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Posaconazole inhibits dengue virus replication by targeting oxysterol-binding protein

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ABSTRACT

Dengue virus (DENV) is associated with an estimated 390 million infections per year, occurring across approximately 100 countries in tropical and sub-tropical regions. To date, there are no antiviral drugs or specific therapies to treat DENV infection. Posaconazole and itraconazole are potent antifungal drugs that inhibit ergosterol biosynthesis in fungal cells, but also target a number of human proteins. Here, we show that itraconazole and posaconazole have antiviral activity against DENV. Posaconazole inhibited replication of multiple serotypes of DENV and the related flavivirus Zika virus, and reduced viral RNA replication, but not translation of the viral genome. We used a combination of knockdown and drug sensitization assays to define the molecular target of posaconazole that mediates its antiviral activity. We found that knockdown of oxysterol-binding protein (OSBP) inhibited DENV replication. Moreover, knockdown of OSBP, but not other known targets of posaconazole, enhanced the inhibitory effect of posaconazole. Our findings imply OSBP as a potential target for the development of antiviral compounds against DENV.

1. Introduction

Dengue virus (DENV) is one of the most important human pathogens that are transmitted by mosquitoes. It is estimated that the virus causes 390 million infections per year, of which 96 million manifest symptoms. The virus occurs across tropical and sub-tropical countries, with potential for further spread (Bhatt et al., 2013). The evolution and interaction of four serotypes (DENV 1–4) account for greater disease severity and contribute to the escalation of the dengue pandemic (Gubler, 2006). Recently, a licensed vaccine has become available in a number of countries (Hadinegoro et al., 2015), but its low efficacy in children under 9 years of age limits the implementation of this vaccine. Antiviral drugs are considered potential tools to combat dengue virus, yet, despite decades-long efforts, a licensed anti-DENV drug is still lacking (Lim et al., 2013; Simmons et al., 2012).

High-throughput screening (HTS) of libraries of small molecules is a powerful tool to identify novel dengue and flavivirus inhibitors (Li et al., 2013; Lo et al., 2003; Puig-Basagoiti et al., 2005; Xie et al., 2011, 2016). We previously used an unbiased replicon-based system to screen the National Institutes of Health (NIH) Clinical Collection for DENV inhibitors, and identified SDM25N, naltrindole, and AM404 as novel inhibitors that target the viral NS4B protein directly or indirectly (van

Abbreviations: AMPK, AMP-activated protein kinase; CPE, cytopathic effect; DENV, dengue virus; DMEM, Dulbecco's modified Eagle medium; EC_{50}, 50% effective concentration; FASN, fatty-acid synthase; FRAP1, FK506-Binding Protein 12-Rapamycin Complex-Associated Protein 1; HCV, hepatitis C virus; hCyp51, Human cytochrome P450, family 51; Hh, Hedgehog; HTS, high throughput screening; ITZ, Itraconazole; mTOR, mechanistic target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MVD, mevalonate diphosphate decarboxylase; NGC, new guinea C; NIH, National Institutes of Health; NPC1, Niemann-Pick C1; NPC2, Niemann-Pick C2; ORP, OSBP-related protein; OSBP, oxysterol binding protein; OSW-1, outer spore wall 1; PI4KIIIα, phosphatidylinositol 4-kinase type III alpha; PI4KIIIβ, phosphatidylinositol 4-kinase type III beta; PI4P, phosphatidylinositol 4-phosphate; POS, posaconazole; shRNA, short-hairpin RNA; siRNA, small interfering RNA; SMO, Smoothened; VDAC1, voltage-dependent anion channel 1; YFV, yellow fever virus; ZIKV, Zika virus

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Cleef et al., 2013, 2016). As replicons contain all the viral elements required for autonomous viral RNA replication, replicon-based screens have the potential to identify broadly acting antiviral compounds (Kato and Hishiki, 2016; Ng et al., 2007).

One of the hits from our screen was itraconazole (ITZ), a triazole drug that is used to treat a broad range of fungal infections in humans (Warnock and Campbell, 1996). Antifungal triazoles inhibit 14α-demethylation of lanosterol in the ergosterol biosynthetic pathway of fungi (Peyton et al., 2015; Vanden Bossche et al., 1993). In addition, these compounds target a number of factors in human cells. For example, ITZ has anti-angiogenic activity by inhibiting the mTOR (mechanistic target of rapamycin) signaling pathway, which regulates cell proliferation and is required for angiogenesis (Chong et al., 2007). Binding of ITZ to voltage-dependent anion channel 1 (VDAC1) triggers AMP-activated protein kinase (AMPK) phosphorylation of regulatory-associated protein of mTOR (raptor) (Head et al., 2015). In addition, ITZ and the related posaconazole (POS) are reported to bind Niemann-Pick C1 (NPC1), a membrane protein responsible for cholesterol transfer from lysosomes to other membranes, such as the plasma membrane and the endoplasmic reticulum (Das et al., 2014; Iaea and Maxfield, 2015; Trinh et al., 2017). The fact that knockdown of NPC1 and NPC2 inhibits mTOR activity in endothelial cells (Xu et al., 2010) provides a potential explanation for the mechanism by which ITZ and POS inhibit the mTOR pathway (Head et al., 2017; Xu et al., 2010). ITZ and POS also act as anticancer agents by inhibiting the hedgehog (Hh) pathway through suppression of the accumulation of Smoothened (SMO) protein, an essential component of this pathway (Chen et al., 2016; Kim et al., 2010).

ITZ was recently found to inhibit enterovirus replication by binding to oxysterol-binding protein (OSBP) (Strating et al., 2015). In agreement, other OSBP antagonists like 25-hydroxycholesterol, OSW-1, and T-00127-HEV2, also showed inhibitory activity against enterovirus replication (Albulescu et al., 2015; Arita et al., 2013; Roulin et al., 2014). OSBP is a transmembrane protein involved in the exchange of sterol and phosphatidylinositol 4-phosphate (PI4P) between ER and Golgi membranes (Mesmin et al., 2013). OSBP is required for efficient replication of a number of RNA viruses, including hepatitis c virus (HCV) and picornaviruses (Amako et al., 2009; Ishikawa-Sasaki et al., 2018; Rhoden et al., 2018; Strating et al., 2015; Wang et al., 2014).

Positive-strand RNA viruses remodel cellular membranes into specific compartments for viral RNA replication, called replication organelles. OSBP plays a critical role in the formation of these organelles, by acting as a sterol transporter in exchange for PI4P, resulting in cholesterol enrichment at these sites (Amako et al., 2009; Arita et al., 2015; Roulin et al., 2014; Strating et al., 2015; Wang et al., 2014). OSBP seems to be actively recruited for the formation of replication organelles. For example, OSBP co-localized with HCV NS5A in the viral ribonucleoprotein complex and, in accordance, silencing of OSBP reduced HCV replication and particle release in human cells (Amako et al., 2009; Wang et al., 2014). OSBP-related protein 4 (ORP4) was also found to interact with HCV NS5B (Park et al., 2013). However, ORP4 overexpression reduced HCV replication in HEK293T cells (Park et al., 2013), suggesting distinct functions from OSBP in the viral replication cycle.

Here, we characterize the antiviral activity of ITZ and POS against DENV and its closely related family member Zika virus (ZIKV). We demonstrate that POS inhibits replication of DENV by targeting OSBP. Together with recent reports (Albulescu et al., 2015, 2017; Strating et al., 2015), our findings broaden the antiviral scope of antifungal triazoles and identify OSBP as a host factor for DENV replication.

2. Material and methods

2.1. Cells and virus

HeLa and Vero cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L D-Glucose and L-Glutamine (Life Technologies). The medium was supplemented with 10% (v/v) fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). HeLa cells containing the stably replicating DENV2 RepDVPacLuc replicon were grown at 37 °C and 5% CO_2 in the medium described above, supplemented with 0.375 μg/ml puromycin. The generation and characterization of HeLa DENV2 replicon cells has been described previously (van Cleef et al., 2013, 2016).

Stocks of DENV2 (strain New Guinea [NGC] and strain 16681), DENV1 (strain 16007), DENV4 (strain H241) and YFV (strain 17D) were prepared on *Aedes albopictus* C6/36 cells. The culture supernatant was harvested when advanced cytopathic effect (CPE) was visible, centrifuged at low speed to clear the supernatant from cell debris, and divided into aliquots. The aliquots were flash-frozen in liquid nitrogen and stored at −80 °C. All virus infections were performed in complete growth medium of the corresponding cells.

2.2. Compounds

POS (CAS No. 171228-49-2), clotrimazole (CAS No. 23593-75-1), ribavirin (CAS No. 36791-04-5), and the Hh inhibitors SANT-1 (CAS No. 304909-07-7) and GANT-61 (CAS No. 500579-04-4) were purchased from Sigma-Aldrich. ITZ (CAS No. 84625-61-6) and ketoconazole (CAS No. 65277-42-1) were purchased from Santa Cruz Biotechnology and Enzo Life Science, respectively. Voriconazole and Fluconazole were kind gifts from Paul Verweij (Radboud University Medical Center). Torin1 (CAS number 1222998-36-8) was purchased from Bio-Vision. OSW-1 was a kind gift from Matthew Shair (Harvard University). All compounds were dissolved in DMSO.

2.3. Subgenomic replicons

DENV2 and ZIKV replicons have been previously described (Kaptein et al., 2010; Mutso et al., 2017; van Cleef et al., 2013). To produce replicon RNA for transient assays, the replicon plasmids pRepDVRLuc2A and pCCI-SP6-ZIKV NanoLuc were linearized with *Xba*I and *Age*I, respectively. Linearized pRepDVRLuc2A was used as template for *in vitro* transcription reactions using the T7 RiboMAX Large Scale RNA Production System (Promega) in the presence of Ribo m7G Cap Analog (Promega) at a cap analog to GTP ratio of 2.5 to 1. Linearized pCCI-SP6-ZIKV NanoLuc was *in vitro* transcribed using mMESSAGE mMACHINE SP6 Transcription Kit (Invitrogen) in the presence of m7G Cap analog at a cap analog to GTP ratio of 4 to 1. pRepDVRLuc2A and pCCI-SP6-ZIKV NanoLuc RNA was purified using the RNeasy Mini Kit (QIAGEN).

2.4. Virus infection in Vero cells

Vero cells were seeded in 24-well plates at a density of 1×10^5 cells/well. The next day, cells were inoculated with DENV1 (16007), DENV2 (16881), DENV4 (H241), and YFV (17D) at an MOI of 0.01. The supernatant was replaced with 500 μl of fresh medium supplemented with compounds, or equal amounts of DMSO at 2 h after infection. At different time points after infection, the culture supernatant was harvested and stored at −80 °C until further processing. Viral genome copy numbers in the culture supernatant were determined by qRT-PCR.

2.5. qRT-PCRs

Total RNA isolation, cDNA synthesis, and qPCR were performed as previously described (van Cleef et al., 2013, 2016). For primer sequences, see Supplemental Table 1.

2.6. Antiviral assays in HeLa DENV2 replicon cells

HeLa DENV2 replicon cells were seeded in either 24-well plates (1×10^5 cells/well) or 96-well plates (1×10^4 cells/well) in culture medium without puromycin. The following day, compounds or equal volumes of DMSO were added to the medium. Luciferase activity and cell viability assays were performed at two days after addition of compounds as described previously (van Cleef et al., 2013), except for the cell viability assay in Supplemental Fig. 4, which was determined using the CellTiter-Glo assay (Promega). The 50% effective concentration (EC_{50}) was defined as the concentration of compound that reduced luciferase activity by 50% relative to DMSO-treated cells. EC_{50} values and 95% confidence intervals were calculated by non-linear regression analysis using GraphPad Prism software (version 5.03).

2.7. Transient translation/replication assay

HeLa cells were seeded in 24-well plates at a density of 1×10^5 cells/well. The following day, the culture medium was supplemented with compounds or equal volumes of DMSO. One day after addition of compounds, 300 ng and 350 ng of *in vitro* transcribed replicon RNA of pRepDVRLuc2A and pCCI-SP6 ZIKV-NanoLuc, respectively, was transfected into the cells using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. Luciferase activity assays were performed at different time points after transfection using Renilla Luciferase Assay System (Promega) and measured on a Modulus luminometer (Turner BioSystems).

2.8. Rescue experiment

HeLa cells were seeded in 24-well plates at a density of 1×10^5 cells/well. The following day, pEGFP-C1 (Clontech) and pEGFP-hOSBP plasmids (Strating et al., 2015) were transfected into the cells using FuGene transfection reagent (Promega). Two days after plasmid transfection, 300 ng of pRepDVRLuc2A replicon RNA was transfected into the cells using Effectene Transfection Reagent. Six hours after RNA transfection, the transfection medium was replaced with medium containing 10 μM of compounds or, as a control, an equal volume of DMSO. Samples were harvested for luciferase activity assays at 48 h after RNA transfection.

2.9. siRNA sensitization assay

Sensitization assays were performed following a previously described approach (Arita et al., 2011; Strating et al., 2015). siRNAs were reverse transfected into HeLa DENV2 replicon cells in 96-well plates (1.5×10^4 cells/well). Before seeding the cells, siRNAs and transfection reagent (Lipofectamine RNAiMax, Invitrogen) were mixed according to the manufacturer's instructions and added to the wells. Compounds were added at 72 h after transfection and samples were harvested for luciferase activity assays and qRT-PCR at 48 h after addition of the compounds. qRT-PCRs were performed to assess knockdown efficiency. siRNAs were purchased from Dharmacon (human siGenome smartpool) or from Sigma-Aldrich, and dissolved in siRNA buffer (60 mM KCl, 6 mM HEPES-pH 7.5, 0.2 mM $MgCl_2$). For siRNA sequences, see Supplemental Table 2.

2.10. Immunofluorescence microscopy

Replicon-containing or non-infected control HeLa cells were grown on coverslips in 24-well plates until they reached ~50% confluency. Cells were transfected with pEGFP-C1 and pEGFP-hOSBP using FuGene (Roche) according to the manufacturer's instructions and incubated for 48 h to obtain optimal expression levels. POS (10 μM) and DMSO were added to the cultures and after an incubation for a period of 1 h, cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% Triton X-100 (PBS-T), and stained for 1 h with primary antibody (dsRNA; SCICONS J2) diluted 1:1000 in blocking buffer (10% bovine serum albumin, 2% normal goat serum, 0.1% Triton X-100, and 10% Tween-20 in PBS). Cells were then washed 3 times with PBS-T for 5 min and incubated with conjugated antibody (Goat-α-Rabbit-Alexa Fluor 594, Life Technologies) diluted 1:200 in blocking buffer for 1 h. After 2 washes with PBS-T and 2 washes with PBS (5 min each), the cells were counterstained with Hoechst and embedded in Mowiol (Omnilabo).

To analyze colocalization between OSBP and the Golgi apparatus, pEGFP-hOSBP was cotransfected with an expression plasmid encoding the Golgi marker GM130 tagged with mCherry (GM130-mCherry, created by cloning GM130 cDNA into pmCherry-C1 [Clontech]), into HeLa and HeLa DENV2 replicon cells and incubated for 48 h before treatment with POS (10 μM) or DMSO. Cells were prepared as described above for confocal microscopy analysis.

For filipin staining, cells were fixed and stained with 50 μg/ml Filipin III (Sigma Aldrich) and incubated for 2 h in the dark, at room temperature. After 3 washes with PBS, the cells were embedded in Mowiol (Omnilabo). Where applicable, cells were stained with an antibody for dsRNA and Hoechst before filipin staining. Images were taken on an Olympus FV1000 confocal laser scanning microscope using a 60x objective after which a Gaussian filter was applied for smoothing and edge detection in FIJI (Schindelin et al., 2012). The intracellular distribution of cholesterol was quantified using FIJI and defined as coefficient of variation. The cytoplasm of 10 cells was traced, and the coefficient of variation (Cv) was calculated from the mean intensity value (μ) and standard deviation (σ) as a measure for signal granularity ($Cv = \sigma/\mu$) using FIJI.

3. Results

3.1. Posaconazole inhibits dengue virus RNA replication

ITZ (PubChem ID 55283, Fig. 1A) was identified as an inhibitor of DENV replication in a previously described screen of the NIH Clinical Collection using a HeLa cell line that contains a stably replicating, non-infectious subgenomic replicon of DENV2 (New Guinea C strain) (referred to as HeLa DENV2 replicon cells) (van Cleef et al., 2013, 2016). To confirm this result, we analyzed the antiviral activity of ITZ and the closely related POS (PubChem ID 468595, Fig. 1A) using the same replicon system (Fig. 1B). In this replicon, the structural genes are replaced by a cassette encoding a puromycin resistance selection marker and a firefly luciferase reporter gene flanked by two NS3 cleavage sites, providing a simple readout for virus replication (Kaptein et al., 2010).

ITZ inhibited DENV replication with a 50% effective concentration (EC_{50}) of 1.7 μM (95% CI, 1.7–1.8 μM, Supplemental Fig. 1), which is in the same order of magnitude as its EC_{50} values against viruses from several genera of picornaviruses (Ishikawa-Sasaki et al., 2018; Rhoden et al., 2018; Strating et al., 2015). Likewise, POS reduced DENV replication in a dose-dependent manner with an EC_{50} of 4.1 μM (95% CI, 3.9–4.2 μM, Fig. 1B, Supplemental Fig. 1). Although ITZ did not induce cell toxicity, as assessed by visual inspection and a colorimetric assay (MTT assay), we noted some precipitation of ITZ during incubation at the highest concentrations, and we therefore chose to use POS for further experiments.

To determine which stage of the viral replication cycle is suppressed by POS, a second DENV2 subgenomic replicon was used for translation/replication assays (van Cleef et al., 2013). Upon transient transfection of *in vitro* transcribed replicon RNA, luciferase activity peaks at 8 h post-transfection (hpt), reflecting translation of the transfected RNA, whereas luciferase activity at later time points reflect translation of newly formed progeny RNA (Alvarez et al., 2005; Holden et al., 2006; van Cleef et al., 2013). We thus analyzed luciferase activity in HeLa cells transiently transfected with replicon RNA and cultured for 8 and 48 h in the presence of 10 μM of POS or, as a control, DMSO. At 8 hpt,

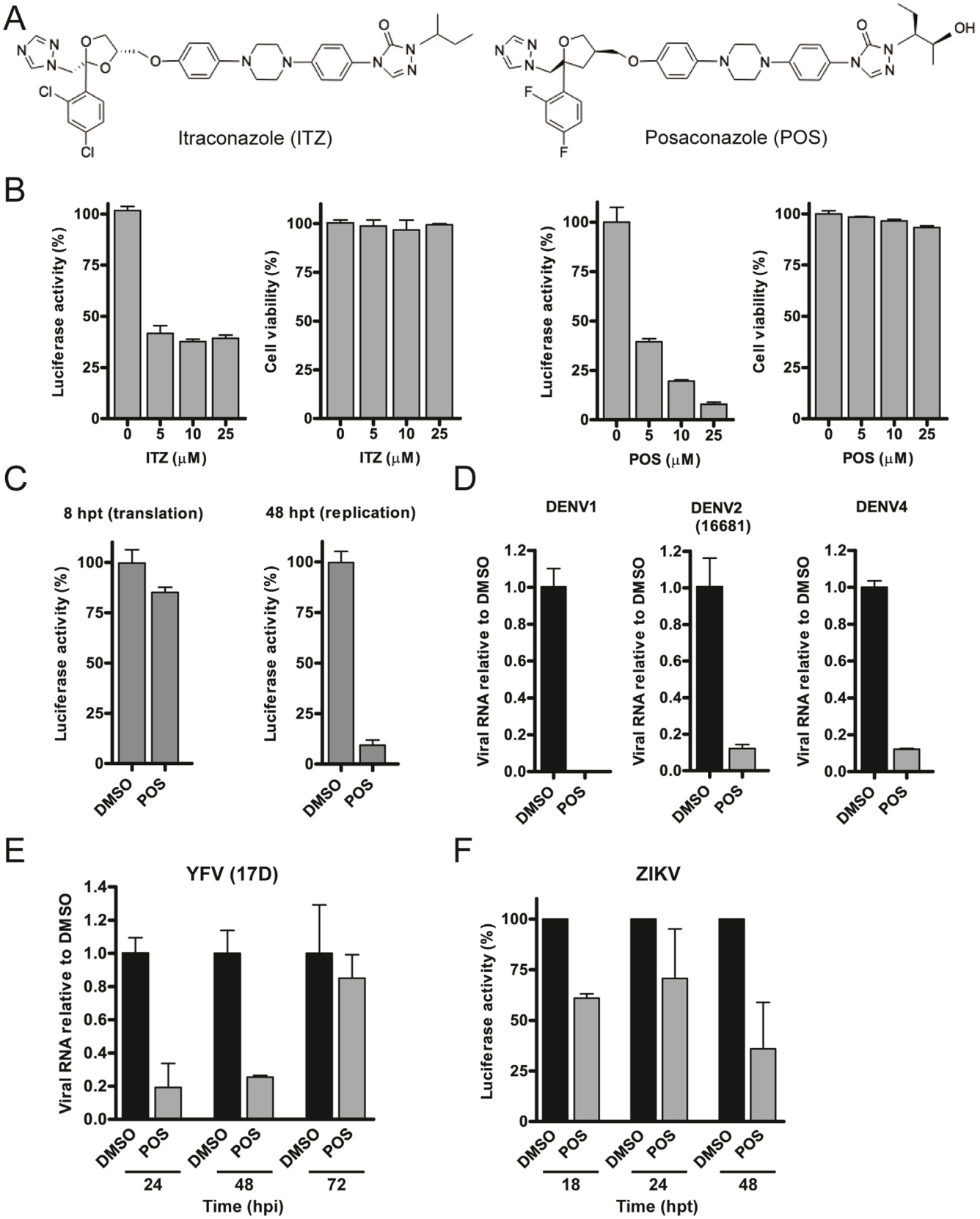

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Fig. 1. Itraconazole and posaconazole inhibit DENV replication. (**A**) Molecular structure of itraconazole (ITZ) and posaconazole (POS). (**B**) Luciferase activity and cell viability of HeLa DENV2 replicon cells at 48 h after addition of the indicated concentrations of ITZ (left panel) and POS (right panel). (**C**) Luciferase activity at 8 h and 48 h after RNA transfection (hpt) of HeLa cells with a DENV2 replicon expressing Renilla luciferase (pRepDVRLuc). The cells had been pre-treated with 10 μM POS for 24 h before transfection. (**D**) Replication of DENV1 (strain 16007), DENV2 (strain 16681), and DENV4 (strain H241) in Vero cells treated with 10 μM POS or control (DMSO). Cells were infected at an MOI of 0.01. Virus copy numbers in the culture supernatant were measured by qRT-PCR at 48 h post infection and expressed relative to copy numbers for the DMSO control. (**E**) Replication of yellow fever virus 17D strain (YFV-17D) in Vero cells treated with 10 μM POS or control (DMSO). Cells were infected at an MOI of 0.01. Virus copy numbers in the culture supernatant were measured by qRT-PCR at the indicated time points post infection and expressed as in (D). (**F**) Luciferase activity at the indicated time points after RNA transfection of HeLa cells with a Zika virus (ZIKV) replicon expressing NanoLuc luciferase. DMSO or 10 μM POS was added at 2 h after RNA transfection. All compounds were dissolved in DMSO which was used as negative control in all experiments. All data were normalized to the DMSO control (0 μM). Bars and error bars represent means and SDs of three independent samples.

luciferase activity in POS treated cells was comparable with luciferase activity of DMSO treated cells (Fig. 1C). In contrast, luciferase activity was 10-fold reduced in POS treated cells at 48 hpt (Fig. 1C). These data imply that POS inhibits RNA replication, but not translation of viral RNA.

To investigate the antiviral spectrum of POS, we analyzed different serotypes of DENV, as well as other members of the genus *Flavivirus*, Zika virus (ZIKV) and yellow fever virus (YFV, 17D vaccine strain). As expected, POS treatment resulted in a > 10-fold reduction in replication of DENV2 (strain 16681), DENV1 (strain 16007), and DENV4 (strain H241) implying that POS inhibits multiple DENV serotypes (Fig. 1D). Likewise, POS treatment reduced YFV replication in Vero cells, especially at early time points after infection (Fig. 1E). To analyze the replication of ZIKV, we transiently transfected RNA of a ZIKV subgenomic replicon expressing NanoLuc (ZIKV-NanoLuc) into HeLa cells (Mutso et al., 2017). POS treatment resulted in 30–65% reduction of luciferase activity in cells transfected with ZIKV-NanoLuc over time as compared to DMSO treated cells (Fig. 1F). Together, these results suggest that POS has pan-Flavivirus activity.

3.2. Antifungal and anticancer targets do not mediate the antiviral activity of posaconazole

ITZ and POS are potent antifungal drugs that inhibit the activity of fungal and human sterol 14-alpha demethylases (Cytochrome P450, family 51, Cyp51), which are crucial for ergosterol biosynthesis in fungi and cholesterol biosynthesis in humans (Kim and Williams, 2014; Sabatelli et al., 2006; Sheehan et al., 1999). Thus, to analyze whether targeting of human Cyp51 (hCyp51) explains the antiviral activity of the antifungal triazoles ITZ and POS, we analyzed the anti-DENV activity of a range of antifungal triazoles that differ in the extent of cross-reactivity to hCyp51 (Lamb et al., 1999; Warrilow et al., 2013). We found that none of the other tested antifungal azoles (ketoconazole, voriconazole, fluconazole, and miconazole) inhibited replication of DENV (Fig. 2A, Supplemental Fig. 2A). Of particular interest in this regard is ketoconazole, which inhibits hCyp51 as efficiently as ITZ (Lamb et al., 1999). The observation that these compounds do not inhibit DENV replication, suggests that this putative target cannot explain the antiviral activity of POS and ITZ.

To extend these observations, we used a drug sensitization assay in which gene knockdown by small interfering RNAs (siRNAs) is combined with POS treatment. This assay assumes that knockdown of a target gene sensitizes the cell to the antiviral activity of the compound of interest. Thus, we transfected siRNAs targeting hCyp51 into HeLa DENV2 replicon cells and treated the cells with increasing concentrations of POS. We verified knockdown efficiency using qRT-PCR and absence of cell toxicity using a cell viability assay (Supplemental Figs. 2B–C). To analyze the extent of sensitization to POS, we normalized the luciferase activity in hCyp51 silenced cells to cells transfected with a non-targeting control siRNA at the same POS concentration. Two conclusions can be drawn from this experiment. First, in the absence of POS, hCyp51 knockdown did not affect DENV replication (Fig. 2B), indicating that hCyp51 is not a major host factor for DENV replication. Second, hCyp51 knockdown did not significantly sensitize the cells to the antiviral activity of POS. Together, these data suggest that the anti-DENV activity of POS cannot be explained by targeting of human Cyp51.

The hedgehog (Hh) signaling pathway is important for development and differentiation, and defects in the pathway are associated with a number of cancers. During normal conditions, the 7-transmembrane protein Smoothened (SMO) activates the transcription factor Gli, which then translocates into the nucleus to initiate transcription of effector genes. ITZ and POS have been shown to antagonize the Hh pathway, resulting in suppression of Hh-dependent tumors (Chen et al., 2016; Kim et al., 2010). The mechanism of action is not fully clear, but has been proposed to be mediated through SMO binding (Kim et al., 2010). To investigate the importance of the Hh pathway in DENV replication, we treated HeLa DENV2 replicon cells with the established Hh pathway inhibitors SANT-1 and GANT61 (Chen et al., 2002; Lauth et al., 2007; Lee et al., 2007). SMO antagonist SANT-1 did not affect DENV replication at any of the concentrations tested (Fig. 2C). Likewise, GANT61, an antagonist of the Gli protein downstream of SMO, only inhibited DENV replication at concentrations that were near to or at toxic levels, as is evident from the reduced cell viability (Fig. 2C). To confirm these results, we analyzed whether siRNA-mediated knockdown of SMO sensitized cells to POS. SMO knockdown resulted in a modest, 20% reduction in luciferase activity in the absence of POS, but did not significantly enhance the antiviral activity of POS (Fig. 2B, Supplemental Figs. 2B–C). These results imply that inhibition of the Hh pathway does not mediate the anti-DENV activity of POS.

ITZ and POS have also been shown to inhibit mTOR complex 1 and 2 in mammalian endothelial cells, resulting in inhibition of angiogenesis. The kinase mTOR, also known as FK506-Binding Protein 12-Rapamycin Complex-Associated Protein 1 (FRAP1), is the core component of both protein complexes (Brown et al., 1994). To investigate the role of the mTOR pathway in DENV replication and its role in the antiviral activity of POS, we used Torin1, a potent inhibitor of mTOR phosphorylation. Interestingly, Torin1 dramatically reduced luciferase activity in HeLa DENV2 replicon cells (Fig. 2D), although this may be partially due to its effect on cell viability. To confirm these findings, a drug sensitization assay was performed using siRNA-mediated knockdown of mTOR/FRAP1. Knockdown of mTOR reduced luciferase activity in HeLa DENV2 replicon cells with 40% in the absence of POS compared to the control knockdown, which was not associated with detectable toxicity (Fig. 2B, Supplemental Fig. 2B), suggesting that an active mTOR pathway is important for DENV replication. However, silencing of mTOR did not enhance the sensitivity of DENV to POS, suggesting that the antiviral activity of POS is not mediated by its effects on the mTOR pathway. Together, these data imply that the anti-DENV activity of POS is independent of its effects on known antifungal and anticancer targets.

3.3. OSBP and OSBP-related proteins (ORPs) are involved in DENV replication

OSBP is required for efficient replication of several RNA viruses, including enteroviruses and HCV (Amako et al., 2009; Strating et al., 2015; Wang et al., 2014). To investigate the role of OSBP in DENV replication, we used siRNAs to silence OSBP expression in HeLa DENV2 replicon cells and measured luciferase activity to assess viral

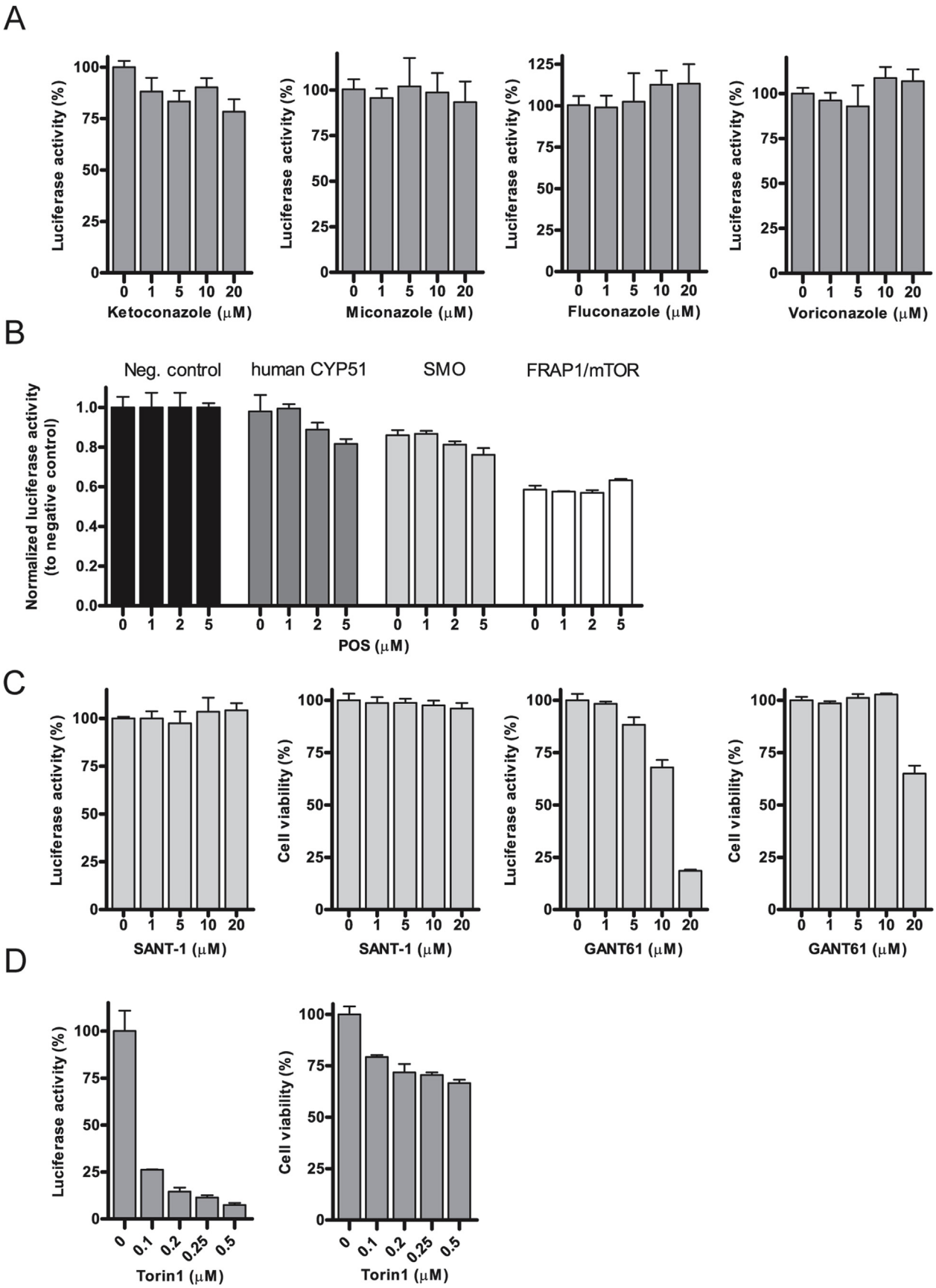

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Fig. 2. Antifungal and anticancer targets do not mediate the antiviral activity of posaconazole. (**A**) Luciferase activity of HeLa DENV2 replicon cells at 48 h after addition of different concentrations of the indicated azole compounds. Data were normalized to the DMSO control (0 μM). (**B**) Target identification using an siRNA sensitization assay. HeLa DENV2 replicon cells were transfected with siRNAs targeting the human CYP51, SMO, and FRAP1 (mTOR) genes or a non-targeting control siRNA (neg. control) in the absence (DMSO, 0 μM) or presence of different concentrations of POS. For each concentration of POS, luciferase activity in cells treated with gene specific siRNAs was normalized to cells treated with the control siRNA and the corresponding POS concentration. (**C** and **D**) Luciferase activity and cell viability of HeLa DENV2 replicon cells at 48 h after addition of different concentrations of the indicated inhibitors of the (C) hedgehog and (D) mTOR pathways. Bars and error bars represent means and SDs of three independent samples. Data were normalized to the DMSO control (0 μM).

replication. siRNA treatment effectively silenced OSBP expression by 66% and resulted in a consistent and significant reduction in luciferase activity in HeLa DENV2 replicon cells (45% reduction, Fig. 3A). As expected, the reduction in luciferase counts was modest when compared to treatment with an siRNA that directly targets the viral genome (NS3, 91% reduction, Fig. 3A), which may be explained by incomplete OSBP silencing, stability of the OSBP protein, or putative redundancy with OSBP gene family members. Our result is at odds with the results of Wang et al., who were unable to detect inhibition of DENV replication by short-hairpin RNA (shRNA)-mediated knockdown of OSBP (Wang et al., 2014).

OSBP is the founding member of a family of lipid transfer proteins, called OSBP-related proteins (ORPs). Silencing of OSBP and ORP4, the closest relative of OSBP, enhanced the sensitivity of picornaviruses to ITZ, although it is unclear whether ORP4 directly affects viral replication (Strating et al., 2015). We thus tested whether knockdown of different ORP genes affects DENV replication. We excluded ORP3 and ORP5 from these analyses, as these genes are not expressed in HeLa DENV2 replicon cells (Supplemental Fig. 3A). We verified efficient knockdown and lack of toxicity in all knockdown conditions, except for the ORP4 knockdown, which reduced cell viability as also observed previously (Charman et al., 2014) (Supplemental Figs. 3B–C). As shown earlier (Fig. 3A), OSBP knockdown resulted in a significant decrease in DENV replication (Fig. 3B). In addition, a modest but significant reduction in luciferase activity was observed upon knockdown of ORP2, ORP4 and ORP11 (Fig. 3B), although the result of ORP4 may be due to the effect of knockdown on cell viability. Together, these results suggest that OSBP, ORP2, ORP11, and perhaps ORP4 are required for efficient DENV replication.

To further investigate whether OSBP is implicated in DENV replication, we treated HeLa DENV2 replicon cells with OSW-1, a ligand of OSBP that also inhibits replication of enteroviruses and HCV (Albulescu et al., 2015; Burgett et al., 2011). As expected, OSW-1 inhibited DENV replication at concentrations at which no cell toxicity was observed (Fig. 3C). These observations extend the results from the RNAi experiments and indicate that OSBP is required for efficient DENV replication.

3.4. OSBP mediates the antiviral activity of posaconazole

The antiviral activity of ITZ against enteroviruses is mediated by targeting OSBP and ORP4 (Strating et al., 2015). To investigate whether OSBP and ORPs explain the anti-DENV activity of POS, we performed a sensitization assay in which the antiviral activity of POS is assessed in HeLa DENV2 replicon cells in which OSBP/ORP expression was silenced using siRNAs. Strikingly, OSBP knockdown increased the sensitivity of DENV to POS in a dose dependent manner (Fig. 4A). In contrast, silencing of OSBP in HeLa DENV2 replicon cells did not enhance DENV sensitivity to ribavirin, a nucleoside analog that is known to inhibit DENV (Fig. 4A) (Te et al., 2007), highlighting that OSBP knockdown does not sensitize to all antiviral compounds. As ITZ and POS have highly similar structures (Fig. 1A), we analyzed whether ITZ, like POS, exerts it anti-DENV activity by targeting OSBP using a similar drug sensitization assay. As expected, OSBP knockdown enhanced the antiviral effect of ITZ compared to control knockdown (Supplemental Fig. 4A).

We observed that knockdown of ORP2, ORP4 and ORP11 significantly affected DENV replication (Fig. 3B). To analyze whether these ORPs contribute to the antiviral activity of POS, we performed a drug sensitization assay and found that knockdown of these ORPs did not sensitize cells to POS (Fig. 4B, Supplemental Fig. 4B). These results suggest that ORP2, ORP4 and ORP11 do not mediate the antiviral activity of POS, although they may be important to establish an optimal intracellular environment for DENV replication.

To further confirm the role of OSBP in mediating the antiviral activity of POS, we analyzed whether expression of an EGFP-tagged OSBP transgene rescues DENV replication under POS treatment. To this end, HeLa cells were sequentially transfected with EGFP-OSBP or EGFP as a control, followed by transfection with replicon RNA and treatment with POS or DMSO. Luciferase activity was significantly higher in cells treated with POS and over-expressing OSBP compared to cells transfected with a control plasmid (Fig. 4C). We attribute the relatively modest rescue to the experimental set-up that requires that cells receive both plasmid and viral RNA after sequential transfection. Importantly however, the effect was specific for POS, as OSBP failed to rescue the antiviral effect of the nucleoside analog ribavirin (Fig. 4C). Together, these data suggest that OSBP is required for efficient DENV RNA replication and mediates the anti-DENV activity of POS.

3.5. POS causes redistribution of OSBP and cholesterol in infected and non-infected cells

ITZ and POS treatment results in the accumulation of (both endogenous and transgenically expressed) OSBP at the Golgi apparatus in HeLa cells (Strating et al., 2015). To confirm these observations, we analyzed the subcellular localization of a GFP-tagged OSBP transgene in HeLa cells (Fig. 5A, Supplemental Fig. 5A). As expected, OSBP-EGFP showed a stronger Golgi-like localization in POS treated cells than in cells treated with the DMSO control (Fig. 5A). Strong colocalization with the Golgi matrix protein GM130 confirms that these structures were indeed the Golgi apparatus (Supplemental Fig. 5B). To study whether DENV infection changes the subcellular localization of OSBP, we next analyzed GFP-tagged OSBP in HeLa DENV2 replicon cells. Like in uninfected cells, POS treatment resulted in the accumulation of OSBP at the Golgi apparatus in these cells (Fig. 5A–B, Supplemental Figs. 5B–C). Moreover, OSBP did not co-localize with dsRNA, neither in POS treated cells nor in DMSO treated cells. These results suggest that OSBP is not actively recruited to replication complexes during DENV infection, unlike picornavirus and HCV infections, during which OSBP co-localizes with respectively the viral 3A and NS5B proteins at the sites of replication (Amako et al., 2009; Arita et al., 2011; Strating et al., 2015). These results therefore imply that OSBP is indirectly involved in DENV replication, likely by altering the intracellular lipid homeostasis required for DENV replication complex formation.

OSBP shuttles sterol into the Golgi and PI4P into the ER; as a consequence POS causes cholesterol redistribution in HeLa cells (Strating et al., 2015). To study whether POS affects the cholesterol localization in DENV infected and non-infected cells, we stained HeLa and HeLa DENV2 replicon cells with filipin, a natural fluorescent molecule that binds cholesterol. In DMSO treated cells, filipin stained vesicles throughout the cytoplasm, with more pronounced staining near the nuclei, presumably the ER and Golgi apparatus. Upon treatment with POS, we observed less disperse cholesterol distribution in the cytoplasm and stronger perinuclear staining, both in DENV infected and non-

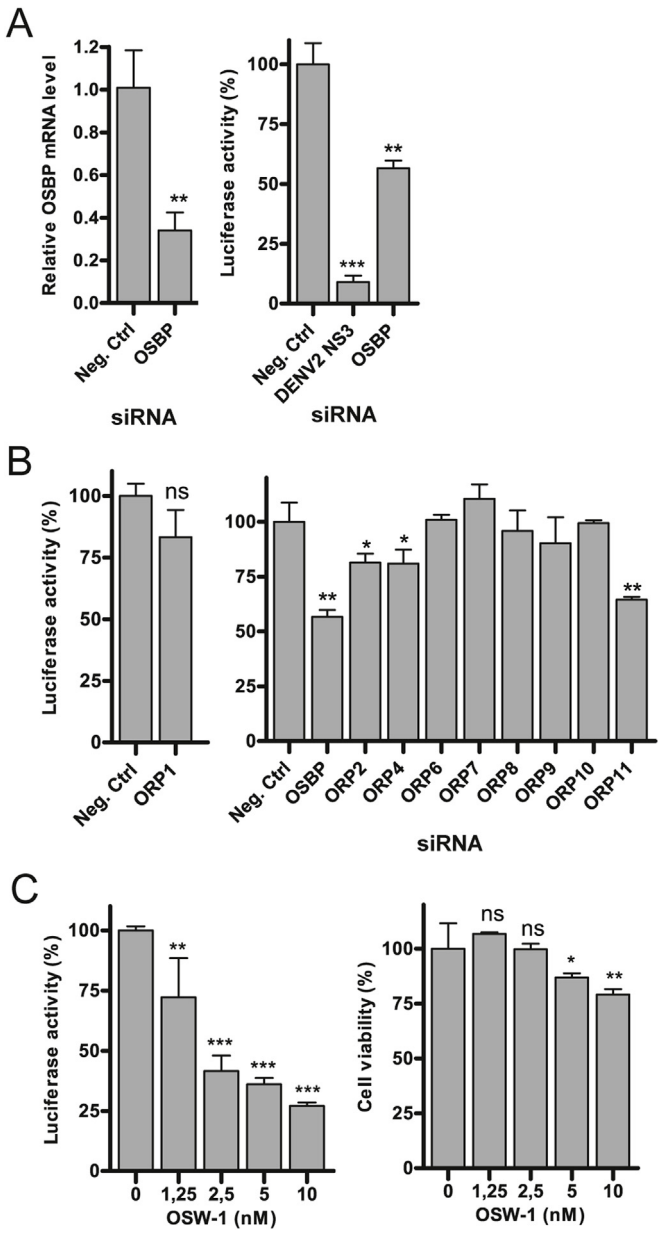

Fig. 3. OSBP is required for efficient DENV replication. (**A**) OSBP expression (left panel) and luciferase activity (right panel) in HeLa DENV2 replicon cells after transfection with siRNAs targeting the gene encoding oxysterol-binding protein (OSBP), DENV2 NS3, or non-targeting control siRNA. OSBP mRNA expression was assessed by qRT-PCR and normalized to cells transfected with control siRNA. Luciferase activity was measured at 48 h after transfection and normalized to control siRNA treated cells. (**B**) Luciferase activity in HeLa DENV2 replicon cells at 48 h after transfection with siRNAs targeting OSBP, the indicated OSBP-related proteins (ORPs), or non-targeting control siRNA. Bars and error bars represent means and SD of three independent samples. (**C**) Luciferase activity and cell viability of HeLa DENV2 replicon cells at 48 h after addition of different concentrations of OSW-1. Data were normalized to the DMSO control (0 nM). Statistical significance was assessed with unpaired *t* tests (A, B, left panel) and one-way ANOVA with Dunnett's post-hoc tests (B, right panels and C). *, $P < 0.05$; **, $P < 0.01$; ***$P < 0.001$.

infected cells (Fig. 5C, quantified in Fig. 5D). We next analyzed whether cholesterol is specifically enriched in viral replication complexes by staining DENV2 replicon cells with filipin and a dsRNA antibody. However, although cholesterol colocalized with dsRNA, cholesterol did not seem to be strongly enriched in viral replication complexes (Supplemental Fig. 5D). These data are consistent with a model in which POS targets OSBP, resulting in an altered intracellular cholesterol distribution, thus affecting DENV replication.

4. Discussion

Antiviral drugs for the treatment of DENV infections are urgently needed, since there are currently no fully protective vaccines and antiviral therapies available. Using a drug-repurposing screen of a library of drug-like small molecules, we identified the antifungal compounds ITZ and POS as inhibitors of DENV replication. Known ITZ/POS targets that are involved in sterol biosynthesis and angiogenesis did not explain their antiviral activity against DENV. Instead, several lines of evidence support the conclusion that POS inhibits DENV replication by targeting OSBP. First, silencing of human OSBP reduced DENV replication; second, knockdown of OSBP sensitized cells to the antiviral activity of POS and ITZ, but not to the nucleoside analog ribavirin; third, inhibition of OSBP by OSW-1, a well-established ligand and inhibitor of OSBP, inhibits viral replication; fourth, transgenic expression of OSBP significantly reduced the antiviral effect of POS.

OSBP is an important protein that controls ER-Golgi membrane tethering and lipid trafficking by exchanging cholesterol and phosphatidylinositol 4-phosphate (PI4P) between these membranes (Mesmin et al., 2013). The 3A protein of enteroviruses recruits phosphatidylinositol 4-kinase type III beta (PI4KIIIβ) to enrich viral replication complexes in PI4P, which is an anchor for OSBP to drive sterol-PI4P exchange between ER and Golgi membranes for the formation of replication organelles (Hsu et al., 2010; Mesmin et al., 2013; Strating et al., 2015). Likewise, HCV replication depends on PI4KIIIα activity, and the recruitment of OSBP to replication complexes is essential for efficient replication (Amako et al., 2009; Wang et al., 2014). The reliance of these viruses on the PI4K-PI4P-OSBP axis explains their sensitivity to ITZ, POS, and other inhibitors of OSBP.

DENV replication seems to be independent of PI4KIIIα and PI4KIIIβ (Heaton et al., 2010; Wang et al., 2014) and our results indicate that OSBP is not recruited to viral replication complexes. Yet, flaviviruses do manipulate lipid homeostasis to support their replication (reviewed in (Chukkapalli et al., 2012)). For example, West Nile virus upregulates cholesterol biosynthesis and induces a dramatic redistribution of cholesterol to viral replication complexes (Mackenzie et al., 2007). In addition, DENV recruits fatty-acid synthase (FASN) to the replication complex and its inhibition by Cerulenin and C75 significantly inhibits DENV replication (Heaton et al., 2010). Likewise, interference with cholesterol biosynthesis by siRNA-mediated knockdown of mevalonate diphosphate decarboxylase (MVD) reduced DENV2 replication (Rothwell et al., 2009) and blocking intracellular trafficking of cholesterol from the late endosomal compartment to the ER with U18666A inhibits entry and post-entry stages of the DENV life cycle (Poh et al., 2012). We observed that POS induces a redistribution of intracellular cholesterol to perinuclear vesicles, presumably the ER, which is in line with the role of OSBP in shuttling cholesterol from the ER to the Golgi. Thus, it is likely that POS interferes with DENV replication through its effects on intracellular cholesterol distribution by targeting OSBP (Lehto et al., 2001).

Viral replication organelles can be subdivided into two classes based on their morphology, namely protrusion-type, which are generated by bending cellular membranes into the cytoplasm, and invagination-type, formed by bending membranes inward into the donor organelle (Strating and van Kuppeveld, 2017). HCV and enteroviruses are PI4K-PI4P-OSBP dependent and generate protrusion-type replication organelles, whereas DENV (PI4K independent) generates invagination-type replication organelles into the ER (Welsch et al., 2009). An interesting parallel can be made with tomato bushy stunt virus (the type species of the genus *Tombusvirus* in the family *Tombusviridae*), a positive strand RNA virus of plants whose replication has been studied extensively in yeast. This virus generates invagination-type replication organelles into peroxisomes in a PI4K and PI4P independent, but ORP dependent

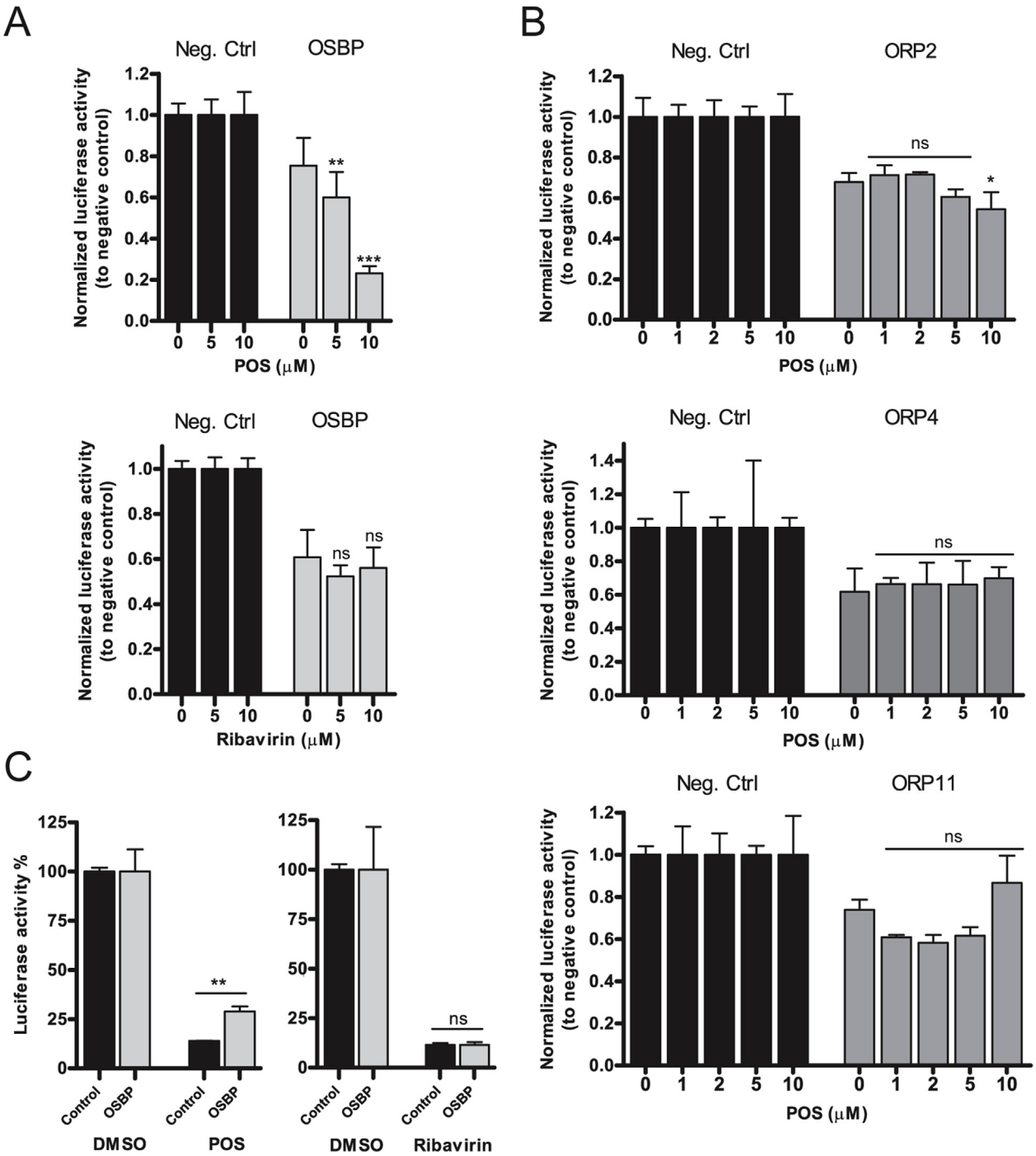

Fig. 4. Posaconazole inhibit DENV replication by targeting OSBP. (**A**) siRNA sensitization assay for OSBP. HeLa DENV2 replicon cells were transfected with siRNA targeting human OSBP, or non-targeting control siRNA. At 72 h after transfection, different concentration of POS (top panel), ribavirin (lower panel), or DMSO as control were added and luciferase activity was measured after an additional 48 h. For each concentration of POS or ribavirin, luciferase activity was normalized to cells transfected with control siRNA at the same concentration of drug. (**B**) siRNA sensitization assay for OSBP related proteins (ORP) 2, 4 and 11. The experiment was performed as described in panel A. (**C**) Rescue experiment with EGFP-OSBP or EGFP. HeLa cells were transfected with expression plasmids encoding EGFP-hOSBP or EGFP (control) and after 48 h transfected with DENV2 replicon RNA expressing Renilla luciferase (pRepDVRLuc) in the presence of 10 μM of POS (left panel), ribavirin (right panel), or DMSO as a control. Luciferase activity was measured at 48 h after RNA transfection. Data were normalized to luciferase activity in cells expressing EGFP. Bars and error bars represent means and SD of three independent samples. Statistical significance was assessed using one-way ANOVA with Dunnett's post-hoc tests (A and B) and unpaired *t* tests (C). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

manner (Fernandez de Castro et al., 2017). Multiple ORPs (named oxysterol-binding homology proteins, Osh, in yeast) are recruited to ER-peroxisome membrane contact sites to shuttle sterols to their replication organelles (Barajas et al., 2014).

In addition to OSBP, knockdown of ORP2, ORP4 and ORP11 reduced DENV replication in our experimental setup (although knockdown of these ORPs did not sensitize the cells to POS). These results suggest new roles for ORP2 and ORP11 in DENV replication; the role of ORP4 in DENV replication remains unclear as its knockdown induced cell toxicity, as was also reported previously (Charman et al., 2014;

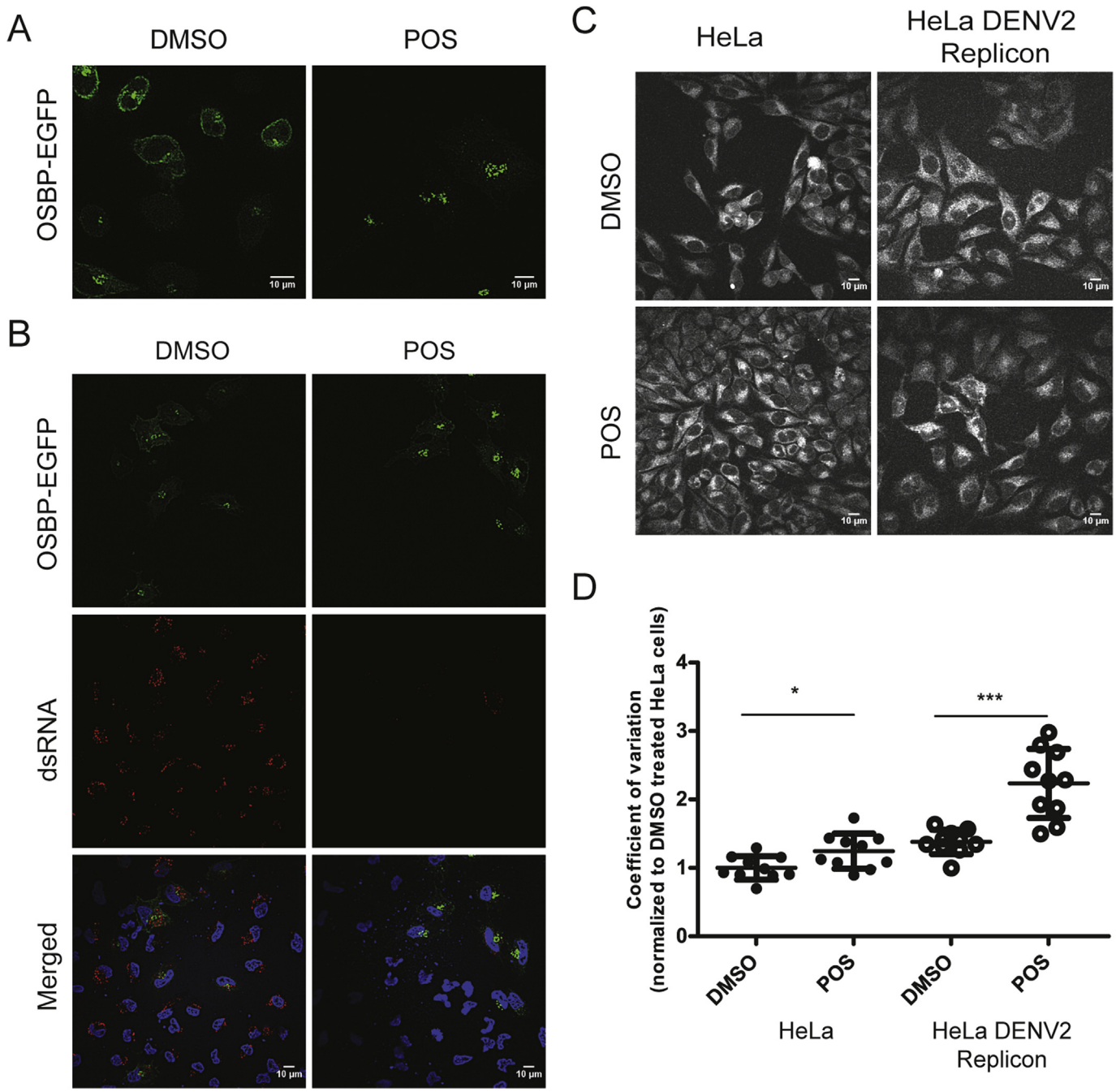

Fig. 5. Posaconazole induces redistribution of OSBP and cholesterol in DENV infected and non-infected cells. (**A** and **B**) Subcellular localization of EGFP-OSBP. (A) Non-infected HeLa cells and (B) HeLa DENV2 replicon cells were transfected with an expression plasmid encoding an OSBP-EGFP fusion protein, and at 48 h post transfection, treated with 10 µM POS or DMSO for 1 h. Cells in (B) were stained with antibodies against dsRNA. (**C**) HeLa and HeLa DENV2 replicon cells were treated with 10 µM POS or DMSO for 1 h before staining with filipin to visualize cholesterol. Images were taken on a confocal laser scanning microscope and processed in FIJI (A and B; for unprocessed images, see Supplemental Figs. 5B and C). Scale bars correspond to 10 µm. (**D**) Signal intensity of filipin was quantified in the cytoplasm of 10 cells for each condition from the experiment in panel (C) and mean and standard deviations were used to calculate the coefficient of variation (Cv) as a measure of granularity. Low Cv values reflect even distribution of cholesterol across the cell, whereas high Cv values reflect uneven distribution across the cell. Statistical significance between DMSO and POS treated cells was assessed with unpaired *t* tests. *, $P < 0.05$; **, $P < 0.01$; ***$P < 0.001$.

Strating et al., 2015). The functions of ORP2 and ORP11 are not fully resolved. ORP11 lacks a known ER targeting signal and localizes on Golgi membranes and late endosomes and was proposed to mediate non-vesicular lipid trafficking between these compartments (Zhou et al., 2010). ORP2, unlike OSBP and ORP11, does not possess a pleckstrin homology (PH) domain, which acts as a phosphoinositide binding domain in other ORP family members. Yet, ORP2 seems to regulate cellular sterol homeostasis and has been proposed to have a role in intracellular cholesterol trafficking, endocytosis, and neutral lipid metabolism (Hynynen et al., 2005, 2009; Laitinen et al., 2002). The precise functions of these ORPs in the DENV replication cycle remain to be studied.

In conclusion, we identified ITZ and POS as specific inhibitor of DENV RNA replication by targeting OSBP. Together with recent reports (Albulescu et al., 2015, 2017; Strating et al., 2015), our work establishes antifungal triazoles as a platform for the development of broadly

active antiviral drugs and identifies OSBP as an important host factor for DENV replication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.antiviral.2018.06.017.

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