



Fatty acid synthase and stearoyl-CoA desaturase-1 are conserved druggable cofactors of Old World *Alphavirus* genome replication

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ABSTRACT

Chikungunya virus (CHIKV) is a rapidly emerging mosquito-borne RNA virus that causes epidemics of debilitating disease in tropical and sub-tropical regions with autochthonous transmission in regions with temperate climate. Currently, there is no licensed vaccine or specific antiviral drug available against CHIKV infection. In this study, we examine the role, in the CHIKV viral cycle, of fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD1), two key lipogenic enzymes required for fatty acid production and early desaturation. We show that both enzymes and their upstream regulator PI3K are required for optimal CHIKV infection. We demonstrate that pharmacologic manipulation of FASN or SCD1 enzymatic activity by non-toxic concentrations of cerulenin or CAY10566 decreases CHIKV genome replication. Interestingly, a similar inhibitory effect was also obtained with Orlistat, an FDA-approved anti-obesity drug that targets FASN activity. These drugs were also effective against Mayaro virus (MAYV), an under-studied arthritogenic Old world *Alphavirus* endemic in South American countries with potential risk of emergence, urbanization and dispersion to other regions. Altogether, our results identify FASN and SCD1 as conserved druggable cofactors of *Alphavirus* genome replication and support the broad-spectrum activity of drugs targeting the host fatty acids metabolism.

1. Introduction

Alphaviruses are widespread arthropod-borne viruses responsible for several medically important emerging diseases. Their impact on human health has been illustrated in the last decade by the rapid spread of the Chikungunya virus (CHIKV) that causes an acute transient febrile arthralgic illness, and may lead to chronic incapacitating articular disease (Burt et al., 2017). At the beginning of the 21st century, CHIKV re-emerged in East Africa and spread to countries neighboring the Indian Ocean before invading India, South-Eastern Asia, Caribbean Islands and South America, with autochthonous transmission episodes also reported in Europe (Pezzi et al., 2019). Over the last 15 years, CHIKV has caused at least 5 million autochthonous cases in > 40 countries. CHIKV belongs to the Semliki Forest virus sero-complex in Old World *Alphaviruses*. In this phylogenetic clade, CHIKV genome displays up to 40% nucleotide divergence with Mayaro virus (MAYV) (Lavergne et al., 2006). First isolated in 1954 in Trinidad and Tobago, MAYV generates a

self-limiting acute viral disease that may evolve towards chronic arthralgia very similar to CHIKV (Anderson and French, 1957; Causey and Maroja, 1957). While responsible for sporadic and small outbreaks in south and central American countries around the Amazon basin (Azevedo et al., 2009; Izurieta et al., 2011; LeDuc et al., 1981; Neumayr et al., 2012; Schmidt et al., 1959; Talarmin et al., 1998), the recent detection of MAYV in Brazil and Haiti raises growing concerns about its potential to spread to other regions similar to CHIKV in the past (Lednický et al., 2016; Mackay and Arden, 2016). Currently, there is no vaccine against any of these *Alphaviruses* and no specific FDA-licensed drug for routine use is available. The limited efficacy of therapies tested so far for the management of CHIKV disease, including ribavirin (Ravichandran and Manian, 2008), the anti-malaria drug chloroquine (Roques et al., 2018) and corticosteroids (Javelle et al., 2015) points at the urgent need for basic antiviral research to identify the first therapeutic targets and molecules effective against *Alphaviruses*.

Cell membranes are critical for almost all steps of (+)RNA virus life

Abbreviations: CHIKV, Chikungunya virus; MAYV, Mayaro virus; FASN, fatty acid synthase; SCD1, stearoyl-CoA desaturase; PI3K, phosphatidylinositol 3-kinase; nsP3, non structural protein 3

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cycle. The exploitation of membranes for initial interactions of the virion with the host cell, penetration and fusion of the viral particle, or for assembly and budding of enveloped progeny virions has long been demonstrated (Altan-Bonnet, 2017). More recently, a key role has been assigned to cell membranes during the replication phase of (+)RNA viruses. This step generally occurs in host membrane-derived replication organelles whose creation and appropriate composition is pivotal for genome replication (Zhang et al., 2019). Remodeling the cell membranes to create autophagosomes, despite being reported as having either pro- or antiviral function according to the viral species, has also found critical for virus replication (Jackson, 2015). Being the major components of membranes, lipids therefore play active roles in the virus life cycle. As such, lipids and associated metabolism are attractive targets for the development of antiviral therapeutics.

Fatty acids are basic building blocks for the majority of cellular lipids and are therefore a source of components necessary for increased membrane production. The core reaction of fatty acid synthesis is catalyzed by the fatty acid synthase (FASN), a homodimeric enzyme that condensates one molecule of acetyl-CoA with seven molecules of malonyl-CoA to generate the 16-carbon saturated fatty acid palmitate. Starting from free fatty acids, stearoyl-CoA desaturase (SCD1) also known as Δ -9-desaturase, catalyzes the biosynthesis of mono-unsaturated fatty acids (MUFAs) used as precursors for the synthesis of various lipids including phospholipids, triglycerides and cholesteryl esters. Once incorporated in membranes, fatty acids, and its derivatives, depending on the length and saturation degree of their hydrocarbon tail, exert a structural effect on membrane fluidity, permeability, and flip-flop dynamics among others, and also alter the function of integral membrane proteins (Bond et al., 2016). Both FASN and SCD1 lipogenic enzymes are controlled by the sterol regulatory element-binding protein (SREBP) family members, the master transcriptional factors placed under control of the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling axis (Furuta et al., 2008). Recently, *Alphaviruses* were reported to manipulate the PI3K/AKT/mTOR signaling pathway through the presence of conserved sequences in virus-encoded nonstructural protein 3 (nsP3), with consequences on their host nucleotide, amino acid and lipid metabolisms (Mazzon et al., 2018). According to this information, this study was therefore conceived to explore the functional role of FASN and SCD1 during Old World *Alphavirus* infection and to investigate their relevance as therapeutic targets for antiviral purpose. Using RNA interference and pharmacological drugs, we demonstrate that FASN and SCD1 expression and enzymatic activity are required for optimal CHIKV genome replication. We demonstrate that their proviral function is conserved for MAYV, thereby reporting among the very first host co-factors for this poorly studied pathogen. Finally, we provide evidence that the US Food and Drug Administration (FDA)-approved anti-obesity drug Orlistat, an inhibitor of FASN activity is active against CHIKV and MAYV infection. Altogether, these data point at the intricate relationship existing between arthritogenic Old World *Alphaviruses* and fatty acid metabolism in their vertebrate host and highlight the therapeutic potential of targeting fatty acid biosynthesis and desaturation to limit the multiplication of a broad range of *Alphaviruses* in human cells.

2. Material and methods

2.1. Cells

HEK293T cells (ATCC number ACS-4500) used for infection experiments and Vero cells (ATCC number CCL-81) used for titration of the CHIKV and MAYV strains were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fischer Scientific) supplemented with penicillin and 10% fetal calf serum (FCS, Lonza) and grown at 37 °C in a 5% CO₂ atmosphere. The C6/36 mosquito cell line, derived from *A. albopictus*, was grown at 28 °C and 5% CO₂ in Minimal Essential Medium (MEM) (Sigma) complemented with 10% inactivated FCS and

1% antibiotics. Cell viability was measured using the Cell Titer 96 Aqueous one solution cell proliferation assay kit (Promega) according to the manufacturer's protocol.

2.2. Reagents and antibodies

Quercetin, cerulenin and Orlistat were purchased from Sigma Aldrich. CAY10566 was from Abcam. Drugs were diluted in DMSO. Appropriate concentration of DMSO was used in control experiments. Antibodies against FASN, SCD1 and GAPDH were purchased from Santa Cruz Biotechnologies, Inc. Anti-tubulin and anti-actin mAbs were from Sigma-Aldrich and Na⁺/K⁺ ATPase mAbs were purchased from Abcam. Rabbit polyclonal serum against nsP1 was a generous gift from Pr. Andres Merits (Tartu University, Estonia). Alexa-647-conjugated wheat germ hemagglutinin (WGA) and secondary antibodies were from Thermo Fisher Scientific.

2.3. Viruses

The CHIKV reporter virus was produced from the pICRES1 full-length CHIKV genomic clone (LR2006_OPY1 strain) containing the renilla luciferase marker inserted into the region encoding nsP3 (Pohjala et al., 2011). The CHIKV-mCherry-377 was previously described (Kummerer et al., 2012). CHIKV RNA was transcribed *in vitro* from the SP6 or T7 promoter using the mMMESSAGE mMACHINE kit (Ambion-Life Technologies). RNA (1 µg) was then transfected with lipofectamine 2000 (Thermo Fisher Scientific) into 10⁵ HEK293T cells. The MAYV reporter virus was obtained from an infectious clone derived from the complete sequence of the TRVL 4675 strain with a nano-luciferase coding sequence inserted in the nsP3 gene and cloned under the control of the CMV promoter (Chuong et al., 2019). The virus was produced by transfection of the infectious clone in Vero cells. Forty-8 h after transfection, the supernatants were collected, filtered through a 0.45 µm membrane, and titered using plaque assay as previously reported (Bernard et al., 2010).

2.4. Plasmids and transfection

The nsP1 sequence was amplified by PCR using pCHIKV-LR-5'GFP (LR-OPY strain) as a template and cloned into the pEGFP-C1 plasmid as previously reported (Matkovic et al., 2018). CHIKV reporter transfection system (kindly provided by Andres Merits) was composed of the CMV-P1234 plasmid encoding a CMV-driven CHIKV nonstructural ORF and of the HSPolI-Fluc-Gluc pseudogenome template containing a Firefly luciferase and a Gaussia luciferase reporter genes under control of CHIKV genomic and subgenomic promoters respectively (Utt et al., 2019). Plasmids were transfected using JetPei reagent (Polyplus Transfection). Twenty-four hrs post-transfection, replication was monitored using the Dual-Glo luciferase assay kit (Promega) and a Spark luminometer (Tecan). Values were normalized according to protein content in the sample determined using the BCA Assay (Pierce).

2.5. RNA interference

siRNA targeting FASN or SCD1 mRNA were purchased from Integrated DNA technologies and Sigma-Aldrich, respectively. Non-targeting siRNA were from Dharmacon. Transfection was achieved using Interferin (Polyplus Transfection). At 48 h post-transfection, an aliquot of the cells was harvested to determine the silencing efficiency by Western blotting. The remaining cells were infected with CHIKV.

2.6. Cell fractionation and membrane flotation assays

Cells were incubated in hypotonic buffer (10 mM Tris/HCl [pH 7.4], 10 mM NaCl supplemented with protease inhibitors) for 10 min on ice and then lysed with a Dounce homogenizer (30–40 strokes). The lysates

were clarified by low-speed centrifugation at 1000g for 10 min. Post-nuclear supernatants were then adjusted to a final concentration of 500 mM NaCl and incubated for 30 min on ice. Cytosolic fraction (supernatant, S25) and membrane fraction (pellet, P25) were obtained by ultracentrifugation at 25,000 g for 20 min in an MLA-150 rotor (Beckman Coulter). P25 samples were solubilized in lysis buffer composed of 1% Brij 96 in 20 mM Tris-Cl pH 7.5 before analysis.

2.7. Immunoblotting

Samples were separated by SDS-PAGE, then transferred to a PVDF membrane (Hybond, Amersham). Membranes were blocked against nonspecific binding by using 5% non-fat milk in PBS, 0.1% Tween 20, and probed with appropriate primary antibodies. After washings in PBS containing 0.1% Tween 20, the membranes were incubated with HRP-conjugated secondary antibodies. After final washes, the detection was performed by incubating the membranes with Luminata Forte (Merck) and then image acquisition was made using a Chemidoc (Bio-Rad). Band intensity has been determined utilizing ImageJ software.

2.8. Immunofluorescence microscopy and image analysis

Cells grown on glass coverslips were washed with PBS and then fixed with 4% paraformaldehyde in PBS (Sigma Aldrich) for 10 min. Then, the cells were permeabilized with 0.1% Triton-X100 in PBS and blocked for 30 min with PBS containing 0.2% bovine serum albumin. Incubation with primary antibody was performed at 37 °C for 1 h at room temperature and secondary reagents were added for 30 min at 37 °C. DAPI (Sigma-Aldrich) was used to stain the nucleus. Staining with WGA-Alexa 647 was performed by incubation at room temperature for 10 min. After final washes, coverslips were mounted with Prolong Gold antifade mounting media (Thermo Fisher Scientific). Images were acquired using a Leica SP5-SMD scanning confocal microscope equipped with a 63 × , 1.4 numerical aperture Leica Aplanochromat oil lense at the Montpellier Resources Imaging platform.

2.9. Statistical analysis

All of the analyses (unpaired Student's t-test) were performed using GraphPad Prism version 6 (GraphPad Software Inc.). * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$; **** $p < 0.0001$.

3. Results

3.1. PI3K activity is essential for CHIKV infection

The PI3K/AKT/mTOR signaling axis is essential for the control of cell proliferation, protein translation and anabolic activities within the cell. Under nutrient abundance, mTOR activation stimulates aerobic glycolysis, and *de novo* lipid synthesis especially through the control of FASN expression (Fig. 1) (Furuta et al., 2008). Starting from the recent observation that Ross River virus (RRV) and Semliki Forest virus (SFV), both grouped with Old World arthritogenic *Alphaviruses*, enhance glycolysis and biosynthesis of C16:0 (palmitate) and C18:0 (stearate) fatty acids in their host by activating the PI3K/AKT/mTOR pathway (Mazzon et al., 2018), we investigated the functional importance of this signaling axis in CHIKV infection. For this purpose, we took advantage of the plant flavonoid quercetin (3,3',4',5,7-pentahydroxyflavone), a well-characterized pharmacological PI3K inhibitor (Agullo et al., 1997). Quercetin, used in a concentration range that was controlled to have no toxic effect (Supplementary Fig. 1A), was added to HEK293T cells 30 min before challenge with a CHIKV reporter virus encoding a luciferase gene inserted in nsP3 sequence (Fig. 2A; upper panel) (multiplicity of infection (MOI) = 1). After 24 h in culture, the infection was monitored by quantification of luciferase expression in cell extracts. In these conditions, quercetin showed a concentration-dependent

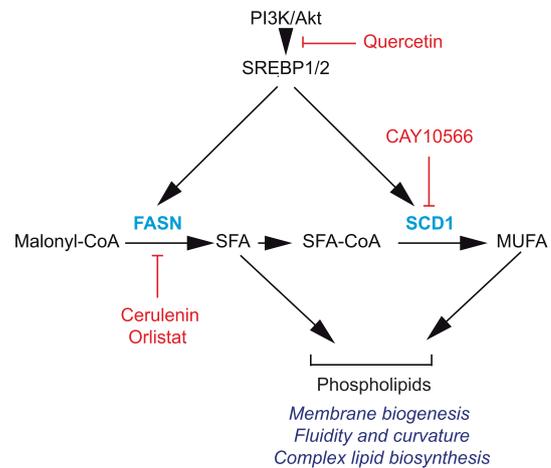


Fig. 1. Fatty acid biosynthesis pathway and inhibitory drugs used in this study.

inhibitory effect on CHIKV infection (Fig. 2A; lower panel). Host lipid metabolism is expected to be pivotal in the various steps of enveloped virus's life cycle. To discriminate between inhibition of viral entry/fusion or subsequent steps in the CHIKV replication cycle, we next used a CHIKV transreplication system (depicted in Fig. 2B; upper panel). Cells were transfected for 2 h with equal amounts of CMV-P1234 and HSPoI-Fluc-Gluc plasmid to reproduce CHIKV post-entry replication (Utt et al., 2019), before the addition of increasing concentrations of quercetin to the culture medium. In these conditions, quercetin displayed a dose-dependent inhibitory effect on virus-encoded luciferase activities (Fig. 2 B, C), supporting that inhibition occurred at the genome replication step. These results were consistent with post-infection experiments performed using a replicating CHIKV (Supplementary Fig. 2A). Altogether, these results, therefore, designate the PI3K/AKT/mTOR signaling axis as essential for CHIKV post-entry events.

3.2. Fatty acid synthase regulates CHIKV genome replication

The PI3K/Akt/mTORC1 signaling has been previously reported to be molecularly connected with FASN expression (Sande et al., 2002). Indeed, in our hands, quercetin-mediated PI3K targeting was able to reduce FASN protein levels in HEK293T cells (Fig. 2D). According to this information and to quercetin anti-CHIKV activity reported above, we explored the direct contribution of FASN in CHIKV genome replication. HEK293T cells were transfected with siRNA specific for FASN or with a non-targeting control duplex. After 48 h, FASN expression was monitored by immunoblotting, showing that FASN protein level was reduced by over 70% by targeting FASN siRNA (Fig. 3A). Then, the cells were transfected with CMV-P1234 and HSPoI-Fluc-Gluc plasmids. In these conditions, reduction of FASN protein level correlated with a 3-fold reduced luciferase activities (Fig. 3B), attesting that FASN expression is required for CHIKV genome replication. Mammalian FASN is a multifunctional enzyme with 7 different catalytical activities that catalyzes the reaction leading to the production of palmitate, a 16-carbon fatty acid. Activity of the N-terminal β -ketoacyl-synthase and C-terminal thio-esterase in FASN can be inhibited using cerulenin antibiotic ((2R)(3S)-2,3-epoxy-4-oxo-7,10-dodecadienolamide) and the FDA-approved anti-obesity drug Orlistat (tetrahydrolipstatin), respectively (Schcolnik-Cabrera et al., 2018) (Fig. 1). Using these drugs, and the CHIKV transreplication system, we next explored whether FASN enzymatic activities are required for genome replication. CHIKV-encoded luciferase activities were dramatically inhibited by cerulenin at concentrations above 15 μ M (Fig. 3) that were non-toxic (Supplementary Fig. 1). A similar effect (> 80% inhibition) was also observed in the presence of 50 or 100 μ M Orlistat (Fig. 3E and F). Despite reducing cell viability by around 20%, this dramatic effect clearly attests of

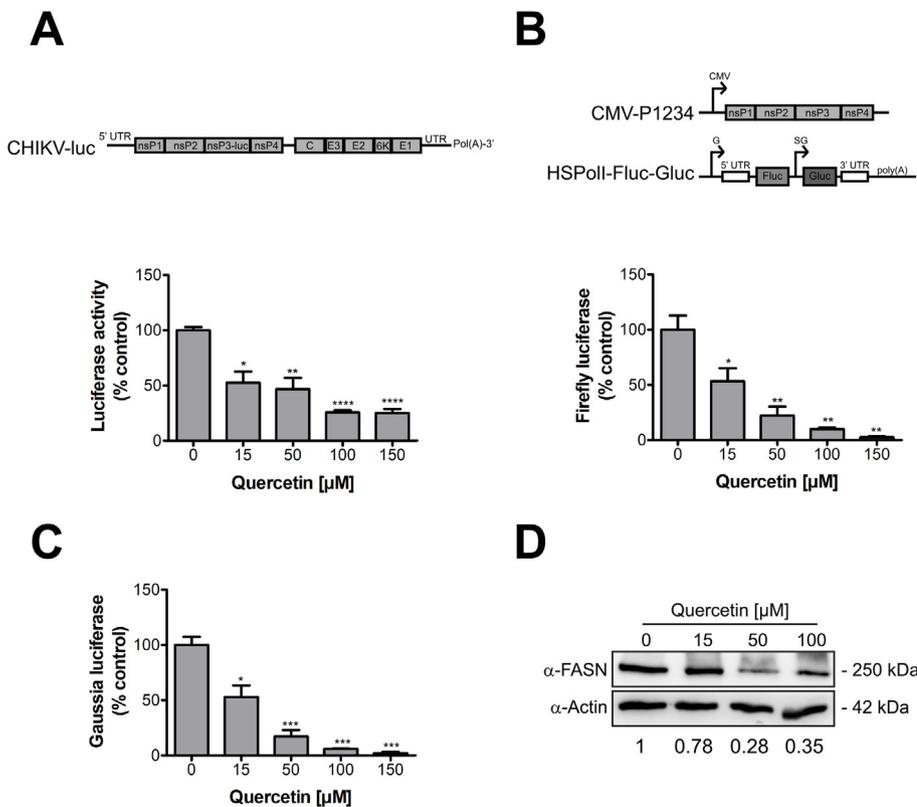


Fig. 2. Pharmacological inhibition of the PI3K/Akt/mTOR/FASN axis and consequences on CHIKV infection. (A) HEK293T cells were pre-incubated with the indicated concentrations of quercetin for 30 min before challenge with a CHIKV-Luc reporter virus (MOI = 1). After 24 h, infection was monitored by quantification of virus-encoded luciferase activity in the cell lysate. (B) (C) Cells were transfected with plasmids depicted in (B) and treated 2 h post-transfection with the indicated concentrations of quercetin. After 24 h, viral genome replication was determined by quantification of Firefly and Gaussia luciferase activities in the cell lysates. Experiments were performed in triplicate and values are expressed as a percentage of the control condition. (D) FASN protein level was monitored in cells incubated for 24 h in the presence of the indicated concentration of quercetin. Actin expression is shown as loading control. Molecular sizes are indicated on the right and relative FASN to actin band intensity is shown below each lane.

Orlistat potent anti-CHIKV activity. Interestingly, both drugs were also effective at inhibiting CHIKV infection in C6/36 cells (Supplementary Fig. 3). Altogether these results support that expression and enzymatic activity of FASN are required for optimal CHIKV genome replication. This result was further validated using CHIKV-infected cells treated with cerulenin or Orlistat after virus exposure (Supplementary Figs. 2B and C).

3.3. Stearoyl-CoA desaturase 1 expression and enzymatic activity are required for CHIKV genome replication

Starting from saturated fatty acids, preferentially palmitoyl-CoA and stearoyl-CoA, the reaction product of FASN, SCD1 introduces a double bond between carbons 9 and 10, to yield palmitoleoyl-CoA and oleoyl-CoA, respectively. To evaluate the impact of fatty acid desaturation on the CHIKV viral cycle, SCD1 was knocked down using siRNA, leading to the almost complete inhibition of SCD1 expression (Fig. 4A). When these cells were transfected with the CHIKV plasmids, expression of the luciferase reporter genes was dramatically reduced as compared to cells transfected with non-targeting control siRNAs (Fig. 4B). SCD1 activity can be manipulated by CAY10566 (3-[4-(2-chloro-5-fluorophenoxy)-1-piperidinyl]-6-(5-methyl-1,3,4-oxadiazol-2-yl)-pyridazine), a potent inhibitor of fatty acyl-CoA conversion to monounsaturated long-chain fatty acids (Liu et al., 2007). Taking advantage of this property, we questioned the contribution of SCD1 enzymatic activity in CHIKV replication. In the presence of increasing concentrations of CAY10566, CHIKV-encoded luciferase activities were significantly decreased up to 50%, indicating that SCD1 activity is required for optimal CHIKV genome replication (Fig. 5C). Altogether, these results identify SCD1 as an essential novel cofactor of CHIKV whose enzymatic activity is required for genome replication events and therefore demonstrate that desaturation of fatty acids is pivotal to post-entry events.

3.4. FASN and SCD1 are conserved cofactors of Old World Alphavirus replication

While reported at risk of spreading to new environments, the biology of MAYV is almost unknown and no antiviral molecule has been identified against this virus. We therefore investigated the inhibitory effect of lipid metabolism inhibitors on MAYV *in vitro*. Because this virus remains understudied, we first established that it actively replicates in human epithelial HEK293T cells (Supplementary Fig. 4). A parallel infection with a MAYV-Luc reporter virus (Fig. 5A) and a CHIKV-Luc (MOI of 1) demonstrated that after 24 h in culture, they generated a comparable luciferase activity in the cell lysate. Using this cell model, MAYV infection was investigated in the presence of quercetin, cerulenin, Orlistat and CAY10566 to determine its requirement for PI3K, FASN and SCD1 activity respectively. Inhibitors were added either 1.5 h before (Fig. 5B–E) or 1.5 h after the viral challenge (Fig. 5F–I). As previously observed for CHIKV, each inhibitor induced a dose-dependent inhibitory effect on MAYV-directed luciferase reporter gene expression independent of the fact that it was added before or after viral challenge. These results, therefore, parallel those obtained with CHIKV, indicating that PI3K, FASN and SCD1 have conserved proviral activity during genome replication of distantly related Old World *Alphaviruses*.

3.5. Outcome of FASN and SCD1 in Alphavirus-replicating cells

We next investigated whether FASN and SCD1 protein level are manipulated in *Alphavirus*-infected cells. Since antibodies are lacking for MAYV, we used CHIKV as a model for subsequent studies. First, FASN and SCD1 expression was analyzed overtime upon CHIKV infection using immunoblotting experiments. As shown in Fig. 6A, FASN and SCD1 protein levels remained almost unchanged until 16 h of infection. At this time, a slight decrease was observed reflecting CHIKV toxicity. FASN was previously reported to be recruited to replication foci formed CHIKV-infected (Karlas et al., 2016), a result that was confirmed by us

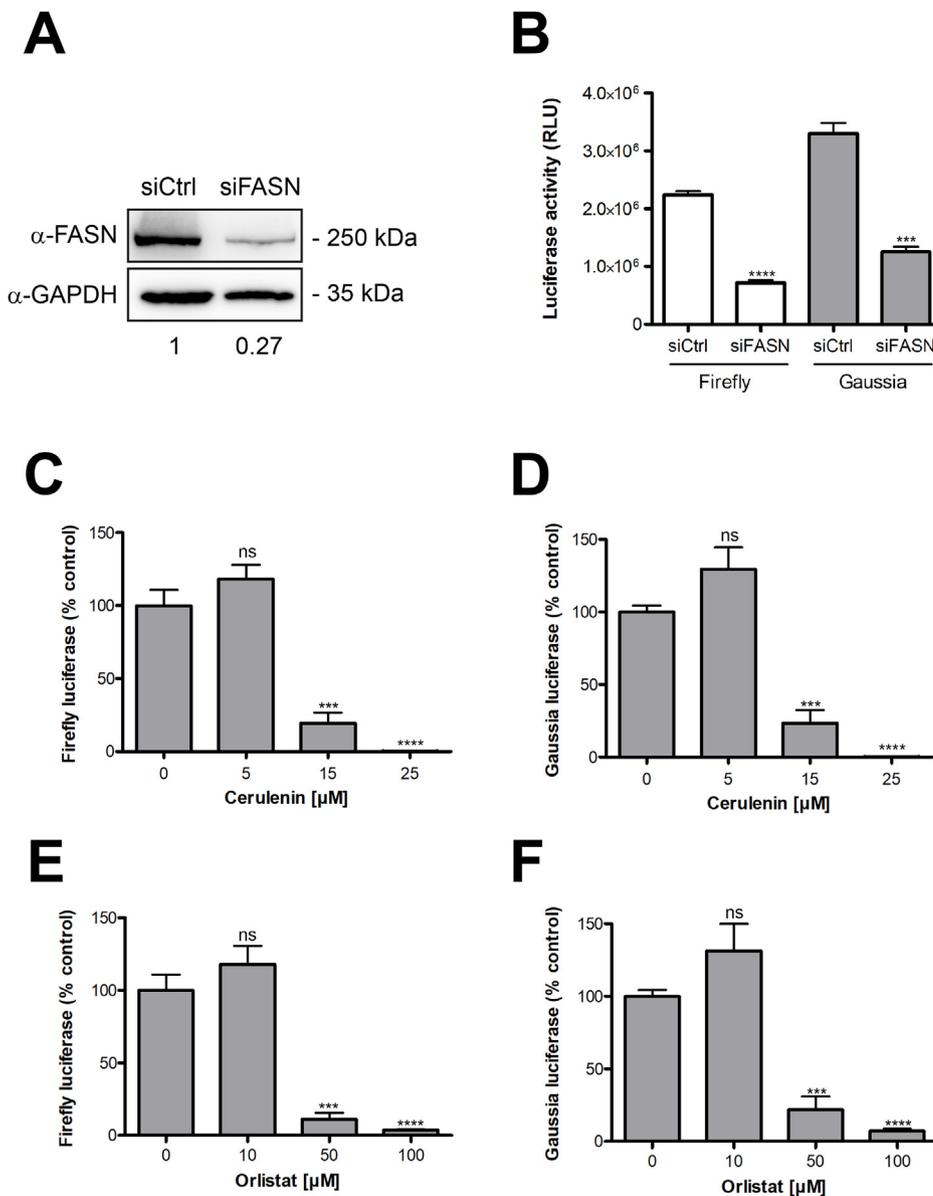


Fig. 3. FASN expression and enzymatic activity are required for CHIKV genome replication. (A) HEK293T cells transfected with siRNA against FASN (siFASN) or non-targeting siRNA (siCtrl) were processed for immunoblot analysis using anti-FASN antibodies. Anti-GAPDH antibodies were used as a loading control. Molecular sizes are indicated on the right. Relative FASN to GAPDH band intensity is indicated below each lane. (B) Cells from (A) were transfected with CHIKV transreplication plasmids. Firefly and Gaussia luciferase activities in the cell lysates were monitored after 24 h in culture. (C–F) Increasing concentrations of cerulenin (C, D) or Orlistat (E, F) were added to cells transfected with CHIKV transreplication plasmids. CHIKV genome replication was determined after 24 h by quantification of virus-encoded Firefly and Gaussia luciferase activities in the cell lysates. Values, expressed as a percentage of the control condition, are means of triplicate experiments \pm SEM.

(data not shown). We therefore investigated the consequences of CHIKV infection on SCD1 subcellular localization. In control cells, SCD1 staining accumulated in the cytoplasm (Fig. 6B) with a distribution coherent with the previously reported endoplasmic reticulum localization of this protein (Nguyen et al., 2014). This pattern persisted in infected cells throughout the time of the infection experiment. No clear colocalization could be observed with the CHIKV non-structural proteins (nsPs) taking part to the replication complex. According to these results, FASN and SCD1 behave differently upon CHIKV infection, with FASN possibly recruited to replication complexes as previously reported (Karlas et al., 2016) while this could not be evidenced for SCD1. These differences may reflect difference in the proviral function of these enzymes in *Alphavirus* replication cycle.

3.6. Consequences of fatty acid metabolism manipulation on CHIKV nsP1 binding to host membranes

Alphavirus genome replication is ensured by the four nsPs complexed with the RNA genome in bulb-shaped membranous organelles derived from the host plasma membrane (Spuul et al., 2011). In these compartments, the replication machinery is tightly anchored to the

lipid bilayer thanks to nsP1, the viral methyl/guanylyltransferase. Membrane anchoring is mediated through a central α -helix (position 244–263 in the CHIKV protein) and reinforced by the palmitoylation of conserved cysteines at the C-terminus (position 417–419 in CHIKV nsP1) (Peranen et al., 1995; Utt et al., 2019). Both nsP1 palmitoylation and the host-encoded ZDHHC2 and ZDHHC19 palmitoyl transferases involved in nsP1 acylation are required for CHIKV infectivity (Utt et al., 2019; N. Zhang et al., 2019). Given the pivotal role of FASN in palmitate synthesis, we investigated the consequences of FASN inhibition on nsP1 membrane binding capacity. HeLa cells expressing a CHIKV GFP-nsP1 protein were left untreated or maintained in the presence of cerulenin or Orlistat at concentrations that were effective against CHIKV replication. In control cells, confocal microscopy imaging detected the GFP-nsP1 fluorescence colocalized with Alexa647-conjugated wheat germ hemagglutinin (WGA) that stains the cell membranes (Fig. 7A). Cell fractionation, clearly evidenced the presence of the GFP-nsP1 protein in the membranous (P25) fraction that also stained positive for the Na^+/K^+ ATPase membrane-associated protein. GFP-nsP1 was in contrast detected at low level in the cytosolic (S25) fraction (Fig. 7B). In the presence of cerulenin, the GFP-nsP1 protein detected by confocal microscopy was more diffuse in the cytoplasm

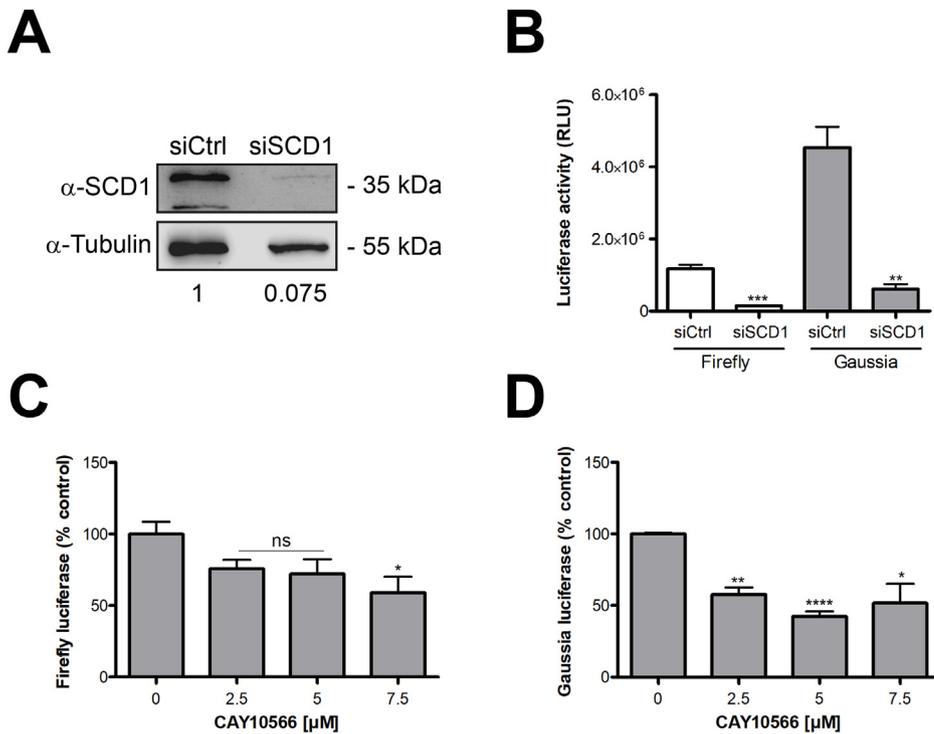


Fig. 4. Consequences of SCD1 inhibition on CHIKV genome replication. (A) HEK293T cells transfected with siRNA against SCD1 (siSCD1) or non-targeting siRNA (siCtrl) were processed for immunoblot analysis using anti-SCD1 antibodies. Anti-tubulin antibodies were used as a loading control. Molecular sizes are indicated on the right. Relative SCD1 to tubulin band intensity is indicated below each lane. (B) Cells from (A) were transfected with CHIKV transreplication plasmids. Firefly and Gaussia luciferase activities were monitored after 24 h in culture. (C–D) Increasing concentrations of CAY10566 were added to HEK293T cells transfected with CHIKV transreplication plasmids. After 24 h, CHIKV-encoded Firefly or Gaussia luciferase activities were determined in the cell lysates. Values are percentages of the DMSO control condition. Means of triplicate experiments ± SEM are shown.

(Fig. 7A). After cell fractionation, an increased proportion of this protein was also detected in the S25 sample and concomitantly slightly reduced in the P25 sample (Fig. 7B). This phenotype was further exacerbated starting from cells cultured in the presence of Orlistat. Therefore, cerulenin- and Orlistat-mediated inhibition of FASN activity and the expected associated decrease in availability of fatty acids in the

cell reduce nsP1 membrane association. According to these results, the reduced CHIKV genome replication generated by FASN inhibitors may partly reflect a decrease in nsP1 anchoring to membranes, a capacity required for optimal activity of CHIKV replication machinery as recently reported (Utt et al., 2019).

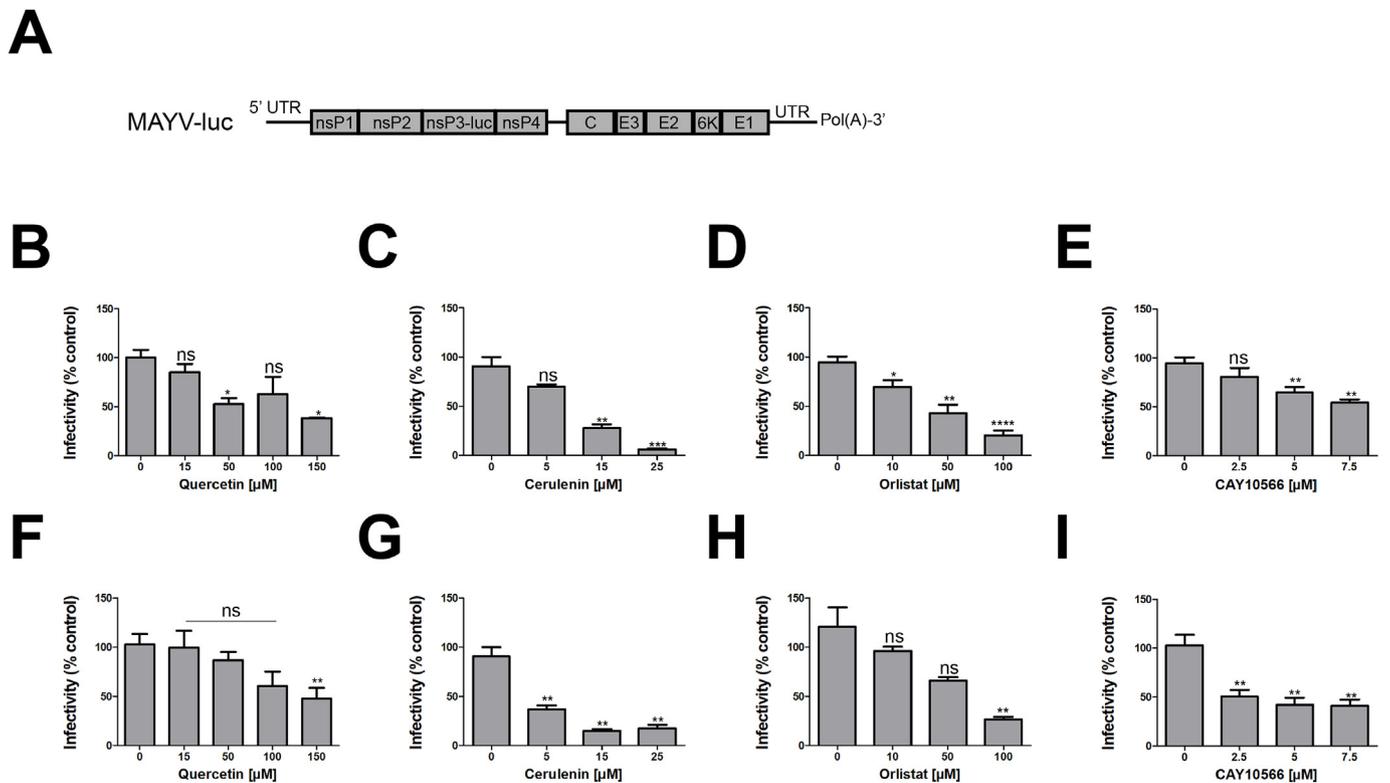


Fig. 5. FASN and SCD1 have proviral activity for MAYV replication. HEK293T cells were infected with the MAYV-luc reporter virus depicted in (A) (MOI = 1). The indicated concentrations of quercetin, cerulenin, Orlistat or CAY10566 were added to the cells either 1.5 h before (B–E) or 1.5 h after (F–I) viral challenge. Virus replication was determined by quantification of luciferase activity in cell extracts. Values (triplicates ± SEM) are expressed as a percentage of the control condition.

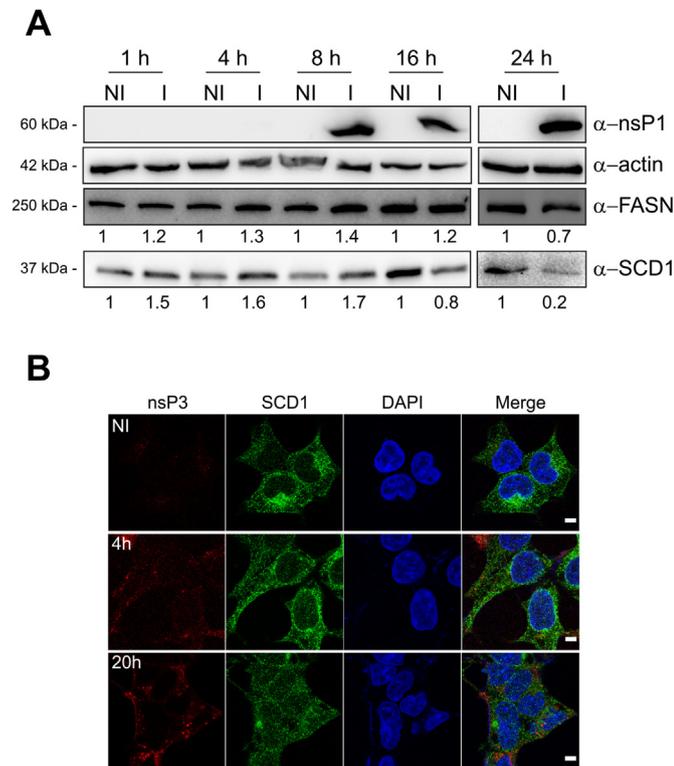


Fig. 6. Consequences of CHIKV infection on FASN and SCD1 expression and localization. (A) Cells infected for the indicated time with CHIKV (I) were subjected to immunoblotting with antibodies against FASN or SCD1. Viral replication in the culture was controlled using anti-nsP1 antibodies and anti-actin antibodies were used as a loading control. Lysate from non-infected cells (NI) cultured in the same conditions were run in parallel. Relative FASN and SCD1 expression level normalized to actin level in the sample is indicated for each condition. (B) Cells were infected with the CHIKV-mCherry-377 virus for the indicated time and labelled with anti-SCD1 antibodies and Alexa-488-conjugated secondary antibodies. Nuclei were stained with DAPI and the cells were analyzed by confocal microscopy. Non-infected cells (NI) are shown as control. Bars: 5 μ m.

4. Discussion

This study was conceived to question the interplay between CHIKV infection and the host fatty acid metabolism in human cells. Using RNA interference and pharmacological inhibitors, we show that FASN and SCD1 expression and activity are required for optimal CHIKV genome replication. FASN is pivotal for various RNA viruses including Respiratory Syncytial Virus (Ohol et al., 2015), West Nile Virus (Martin-Acebes et al., 2011), Astrovirus (Murillo et al., 2015), hepatitis C virus (HCV) (Tongluan et al., 2017; Yang et al., 2008) and dengue virus (DENV) (Huang et al., 2013; Tongluan et al., 2017). Its enzymatic activity regulates important metabolic processes. First, palmitate, the byproduct of FASN is required for post-translational protein acylation. Here, we show that cerulenin and Orlistat, two well known FASN inhibitors that reduce palmitate availability, decrease nsP1 membrane binding capacity. The role of nsP1 acylation in membrane anchoring of the *Alphavirus* replication complex has been long reported (Laakkonen et al., 1996). While dispensable for Sindbis virus infectious cycle (Ahola et al., 2000; Zusinaite et al., 2007), the conserved nsP1 acylated cysteines have been recently found critical for CHIKV replication (Utt et al., 2019). Moreover, FASN inhibition by C75 was previously reported to reduce CHIKV nsP1 palmitoylation (Zhang et al., 2019). Accordingly, cerulenin and Orlistat anti-CHIKV activity reported herein may partly result from the decreased nsP1 membrane binding capacity. Besides, it may also directly result from a reduced availability of FASN

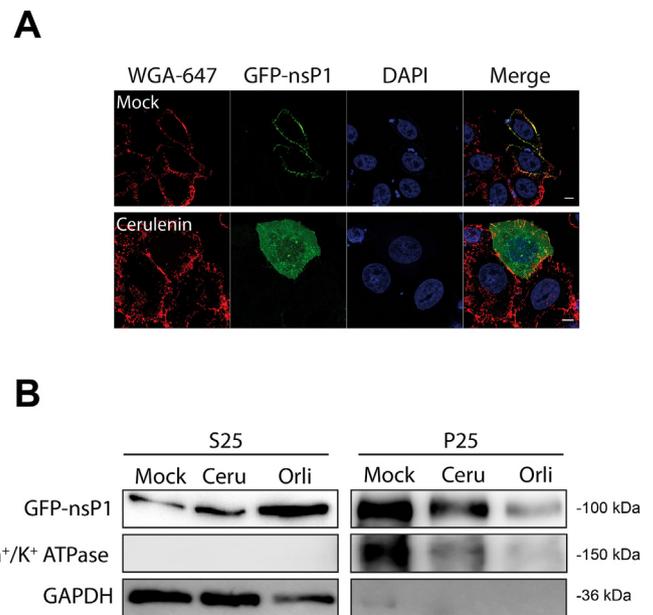


Fig. 7. FASN inhibitors decrease nsP1 membrane binding capacity. (A) HEK293T cells expressing the GFP-nsP1 protein, cultured in the presence of DMSO (Mock) or 15 μ M cerulenin were labelled with Alexa647-conjugated WGA, DAPI and processed for confocal imaging. Bars: 5 μ m. (B) GFP-nsP1 expressing cells cultured in the presence of DMSO (Mock) or 15 μ M cerulenin or 50 μ M Orlistat were subjected to cell fractionation. Cytosolic (S25) and membrane (P25) fractions were separated on SDS-PAGE and probed with anti-nsP1, anti-Na⁺/K⁺ ATPase and anti-GAPDH antibodies. Molecular sizes are indicated on the right.

reaction products, palmitate (16:0) and stearate (18:0), for complex lipid biosynthesis. By catalyzing their conversion into palmitoleate (16:1n-7) and oleate (18:1n-9), SCD1 regulates the saturated fatty acids to MUFA balance with consequences for ATP production via β -oxidation and energy storage in the form of triglycerides. It also regulates for the biosynthesis of phospholipid fatty-acyl chains, triglycerides and cholesterol esters pivotal for membrane extension, fluidity and curvature (Ralston and Mutch, 2015). Here, by using siRNA and taking advantage of the inhibitor CAY10566, we demonstrate that SCD1 expression and desaturase activity are required for CHIKV post-entry events thereby identifying SCD1 as a new CHIKV proviral factor. SCD1 has already been identified as an important cofactor for the replication of *Flaviviruses*, namely DENV (Hishiki et al., 2019), Zika virus and yellow fever virus (Gullberg et al., 2018) in mammalian cells. A screen performed to identify cellular factors and functions required for Brome mosaic virus (BMV) RNA replication in yeast also designated OLE1 the yeast homolog of SCD1 as a proviral factor for viral RNA synthesis. (Lee et al., 2001). For HCV, genome replication defect observed upon SCD-1 blockade was directly correlated with an alteration virus-induced membranous replication organelles as a consequence of modifications in membrane composition (Lyn et al., 2014; Nguyen et al., 2014). The exact function of SCD1 and MUFA in CHIKV post-entry replication events remains to be determined. Nevertheless, requirement for a functional SCD1 suggests that cerulenin and Orlistat antiviral effect not only results from the above discussed reduced nsP1 membrane binding capacity, but may also a reflect a decreased fatty acids availability for MUFA biosynthesis. This result therefore prompts the investigation of MUFA contribution in CHIKV life cycle. Interestingly, we observed a similar requirement for an enzymatically active FASN and SCD1 for MAYV genome replication as found for CHIKV. Despite a generally good conservation of virus-host interactions among Old world *Alphaviruses*, some differences have been reported (Thaa et al., 2015). Our results provide among the very first information on an understudied *Alphavirus* with high risk of emergence and suggest the possibility to transpose

some knowledge acquired from CHIKV.

Altogether these results provide evidence that Old World *Alphavirus* genome replication is tightly interconnected with host lipid metabolism. This observation may open novel avenues for the elaboration of antiviral strategies with broad spectrum application in this group of viruses. *De novo* lipogenesis is currently considered an emerging therapeutic target (Escribá, 2017). More specifically, FASN activity can be modulated through pharmacological drugs that are well tolerated in humans. Mainly, Orlistat, a derivative of lipstatin, approved by the FDA (Ballinger and Peikin, 2002), active against CHIKV and MAYV in our hands, displays antiviral activity against Cocksackievirus B3 (Ammer et al., 2015), HCV (Nasheri et al., 2013) and DENV (Tongluan et al., 2017). The wide spectrum antiviral properties of this anti-obesity drug suggests its possible repurposing for anti-infectious applications. The recent development of liver-targeted SCD1 inhibitors (Lachance et al., 2012) and of the oral SCD1 inhibitor Aramchol, evaluated in patients with primary nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (Ajmera et al., n.d.; Safadi et al., 2014) further supports the possibility to target lipogenic enzymes as a promising avenue for the elaboration of antiviral strategy applicable to medically important *Alphaviruses*.

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Declaration of competing interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2019.104642>.

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