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Serine protease inhibitor AEBSF reduces dengue virus infection via decreased cholesterol synthesis

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ABSTRACT

Dengue virus (DENV) infection has evolved into a major global health menace and economic burden due to its intensity and geographic distribution. DENV infection in humans can cause a wide range of symptoms including dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). An antiviral agent that is effective against all four serotypes of DENV is urgently needed to prevent and to manage this condition. Reducing the viral load during the early phase of infection may minimize the chance of patients progressing to more severe DHF or DSS. In this study, we set forth to investigate the anti-viral effect of five commercially available protease inhibitors on DENV infection since both viral and host proteases can contribute to effective viral replication. Previously, the serine protease inhibitor AEBSF [4-(2-aminoethyl) benzene sulfonyl fluoride] has been shown to inhibit DENV NS3 protease activity. The results of the present study revealed that DENV genome replication and protein synthesis were significantly inhibited by AEBSF in a dose-dependent manner. AEBSF inhibited the expression of genes such as 3-hydroxy 3-methyl-glutaryl-CoA synthase (*HMGCS*), 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMGCR*), and low-density lipoprotein receptor (*LDLR*). Moreover, AEBSF significantly inhibited HMGCR activity and intracellular cholesterol synthesis after DENV infection. The anti-DENV effect of AEBSF was confirmed in all four DENV serotypes and in three different cell lines. These results indicate that AEBSF reduces DENV infection *via* both viral and host protease activities.

1. Introduction

DENV is a flavivirus that belongs to the *Flaviviridae* family, and that comprises four serologically related, but antigenically distinct serotypes. The viral genome is composed of positive-sense RNA. DENV infection can cause a broad spectrum of clinical symptoms including dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (Kautner et al., 1997). Unplanned urbanization and global travel contribute to the global distribution of *Aedes* mosquito vectors across the world (Guzmán and Kouri, 2002). It is estimated that DENV infection affects approximately 100 million people annually in over 120 countries (Bhatt et al., 2013).

Despite tremendous efforts to develop anti-DENV therapy, none have been approved for clinical use. An antiviral agent against DENV should achieve comparable viral inhibition against all DENV serotypes. Pre-clinical and clinical studies demonstrated the involvement of both viral and host factors in the severity of this disease. Host protease is shown to play important roles in virus entry, uncoating, viral protein production, maturation, and disease induction (Bartenschlager and Miller, 2008) while viral protease facilitates viral genome replication and polyprotein processing (Murthy et al., 1999). Interventions that can inhibit or obstruct the aforementioned functions of these proteases may be an effective approach to prevent or to treat DENV infection. Anti-DENV therapy research suggests that antiviral agents can inhibit viral

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proteins including viral capsid, viral protease, viral helicase, viral methyltransferase, viral polymerase, and host targets (Lim et al., 2013). Of the antiviral agents that have been investigated, protease inhibitors showed promise as an effective treatment approach. The antiviral activity of protease inhibitors has been documented in human immunodeficiency virus (HIV) and hepatitis C virus (HCV) by targeting viral enzymes (Agbowuro et al., 2017; Menéndez-Arias, 2010; Patick and Potts, 1998; Soriano et al., 2008). Most of the protease inhibitors developed against DENV are targeted at viral enzymes, particularly viral NS2B/NS3 protease (Wu et al., 2015; Yang et al., 2011).

In this study, five protease inhibitors representing different classes of host proteases were tested for their anti-DENV properties. Serine protease inhibitors [4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF) and aprotinin], a cysteine protease inhibitor [trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64)], a metalloprotease inhibitor [ethylene diamine tetra acetic acid, disodium salt (EDTA)], and a cysteine-serine protease inhibitor (leupeptin hemisulfate) were evaluated in this study. The antiviral activities of these protease inhibitors against DENV were screened by ELISA, western blotting and focus forming unit (FFU) assay to determine intracellular DENV E antigen, DENV NS3 protein and mature virus production, respectively. Our results demonstrated that AEBSF treatment could inhibit DENV infection by suppressing both viral RNA and protein synthesis in a dose-dependent manner. Importantly, AEBSF inhibited all four serotypes of DENV regardless of the cell types being infected. AEBSF inhibited the expression of genes such as 3-hydroxy 3-methyl-glutaryl-CoA synthase (*HMGCS*), 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMGCR*), and low-density lipoprotein receptor (*LDLR*). Moreover, AEBSF significantly inhibited HMGCR activity and intracellular cholesterol synthesis after DENV infection. Taken together, these findings portend a novel strategy for the development of new anti-DENV therapy.

2. Materials and methods

2.1. Cell culture and viruses

HepG2 (ATCC® HB-8065™), A549 (ATCC® CCL-185™), and EA.hy926 (ATCC® CRL-2922™) cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2 and A549 cell lines were maintained in Dulbecco's Minimum Essential Medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acid (NEAA), 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. EA.hy926 cell line was maintained in DMEM F-12 media supplemented with 10% FBS and antibiotics. Vero cell line (ATCC® CCL-81™) was maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS, 2 mM L-glutamine, 1% NEAA, and antibiotics. All cell lines were maintained in an incubator at 37 °C in a humidified atmosphere that contained 5% CO_2. DENV1 (strain: Hawaii), DENV2 (strain: 16,681), DENV3 (strain: H-87), and DENV4 (strain: H241) were propagated in C6/36 cells (ATCC® CRL-1660™) in Leibovitz's L-15 Medium supplemented with 10% FBS, 10% tryptose phosphate broth (TPB), and antibiotics.

2.2. Protease inhibitors

Protease inhibitors 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF), aprotinin, trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64), ethylene diamine tetra acetic acid, disodium salt (EDTA), and leupeptin hemisulfate were purchased from Merck Millipore (Burlington, MA, USA). All protease inhibitors were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corporation, St. Louis, MO, USA) except aprotinin, which was in solution. Working solutions were freshly made at the time of each experiment by diluting the stock solution in maintenance medium containing 2% FBS, 2 mM L-glutamine, 1% Non-Essential Amino Acid (NEAA), 1 mM sodium pyruvate, and antibiotics.

2.3. Cell viability assay

To evaluate inhibitor cytotoxicity, cells were treated with serially diluted protease inhibitors in a 96-well plate for 24 h at 37 °C with 5% CO_2. To assess cell viability, cells were inoculated with DENV at a multiplicity of infection (MOI) of 1 for 2 h. After incubation, cells were washed twice with stock medium and treated with protease inhibitors at different concentrations for 24 h. After incubation, 10 µl of Prestoblue® Reagent (Invitrogen, Carlsbad, CA, USA) was added to each well. Plates were then incubated for 30 min. at 37 °C after which cell viability was determined by measuring the absorbance at 570/595 nm in a microtiter plate reader (BioTek Instruments, Inc., Winooski, VT, USA). Mock treated cells were used as baseline for the comparison with protease inhibitor-treated cells. To measure cytotoxicity, dose response curves were plotted with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

2.4. Enzyme-linked immunosorbent assay

Cells were inoculated with DENV2 at an MOI of 1 for 2 h in a 96-well plate. After incubation, cells were washed and treated with protease inhibitors at different concentrations for 24 h. The cells were then fixed with 3.6% formaldehyde, permeabilized with 0.1% Triton-X 100, and blocked endogenous peroxidase with 3% hydrogen peroxide. Cells were incubated with antibody against DENV E protein (4G2) for 1 h followed by incubation with HRP-conjugated secondary antibody for 30 min. at 37 °C. Substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Invitrogen) was added to the cells followed by sulfuric acid (H_2SO_4) to stop the reaction. The absorbance, which represents intracellular DENV E, was measured at 450/620 nm in a microtiter plate reader (BioTek Instruments).

2.5. Focus forming unit (FFU) assay

Virus supernatants collected from protease inhibitors-treated and untreated DENV-infected cells were serially diluted and inoculated into Vero cells for 2 h. Overlay medium 1.5% carboxy methyl cellulose (Sigma, USA) was added, and the cells were cultured for 72 h at 37 °C. Cells were fixed with 3.6% formaldehyde and permeabilized with 0.1% Triton-X 100. Thereafter, cells were incubated with anti-DENV E antibody (4G2) for 1 h followed by the addition of HRP-conjugated secondary antibody for 30 min. 3-3′-diaminobenzidine (DAB) solution containing $NiCl_2$ and H_2O_2 was added for foci development. Foci were counted under a light microscope, and the virus titer was expressed as FFU/ml.

2.6. Real- time reverse transcription polymerase chain reaction

Total RNA was collected from DENV-infected HepG2 cells after treatment with or without AEBSF for 24 h using TRIzol Reagent (Invitrogen). RNA was reverse transcribed to cDNA using SuperScript III First Strand Synthesis Kit (Invitrogen). cDNA was amplified using specific primers (Table. 1) and SYBR Green I Reaction Mix (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Real-time RT-PCR was performed on a Roche LightCycler 480 System II. The threshold cycle (Ct) values of genes of interest were normalized with Ct of GAPDH, which was used as housekeeping gene. The data was calculated by $2^{-\Delta\Delta Ct}$ method. Results were compared between AEBSF-treated and untreated samples.

2.7. Western blotting

Cell lysates from DENV2-infected HepG2 cells were collected after

Table 1
Specific primers for Real-time RT-PCR analysis.

Genes	Forward (5'-3')	Reverse (5'-3')
DENV E	ATCCAGATGTCATCAGGAAAC	CCGGCTCTACTCCTATGATG
HMGCS	CAAAAAGATCCATGCACCGT	AAAGGCTTCCAGGCCACTAT
HMGCR	GTCATTCCAGCCAAGGTTGT	GGGACCACTTGCTTCCATTA
LDLR	GCTTGTCTGTCACCTGCAAA	AACTGCCGAGAGATGCACTT
GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATCGCAACTG

treatment with or without protease inhibitors for 24 h using radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were determined using a Bradford Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane using a semi-dry blotting system. The membrane was blocked with 5% skim milk followed by overnight incubation of primary antibodies. Respective primary antibodies used are as follows: - anti-DENV NS3 (GT2811, Thermo Fisher Scientific, Rockford, IL, USA) anti-DENV NS1 (clone: 2G6), anti-DENV E (clone: 4G2), anti-DENV PrM (clone: 1C3), anti-DENV C (clone: D2-C1), and anti-GAPDH (all Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Membranes were then incubated with HRP-conjugated secondary antibodies for 2 h. Supersignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect protein chemiluminescence. Protein bands were visualized by exposing the membrane to X-ray film. The band intensities were determined and compared with the control using ImageJ software (http://imagej.nih.gov/ij).

2.8. Intracellular cholesterol detection assay

Intracellular cholesterol level was quantified using Amplex™ Red Cholesterol Assay Kit (Invitrogen). Briefly, DENV-infected or uninfected HepG2 cells that were treated with or without AEBSF were harvested, and equal numbers of cells were lysed with 50 µl of RIPA buffer. Cell lysates were 10-fold serially diluted with a kit derived reaction buffer. The diluted lysate was added to 50 µl of Amplex solution containing cholesterol esterase and oxidase, and that solution was incubated for 30 min. at 37 °C. Thereafter, the fluorescent intensity that represents the intracellular cholesterol level was measured at 485 nm with a microtiter plate reader (Biotek Instruments).

2.9. HMGCR activity assay

HMGCR activity was determined by using a HMGCR activity kit (Biovision, Milpitas, CA, USA). Cell lysates collected from DENV-infected HepG2 cells with or without AEBSF treated for 24 h were used for this enzymatic assay. Briefly, cell lysates were freshly prepared and

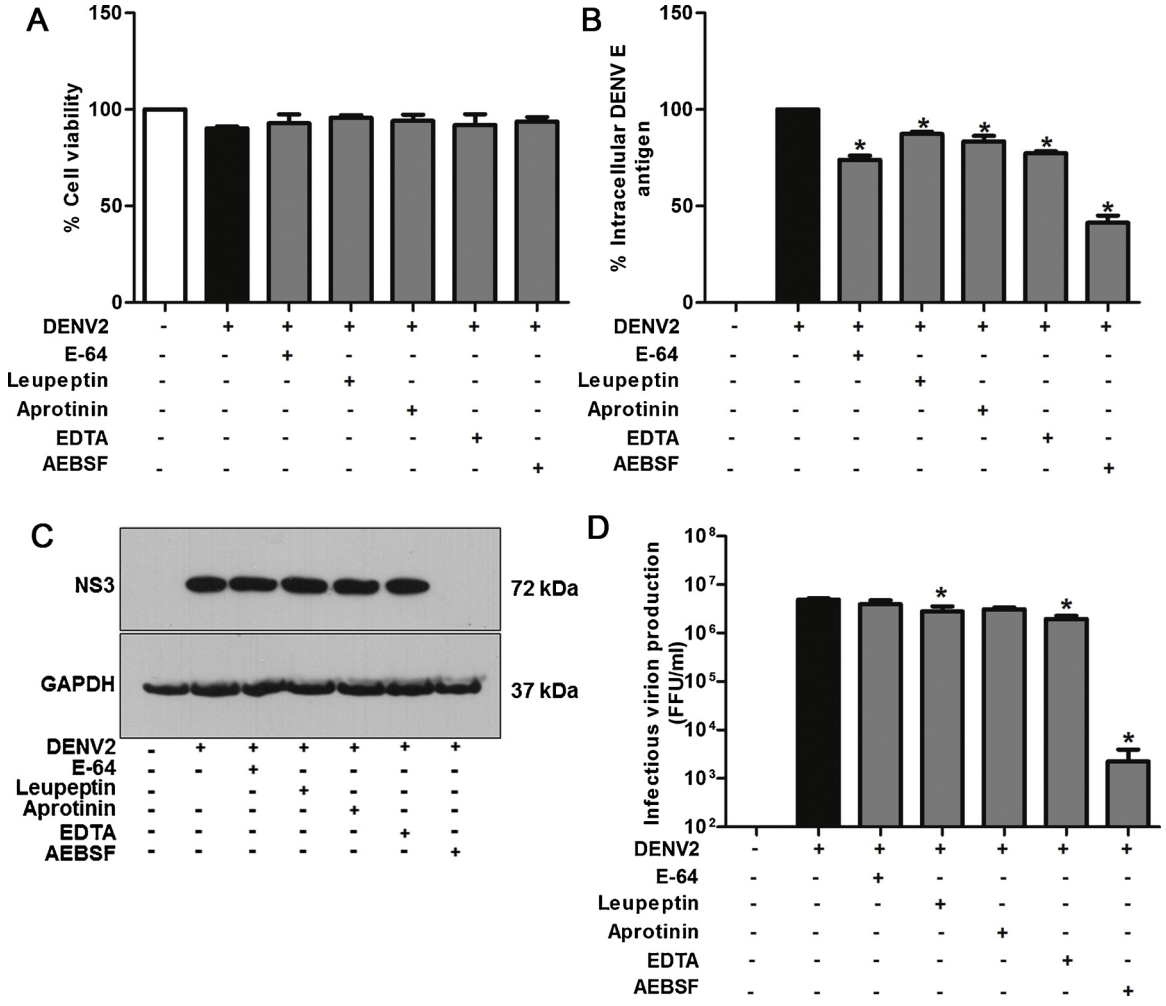

Fig. 1. Screening of protease inhibitors against DENV infection. Effect of protease inhibitors on cell viability (A), Intracellular DENV E antigen (B), DENV NS3 protein (C) and mature virus production (D) in DENV2-infected HepG2 cell line. HepG2 cells seeded in a 96-well plate were infected with DENV2 at an MOI of 1 for 2 h, and treated with the following protease inhibitors for 24 h: 20 µM E-64, 200 µM leupeptin, 1 µM aprotinin, 1000 µM EDTA, and 200 µM AEBSF. Supernatants from DENV2-infected HepG2 cells were collected to evaluate mature virus production. Data represents the results from 3 independent experiments. The asterisks (*) indicate statistical significance between the control and treatment groups which is indicated for $p < 0.05$.

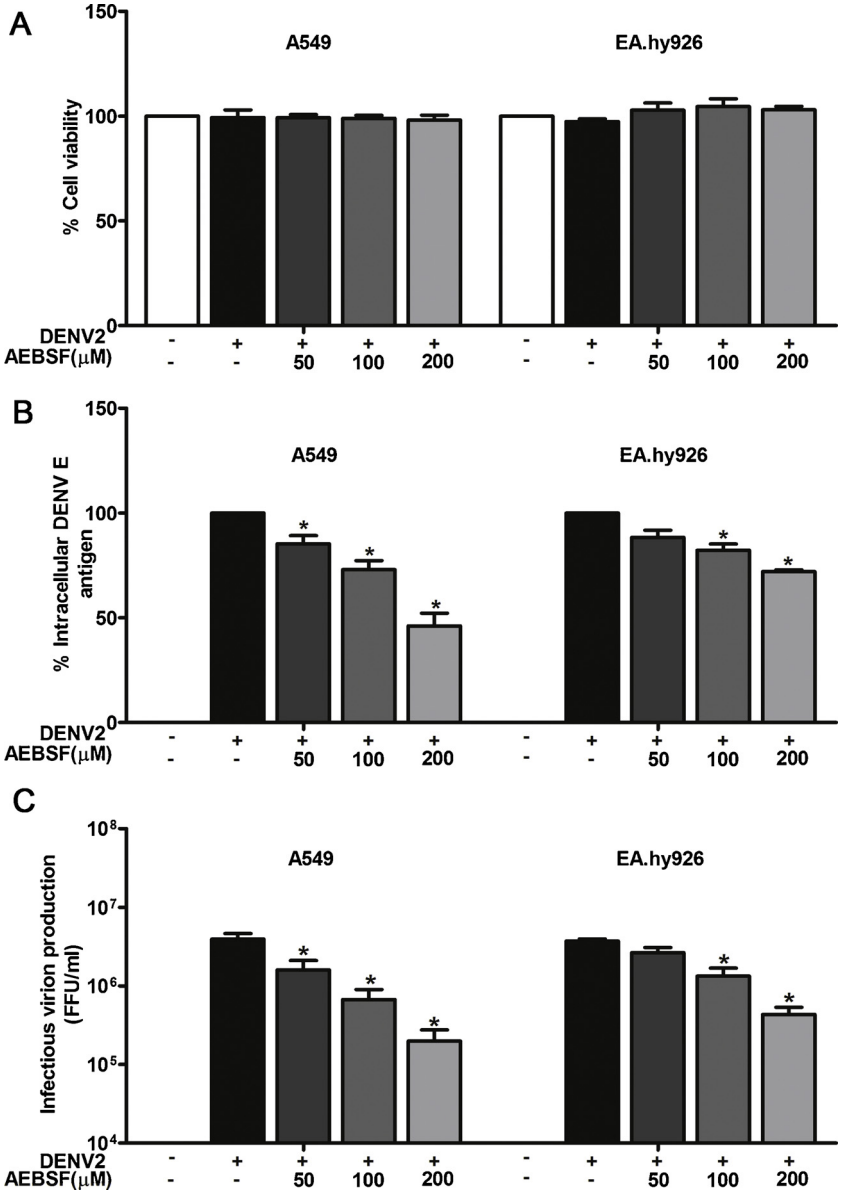

Fig. 2. Anti-DENV effect of AEBSF on A549 and EA.hy926 cell lines. (A) Effect of AEBSF on percentage of cell viability, (B) intracellular DENV E antigen production, and (C) percentage of virus production in DENV2-infected A549 and EA.hy926 cells. DMSO was used as the vehicle control.

protein concentrations were determined. Equal amount of protein from each samples were diluted with a kit-derived assay buffer. Reaction mix containing HMGCoA, NADPH and assay buffer was added to the samples, mixed and immediately measured the absorbance at 340 nm at every 2 min. interval for 30 min. at 37 °C. HMGCR activity is expressed as relative HMGCR activity compared to the mock cells.

2.10. Statistical analysis

All data are the result of three independent experiments, with the results expressed as mean ± standard error of the mean (SEM). Data were analyzed by either one-way analysis of variance (ANOVA) or two-way ANOVA using GraphPad Prism Version 5.0 (GraphPad Software Inc., San Diego, CA, USA). A *p*-value less than 0.05 was regarded as being statistically significant.

3. Results

3.1. Identification of protease inhibitors with anti-DENV property

Prior to determine the anti-DENV effect of various commercially available protease inhibitors, we determined the 50% cytotoxic concentration (CC_{50}) of each protease inhibitor. Briefly, we treated HepG2 cells with protease inhibitors at different concentrations over a period of 24 h. The CC_{50} of E-64, EDTA, and AEBSF in HepG2 cells were 101.5 μM, 2027 μM, and 973.7 μM, respectively (data not shown). Aprotinin and leupeptin did not exhibit any cytotoxicity at the maximum tested concentration. To analyze the anti-DENV effect of these candidate protease inhibitors, we firstly analyzed cell viability. No significant change in cell viability was observed in DENV2-infected HepG2 cells after treatment with these inhibitors for 24 h (Fig. 1A). We then simultaneously determined the anti-DENV effect of protease inhibitors using ELISA, western blotting and FFU assay to evaluate intracellular DENV antigen expression, DENV NS3 protein expression and infectious virion production, respectively. Treatment with E-64, leupeptin,

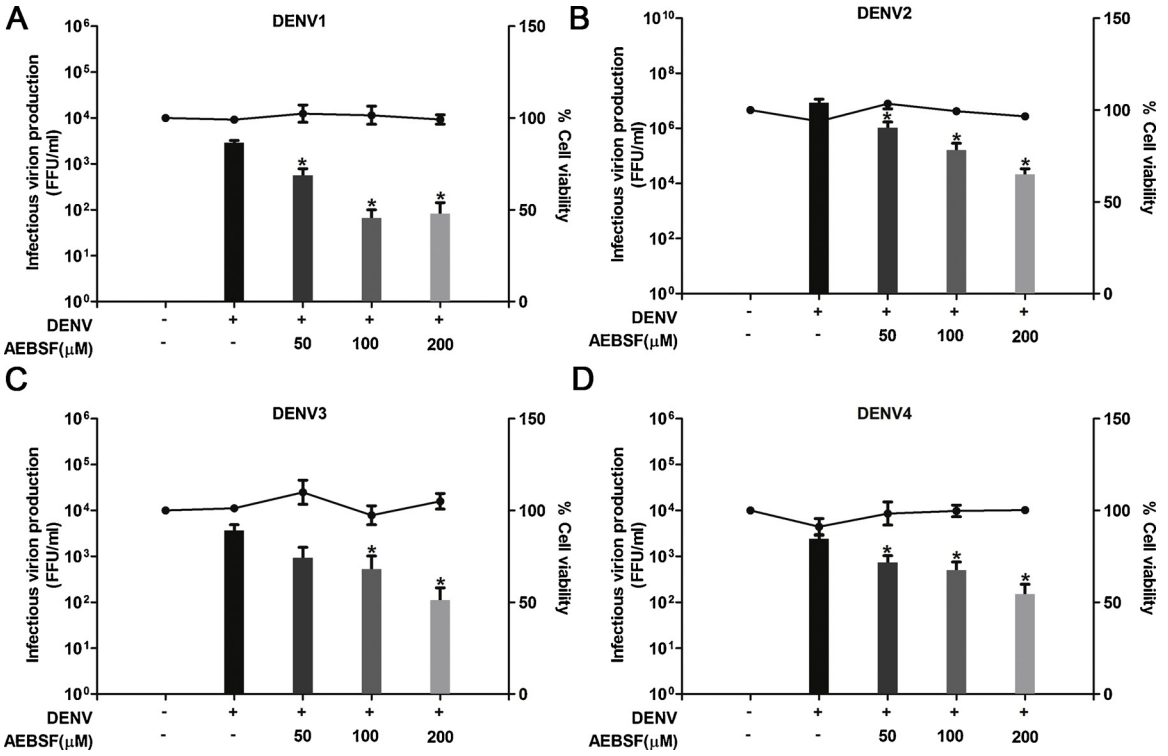

Fig. 3. Effect of AEBSF on the cell viability and virus production in (A) DENV1, (B) DENV2, (C) DENV3 and (D) DENV4 infected HepG2 cells.

aprotinin, and EDTA reduced intracellular DENV E antigen production by less than 20%, whereas treatment with AEBSF significantly reduced viral antigen up to 60% (Fig. 1B). While, only AEBSF abolished the DENV NS3 protein compared to that of the other four protease inhibitors (Fig. 1C). In parallel and among the 5 protease inhibitors screened, only treatment with AEBSF substantially inhibited infectious virion production (Fig. 1D). AEBSF was, therefore, selected for further experiments since it demonstrated the strongest anti-DENV effect.

3.2. AEBSF dose-dependently inhibited virus production in different cell lines, and in all 4 DENV serotypes

To determine the anti-DENV effect of AEBSF in cells from different origins, A549 lung epithelial carcinoma cell line and EA.hy926 endothelial cell line were used in this experiment. The CC_{50} of AEBSF in A549 cells and EA.hy926 cells were 815.30 μM and 951.50 μM, respectively (data not shown). The Prestoblue assay result showed no cytotoxicity at the tested AEBSF concentrations of 50, 100 and 200 μM (Fig. 2A). Similar to the results observed in HepG2 cells, the results from the ELISA and FFU assays revealed the dose-dependent anti-DENV effect of AEBSF in A549 and EA.hy926 cells. Treatment with 200 μM of AEBSF effectuated a 56% and 28% reduction in intracellular DENV E protein in A549 and EA.hy926 cells, respectively (Fig. 2B). Consistent with those results, AEBSF treatment significantly inhibited virus production in both tested cell lines (Fig. 2C).

To evaluate the anti-DENV effect of AEBSF in other serotypes, HepG2 cells were infected with each serotype of DENV (DENV1-4) at an MOI of 1, followed by treatment with AEBSF. AEBSF treatment significantly inhibited all four serotypes of DENV production in HepG2 cells in a dose-dependent manner without adversely affecting cell viability (Fig. 3A–D). The highest percentage of inhibition was observed at an AEBSF dose of 200 μM, which caused inhibition in every serotype of DENV by at least 1 logarithm of FFU. Taken together, these results demonstrate the broad anti-DENV effect of AEBSF given its DENV inhibitory effect in different cell types and in different DENV serotypes.

3.3. AEBSF suppressed viral genome replication and protein synthesis in DENV2-infected HepG2 cells

We next investigated AEBSF-induced interference of viral genome replication and protein synthesis. The results of real-time RT-PCR showed greater than 80% inhibition of RNA synthesis after treatment with AEBSF at all three tested concentrations (Fig. 4A). To evaluate the effect of AEBSF on viral protein production, we analyzed the intracellular DENV E antigen using two confirmative techniques. As determined by ELISA, AEBSF dose-dependently inhibited DENV E expression, with 60% inhibition observed at 200 μM (Fig. 4B). The reduction in viral protein synthesis was then affirmed by Western blot analysis. Viral structural proteins E, PrM, and C, and non-structural protein NS1 were significantly inhibited by AEBSF treatment (Fig. 4C). Relative dose-dependent reduction in the expression of each viral protein after normalization with GAPDH is shown in Fig. 4D.

3.4. AEBSF treatment reduced HMGCR activity and intracellular cholesterol synthesis

Previous study reported that DENV infection increased intracellular cholesterol *in vitro* to facilitate DENV replication (Soto-Acosta et al., 2013). To determine whether the expression of genes involved in cholesterol biosynthesis and uptake were altered by AEBSF treatment, we performed real-time RT-PCR analysis. The upregulation of 3-hydroxy-3-methyl-glutaryl-CoA synthase (*HMGCS*), 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMGCR*), and low-density lipoprotein receptor (*LDLR*) genes in DENV-infected cells was attenuated by administration of AEBSF (Fig. 5A–C). Moreover, an increase in the HMGCR activity during DENV infection was reduced by AEBSF treatment at all the three concentrations used (Fig. 5D). Hence, after quantifying the intracellular cholesterol level, we found that the cholesterol level in DENV-infected cells increased significantly after 24 h of infection compared to uninfected cells, and that AEBSF notably decreased the cholesterol level compared to the untreated group (Fig. 5E).

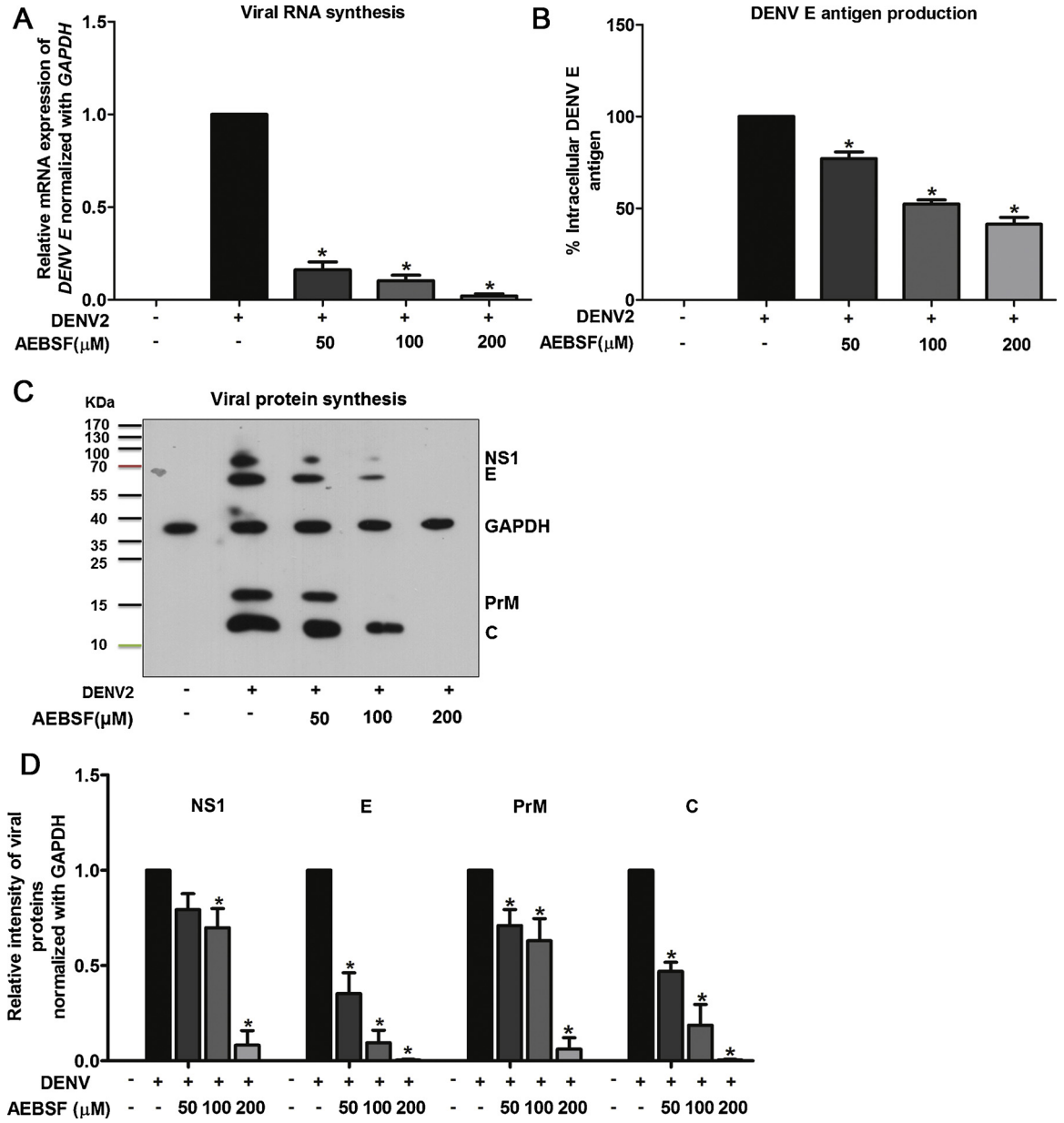

Fig. 4. Effect of AEBSF on DENV RNA replication and protein synthesis. Totally, 5×10^5 HepG2 cells were infected with DENV2 at an MOI of 1 for 2 h followed by the addition of AEBSF at 3 different concentrations. After 24 h, (A) viral RNA was quantified by real-time RT-PCR. (B) Effect of AEBSF on the percentage of intracellular DENV E antigen production as evaluated by ELISA and (C) Western blot analysis was performed for viral E, PrM, C, and NS1 proteins. (D) Densitometric analysis of viral protein synthesis normalized with GAPDH. Data represents the mean result of 3 independent experiments. An asterisk indicates statistical significance between the AEBSF-treated and untreated groups.

4. Discussion

Several host proteases are found to assist certain steps of DENV replication. Targeting these host proteases together with viral protease are quite an excellent target to tackle DENV infection. In this scenario, protease inhibitors that can inhibit proteases, which aid for DENV replication worth a try to seek for their antiviral activity. Here in this study, we showed AEBSF; an irreversible broad spectrum serine protease inhibitor, exhibits anti-DENV activity. Amongst the four protease inhibitors screened, only AEBSF could inhibit intracellular DENV E and NS3 proteins along with the inhibition on mature virus production. The inefficiency of other candidate protease inhibitors may be due to its low specificity, reversible nature or the doses, which we selected in our experiments. In addition, we analyzed the uncleaved DENV polyprotein using western blot probed with pooled convalescent sera; however, we did not observe any protein band that represents the uncleaved DENV polyprotein.

The antiviral activity of AEBSF has previously documented in herpes simplex virus type 1 (HSV-1) (Wechuck et al., 2000), influenza (Bahgat et al., 2011), and respiratory syncytial virus (Van der Gucht et al., 2017). It has also been reported to inhibit hepatitis C virus (HCV) and DENV NS2B/NS3 protease in a cell free assay system (Hahm et al., 1995; Leung et al., 2001). And here in this study, we found the inhibition of DENV replication by AEBSF treatment. We confirmed the anti-DENV effect of AEBSF in three different cell lines. Yet, the ability to inhibit DENV production in these three cell lines varied. Since liver plays a pivotal role in cholesterol biosynthesis, the high reduction in DENV production observed in HepG2 cell line may be due to the effect of AEBSF in inhibiting the intracellular cholesterol. AEBSF inhibited all the four serotypes of DENV. The infectivity of each serotype differed

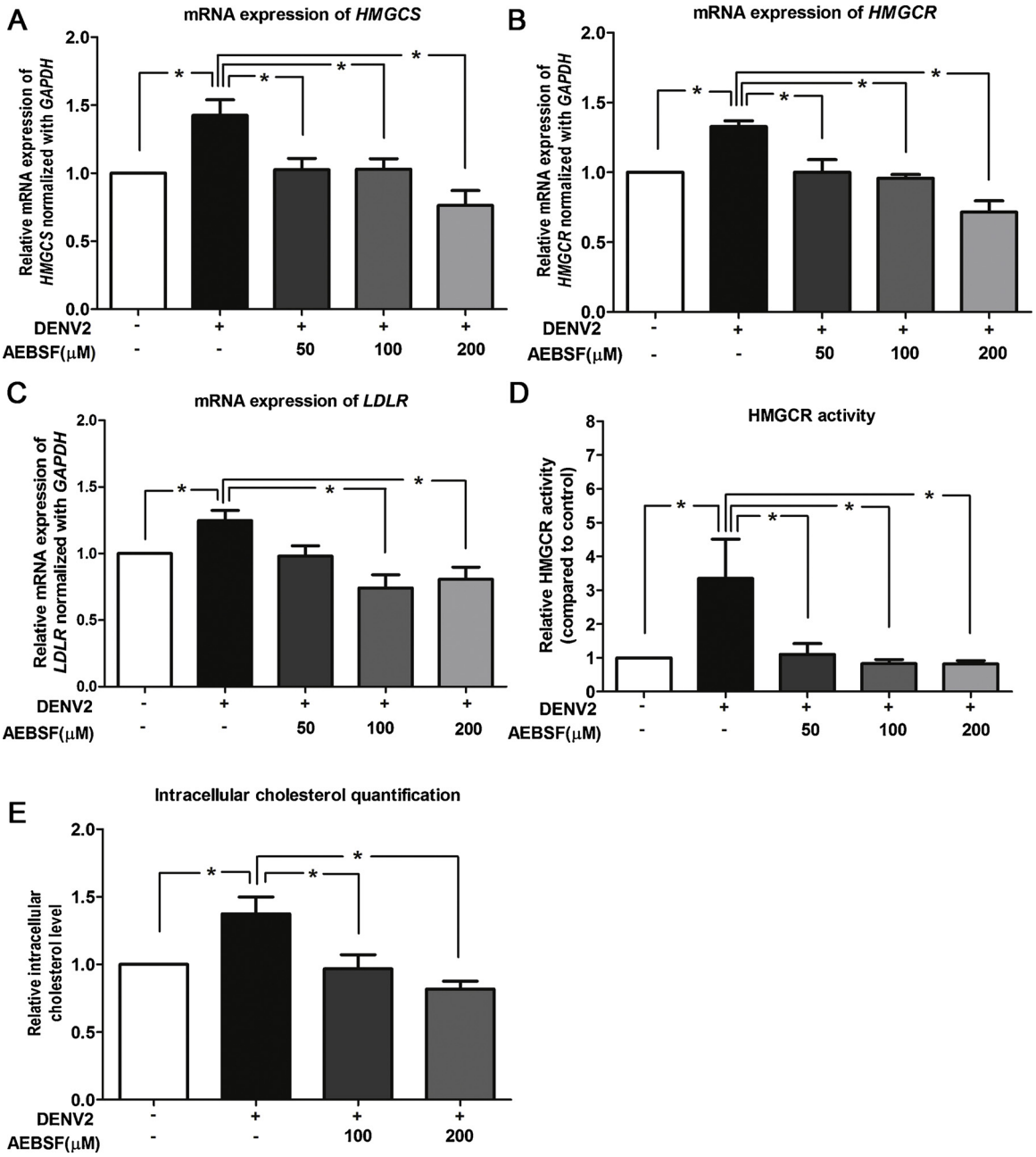

Fig. 5. Effect of AEBSF on cholesterol biosynthesis in DENV-infected HepG2 cells. Totally, 5×10^5 HepG2 cells were infected with DENV2 at an MOI of 1 for 2 h followed by the addition of AEBSF at different concentrations. mRNA expression of (A) HMGCS, (B) HMGCR, and (C) LDLR were quantified by real-time RT-PCR. (D) HMGCR activity relative to the control and (E) Intracellular cholesterol level was evaluated by Amplex Red Cholesterol Assay. DMSO was used as the vehicle control.

where DENV2 exhibited high virus production when infected with MOI 1 for 24 h compared to other serotypes. In this study, we used DENV2 strain 16,681, considering it as one among the strains, which has the lowest threshold for infection and requires least input to attain maximum infection (Diamond et al., 2000). Both DENV serotype and genotype play an important role in the disease severity (Yung et al., 2015).

We further demonstrated the inhibition of AEBSF in DENV genome replication and protein synthesis. A robust decrease in RNA synthesis was observed suggesting the interruption of AEBSF during the early phase of infection. Consistent with the result of RNA replication, treatment with AEBSF inhibited protein synthesis in a dose-dependent manner. Since AEBSF suppressed both viral genome replication and translation in this post treatment condition, we assume that AEBSF may target some of the host factors necessary for DENV replication, which assist in the early stage of DENV life cycle.

Many intracellular pathogens including flaviviruses depend on cellular lipids at multiple stages of their life cycle by manupulating lipid metabolic pathways to enhance virus production (Fischl and Bartenschlager, 2011; Jordan and Randall, 2016; Syed et al., 2010a). Cholesterol biosynthetic pathway is one among the major host cellular pathways targeted by DENV for replication and infectivity. Involvement of cholesterol in viral infection seems to be a critical factor for every steps of replication (Heaton and Randall, 2010; Syed et al., 2010b; Villareal et al., 2015). Metabolism of cholesterol is necessary for flavivirus entry, replication complex formation, viral assembly, energy production and host immune response to the virus. Pharmacological and genetic modulation of cholesterol biosynthetic pathway has been shown to modulate DENV replication. Several statins have been noted to inhibit DENV replication by inhibiting the enzymes that are essential for cholesterol biosynthesis (Bryan-Marrugo et al., 2016; Martinez-

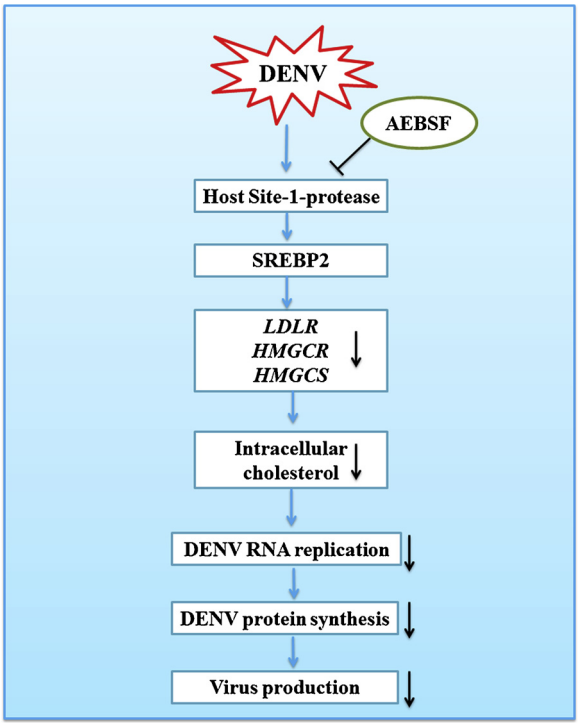

Fig. 6. Proposed mechanism of action of AEBSF in DENV infection. AEBSF decreases DENV production by inhibiting cholesterol biosynthesis.

Gutierrez et al., 2011, 2014). Here we demonstrated that AEBSF could decrease intracellular cholesterol level in DENV infection by inhibiting HMGCR activity and the expression of genes responsible for cholesterol uptake and biosynthesis.

Site-1-protease (S-1-P) is one of the primary targets of AEBSF (Basak et al., 2004) that plays a pivotal role in cholesterol metabolism, fatty acid synthesis, and ER stress regulation (Brown and Goldstein, 1999; Sun et al., 2015; Ye et al., 2000). Previous studies demonstrated that the inhibition of S-1-P significantly attenuated DENV propagation (Hyrina et al., 2017; Uchida et al., 2016). This led us to hypothesize that the anti-DENV property of AEBSF may be because of its inhibitory effect on S-1-P. Sterol regulatory element binding protein 2 (SREBP2) is one of the substrates for S-1-P, which functions to control the intracellular cholesterol level by activating the expression of cholesterol biosynthesis and uptake-related genes. Our results demonstrated the downregulation of three SREBP-2-targeted genes (*HMGCS*, *HMCR*, and *LDLR*) which correlated with the observed reduction in the intracellular cholesterol level after treatment with AEBSF. Similar to previous reports, we observed an upregulation in HMGCR activity at 24 h post infection and AEBSF could inhibit the activity. Enhanced HMGCR activity has been reported through the inactivation of 5′ adenosine-monophosphate activated kinase (AMPK) (Soto-Acosta et al., 2017). However, activation of AMPK and induction of autophagy to facilitate DENV replication has been previously reported (Jordan and Randall, 2017).Therefore, future works are needed to be performed to clearly dissect the role of AMPK in AEBSF-mediated inhibition of DENV replication.

The interruption on the activation of activating transcription factor-6α (ATF-6α) by S-1-P (Okada et al., 2003) may subsequently lead to a reduced expression of GRP78 [an endoplasmic reticulum (ER)-chaperone protein], which plays a critical role in DENV infection by interacting with the DENV E and NS1 proteins (Limjindaporn et al., 2009; Songprakhon et al., 2018) may contribute to the inhibition of DENV infection by AEBSF. The anti-DENV mechanism of AEBSF that is being proposed in the present study is illustrated in Fig. 6.

In spite of various pharmacological activites exhibited by AEBSF, it is important to acknowledge the potential cytotoxicity of using AEBSF *in vivo* or in clinical use due to its non-specificity and multiple cellular actions. Nevertheless, we selected the doses less than CC_{50} value and none of the concentrations exhibited significant toxicity for a period of 24 h as observed from cell viability assay. A recent study has shown the possibility of using gold nanoparticle coated AEBSF, which could increase the half-life and could decrease toxicity exerted by AEBSF (Limon et al., 2018). Considering the fact on the inhibtion of AEBSF on coagulation factors such as plasmin and thrombin, we must thoroughly review the implementation of AEBSF treatment in the pre-clinical and clinical trials. Hence, further works are needed to test its efficacy *in vivo*. Several studies including one in virus, have shown that the *in vivo* administrations of AEBSF have no overt toxicity (Bahgat et al., 2011; Buitrago-Rey, 2002; Megyeri et al., 1999).

5. Conclusion

AEBSF inhibits DENV infection *in vitro* and the anti-DENV property of AEBSF is independent of the serotypes and cells lines. This efficacy of AEBSF to suppress DENV production may attribute to its effect in decreasing intracellular cholesterol synthesis.

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Declaration of Competing Interest

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