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Stearoyl-CoA desaturase-1 is required for flavivirus RNA replication

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ABSTRACT

Dengue virus (DENV) is the most prevalent human arthropod-borne virus and causes severe problems worldwide, mainly in tropical and sub-tropical regions. However, there is no specific antiviral drug against DENV infection. We and others recently reported that stearoyl-CoA desaturase-1 (SCD1) inhibitor showed potent suppression of hepatitis C virus replication. In this study, we examined the impact of SCD1 on DENV replication. We found that SCD1 inhibitors (MK8245 and #1716) dramatically suppressed DENV replication in a dose-dependent manner without cytotoxicity. This anti-DENV efficacy was observed against all four DENV serotypes and other flaviviruses, including Zika virus and Japanese encephalitis virus. A subgenomic replicon system of DENV was used to confirm that SCD1 inhibitor suppressed viral RNA replication. Interestingly, exogenous supplementation of unsaturated fatty acids resulted in recovery of the DENV titer even in the presence of SCD1 inhibitor, suggesting that fatty acid biosynthesis contributes to DENV genome replication. These findings indicate that SCD1 is a novel host factor required for DENV replication, and SCD1 inhibitor is a potential candidate for treating dengue fever.

Dengue virus (DENV) is transmitted to humans by *Aedes* mosquitoes and causes dengue fever and dengue hemorrhagic fever (Gubler, 1998). DENV is mainly found in tropical and sub-tropical countries and is considered a major public health issue in more than 100 countries (WHO, 2018). Recent estimates suggest that more than 390 million dengue infections with nearly 96 million clinical manifestations occur annually (Bhatt et al., 2013).

DENV belongs to the family *Flaviviridae*, which includes three genera: *Flavivirus*, *Pestivirus*, and *Hepacivirus*. Members of the *Flavivirus* genus include the four serotypes of DENV (DENV-1 to 4), Zika virus (ZIKV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV). All are enveloped viruses ~50 nm in diameter, containing a single-stranded positive-sense genomic RNA (gRNA) of approximately 11 kb packaged as a nucleocapsid (Lindenbach et al., 2007). A single long open reading frame in the gRNA encodes a polyprotein that is processed by viral and host cellular proteases into three structural (capsid [C], precursor membrane [prM], and envelope [E]) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form the viral particles. NS proteins are necessary for viral genome replication and evasion of the host immune response (Chambers et al., 1990; Chen et al., 2017).

Development of anti-DENV agents has been focused on the targeting of both viral factors (directly acting antivirals; DAAs) and host cellular

Abbreviations: CC_{50}, cytotoxicity concentration; DENV, dengue virus; EC_{50}, half-maximal effective concentration; FAS, fatty acid synthesis; gRNA, genomic RNA; HCV, hepatitis C virus; hpi, hours post-infection; JEV, Japanese encephalitis virus; MOI, multiplicity of infection; MPA, mycophenolic acid; NS, non-structural; SCD1, stearoyl-CoA desaturase-1; ZIKV, Zika virus

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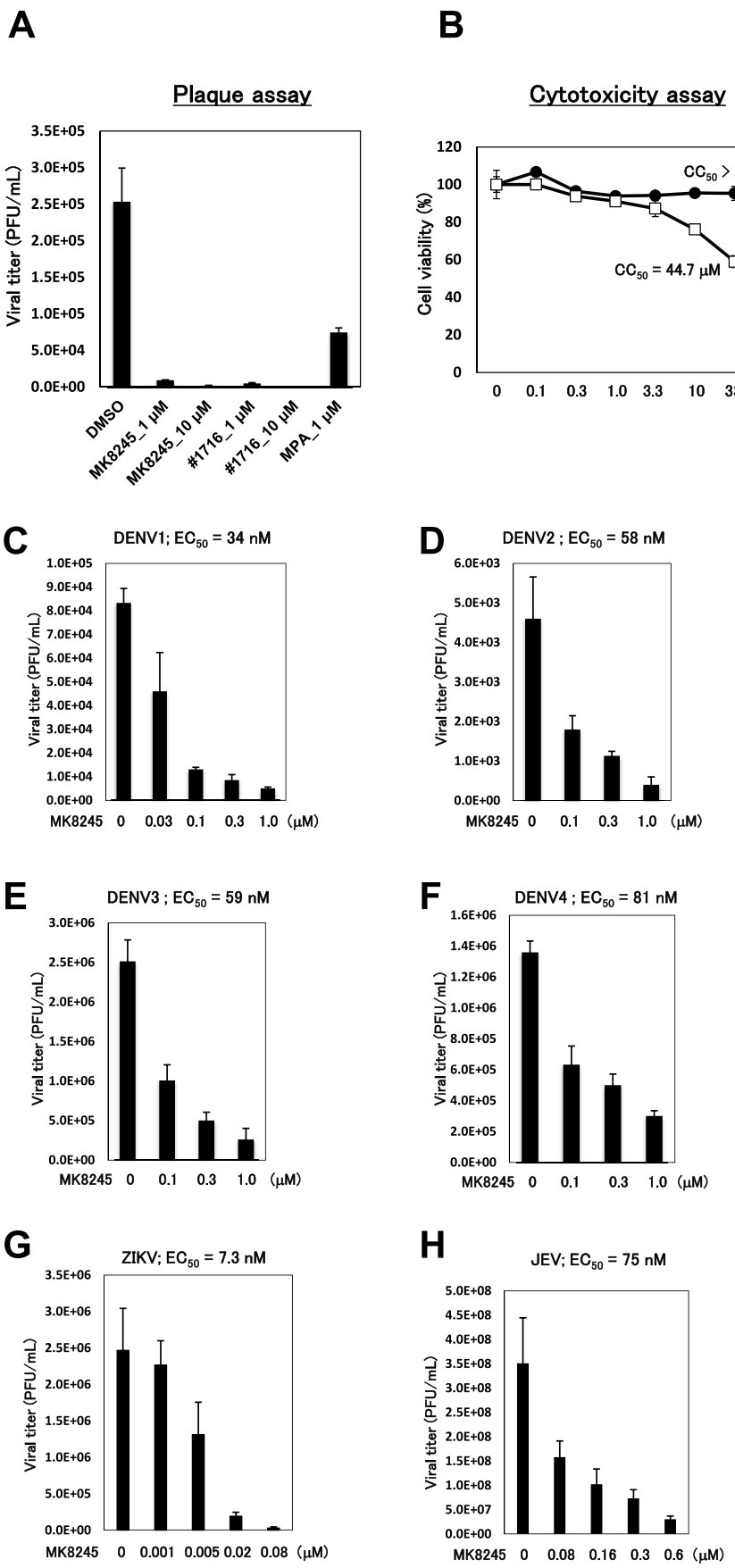

Fig. 1. SCD1 inhibitor inhibits replication of all DENV serotypes and other flaviviruses. (A) Huh7 cells were infected with DENV-1 (02-20) at an MOI of 0.01 in the presence of SCD1 inhibitor MK8245 or #1716 (1 μM or 10 μM). Cell culture supernatants were collected at 72 hpi, and viral titers were determined by plaque assay. As a positive control, anti-DENV compound mycophenolic acid (MPA) was used at 1 μM. (B) Huh7 cells were cultured with the SCD1 inhibitor MK8245 (closed circle) or #1716 (open square). After 72 h, cell proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay. Data were normalized to a DMSO control (0 μM of SCD1 inhibitor). Huh7 cells were infected with DENV-1 (C), DENV-2 (D), DENV-3 (E), DENV-4 (F), ZIKV (G), and JEV (H) at an MOI of 0.01 in the presence of SCD1 inhibitor (MK8245). Cell culture supernatants were collected at 72 hpi, and viral titers were determined by plaque assay. The half-maximal effective concentration (EC_{50}) and cytotoxicity concentration (CC_{50}) were calculated using the Reed and Muench method (Reed and Muench, 1938). Each data point represents the mean ± standard deviation of triplicate experiments.

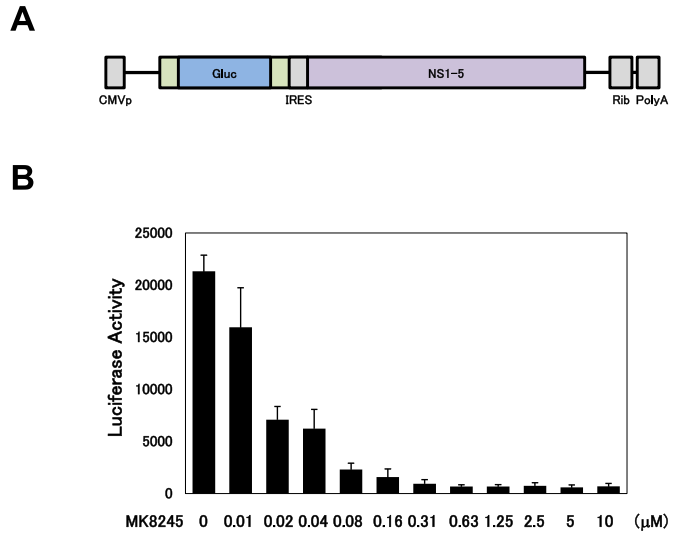

Fig. 2. SCD1 inhibitor suppresses subgenomic reporter replicon activity. (A) Schematic representation of the replicon system. CMVp, cytomegalovirus promoter. Gluc, secretory Gaussia luciferase. IRES, internal ribosome entry site. Rib, ribozyme sequence. (B) Replicon plasmid DGL2 was transfected into Huh7 cells in the presence of SCD1 inhibitor MK8245 (0.01 μM–10 μM). After 72 h, luciferase activity in the culture supernatant was measured. Each data point represents the mean ± standard deviation of triplicate experiments.

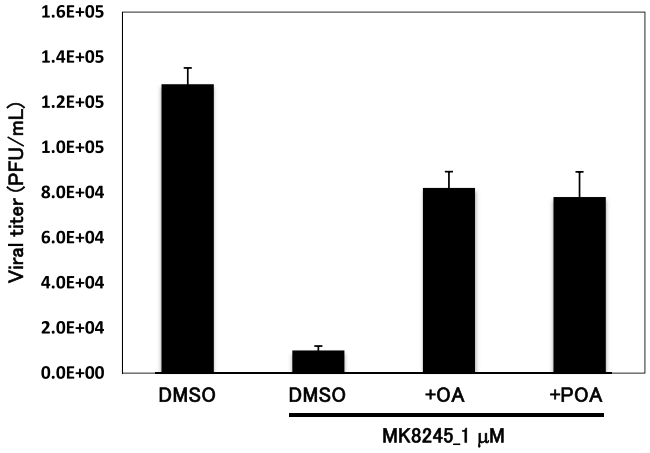

Fig. 3. Unsaturated fatty acids recover DENV replication suppressed by SCD1 inhibitor. Huh7 cells were infected with DENV-1 and then treated with 1.0 μM MK8245 in the presence or absence of oleic acid (OA; 200 μM) or palmitoleic acid (POA; 200 μM). At 72 hpi, viral titers in the cell culture supernatant were measured by plaque assay. Each data point represents the mean ± standard deviation of triplicate experiments.

factors. Several antiviral compounds identified using viral enzyme activity assay, replicon assay, and DENV infection assay have been reported (Noble et al., 2010; Lim et al., 2013; Yang et al., 2014; Kato and Hishiki, 2016; Kato et al., 2016; Hishiki et al., 2017; Low et al., 2017). Although many chemical compounds and natural products inhibit DENV replication *in vitro* and *in vivo*, no antiviral drugs specific for DENV infection are currently available (Low et al., 2017).

DENV infection induces fatty acid synthesis (FAS), and therefore, FAS inhibitors have been found to significantly suppress DENV replication (Heaton et al., 2010). Stearoyl-CoA desaturase-1 (SCD1) is an enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids. The substrates of SCDs are stearoyl- and palmitoyl-CoA; the resulting unsaturated fatty acids, oleoyl- and palmitoleoyl-CoA, serve as the main components in the biosynthesis of phospholipids, triglycerides, cholesterol esters, and wax esters. Unsaturated fatty acids play key roles in the membrane curvature and fluidity required to form hepatitis C virus (HCV) replication complexes (Lyn et al., 2014). We and others recently reported that SCD1 and FAS inhibitors show potent suppression of HCV and DENV replication (Rothwell et al., 2009; Heaton et al., 2010; Lyn et al., 2014; Nyguyen et al., 2014; Nio et al., 2016; Gullberg et al., 2018). In this study, to further clarify the relationship between SCD1 and DENV replication, we assessed the antiviral efficacy of SCD1 inhibitors and validated the viral target by gene knockdown using siRNA that targets SCD1.

To evaluate the anti-DENV activity of SCD1 inhibitors, we first used an *in vitro* cell-based infection assay in conjunction with plaque assay. The compounds MK8245 (Wako Pure Chemical Industries Ltd., Osaka, Japan), 4-(2-chlorophenoxy)-N-(3-(3-methylcarbamoyl)phenyl)piperidine-1-carboxamide (#1716; BioVision, Milpitas, CA, USA), and mycophenolic acid (MPA), a known inhibitor of DENV gRNA replication, were examined. DENV-1 (02-20 strain)-infected human hepatoma cells (Huh7) were co-cultured with each compound. Three days post-infection, the viral titer of the supernatant was significantly suppressed by not only MPA but also SCD1 inhibitors (Fig. 1A). Next, to exclude the possibility of cytotoxicity of the tested compounds, the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was conducted. As a result, the cytotoxicity concentration (CC_{50}) of MK8245 and #1716 was > 100 μM and 44.7 μM, respectively (Fig. 1B). Furthermore, we found MK8245 suppressed DENV-1 replication in human lung carcinoma cells (A549) and baby hamster kidney cells (BHK-21) (Figs. S1A–S1D). These results show that both SCD1 inhibitors, MK8245 and #1716, suppressed DENV-1 replication without apparent cytotoxicity.

We then analyzed the antiviral activity of MK8245 against all four DENV serotypes—DENV-1 (02-20 strain, GenBank accession no. AB178040) (Tajima et al., 2006), DENV-2 (09-74 strain, GenBank accession no. LC367234), DENV-3 (00-40 strain, GenBank accession no. AB111082) (Ito et al., 2007), and DENV-4 (09-48 strain, GenBank accession no. LC069810) (Kato et al., 2018)—using the plaque assay. The virus titer of all four DENV serotypes was remarkably decreased by MK8245 in a dose-dependent manner. The half-maximal effective concentration (EC_{50}) against DENV-1, 2, 3, and 4 was 34, 58, 59, and 81 nM, respectively (Fig. 1C–F). Furthermore, we investigated the antiviral activity of MK8245 against flaviviruses ZIKV (MR766-NIID, GenBank accession no. LC002520) (Kato et al., 2017) and JEV (Mie/41/2002, GenBank accession no. AB241119) (Nerome et al., 2007). As expected, the infectious viral titer in supernatant was significantly suppressed by MK8245. The EC_{50} against ZIKV and JEV was 7.3 and 75 nM, respectively (Fig. 1G and H). These results suggest that MK8245 is potent and broad inhibitor against flavivirus replication because selectivity index, ratio of CC_{50} to EC_{50}, is > 1000.

To determine the stage of the DENV life cycle that is targeted by MK8245, we performed a reporter subgenomic replicon assay. The subgenomic replicon system was deleted almost the structural region of DENV-1; therefore, the assay could only be used for analysis of the viral genome translation and replication steps (Kato et al., 2014; Kato and Hishiki, 2016) (Fig. 2A). After 72 h of replicon plasmid transfection, the Gaussia luciferase activity in the culture supernatant was analyzed. As shown in Fig. 2B, MK8245 reduced luciferase activity levels in a dose-dependent manner. This result indicates that MK8245 inhibits DENV replication during viral gRNA translation and synthesis.

Next, to confirm the contribution of unsaturated fatty acids to the life cycle of DENV, we conducted an additional experiment with monounsaturated fatty acids in the presence of MK8245. Huh7 cells were infected with DENV-1 and co-cultured with 1.0 μM of MK8245 in the presence of oleic acid (200 μM) or palmitoleic acid (200 μM). At 72 h post-infection (hpi), viral titers in the cell culture supernatant were measured by plaque assay. As shown in Fig. 3, supplementation of oleic acid or palmitoleic acid resulted in recovery of the viral titer, suggesting that fatty acid biosynthesis mainly contributes to DENV replication.

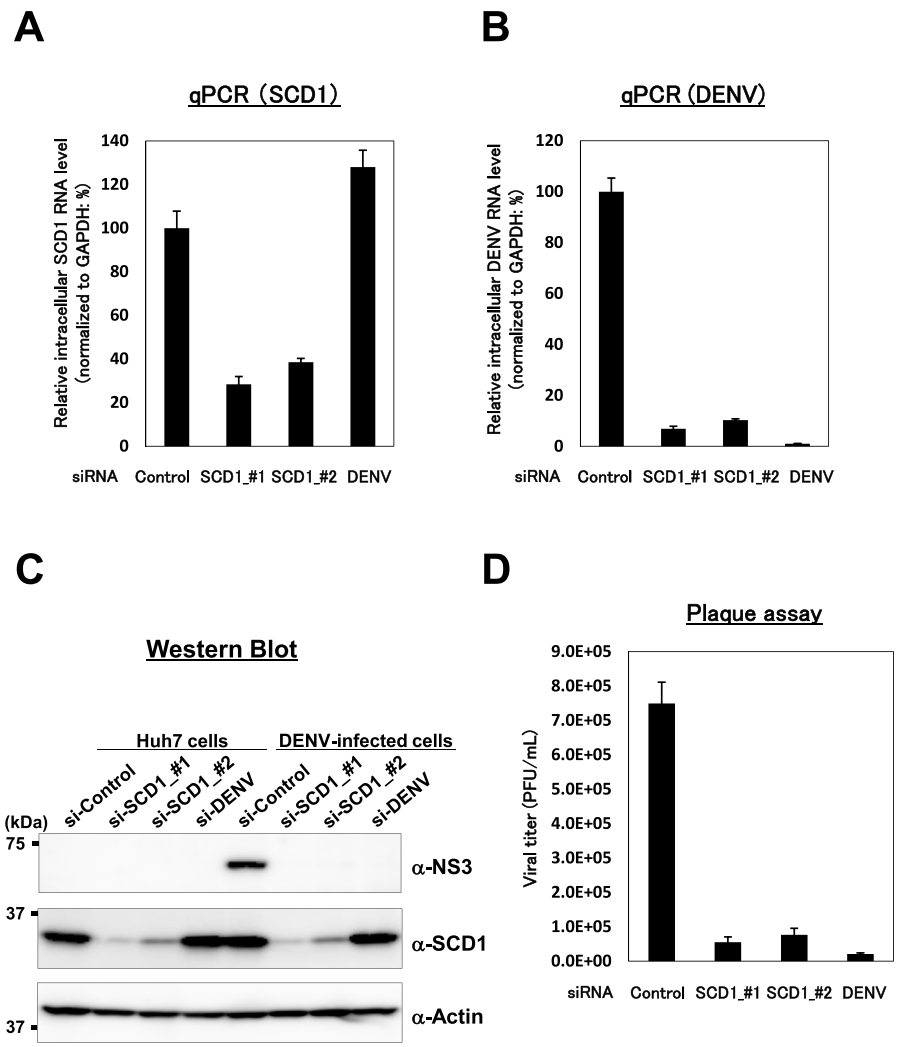

Fig. 4. SCD1 is an essential host factor for DENV replication. (A, B) Huh7 cells were transfected with siRNA targeting two different sites of SCD1 mRNA and then infected with DENV-1 at an MOI of 0.1. At 48 hpi, the intracellular RNA levels of SCD1 and DENV were quantified by qPCR. As a positive control, siRNA targeting DENV genome was used. (C) Cell lysates were immunoblotted with the indicated antibodies. (D) The viral titer in the cell culture supernatant was analyzed by plaque assay. Each data point represents the mean ± standard deviation of triplicate experiments.

Finally, to clarify the importance of SCD1 in the DENV life cycle, we conducted a knockdown experiment with siRNA targeting SCD1 mRNA or DENV genome. After 24-h transfection of siRNA, the cells were infected with DENV-1 at an MOI of 0.1. At 48 hpi, the intracellular RNA level of SCD1 and DENV was quantified by qPCR (Fig. 4A and B). Moreover, the protein expression level of SCD1 and DENV-NS3 in the cell lysate was analyzed by immunoblot (Fig. 4C). As a result, the expression level of DENV RNA and DENV-NS3 protein was significantly suppressed by siRNA targeting SCD1. Furthermore, the viral titer in the culture supernatant was assessed by plaque assay. As shown in Fig. 4D, SCD1 mRNA knockdown reduced the virus titer significantly. Moreover, its reduction was recovered by supplementation of unsaturated fatty acids (Fig. S2). Collectively, these results suggest that SCD1 is an important cellular factor for DENV replication.

In this work, we examined SCD1, an enzyme involved in monounsaturated FAS, and found that it is a key factor that regulates DENV replication efficiency, which was consistent with a recent report (Gullberg et al., 2018). However, MK8245 also may suppress viral particle release and infectivity similarly to recent study (Gullberg et al., 2018). Unsaturated fatty acids also play key roles in HCV replication (Lyn et al., 2014; Nyguyen et al., 2014; Nio et al., 2016). Because HCV and DENV belong to the same family, the viruses may share many common features of their life cycles. However, the emergence of viruses resistant to antiviral agents targeting viral factors directly is an unresolved issue. One possible strategy to suppress the emergence of drug-resistant viruses is the use of drugs that inhibit the host factor that contributes to DENV proliferation. The replication complex of DENV was reported to be present in the membranous compartments of cells. Furthermore, DENV infection leads to remarkable changes in intracellular membranes and fatty acid metabolism (Heaton et al., 2010; Perera et al., 2012). Thus, lipid metabolism modulators might be candidate targets for anti-DENV agents.

Systemic exposure to SCD1 inhibitor has been found to cause some side effects in the eyes and skin. To avoid these side effects, the liver-specific SCD1 inhibitor MK8245 was synthesized; this inhibitor showed antidiabetic effects in a diabetic mouse model without side effects (Oballa et al., 2011). Furthermore, it was reported that MK8245 did not show any remarkable side effects in a phase 1 clinical trial (ClinicalTrials.gov Identifier: NCT00790556). Therefore, we hypothesize that the liver-specific SCD1 inhibitor MK8245 may be a suitable agent for treating DENV-infected patients and that it might pose a low risk of emergence of drug-resistant DENV since the drug dosing regimen would be similar to that of other acute viral diseases such as influenza.

Animal models of DENV infection have been developed for assessment of the efficacy of antiviral compounds or vaccines (Moi et al., 2014; Chan et al., 2015; Kato et al., 2018; Watanabe et al., 2018).

Preliminary evaluation of the *in vivo* efficacy of MK8245 in a DENV lethal AG129 mouse model (deficient in interferon alpha/beta and gamma receptors) (Watanabe et al., 2012) did not result in viremia reduction or mouse survival (data not shown). The discrepancy between the *in vitro* and *in vivo* efficacies might be because MK8245 did not act on DENV infected tissue or associated with *in vivo* metabolic clearance. Thus, further improvement of the compound and careful *in vivo* observation may be required if our finding is translated into clinical settings.

Taken together, our findings support the use of SCD1 inhibitor to treat DENV infection. We found that MK8245 exerts antiviral activity against flaviviruses, especially DENV, and inhibits viral gRNA replication. However, the detailed mechanisms of the antiviral activity need to be identified for development of an anti-DENV drug. Further studies are ongoing to elucidate the mechanism of antiviral activity of the SCD1 inhibitor.

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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.2019.03.002.

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