TaqMan Reverse Transcription Reagents (Life Technologies) and random hexamers. Following cDNA synthesis, qPCRs were performed on a LightCycler 480 (Roche) using LightCycler 480 SYBR Green I Master reagents (Roche) and DENV2 primers KC54 (AGAACTGAAGTGTGGCAGTGGGAT) and KC55 (TGCCCTCTTCATGAGCTTTCTGGA). qPCRs with human β-actin primers 453.1 (CCTTCCTGGGCATGGAGTCCTG) and 453.2 (GGAGCAATGATCTTGATCTTC) were run in parallel to normalize the data.

2.8. Luciferase activity and cell viability assays

Firefly luciferase activity was measured with the Steady-Glo Luciferase Assay System (Promega) and a VICTOR^3 1420 Multilabel Counter plate reader (PerkinElmer). *Renilla* luciferase activity was measured with the *Renilla* Luciferase Assay System (Promega) and a Modulus luminometer (Turner BioSystems). Cell viability was determined with the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega) using the VICTOR^3 1420 Multilabel Counter plate reader.

3. Results

3.1. Identification of a compound with anti-DENV activity in the NCC

To screen the NCC for compounds with antiviral activity against DENV, we generated and validated a Hela cell line carrying a stably replicating, non-infectious DENV2 subgenomic replicon (see Supplementary data). In the replicon (RepDVPacLuc), a large part of the genomic region encoding the structural proteins is replaced by a cassette encoding a puromycin resistance selection marker and a firefly luciferase reporter flanked by two NS3 cleavage sites (Kaptein et al., 2010) (Fig. 1A). Hence, luciferase activity in the replicon-containing cells correlates with viral replication.

Hela DENV2 replicon cells were treated for two days with the NCC compounds at a single concentration of 10 μM before cell viability and luciferase activity were measured (Fig. 1B). Compounds that reduced cell viability to <80% of the DMSO control were excluded from further analyses. Remaining compounds that reduced luciferase activity to <50% of the DMSO control were selected as potential DENV inhibitors. The most potent DENV inhibitor identified by the screen was δ opioid receptor antagonist SDM25N (Fig. 1C). The anti-DENV activity of SDM25N was confirmed by treatment of the Hela DENV2 replicon cells with increasing SDM25N concentrations, which resulted in a dose-dependent reduction in viral replication (Fig. 1D). Interestingly, naltrindole, a δ opioid receptor antagonist with a structure very similar to SDM25N (Fig. 1C), also inhibited viral replication in a dose-dependent manner (Fig. 1D). Since naltrindole (EC_{50} = 13.3 μM; 95% CI = 12.1–14.7 μM) was less potent than SDM25N (EC_{50} = 1.9 μM; 95% CI = 1.7–2.1 μM), we decided to focus on SDM25N for additional mode-of-action studies.

To further verify the antiviral activity of SDM25N, we tested its potency in viral titer reduction assays with wild-type DENV2 in Hela cells. We analyzed the effect of SDM25N (10 μM) on the production of infectious virus at 24 and 48 h post-infection (hpi). At 24 hpi, SDM25N reduced viral titers more than 400-fold when compared to the DMSO control, to a level below the detection limit (Fig. 2A). At 48 hpi, the titers were 2.1 logs lower for cells treated with SDM25N than for cells treated with DMSO (Fig. 2A). The drop in viral titers at 48 hpi coincided with a 15-fold reduction in viral genome copies (Fig. 2B). These results confirm the antiviral activity of SDM25N during infections with wild-type DENV2.

3.2. SDM25N inhibits DENV in a cell type-specific manner

Next, we investigated the anti-DENV activity of SDM25N in different cell types. The production of infectious virus by wild-type DENV2-infected BHK-21 and C6/36 cells treated with DMSO or SDM25N (10 μM) was examined by viral titer reduction assays. Like in Hela cells, SDM25N displayed antiviral activity against DENV2 in BHK-21 cells, leading to a reduction of titers with 3.6 and 1.2 logs at 24 and 48 hpi, respectively (Fig. 3). However, SDM25N did not inhibit DENV2 in C6/36 cells (Fig. 3). This observation suggests that the anti-DENV activity of SDM25N differs between mammalian and mosquito cells.

3.3. SDM25N inhibits genomic RNA replication

Replicon-based screening approaches may identify antiviral compounds that target different post-entry stages of the viral replication cycle, such as translation of the viral genome, proteolytic cleavage of the polyprotein, and genomic RNA replication. To gain more insight into the mechanism of SDM25N-mediated DENV suppression, we generated a second DENV2 subgenomic replicon, designed for transient translation/replication assays. In this replicon (RepDVRLuc), the coding sequences for the structural proteins are replaced by a cassette encoding a *Renilla* luciferase reporter and an FMDV 2A sequence (Fig. 4A). Transfection of *in vitro* transcribed replicon RNA into Hela cells results in an initial peak of luciferase activity that reaches maximum levels at 8 h post-transfection (hpt) (Fig. 4B). Luciferase activity then drops, reaching minimum levels at 24 hpt, to rebound again at 48 hpt (Fig. 4B). The initial peak of luciferase activity reflects translation of the input RNA, whereas the increase in luciferase activity at later time-points reflects translation of newly formed progeny RNA (Alvarez et al., 2005; Holden et al., 2006). To determine which stage of the viral replication cycle is repressed by SDM25N, we measured luciferase activity in replicon-transfected Hela cells that were cultured in the presence of DMSO or SDM25N (10 μM). At 8 hpt, luciferase activity was comparable in DMSO- and SDM25N-treated cells (Fig. 4C). However, when compared to DMSO-treated cells, luciferase activity was severely suppressed (33-fold) in SDM25N-treated cells at 48 hpt (Fig. 4C). These data indicate that SDM25N inhibits viral RNA replication rather than translation of the viral genome.

3.4. SDM25N targets the viral NS4B protein

To identify potential viral targets of SDM25N, we set out to generate SDM25N-resistant replicons. To this end, we cultured the Hela DENV2 replicon cells for several passages under selection of both SDM25N and puromycin. Following this procedure, three independent cell lines were obtained. To map the mutations that

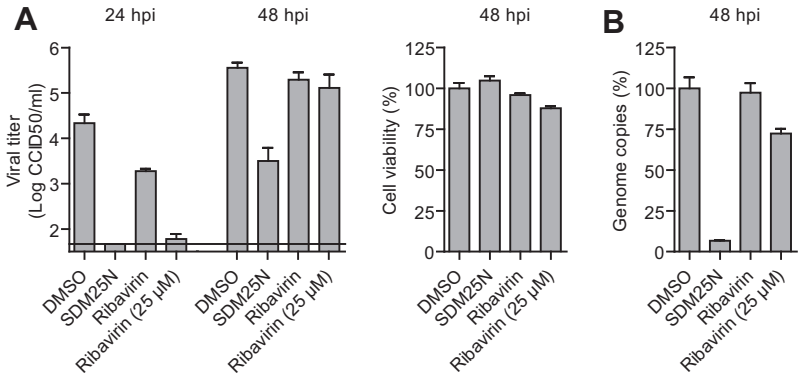

Fig. 2. SDM25N inhibits wild-type DENV2. (A) Viral titers at 24 and 48 h post-infection (hpi) in the culture supernatant of Hela cells infected with wild-type DENV2 and treated with DMSO, SDM25N or ribavirin (left panel). The concentration of both compounds was 10 μM, unless indicated otherwise. The horizontal line reflects the detection limit of the assay. Cell viability at 48 hpi is shown in the right panel. (B) DENV2 genome copies in Hela cells treated with DMSO, SDM25N or ribavirin at 48 hpi. Cell viability (A) and DENV2 genome copies (B) were normalized to the DMSO control. Bars and error bars represent mean and standard error of the mean of three independent samples.

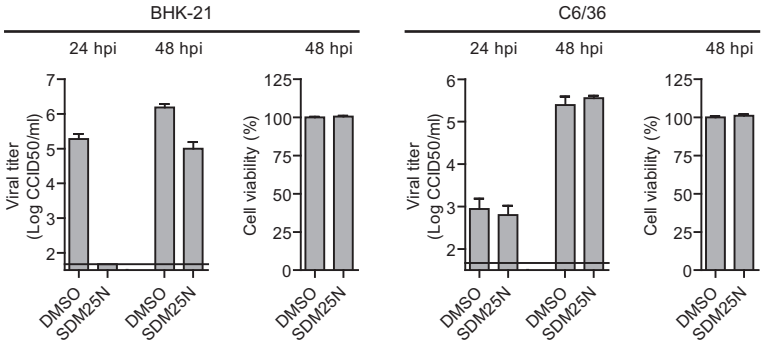

Fig. 3. SDM25N inhibits DENV in a cell type-specific manner. Viral titers at 24 and 48 h post-infection (hpi) in the culture supernatant of BHK-21 and C6/36 cells infected with wild-type DENV2 and treated with DMSO or 10 μM of SDM25N (left panel for each cell type). The horizontal line reflects the detection limit of the assay. Cell viability for each cell type at 48 hpi is shown in the right panel. Cell viability was normalized to the DMSO control. Bars and error bars represent mean and standard error of the mean of three independent samples.

are responsible for SDM25N resistance, we compared the sequences of the three SDM25N-selected replicons to that of the parental replicon. A prominent mutation was found in all three replicons. This mutation, a T to C substitution at position 7315 (relative to the DENV2 NGC genome), results in a Phenylalanine to Leucine substitution at position 164 (F164L) of the NS4B protein. According to a membrane topology model of the NS4B protein (Miller et al., 2006), this residue is located in the cytoplasmic loop preceding the predicted trans-membrane domain 4 (Fig. 5A).

Given that the NS4B F164L mutation was present in the replicons in all three SDM25N-selected cell lines, but absent from the replicon in the parental cell line, we deemed it likely that this mutation accounted for the SDM25N resistance. To test this hypothesis, we inserted this mutation into the transient replicon. We then compared the SDM25N sensitivity of the NS4B F164L mutant replicon to that of the wild-type replicon. Again, replication of the wild-type replicon was suppressed (74-fold) by SDM25N (Fig. 5B). In contrast, SDM25N did not inhibit, but even slightly enhanced (1.5-fold) replication of the mutant replicon (Fig. 5B). As expected, the mutant replicon remained sensitive to the antiviral activity of ribavirin. These results confirm that SDM25N (directly or indirectly) targets the viral NS4B protein and that the F164L mutation in this protein confers SDM25N resistance.

Since replication of the NS4B F164L mutant replicon seemed to be enhanced in the presence of SDM25N, we compared the replication kinetics of the wild-type and mutant replicons in the absence of drug. At 8 hpt, luciferase activity was comparable between the two replicons (Fig. 5C). However, luciferase activity of the mutant replicon was considerably lower (5.4-fold) than that of the wild-type replicon at 48 hpt (Fig. 5C). These data show that replication, but not translation of the NS4B F164L mutant replicon is attenuated. Although a single amino acid substitution is sufficient to confer resistance, the reduced fitness of the escape mutant may restrict the emergence of the NS4B F164L resistance mutation.

Recently another inhibitor of DENV replication (NITD-618) was identified in a high-throughput screen, resistance to which was also attributed to mutations in NS4B (Xie et al., 2011). We therefore tested whether these mutations (P104L and A119T), which are both located in the predicted trans-membrane domain 3 of NS4B (Miller et al., 2006) (Fig. 5A), also confer resistance to SDM25N. Remarkably, similar to our observations with the NS4B F164L replicon, SDM25N did not inhibit, but even slightly enhanced (2.6-fold) replication of the NS4B P104L mutant replicon (Fig. 5D). In contrast, the NS4B A119T mutant replicon remained fully sensitive to SDM25N, with a strong 112-fold suppression of luciferase activity (Fig. 5D). Thus, two distinct amino acid substitutions in NS4B (F164L and P104L) confer resistance to SDM25N. Although the amino acids are located in different subcellular compartments, we expect that these substitutions induce similar conformational changes that prevent the antiviral activity of SDM25N.

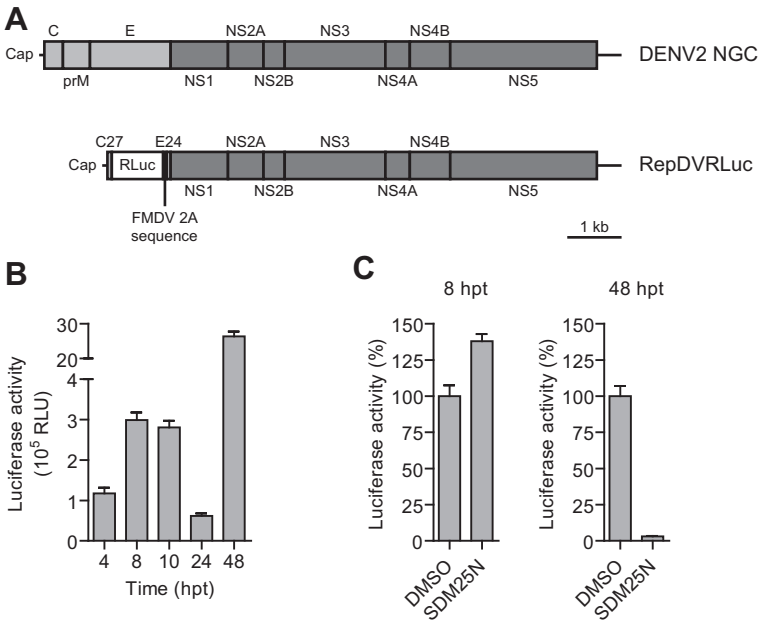

Fig. 4. SDM25N inhibits genomic RNA replication. (A) Schematic representations of the DENV2 NGC genome and the subgenomic replicon RepDVRLuc. The structural and non-structural regions are shown in light and dark grey, respectively. RLuc, *Renilla* luciferase. (B) Luciferase activity in Hela cells transfected with RepDVRLuc over time. RLU, relative light units; hpt, hours post-transfection. (C) Luciferase activity at 8 (left panel) and 48 (right panel) hpt in RepDVRLuc-transfected Hela cells treated with DMSO or 10 μM of SDM25N. Luciferase activity in (C) was normalized to the DMSO control. Bars and error bars represent mean and standard error of the mean of three independent samples.

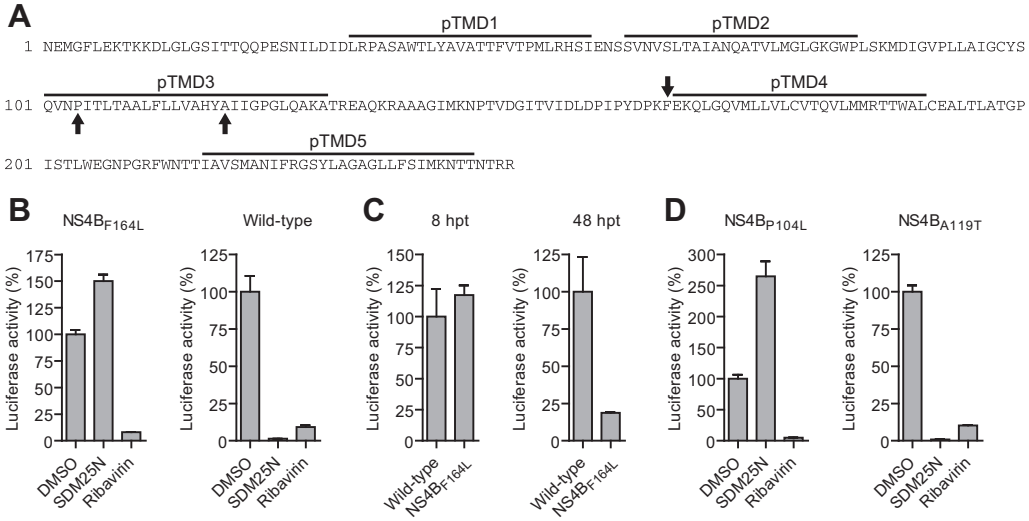

Fig. 5. SDM25N targets the viral NS4B protein. (A) Amino acid sequence of the DENV2 NGC NS4B protein (GenBank accession No. AF038403.1). Horizontal bars show the location of the predicted trans-membrane domains (pTMD1-5) (Miller et al., 2006). Positions F164, P104 and A119 are depicted by arrows. (B) Luciferase activity at 48 h post-transfection (hpt) in Hela cells transfected with RepDVRLuc-NS4B$_{F164L}$ (left panel) or wild-type RepDVRLuc (right panel) and treated with DMSO or with 10 μM of SDM25N or ribavirin. (C) Luciferase activity at 8 (left panel) and 48 (right panel) hpt in wild-type and NS4B$_{F164L}$ mutant RepDVRLuc-transfected Hela cells. (D) Luciferase activity at 48 hpt in Hela cells transfected with RepDVRLuc-NS4B$_{P104L}$ (left panel) or RepDVRLuc-NS4B$_{A119T}$ (right panel) and treated with DMSO or with 10 μM of SDM25N or ribavirin. All data were normalized to their controls (DMSO in (B) and (D); wild-type RepDVRLuc replicon in (C)). Bars and error bars represent mean and standard error of the mean of three independent samples.

4. Discussion

DENV is the most common human arthropod-borne virus and a major public health concern. However, a licensed vaccine or specific antiviral treatment is not available. Drugs with anti-DENV activity are therefore urgently needed. Using a replication-based screening assay, we have identified a DENV inhibitor in a library of drug-like small molecules. This inhibitor, SDM25N, restricts genomic RNA replication and single amino acid substitutions (F164L and P104L) in the viral NS4B protein confer resistance to SDM25N. Our studies, together with a recent report (Xie et al., 2011), establish NS4B as a (direct or indirect) target for specific anti-DENV drug development. The potential of NS4B as a target for the development of anti-flavivirus therapy is further exemplified by the identification of NS4B inhibitors for yellow fever virus (Patkar et al., 2009).

SDM25N, an analog of naltrindole, is a potent and highly selective δ opioid receptor antagonist (McLamore et al., 2001). When compared to SDM25N, naltrindole has a lower selectivity, but a considerably higher affinity and antagonist activity at the δ

receptor (McLamore et al., 2001). It is therefore unlikely that SDM25N exerts its antiviral activity through δ receptor antagonism, since it inhibited DENV replication more potently than naltrindole (Fig. 1D).

The fact that SDM25N inhibits the replication of DENV in Hela and BHK-21 cells, but not in C6/36 cells suggests that SDM25N targets a function of NS4B that is only required for efficient replication in mammalian cells and not in mosquito cells. NS4B interferes with interferon (IFN) signaling by blocking the activation of signal transducer and activator of transcription 1 (Muñoz-Jordán et al., 2003, 2005). This function of NS4B is restricted to mammalian cells, since insects lack IFN responses. Given that SDM25N only seems to inhibit DENV in mammalian cells, it is tempting to speculate that it interferes with the IFN-suppressive activity of NS4B. Although plausible, SDM25N exhibits anti-DENV activity in Vero (data not shown) and BHK-21 cells, two cell lines with defects in the type I IFN system (Desmyter et al., 1968; Diaz et al., 1988; Emeny and Morgan, 1979; Habjan et al., 2008; Mosca and Pitha, 1986). Its ability to restrict virus replication in type I IFN-defective cells indicates that SDM25N likely targets other functions of NS4B than its IFN-antagonistic activity. The identity of these functions awaits further investigation. It also remains to be established whether SDM25N targets NS4B directly or indirectly via a specific host factor or via other viral proteins that interact with NS4B.

Taken together, SDM25N is a new DENV inhibitor that restricts genomic RNA replication. Our data, as well as two recent papers (Patkar et al., 2009; Xie et al., 2011), establish NS4B as a target for anti-flavivirus drugs. Further studies will be required to identify the mechanisms that are responsible for the antiviral properties of SDM25N. Furthermore, *in vivo* studies in animal models will be crucial to evaluate the potential of SDM25N as a DENV drug candidate.

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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.2013.05.011.

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