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Anti-Dengue Activity of Revaprazan Hydrochloride: A Repurposed Drug Candidate

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Abstract: Dengue fever is a major health problem, and there are no vaccines or effective antiviral treatments. Drug repurposing is a quick and appealing innovative therapeutic strategy. This study aims to develop a potent anti-dengue drug for the treatment of dengue infections. In this study, we screened a library containing 174 compounds using foci-forming unit reduction and quantitative real-time polymerase chain reaction assays. Revaprazan hydrochloride showed the most potent anti-dengue activity and low cytotoxicity in Vero cells

with a half-maximal inhibitory concentration (IC_{50}) of 1.36 ± 0.16 µM, and a half-maximal cytotoxicity concentration (CC_{50}) of 42.41 ± 0.05 µM. Revaprazan hydrochloride revealed 98.31% virucidal activity and 99.80% for post-infection activity against dengue virus serotype 3 (DENV-3). Revaprazan hydrochloride required only 5 min to exert its virucidal effects after contact with DENV-3. Time-of-drug addition and time-of-drug elimination assays indicated that revaprazan hydrochloride was able to interfere with the early stage of DENV-3 infection. *In silico* studies demonstrated that revaprazan hydrochloride had strong binding energy to the RNA-dependent RNA polymerase domain of the NS5 and the helicase domain of the NS2B/NS3 protein. DENV-3 showed no resistance up to 25 passages in the presence of revaprazan hydrochloride. Revaprazan hydrochloride showed varying inhibitory effects against all four dengue virus serotypes. Overall, revaprazan hydrochloride is a novel dengue virus inhibitor and could serve as a promising antiviral drug for further preclinical evaluations.

Keywords: Revaprazan hydrochloride, repurposed drug, dengue virus, mode of action, resistance, antiviral drug; SDG 3 Good health and well-being

1. Introduction

Dengue fever (DF) is a serious public health hazard in the tropical and subtropical regions with *Aedes aegypti* and *Aedes albopictus* being the primary vectors [1, 2]. Dengue virus (DENV), a member of the *Flavivirus* genus is a small, spherical virus that measures 40–50 nm in diameter and comprises a single-stranded positive-sense RNA genome of nearly 11 kb in length [3, 4]. There are four distinct serotypes of DENV (1-4) that share approximately 65-70% of their amino acid sequences [5, 6]. Dengue fever is classified into three types by the World Health Organization (WHO) based on the level of severity: dengue without warning signs; dengue with warning signs; and severe dengue which includes severe plasma leakage, severe hemorrhage, and severe organ dysfunction [7, 8]. According to WHO estimation, 2.5 billion people are at risk for DF, with a global annual infection of 390 million cases, resulting in 25,000 deaths [9]. Worryingly over the last 50 years, the incidence of dengue fever has increased ~30-fold in over 100 endemic countries, with South-East Asia being the most severely affected region due to the lack of effective treatment strategies and comprehensive long-term vector control [10, 11]. The Coronavirus disease 2019 (COVID-19) pandemic has further underscored the global impact of viral diseases on public health and highlighted the urgent need for effective antiviral therapies and comprehensive management strategies [12, 13].

Currently, despite global efforts, there is no clinically approved antiviral treatment for DENV infections [14]. Several compounds have been reported to hinder DENV replication *in vitro* and *in vivo* [15-18]. However, there are still no approved drugs available to treat dengue. The tetravalent, live attenuated, chimeric dengue vaccine (CYD-TDV, Dengvaxia®; Sanofi Pasteur) is licensed in 20 countries for individuals aged 9-45 years, however, its efficacy against all four DENV serotypes is limited, and adverse effects in young children led to the withdrawal-licensing of Dengvaxia in the Philippines [19-21]. Qdenga, another dengue vaccine which was recently approved by the European Medicines Agency, was not able to protect dengue-naïve individuals against DENV-3 and DENV-4 infections [22, 23]. Given the rapid spread of DENV and the typical time frame required for bringing a new drug into the market, repurposing existing drugs signifies a promising alternative technique for a rapid therapeutic approach [24, 25]. Repurposed drugs are approved or marketed compounds used in various clinical settings, or compounds that failed in clinical trials [26-28]. Pharmaceutical companies show great interest in the drug repurposing approach as it provides a faster and safer way to develop drugs for diseases with no pre-existing treatment options [29, 30].

In this study, we screened a library containing 174 repurposed compounds against DENV-3 with the foci-forming unit reduction assay (FFURA) and quantitative real-time polymerase chain reaction (qRT-PCR). Revaprazan hydrochloride was identified to demonstrate antiviral activity against DENV-3, by requiring only a short period to exert its virucidal effects, and could inhibit the early stages of the DENV replication. Furthermore, serial passages of DENV in the presence of revaprazan hydrochloride did not yield resistance to the virus. Overall, these findings suggest that revaprazan hydrochloride could be repurposed to inhibit DENV and provide insights for future development of a suitable anti-DENV therapeutic agent.

2. Materials and Methods

2.1. Cell and viruses

DENV prototype viral strains DENV-1 (Hawaii), DENV-2 New Guinea C (NGC), DENV-3 (H87), and DENV-4 (H241) and Vero C1008 (African green monkey kidney, ATCC®CCL-81) cells were purchased from the American Type Culture Collection (ATCC, USA). The Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA; Catalog number: FBSEU500) and 1% penicillin-streptomycin (Gibco, USA). In a T-75 flask, a monolayer of confluent Vero cells was infected with DENV, and adsorption was permitted for 4 h at 37°C in the presence of 5% CO_2. After incubation, maintenance media (DMEM

supplemented with 2% FBS) was added. The cytopathic effect of DENV in Vero cells was observed for 5-10 days, and the virus was harvested using three freeze-thaw cycles. The virus was diluted in DMEM containing 20% FBS, and stored at -80°C until use.

2.2. Antiviral Compound Library

An anti-virus compound library containing 174 repurposed compounds, purchased from TargetMol, US (Catalogue No. L1700) was used for the screening against DENV-3. All compounds were dissolved in 100% dimethyl sulfoxide (ATCC, USA) at a final concentration of 10 mM, and stored at -80°C. Compound concentrations were determined by dilution with serum-free DMEM media or DMEM supplemented with 2% FBS and 1% penicillin-streptomycin.

2.3. Foci-forming unit reduction assay (FFURA)

Foci-forming unit reduction assay (FFURA) was carried out by the previously stated procedure [31]. Vero cells (1 x 10^5/mL) were seeded in the wells of a 24-well plate overnight and then infected with 10-fold serial dilutions of DENV. Non-infected cells served as a negative control. After 1 h of virus incubation at 37°C, the cells were washed with phosphate buffered saline (PBS) to eliminate the unbound DENV. Subsequently, 1 mL of 1.5% medium viscosity carboxymethylcellulose (CMC) (Sigma Aldrich, MO, USA) in DMEM (overlay media) supplemented with 2% FBS and 1% penicillin-streptomycin was added to each well and the plate was incubated at 37°C, in the presence of 5% CO_2. After 4 days, the overlaying media was removed and the cells were fixed in a mixture of 50% methanol and 50% acetone for 1 h at 4°C. Next, the cells were labeled with a monoclonal antibody against the *Flavivirus* group antigen (D1-4G2-4-15) generated from mouse hybridoma cells (HB112; ATCC), diluted in PBS with 5% skim milk (Thermo Fisher, USA) for 1 h at 37°C. The primary antibodies were tagged with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Catalog number: 31430) (Invitrogen, USA) diluted in PBS with 5% skim milk powder for 1 h at 37°C. Cells positive for the DENV E protein were quantified. Lastly, the cells were stained with KPL TrueBlue™ Peroxidase substrate (Seracare, USA). The foci were manually counted and images were taken using the CTL Immunospot® S6 Versa Analyser (Cleveland, OH, USA). The virus titer was calculated in foci-forming units per milliliter (FFU/mL) using the formula: FFU/mL = (number of foci x dilution factor) / volume of inoculum (mL).

2.4. Viral RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction was carried out using the QIAamp Viral RNA Mini QIAcube Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To run qRT-PCR on Dengue Virus subtypes 1, 2, 3, and 4, the 3' Untranslated Region (3' UTR) genesig® Standard Kit was utilized (Primerdesign, England) with a CFX96 Touch™ Real-Time PCR Detection system (Bio-Rad, California, USA). Each 20-µL reaction mixture contained the RNA template, oasig™ OneStep or Precision™ OneStepPLUS 2x qRT-PCR Mastermix, Dengue primer/probe mix, and RNAse/DNAse free water. Reverse transcription was initiated by heating the samples to 55°C for 10 min, followed by an initial denaturation step at 95°C for 2 min, followed by 50 cycles of amplification at 95°C for 10 s and annealing at 60°C for 60 s. Using serial dilutions of known dengue RNA copy numbers, a standard graph was created (10^1-10^6). The threshold quantitation cycle (Cq) value was computed using the default settings of the CFX96 Touch™ Real-Time PCR Detection System.

2.5. Cytotoxicity assay

The cytotoxicity of antiviral compounds was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Briefly, Vero cells (2 x 10^5/mL) were seeded in the wells of a 96-well plate overnight and incubated at 37°C in the presence of 5% CO_2. Vero cells were treated with various concentrations of compounds (1.5, 3.1, 6.2, 12.5, 25.0, 50.0, 100.0, 200.0 µM) prepared in a maintenance medium supplemented with 2% FBS. As a negative control, Vero cells were grown in the absence of the compound. The plate was then incubated for 48 h at 37°C in the presence of 5% CO_2. Then, a 20-µL aliquot of colorimetric solution was added to each well and the plate was incubated for 1 h at 37°C in the presence of 5% CO_2. An Infinite® 200 Pro Multiplate Reader (Tecan, Männdedorf, Switzerland) was used to measure absorbance at 490 nm. The half-maximum cytotoxic concentration (CC_{50}) was calculated using GraphPad Prism software, version 7 (GraphPad Software, California, USA).

2.6. Cell protection assay

Vero cells (2 x 10^5/mL) were seeded in the wells of a 96-well plate overnight. Cells were then treated with revaprazan hydrochloride (35 µM) for 1 h at 37°C in the presence of 5% CO_2. Following incubation, the compound-containing medium was withdrawn and the cells were washed in PBS, then infected with DENV-3 (MOI = 0.1) at 37°C for 1 h. After the virus had been absorbed, the cells were rinsed with PBS and then resuspended in DMEM

supplemented with 2% FBS. The supernatant was collected after 48 h, and the decrease in foci and mRNA copy number were measured using FFURA and qRT-PCR, respectively.

2.7. Virucidal assay

Vero cells (2 x 10^5/mL) were seeded in the wells of a 96-well plate overnight. DENV-1-4 (MOI = 0.1) was pre-treated for 1 h with revaprazan hydrochloride (35 µM). Vero cells were then infected with the pre-treated mixture of the virus-containing compound for 1 h at 37°C in the presence of 5% CO_2. After the virus had been absorbed, the medium was removed, and the cells were rinsed with PBS and then resuspended in DMEM supplemented with 2% FBS. The supernatant was collected after 48 h, and the decrease in foci and mRNA copy number were measured using FFURA and qRT-PCR, respectively.

2.8. Post-infection assay

Vero cells (2 x 10^5/mL) were seeded in the wells of a 96-well plate overnight and then infected with DENV-1-4 (MOI = 0.1) for 1 h at 37°C in the presence of 5% CO_2. After the virus had been absorbed, the medium was removed and rinsed with PBS and the virus-infected cells were incubated with revaprazan hydrochloride (35 µM) in DMEM supplemented with 2% FBS and incubated for 48 h at 37°C in the presence of 5% CO_2. The supernatant was collected after 48 h, and the decrease in foci and mRNA copy number were measured using FFURA and qRT-PCR, respectively.

2.9. Determination of 50% inhibitory concentration (IC_{50})

The IC_{50} of antiviral compounds against DENV-3 was determined using the comprehensive assay (a combination of cell protection, virucidal and post-infection assays). Briefly, Vero cells (2 x 10^5/mL) were seeded in the wells of a 96-well plate overnight. Vero cells were then pre-treated with various concentrations of compounds (0.39, 0.78, 1.56, 3.12, 6.23, 12.50, 25.00 µM) for 1 h at 37°C in the presence of 5% CO_2. Simultaneously, equivalent volumes of DENV-3 suspensions (multiplicity of infection [MOI] = 0.1) were cultured for 1 h at 37°C in equal volumes of DMEM-containing compounds. After incubation, the medium containing compounds were removed and the cells were rinsed with PBS, then incubated with compound-treated DENV-3 for 1 h at 37°C. The medium was removed after 1 h, and the cells were rinsed with PBS and incubated with compounds at different concentrations in DMEM supplemented with 2% FBS. The supernatant was collected after 48 h and the decrease in foci and mRNA copy number were measured using FFURA and qRT-PCR, respectively. The IC_{50} was calculated with GraphPad Prism software, version 7.

2.10. Time course assay for virucidal activity

Vero cells (2 x 10^5/mL) were seeded in the wells of a 96-well plate overnight. DENV-3 (MOI = 0.1) in 1.5 mL microcentrifuge tubes were pre-treated with revaprazan hydrochloride (35 µM) for 0, 5, 15, 30, 45 and, 60 min. Vero cells were incubated with a pre-treated mixture of the virus-containing compound for 1 h at 37°C in the presence of 5% CO_2. The medium was removed and the cells were rinsed with PBS resuspended in DMEM supplemented with 2% FBS. The supernatant was collected after 48 h, and the decrease in foci and mRNA copy number were measured using FFURA and qRT-PCR, respectively.

2.11. Attachment assay

Vero cells (1 x 10^5/mL) were seeded in the wells of a 24-well plate overnight. Revaprazan hydrochloride (35 µM) was pre-incubated with DENV-3 (MOI = 0.1) at 37°C in the presence of 5% CO_2 for 1 h. Both the Vero cells and compound-treated virus were pre-chilled at 4°C for 1 h. To allow attachment, pre-chilled cells were incubated with the pre-chilled compound-treated virus and at 4°C for 1 h. After 1 h, the medium was removed, the cells were washed with PBS and each well was coated with 1.5% medium viscosity CMC. After 4 days, of incubation, the attachment of DENV-3 to Vero cells was evaluated using FFURA as previously described.

2.12. Entry assay

Vero cells (1 x 10^5/mL) were seeded in the wells of a 24-well plate overnight. Both the Vero cells and DENV-3 were pre-chilled at 4°C for 1 h. In the absence of revaprazan hydrochloride, pre-chilled cells were incubated with pre-chilled DENV-3 (MOI = 0.1) at 4°C for 1 h to facilitate virus attachment. The medium was removed after 1 h, and Vero cells were washed with PBS to eliminate any unattached virus. To allow virus penetration, revaprazan hydrochloride (35 µM) was added to the cells and the temperature was raised to 37°C for 1 h. After 1 h, the medium was withdrawn and the cells were treated for 60 s with acidic PBS (pH 3) to inactivate any viruses that had become attached. After 60 s, the acidic pH was neutralized by adding PBS (pH 11) to each well. The cells in each well were then washed with serum-free DMEM and covered with 1.5% medium viscosity CMC. After 4 days of incubation, the entry of DENV-3 into Vero cells was determined with the FFURA as previously described.

2.13. Time-of-drug addition assay

The time-of-drug-addition experiment was carried out as described with slight modifications [32]. Vero cells (2 x 10^5/mL) were seeded in the wells of a 96-well plate overnight. Vero cells were infected with DENV-3 (MOI = 0.1) for 1 h at 37°C in the presence of 5% CO_2. The medium was removed after 1 h and rinsed with PBS. Revaprazan hydrochloride (35 µM) was added to the infected cells at 0, 1, 2, 4, 6, 8, 12, 18, and 24 h post-infection. The supernatant was collected at 48 h post-infection, and the decrease in foci and mRNA copy number was evaluated using FFURA and qRT-PCR, respectively.

2.14. Time-of-drug elimination assay

The time-of-drug-elimination assay was carried out as described with slight modifications [32]. Vero cells (2 x 10^5/mL) were seeded in the wells of a 96-well plate overnight. Vero cells were infected with DENV-3 (MOI = 0.1) for 1 h at 37°C in the presence of 5% CO_2. The medium was withdrawn after 1 h, and the cells were rinsed with PBS and then incubated with revaprazan hydrochloride (35 µM) for 1 h at 37°C in the presence of 5% CO_2. The medium was removed after 1, 2, 4, 6, and 8 h of infection, and cells were rinsed with PBS, and then resuspended in DMEM supplemented with 2% FBS. The supernatant was collected 48 h post-infection, and the decrease in foci and mRNA copy number was evaluated using FFURA and qRT-PCR, respectively.

2.15. Resistance of DENV-3 in the presence of revaprazan hydrochloride

Revaprazan hydrochloride-resistant DENV-3 was conducted as described with modifications [33]. Drug resistance was generated by passaging DENV-3 with Vero cells in the presence of the drug. Briefly, Vero cells (1 x 10^5/mL) were seeded in the wells of a 24-well plate overnight and infected with wild-type DENV-3 (MOI = 0.1) for 1 h at 37°C in the presence of 5% CO_2. The medium was removed and Vero cells were allowed to grow in the presence or absence of revaprazan hydrochloride (10 µM). The virus present in the supernatant was collected at 72 h post-infection. The supernatant was collected and centrifuged at 4000 rpm for 10 min at 4°C to remove cell debris. For the adaptation of the virus to revaprazan hydrochloride, 200-µL portions of virus-containing supernatants were used to infect fresh Vero cells. DENV-3 passaged in the absence of revaprazan hydrochloride was used as a control to validate the mutations caused by serial passaging in the presence of the drug. Viral titers were quantified for every five passages with the FFURA to monitor for resistance. The selection was terminated at passage 25 when no further improvement of the resistance was observed.

2.16. Three-dimensional (3D) structure prediction of DENV-3 proteins

2.16.1. Model building

The PSI-BLAST of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) was used to search for a suitable crystal structure in the protein structure database for use as a template. The 3D structure of proteins was predicted by homology modeling procedure using four sequential steps through the YASARA program (www.yasara.org) (v21.6.17) using the i) chain 'A' of the 3D structures of 5 templates (1UZG, 3J27, 4GT0, 6ZQU, 7KV8) for E protein, ii) chain 'A'='B' of the 3D structures of 3 templates (5YVU, 5YW1, 5YVV) and chain 'A' of 2 templates (2WHX, 2WV9) for NS2B/NS3 protein and iii) chain 'A' of the 3D structure of 4 templates (5CCV, 4V0Q, 4V0R, 5DTO) for NS5 protein.

2.16.2. Model refinement

Using YASARA force fields 03, an unrestrained high-resolution refinement with explicit solvent molecules was carried out. The results were validated by the YASARA program to ensure that the refinement did not change the model in the wrong direction. Based on the templates used to predict the structure, 12 models for E protein, 15 models for NS2B/NS3, and 4 models for NS5 were built. The highest-ranking models were then refined using the YASARA program to refine the geometry and orientation of the binding model.

2.16.3. Molecular docking

An *in silico* study was used to predict the position and orientation of the compound binding to the E, NS2B/NS3, and NS5 proteins. The 3D structure of revaprazan hydrochloride was retrieved from PubChem (Chemical ID: 204103). The receptor was prepared by removing the water molecules, ions, and inhibitors. Protein-ligand docking was performed using the YASARA program, and VINA approach (Vienna, Austria). Binding energy and dissociation constant (Kd) were calculated from atomic B-factor and atomic property using the YASARA docking program. Positive binding energies suggest stronger binding, while negative binding energies indicate weaker or no binding. Each protein had 25 runs, and the docking findings clustered around certain hotspot conformations. The lowest energy complex in each cluster was preserved, and the final docked structure was analyzed with Dassault Systèmes' discovery studio (Vélizy-Villacoublay, France) software.

2.17. Data analysis

GraphPad Prism, version 7 (GraphPad Software, California, USA) was used to analyze the data to obtain the 50% inhibitory concentration (IC_{50}) and half maximum cytotoxic concentration (CC_{50}). The selectivity index value (SI) for each drug was calculated as the ratio of CC_{50} to IC_{50}. Each assay was performed in triplicates and the mean standard deviation (SD) was plotted in the figure. The student's *t*-test was used to assess differences between the revaprazan hydrochloride-treated groups and the untreated group. A probability (*p*) value of < 0.05 was considered statistically significant.

3. Results

3.1. Identification of compounds with anti-DENV-3 activity from the chemical library

Novel anti-DENV compounds were identified by screening an anti-virus compound library containing 174 repurposed compounds. In the preliminary screening, Vero cells infected with DENV-3 at MOI 0.1 were cultured in the presence of each compound at a final concentration of 25 µM and the reduction of viral foci formation was determined using the foci-forming unit reduction assay. Next, compounds showing cell toxicity were excluded based on the MTS assay (Figure 1A) and revaprazan hydrochloride (Figure 1B) was identified as one of the potent inhibitors for DENV-3, with CC_{50} of 42.41 ± 0.05 µM (Figure 1C) and IC_{50} values of 1.36 ± 0.16 µM and 1.10 ± 0.03 µM determined by FFURA (Figure 1D) and qRT-PCR (Figure 1E) respectively. CC_{20} of revaprazan hydrochloride (35 µM) was chosen as the maximum dose for further antiviral assays against DENV-3. The selective index (SI) of revaprazan hydrochloride evaluated by FFURA and qRT-PCR were 31.18 and 38.55, respectively (Table 1).

3.2. Antiviral activity of revaprazan hydrochloride against DENV-3

To determine the anti-DENV-3 activity of revaprazan hydrochloride, cell protection, virucidal, and post-infection assays were performed. Revaprazan hydrochloride exhibited the highest anti-DENV-3 effect of 99.80 ± 0.29% in post-infection treatment and demonstrated 98.31 ± 2.40% for virucidal assay by foci-forming unit reduction assay (Figure 2A, B and Table 2). Consistently, DENV-3 treated with revaprazan hydrochloride demonstrated 99.84 ± 0.22% inhibition during post-infection treatment and 99.37 ± 0.89% in virucidal activity when the viral RNA particles were quantified by qRT-PCR (Figure 2C and Table 2).

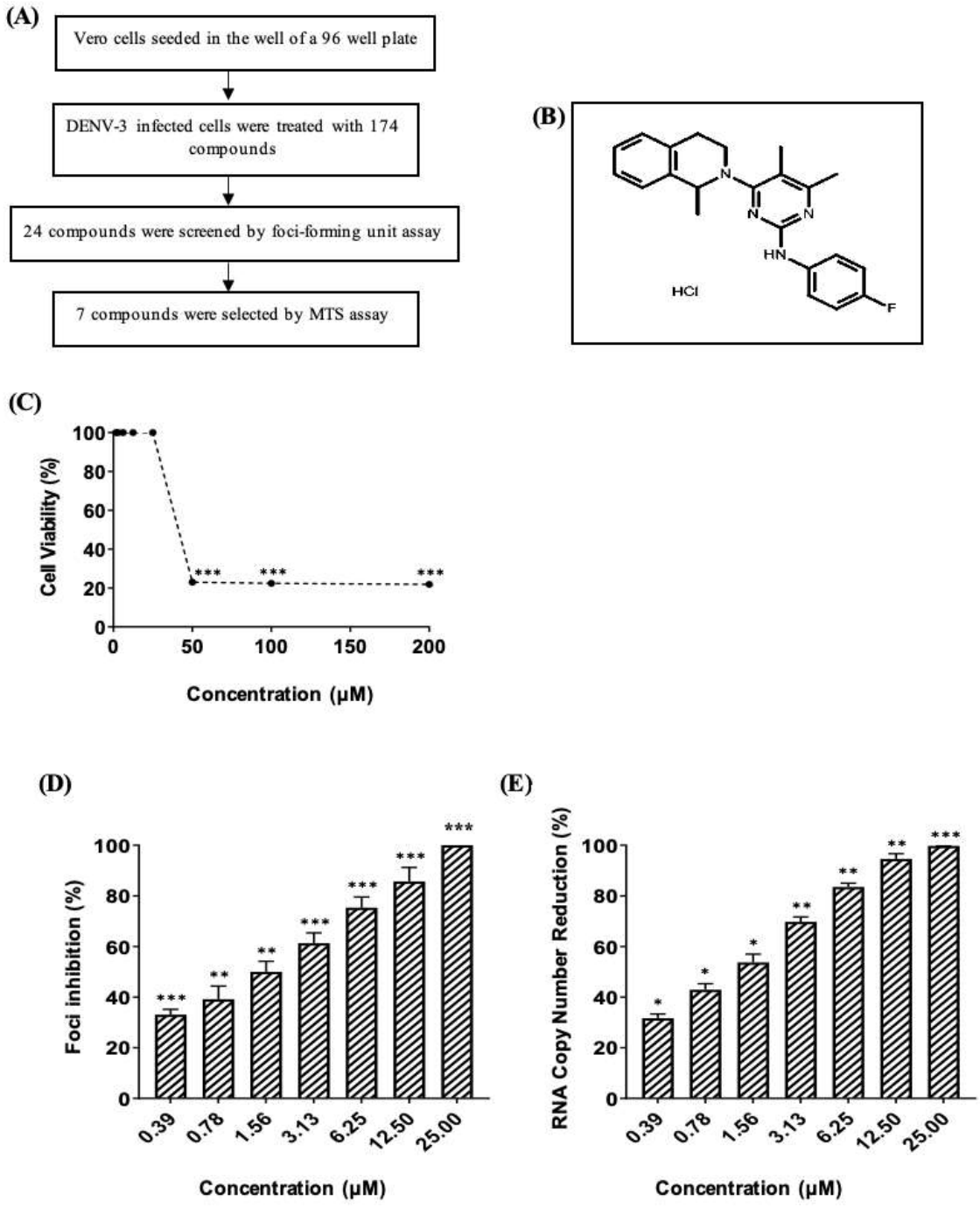

Figure 1. Revaprazan hydrochloride inhibits DENV-3. (A) Flow chart of the screening assay. (B) Structural formula of revaprazan hydrochloride. (C) The CellTiter 96® Aqueous One Solution Cell Proliferation Assay was used to determine the cytotoxicity of revaprazan hydrochloride. Absorbance was measured at 490 nm using a microplate reader. Revaprazan hydrochloride was serially diluted and Vero cells were infected with DENV-3 (MOI = 0.1) using a comprehensive assay. The inhibitory effects of revaprazan hydrochloride against DENV-3 were investigated with (D) the FFURA and (E) the quantification of RNA copy number. The data are presented as mean (SD). The error bars denote the range of values obtained in triplicate experiments. The student's *t*-test was used to compare differences between revaprazan hydrochloride-treated groups and the untreated group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Table 1. CC_{50}, IC_{50}, and SI of revaprazan hydrochloride. The data are presented as mean (SD).

Compound	CC_{50} [a]	IC_{50} (μM) [b]		SI [c]	
		FFURA	qRT-PCR	FFURA	qRT-PCR
Revaprazan hydrochloride	42.41 ± 0.05 μM	1.36 ± 0.16	1.10 ± 0.03	31.18	38.55

[a] CC_{50} (Cytotoxicity concentration 50%): concentration required to reduce 50% of the number of viable Vero cells after 48 h incubation with revaprazan hydrochloride.

[b] IC_{50} (Inhibitory concentration): concentration required to reduce foci number in Vero-infected cells by 50%

[c] SI (selectivity index): CC_{50}/IC_{50}

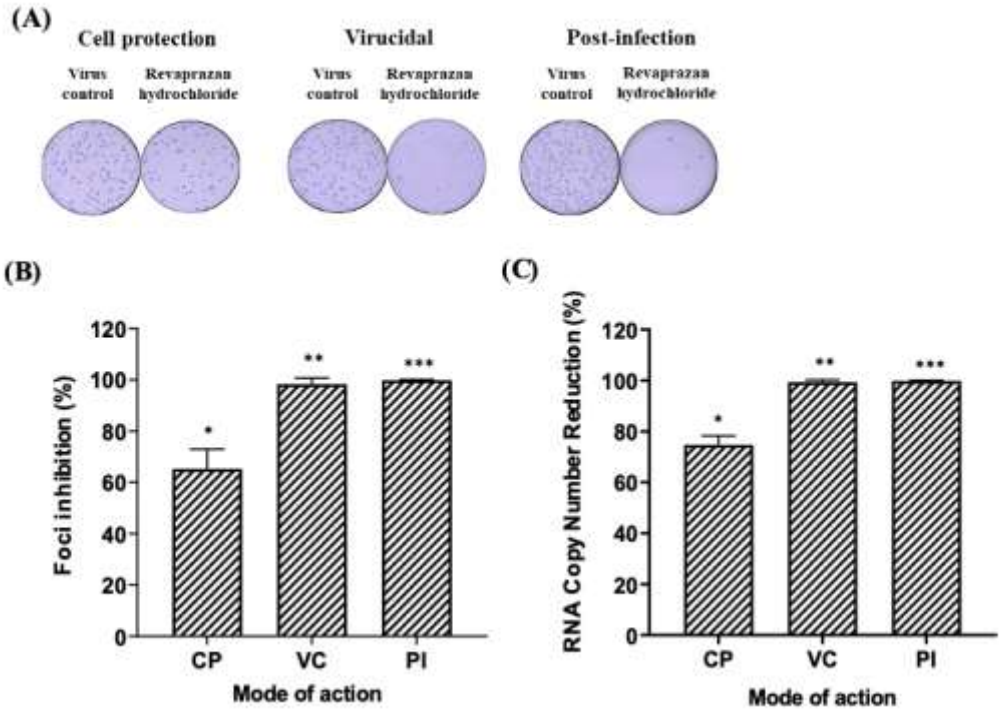

Figure 2. Antiviral activity of revaprazan hydrochloride against DENV-3. (A) The reduction in foci produced was manually counted and images were captured using the CTL Immunospot® S6 Versa Analyser. Vero cells were pre-treated with revaprazan hydrochloride at 35 μM followed by infection with virus (CP, cell protection); DENV-3 was pre-treated with revaprazan hydrochloride, and cells were infected with the treated virus (VC, virucidal); Vero cells were infected with a virus and then treated with revaprazan hydrochloride (PI, post-infection). The inhibitory effects of revaprazan hydrochloride against DENV-3 were measured with (B) the FFURA and (C) quantification of RNA copy number. The data are presented as mean (SD). The error bars denote the range of values obtained in triplicate experiments. The student's *t*-test was used to compare differences between revaprazan hydrochloride-treated groups and the untreated group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Table 2. Inhibitory effects of revaprazan hydrochloride against DENV-3, as determined by the cell protection, virucidal, and post-infection assays. The data are presented as mean (SD).

Compound	Quantification assays	Cell protection	Virucidal	Post-infection
Revaprazan hydrochloride	FFURA	65.26 ± 7.65	98.31 ± 2.40	99.80 ± 0.29
	qRT-PCR	74.71 ± 3.53	99.37 ± 0.89	99.84 ± 0.22

3.3. Revaprazan hydrochloride required a short period of contact with DENV-3 to exert its virucidal effects

To assess the effective time required for revaprazan hydrochloride to exert its virucidal effects on DENV-3, the compound was co-incubated with DENV-3 at MOI 0.1 for 0, 5, 15, 30, 45, and 60 min at 37 °C in the presence of 5% CO_2. Revaprazan hydrochloride demonstrated >80% inhibition at a minimal co-incubation period of 5 min and exhibited >90% inhibitory effect in just 30 min using foci and qRT-PCR assays (Figure 3). Thus, revaprazan hydrochloride only required a short period of contact with DENV-3 to effectively reduce the virus growth (Figure S1).

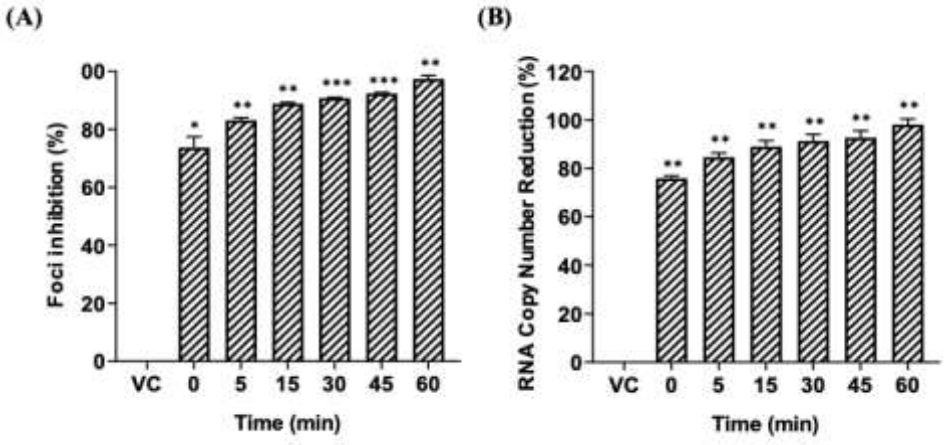

Figure 3. Extracellular virucidal activity of revaprazan hydrochloride against DENV-3 at different co-incubation times. Revaprazan hydrochloride at 35 μM was co-incubated with DENV-3 at 37°C for 0, 5, 15, 30, 45, and 60 min. Following that, Vero cells were infected with the mixtures from each time point. The inhibitory effects of revaprazan hydrochloride against DENV-3 were measured with (A) the FFURA and (B) quantification of RNA copy number. VC represents virus control. The data are presented as mean (SD). The error bars denote the range of values obtained in triplicate experiments. The student's *t*-test was used to compare differences between revaprazan hydrochloride-treated groups and the untreated group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

3.4. Revaprazan hydrochloride did not block cell attachment and entry of DENV-3

Based on the attachment and entry assay, revaprazan hydrochloride inhibited 77.84 % on viral attachment and 63.23 % entry of DENV-3 into Vero cells. This suggest that revaprazan hydrochloride had potent inhibitory effect against DENV-3 (Figure S2).

3.5. Revaprazan hydrochloride interferes the DENV-3 replication

The stage of the DENV replication that is affected by revaprazan hydrochloride was determined by performing a time-of-drug addition assay. In the replication of DENV, the viral proteins were translated from the genomic RNA during the first 5 h post-infection, followed by RNA synthesis and the assembly and release of progeny virions after 12 h (Figure 4A) [34]. The assay showed that the addition of revaprazan hydrochloride up to 8 h post-infection significantly reduced the viral yield by >80%, as determined with the FFURA (Figure 4B) and >90% of RNA copy number as determined by qRT-PCR and dropped to <50% inhibition when revaprazan hydrochloride was added at 12 h post-infection (Figure 4C). The findings suggest that revaprazan hydrochloride was able to inhibit DENV-3 replication at an early stage of infection. The target of revaprazan hydrochloride to the DENV replication stage was also assessed by performing a time-of-drug elimination assay. The results showed that revaprazan hydrochloride significantly suppressed replication of DENV-3 by >80% when treatment commenced at 4 h post-infection measured by the FFURA (Figure 4D) and at 2 h post-infection by qRT-PCR (Figure 4E).

3.6. DENV-3 showed no resistance in the presence of revaprazan hydrochloride-treated Vero cells

The discovery of drug-resistant viruses not only revealed how DENV could evade inhibition, but also the components likely targeted by the antiviral compound. To investigate if revaprazan hydrochloride-resistant DENV-3 could emerge, the adaptation assay was performed. DENV-3 was passaged 25 times in Vero cells cultured in a medium containing 10 µM revaprazan hydrochloride. The infectivity of culture supernatants recovered after every 5 passages was analysed with the FFURA. The results showed that DENV-3 did not reveal any resistance up to 25 passages in the presence of revaprazan hydrochloride-treated Vero cells (Figure S3).

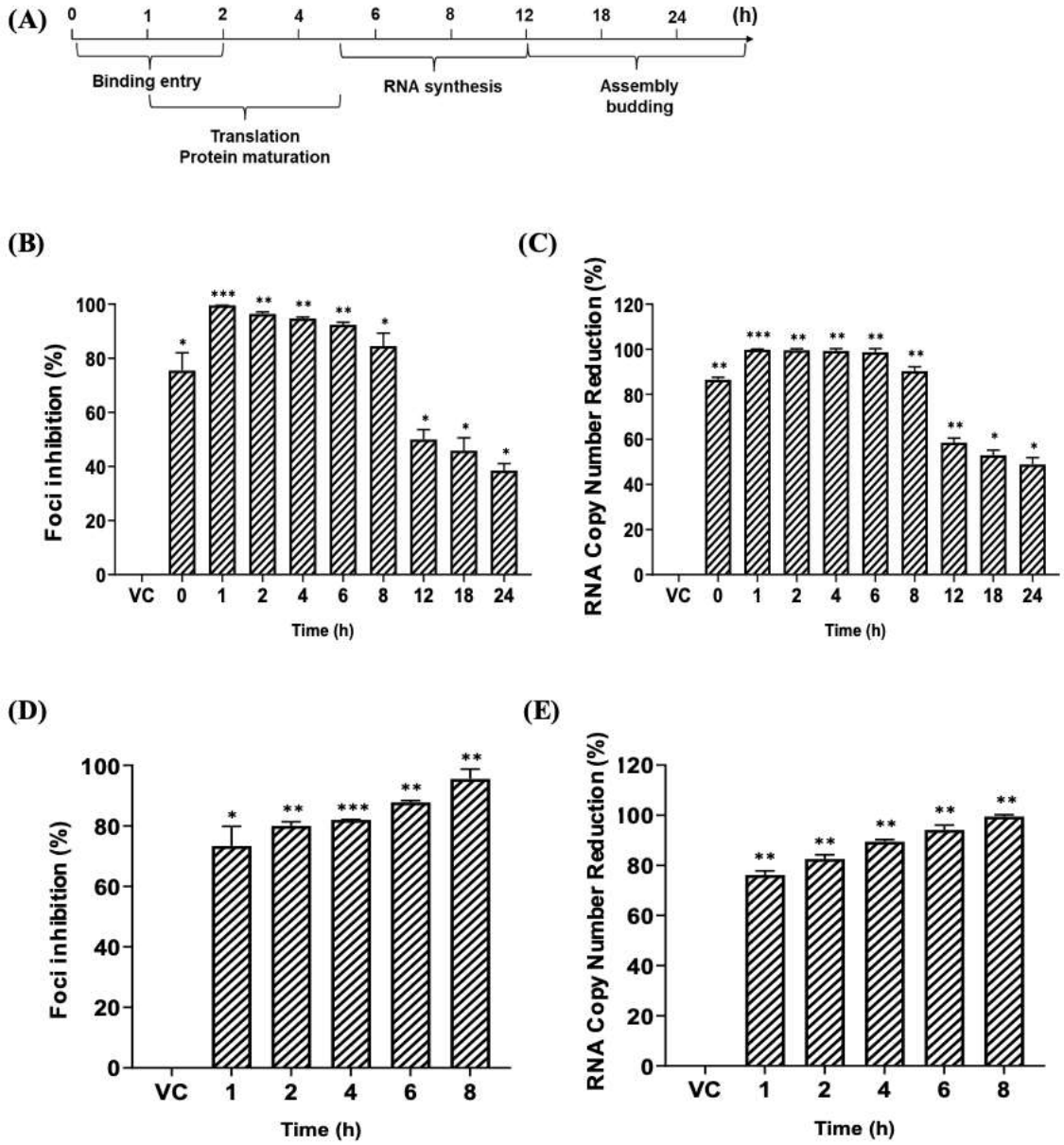

Figure 4. Revaprazan hydrochloride inhibits DENV-3 replication. (A) Time course of DENV propagation. Time-of-drug addition assay of revaprazan hydrochloride at 35 μM against DENV-3. Revaprazan hydrochloride was administered at 0, 1, 2, 4, 6, 8, 12, 18, and 24 h post-infection. The suppression of revaprazan hydrochloride viral infectivity against DENV-3 was measured 48 h after infection using (B) the FFURA and (C) quantification of RNA copy number. In the time-of-drug elimination assay of revaprazan hydrochloride at 35 μM against DENV-3, Revaprazan hydrochloride was removed after 1, 2, 4, 6, and 8 h infection. The inhibitory effects of revaprazan hydrochloride against DENV-3 at 48 h post-infection were determined with (D) the FFURA and (E) quantification of RNA copy number. VC represents virus control. The data are presented as mean (SD). The error bars denote the range of values obtained in triplicate experiments. The student's *t*-test was used to compare differences between revaprazan hydrochloride-treated groups and the untreated group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

3.7. Revaprazan hydrochloride exhibits antiviral activity against all four DENV serotypes

Revaprazan hydrochloride was assessed for both virucidal and post-infection activity against the other 3 DENV serotypes. The inhibition efficiency of revaprazan hydrochloride against DENV-1, -2 and -4 were 91.34 ± 3.46%, 95.60 ± 2.24% and 62.36 ± 1.25% with the post-infection assay and showed 64.63 ± 4.91%, 78.94 ± 5.02% and 82.06 ± 7.52% with the virucidal assay (Figure 5A and Table 3). Consistently, the qRT-PCR results demonstrated inhibition of DENV-1, -2 and -4 by 92.63 ± 2.44%, 96.43 ± 2.43%, and 70.10 ± 1.81% by the post-infection assay, and exerted 71.79 ± 1.64%, 85.83 ± 3.84% and 85.07 ± 3.06%, as determined in the virucidal activity (Figure 5B and Table 3). Thus, it can be concluded that revaprazan hydrochloride is a potential antiviral agent against all four DENV serotypes, albeit with varying viral inhibitory effect from both virucidal and post-infection assays, and could serve as a potential antiviral candidate for the treatment of DENV infection (Figure S4).

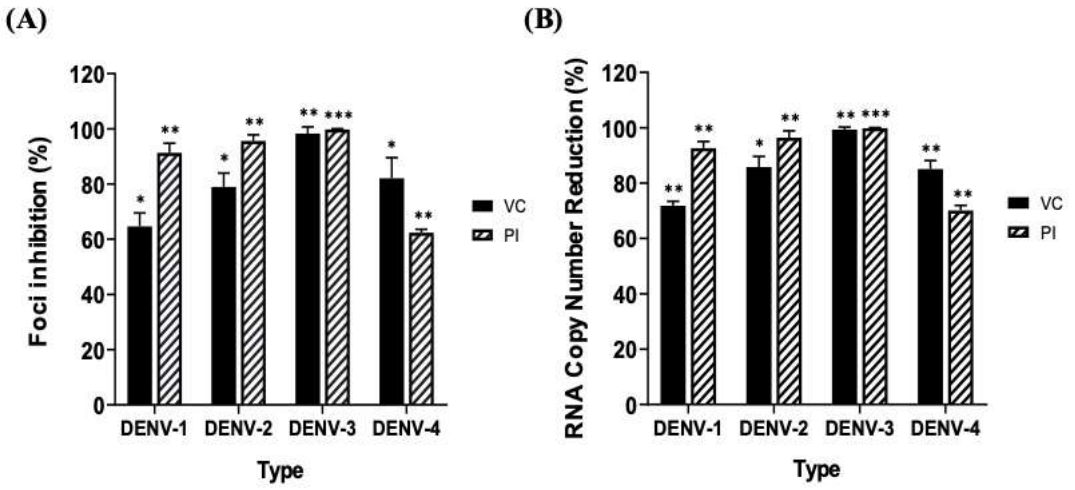

Figure 5. Antiviral activity of revaprazan hydrochloride against all DENV serotypes. DENV-1-4 was pre-treated with revaprazan hydrochloride (VC-Virucidal); Vero cells were infected with virus and then treated with revaprazan hydrochloride (PI-Post-infection). The inhibitory effects of revaprazan hydrochloride against DENV-1–4 was determined by (A) the FFURA and (B) quantification of RNA copy number. The data are presented as mean (SD). The error bars denote the range of values obtained in triplicate experiments. The student's *t*-test was used to compare differences between revaprazan hydrochloride-treated groups and the untreated group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Table 3. Virucidal and post-infection activity of revaprazan hydrochloride against all four dengue serotypes. The data are presented as mean (SD).

Compound	DENV serotype	Quantification method	Virucidal	Post-infection
Revaprazan hydrochloride	DENV-1	FFURA	64.63 ± 4.91	91.34 ± 3.46
		RT-PCR	71.79 ± 1.64	92.63 ± 2.44
	DENV-2	FFURA	78.94 ± 5.02	95.60 ± 2.24
		RT-PCR	85.83 ± 3.84	96.43 ± 2.43
	DENV-3	FFURA	98.31 ± 2.40	99.80 ± 0.29
		RT-PCR	99.37 ± 0.89	99.84 ± 0.22
	DENV-4	FFURA	82.06 ± 7.52	62.36 ± 1.25
		RT-PCR	85.07 ± 3.06	70.10 ± 1.81

3.8. Structural prediction of DENV-3 proteins

To further understand the potential molecular mechanisms underlying the antiviral activity of revaprazan hydrochloride, we performed *in silico* structural prediction and docking analyses with key DENV-3 proteins. The amino acid sequences of the E, NS2B/NS3 and NS5 of DENV-3 proteins were blast using PSI-BLAST tool in NCBI. The database used in this study was Protein Data Bank proteins (PDB) (Table 4).

Table 4. PSI blast of different templates of E, NS2B/NS3 and NS5 proteins for DENV-3.

Crystal structure	Length (aa)	Identity (%)	Resolution (Å)
Envelope protein			
|pdb|1UZG |A	392	98.46	3.50
|pdb|7KV8 |A	495	97.14	2.50
|pdb|3J27 |A	495	67.68	3.60
|pdb|4GT0 |A	437	77.99	2.57
|pdb|6ZQU |A	495	67.89	3.10
NS2B/NS3			
|pdb|5YVU |A=B	627	79.13	2.49
|pdb|5YW1 |A=B	618	79.23	2.60

|pdb|5YVV |A=B	599	79.24	3.10
|pdb|2WHX |A	618	78.74	2.20
|pdb|2WV9 |A	673	57.33	2.75
NS5			
|pdb|5CCV |A	905	99.11	3.60
|pdb|4V0Q |A	892	98.21	2.30
|pdb|4V0R |A	892	98.09	2.40
|pdb|5DTO |A	892	98.09	2.60

3.9. Model analysis

The E protein contains 3 domains and Flavi-E-stem at the C-terminal region (Figure S5A). Domain I include amino acids 1-52, 133-191and 275-296 and is marked in pink, domain II comprises amino acids 53-132 and 192-274 that is marked in green, domain III contains amino acids 297-395 that is marked in orange and the Flavi-E-stem includes amino acids 396-471 that is marked in grey. The E protein has two binding sites, 1 and 2, located between domain I/II and domain I/III, respectively.

The NS2B/NS3 protein contains the NS2B region and 2 domains in NS3, trypsin-like serine protease (pink) and helicase domain (yellow) (Figure S5B). The NS2B comprises amino acids 1-127 that are marked in grey. Trypsin-like serine protease has 2 subdomains (subdomain I include amino acids 128-215 and subdomain II comprises amino acids 216-303). Trypsin-like serine protease (amino acids 229-286) is in domain I. The helicase domain contains 4 subdomains. Subdomain I contain amino acids 304-451 and 610-690. DEXH-box helicase domain (amino acids 314-464) and putative ATP binding site (amino acids 323-329 and 412) are in subdomain I. Subdomain II includes amino acids 452-553 and 593-609. The C-terminal helicase domain of viral helicase (amino acids 460-604) is in subdomain II. Subdomain III comprises amino acids 554-592 and the ATP binding site (amino acids 588) is in subdomain III. Subdomain IV includes amino acids 691-750.

The NS5 protein contains 2 domains, MTase (1-249 amino acids) which is marked in red and RdRp (250-875 amino acids) is marked in yellow (Figure S5C). MTase domain has two subdomains. Subdomain I include amino acids 1-52 and 225-265. The capping-2-MTase (amino acids 9-239) is in subdomain I. Subdomain II contains amino acids 53-224. The active

site comprises amino acids 146-180, 216, the nucleic acid substrate binding site comprises amino acids 59, 61, 148, 180, 214, and 216, SAM binding site contains amino acids 59, 62, 79-63, 67, 104-106, 130, 131, 146-148 and 166) which are in subdomain II. The RdRp domain contains two subdomains (subdomain I include amino acids 266-312 and 362-698 and subdomain II contains amino acids 313-361 and 699-875).

3.10. Molecular docking

The binding affinity of revaprazan hydrochloride to E, NS2B/NS3, and NS5 proteins of DENV-3 was simulated *in silico* using the YASARA program and the results showed that the compound was able to interact with all three vital proteins. However, the complex of revaprazan hydrochloride-NS5 showed the highest binding energy and lowest dissociation constant compared to other proteins. Table 5 shows the binding energy, dissociation constant, and contacting receptor residues of E, NS2B/NS3, and NS5 proteins against revaprazan hydrochloride.

Table 5. Amino acid residues found in proximity with the target proteins with the binding energy and the dissociation constant.

Target Protein	Binding energy [kcal/mol]	Dissociation constant [pM]	Contacting receptor residues
Envelope	8.03	1,291,219.00	Thr184, Gly185, Leu186, Asp187, Asn189, Glu190, Met191, Met202, Gly276, His277, Ala408, Ile409, Leu410, Gly411, Asp412, Thr413, Tyr439, Thr440, Lys451, Leu484, Gln489
NS2B/NS3	9.17	188,524.95	Ala37, Tyr39, Ser45, Ala46, Asp47, Thr180, Arg181, Ala183, Val184, Phe482, Gly484, Asp548, Gln594, Lys595, Glu596, Asn597, Gln599
NS5	9.33	146,111.48	Phe395, Val399, Asn402, Gln414, Trp415, Ala418, Lys453, Lys456, Trp471, Trp474, Leu475, Arg478, Phe482, Asn489, Ser597, Gly598, Gln599, Val600, Gly601, Thr602, Tyr603, Trp792, Ser793, Ile794

The complexes formed between revaprazan hydrochloride and E protein exhibited binding energy of 6.16 to 8.03 kcal/mol. Revaprazan hydrochloride was able to bind to four different areas in the E protein (Figure 6A). The highest binding energy and lower dissociation constant were calculated when the revaprazan hydrochloride binds to site 1 of the E protein (between domain I/II) (Figure 6B). The amino acids involved in hydrogen bonds were GLN489 and ILE409 and amino acids that formed 3 hydrophobic interactions were

MET191 and HIS277 (Figure 6C, D). Analysis of the revaprazan hydrochloride-E complex showed that the compound binds to the E protein through 2 hydrogen bonds and 3 hydrophobic interactions (Table S1).

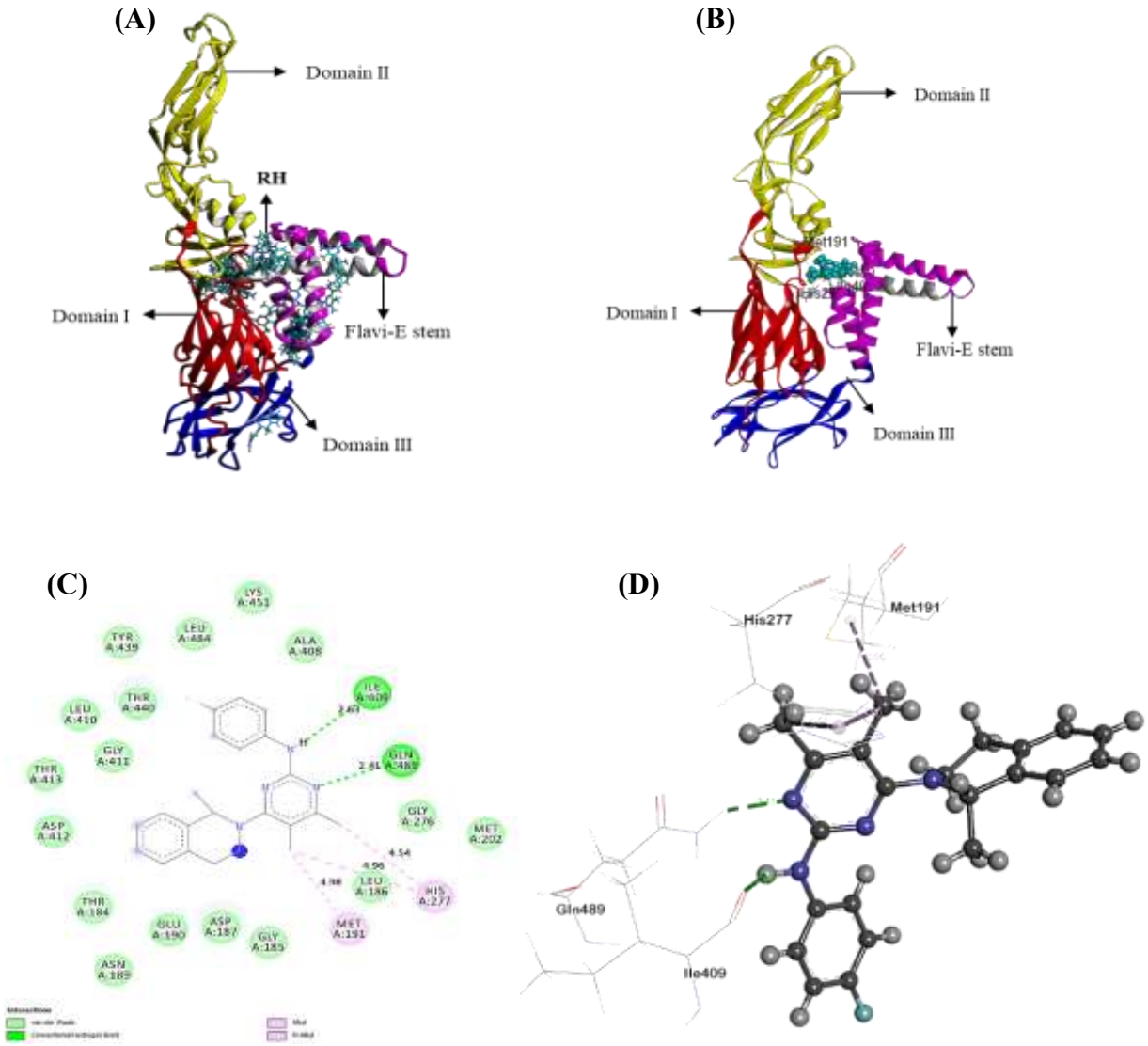

Figure 6. Molecular docking of DENV-3 E protein docking with revaprazan hydrochloride. (A) Structure of the DENV-3 E protein docking with revaprazan hydrochloride. (B) Binding pose of revaprazan hydrochloride's molecular structure on DENV-3 E protein with four different possible binding sites. (C) 2D scheme of the interaction of revaprazan hydrochloride with the amino acid residues of DENV-3 E protein, including H-bonding and hydrophobic interactions as shown by docking analysis. (D) 3D scheme of the interaction of revaprazan hydrochloride with the DENV-3 E protein, with H-bonds at the E protein Gln489 and Ile409 and hydrophobic interactions at Met 191, and His277.

Besides, the complexes formed between revaprazan hydrochloride and NS2B/NS3 protein exhibited binding energy of 6.25 to 9.17 kcal/mol. Revaprazan hydrochloride was able to bind to six different regions in the NS2B/NS3 protein (Figure 7A). The highest binding energy and lower dissociation constant were calculated when the revaprazan hydrochloride bound to the area between domain I and IV of the NS2B/NS3 protein (Figure 7B). The amino acids involved in the hydrogen bond was ALA183 and amino acids included in the electrostatic interaction was ASP548. The amino acids that formed 8 hydrophobic interactions were ALA37, ALA183, LYS595, VAL184, and ARG181 (Figure 7C, D). Analysis of the revaprazan hydrochloride-NS2B/NS3 complex showed that the compound binds to the NS2B/NS3 protein through 1 hydrogen bond, 1 electrostatic, and 8 hydrophobic interactions (Table S2).

Remarkably, the complexes formed between revaprazan hydrochloride and NS5 protein exhibited binding energy of 6.07 to 9.37 kcal/mol, where the higher the binding energy the better the bond between the targeted protein and the drug. Revaprazan hydrochloride was able to bind to eight different areas in the NS5 protein (Figure 8A). The highest binding energy and lower dissociation constant were obtained when revaprazan hydrochloride binds to the domain III of NS5 protein which is the RdRp (Figure 8B). The amino acids involved in the hydrogen bond were GLN414 and GLN599 and amino acids that formed 4 hydrophobic interactions were TRP415, TRP474, ARG478 and VAL600 (Figure 8C, D). Analysis of the revaprazan hydrochloride-NS5 complex showed that the compound binds to NS5 protein through 2 hydrogen bonds and 4 hydrophobic interactions (Table S3).

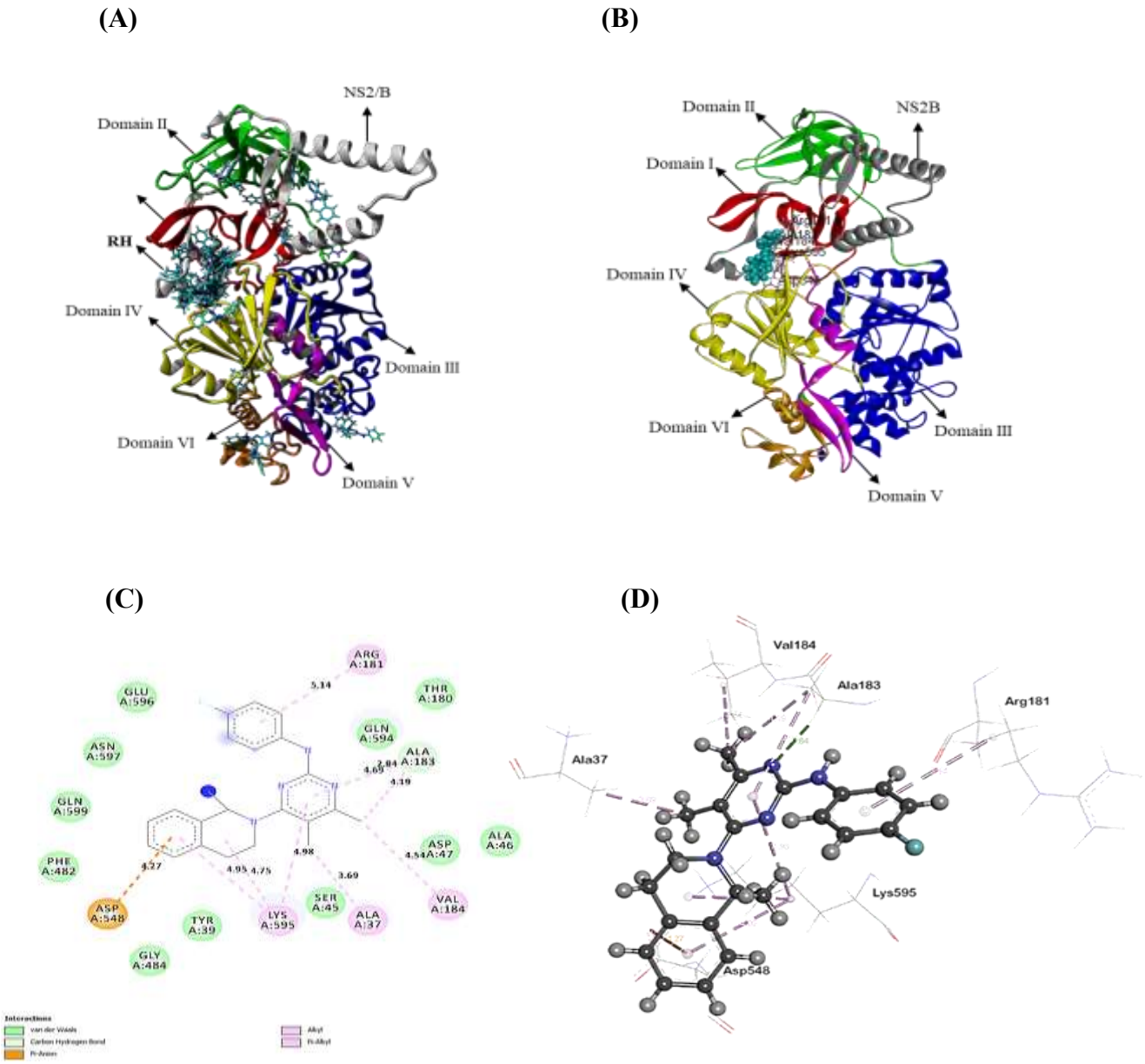

Figure 7. Molecular docking of DENV-3 NS2B/NS3 protein docking with revaprazan hydrochloride. (A) Structure of the DENV-3 NS2B/NS3 protein docking with revaprazan hydrochloride. (B) Binding pose of revaprazan hydrochloride's molecular structure on DENV-3 NS2B/NS3 protein with seven different possible binding sites. (C) 2D scheme of the interaction of revaprazan hydrochloride with the amino acid residues of DENV-3 NS2B/NS3 protein, including H-bonding, electrostatic and hydrophobic interactions as shown by docking analysis. (D) 3D scheme of the interaction of revaprazan hydrochloride with the DENV-3 NS2B/NS3 protein, with electrostatic interaction at residue Asp548 and hydrophobic interactions at Ala37, Ala183, Lys595, Val184, and Arg181.

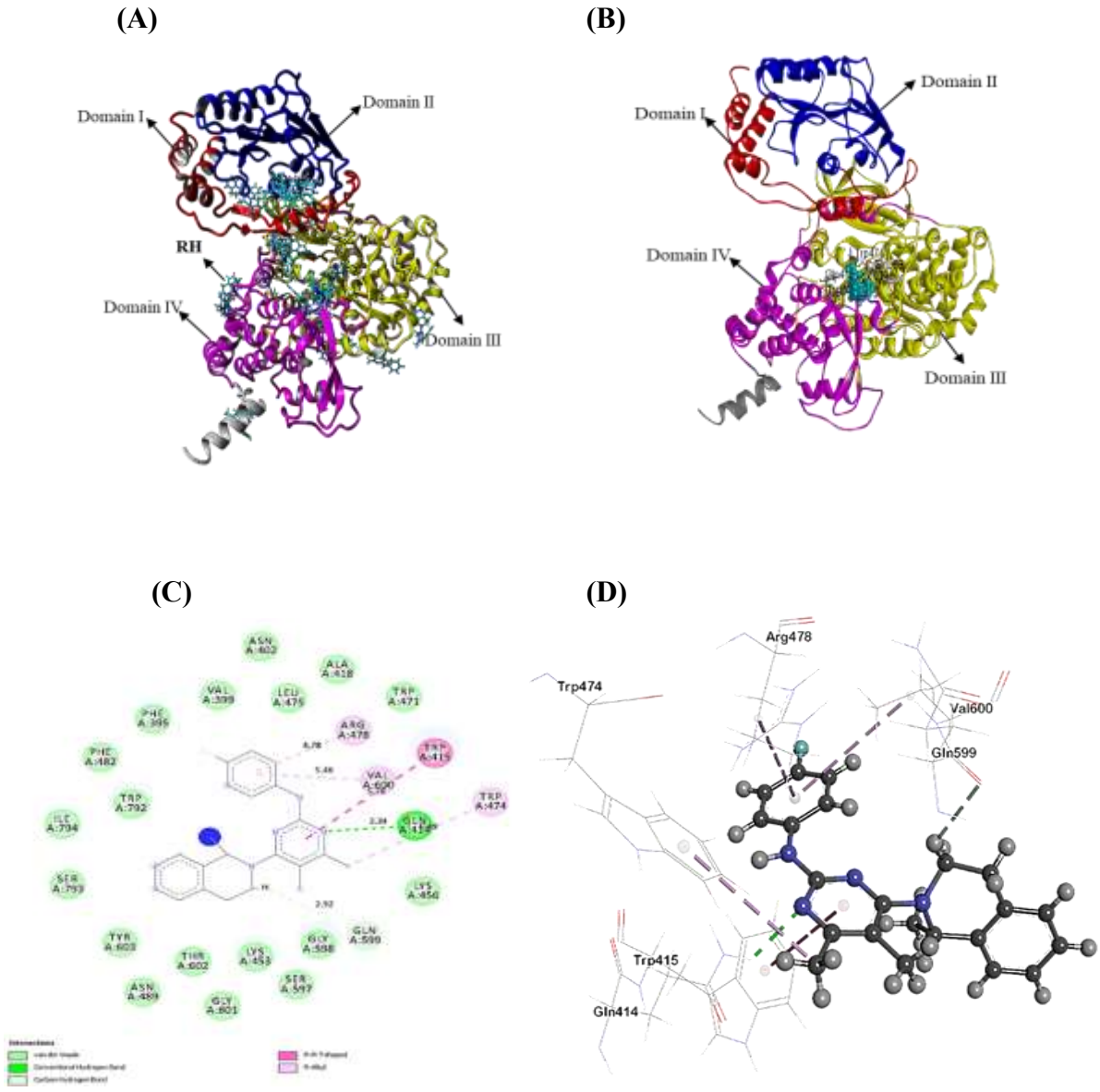

Figure 8. Molecular docking of DENV-3 NS5 protein docking with revaprazan hydrochloride. (A) Structure of the DENV-3 NS5 protein docking with revaprazan hydrochloride. (B) Binding pose of revaprazan hydrochloride's molecular structure on DENV-3 NS5 protein with eight different possible binding sites. (C) 2D scheme of the interaction of revaprazan hydrochloride with the amino acid residues of DENV-3 NS5 protein, including H-bonding and hydrophobic interactions as shown by docking analysis. (D) 3D scheme of the interaction of revaprazan hydrochloride with the DENV-3 NS5 protein, H-bonds at the NS5 protein Gln414, and Gln599 and hydrophobic interactions at Trp415, Trp474, Arg478, and Val600.

4. Discussion

Dengue fever is the second most prevalent vector-borne disease, trailing behind malaria [1, 35]. Currently, no anti-DENV drugs have been developed to treat dengue infections, and patients rely primarily on symptomatic treatment and supportive medical care [36, 37].

Given the global surge in DENV infections, repurposing existing drugs is of great interest to treat or prevent the advancement of dengue. Furthermore, the use of repurposed drugs allows for a much faster and safer method of producing treatments for a wide range of infectious diseases [26, 38, 39].

We screened a chemical library containing 174 repurposed compounds against DENV-3 infected Vero cells using a foci-forming unit reduction assay to identify novel anti-DENV compounds. At the time this research was initiated, DENV-3 was the predominant circulating serotype, making it a relevant choice for initial compound screening and characterization [40, 41]. Through the screening, revaprazan hydrochloride was identified as the novel DENV-3 inhibitor. Revaprazan hydrochloride, a potassium-competitive acid blocker (P-CABs) was discovered by Yuhan in South Korea in 2007 and was utilized as a medicinal drug for treating gastric and acid-related illnesses [42]. There is no scientific investigation on the use of the repurposed drug, revaprazan hydrochloride against dengue and other viruses, and this study provides insights into the antiviral activity of this drug against DENV. Based on our study, the SI values for revaprazan hydrochloride evaluated via the reduction in foci and viral RNA copy suggested that this drug is safe and can be employed as an effective antiviral agent against DENV-3. Our cell protection assay revealed that pre-treatment of Vero cells with revaprazan hydrochloride had a minimal inhibitory effect against DENV-3 indicating that the drug might have a little interaction with the cellular receptor(s) and might be acting directly on the virus or disrupting the translation or RNA synthesis steps by targeting virally encoded proteins such as the E, NS2B/NS3, and NS5 proteins.

Antiviral compounds that serve as virucidal agents are capable of inactivating extracellular viral particles by either destroying the viral protein coat or penetrating the virion and destroying the viral genome [43]. Revaprazan hydrochloride at 35 µM demonstrated virucidal effect against DENV-3 with a 98.64 % inhibitory effect and required only 30 min to limit viral replication by 90%. Other compounds have been described to have similar virucidal effects, such as baicalein which exhibited potent direct virucidal activity against DENV-2 containing 200 FFU at IC_{50} = 1.55 µg/mL, as well as a 99.2 % decrease in DENV-2 RNA production by qRT-PCR when infected cells were treated with baicalein at 12.5 µg/mL [44]. This finding is consistent with baicalin's virucidal effect against DENV-2 at IC_{50} = 8.74±0.08 µg/mL. FFURA and qRT-PCR analysis demonstrated that baicalin inhibited DENV-2 replication at more than 90% when tested at 50 µg/mL, but also at 96.1 % when tested at 25 µg/mL, with both tested concentrations remaining lower than MNTD (62.5 g/mL) [44]. An *in silico* study previously found that baicalein demonstrated binding interactions with DENV E protein, which possibly contributed to its virucidal effect [45]. A recent study also

demonstrated that baicalein could inhibit 99.78% virucidal effect at its highest non-toxic concentration, and required only 15 min to show 75% viral infectivity inhibition against DENV-3 [46].

Time-of-drug addition and elimination upon virus infection revealed that revaprazan hydrochloride may inhibit the translation or RNA synthesis steps in the DENV replication. A similar finding was reported on bromocriptine's post-infection activity on BHK-21 cells infected with DENV-1 [32]. Addition of bromocriptine at 10 µM up to 6 h post-infection dramatically reduced DENV-1 production at MOI 1 by more than 2 logs, indicating the molecule might impede the early stages of the DENV replication. The elimination of bromocriptine dramatically decreased viral replication by more than 2 logs when treatment began at 4 h after infection [32]. Besides, the addition of andrographolide at 100 µM reduced DENV-2 virus titer on HepG2 (MOI = 20) or HeLa (MOI = 1) cells when the compound was added up to 3 h post-infection [47]. The addition of hirsutine at 10 µM against DENV-1 (MOI = 1) significantly reduced the virus titers up to 12 h post-infection, suggesting that hirsutine might have inhibited the pre-release step of the viral particle. The elimination of hirsutine did not decrease the viral titre after a 2 h treatment, indicating the attachment and entry stage was not hirsutine's target of interest [48]. Comparably, when DENV-2 infected Vero cells were treated 1 h post-infection, the addition of quinolines 1 and 2 at 3 µM showed maximal impairment of the DENV-2 infection, suggesting that these compounds might have affected the early events of the viral replication [49].

A major concern in the development of antiviral compounds is the emergence of drug-resistant viruses [50]. In a recent study, DENV-4 resistance to ciprofloxacin was detected by the seventh passage and to difloxacin by the tenth passage in HEK-293 cells. Two putative resistance-conferring mutations were detected in the E gene of ciprofloxacin and difloxacin-resistant DENV-4 [51]. Besides, DENV-2 developed resistance in the presence of the nucleoside analog 2′-C-methyl-7-deaza-adenosine (7-DMA) after 17 passages in BHK-21 cell line [52]. Bromocriptine (BRC)-resistant DENV-1 was generated by performing 16 serial passages and, results demonstrated that a single amino acid substitution (NS74H) was found in the NS3 protein that conferred resistance to BRC [32]. Therefore, it is vital to monitor the emergence of resistant viruses during antiviral drug development. In this study, 25 rounds of serial passage of DENV-3 in Vero cells did not demonstrate revaprazan hydrochloride-resistant viruses, suggesting that this compound was not interacting with any viral proteins that could have mutated.

Revaprazan hydrochloride exhibited antiviral activity against all four DENV serotypes in both virucidal and post-infection assays, although the degree of inhibition varied among the serotypes. Notably, DENV-1 and DENV-2 were more susceptible to revaprazan hydrochloride, while DENV-4 showed relatively lower sensitivity, particularly in the post-infection context. These differences may be attributed to serotype-specific variations in viral structure, replication dynamics, or host-virus interactions that could influence the drug's binding affinity or antiviral mechanism. The consistent trend observed across both immunofluorescence and qRT-PCR analyses supports the reliability of these findings. Overall, while revaprazan shows promise as a broad-spectrum anti-dengue agent, its varying efficacy highlights the importance of further mechanistic studies to understand the molecular basis of serotype-specific responses and guide the rational development of more effective dengue therapeutics.

Computational docking studies were carried out to predict and characterize the binding ability of revaprazan hydrochloride with the E, NS2B/NS3, and NS5 proteins to explain the compound's inhibitory mechanism against DENV-3. Our *in silico* analysis showed that revaprazan hydrochloride had the highest binding affinity towards NS5 (9.37 kcal/mol), followed by NS2B/NS3 (9.17 kcal/mol) and E (8.03 kcal/mol) protein. NS5 protein has both RdRp domain at the C-terminal which is important for viral RNA synthesis and MTase domain at the N-terminal which is vital in catalyzing the 5' RNA capping and methylation to enhance RdRp activity [53]. One of the most attractive antiviral targets is the RdRp domain, a clinically proven therapeutic target [54, 55]. Our docking analysis revealed that revaprazan hydrochloride had the greatest binding energy to RdRp at domain III of the NS5 protein. This domain lies between 320-900 amino acids at its C-terminal end, and residues 320-368 are highly conserved among the flaviviruses [56, 57]. This compound also binds to the RdRp domain between 250-875, which contains the conserved residues, suggesting that revaprazan hydrochloride targeted a domain that is profoundly conserved among DENV serotypes.

The NS3 protein has two domains, the serine protease at the N-terminal which is important for polyprotein processing, and the helicase domain (NS3hel) situated at the C-terminal for unwinding the RNA duplex during RNA replication [56]. The NS3hel domain is a potential drug target to design novel effective antiviral compounds against DENV. Studies have revealed that the NS2B co-factor is a prerequisite for the catalytic activity of the NS3 protease [58], therefore the complex NS2B/NS3 resembles a much more structurally relevant target than NS3 alone for functional studies and drug discovery research. Our *in silico* analysis demonstrated that revaprazan hydrochloride binds to the NS2B/NS3 protein with the

highest binding energy between domain I and domain IV with the NS3 helicase domain residing between 460-604 residues at domain IV. Similarly, the NS3hel domain resides within the region 180-618 residue of the NS3 protein [59]. Our docking data indicated that revaprazan hydrochloride might have hindered the activity of the NS3hel domain, and might target the unwinding stage of the RNA duplex which is required as a template in viral replication, thus hindering the replication and translational process.

The E protein consists of three structural domains, the central domain I, extended finger-like domain II and immunoglobulin-like domain III [60, 61]. Domain III links the N-terminal domains to the stem region, which is part of the outer lipid leaflet of the viral membrane and consists of two α-helices associated with a conserved sequence [61]. Our docking studies showed that revaprazan hydrochloride could bind to E protein at binding site 1 (between domain I/II) with the least binding energy. Although the binding affinity of the revaprazan hydrochloride–E protein complex was lower compared to its interactions with NS2B/NS3 and NS5, functional assays showed that revaprazan hydrochloride significantly inhibited both viral attachment and entry of DENV-3 into Vero cells. These results suggest that the compound can interfere with early stages of viral infection, potentially through partial inhibition of E protein function. However, the comparatively stronger binding affinities observed with NS2B/NS3 and NS5 imply that revaprazan hydrochloride primarily targets these multifunctional proteins, which are essential for viral RNA replication, contributing to its overall antiviral activity.

While our findings highlight the promising antiviral activity of revaprazan hydrochloride against all four DENV serotypes, several limitations should be acknowledged. First, the current study was limited to *in vitro* assays and *in silico* analyses; therefore, the efficacy and safety of revaprazan hydrochloride *in vivo* remain to be determined. Second, the precise molecular mechanism underlying revaprazan hydrochloride's antiviral activity, particularly its interactions with viral or host targets, has not yet been fully elucidated and warrants further biochemical and structural studies. Although the molecular docking analysis was conducted using DENV-3 proteins, the conserved nature of the viral targets suggests potential cross-serotype activity; however, this requires further validation through structural and functional studies across other serotypes. Additionally, potential off-target effects and cytotoxicity profiles need to be thoroughly assessed in animal models. Despite these limitations, the repurposing of revaprazan hydrochloride, an already clinically approved gastroprotective agent, offers a practical advantage in terms of safety profiling and regulatory pathways. Future work should explore its pharmacokinetics, antiviral efficacy, and toxicity in preclinical models of dengue infection. If supported by further *in vivo* and clinical studies,

revaprazan hydrochloride could emerge as a cost-effective, orally available antiviral candidate for dengue treatment.

5. Conclusion

In conclusion, this study showed that revaprazan hydrochloride efficiently inhibited DENV-3, showed no resistance up to 25 passages in Vero cells and interfered with the early stage of the DENV-3 replication. *In silico* analyses also indicated that revaprazan hydrochloride could interact with E, NS2B/NS3, and NS5 proteins of DENV, with a strong binding affinity towards the RdRp domain of NS5 and helicase domain of the NS2B/NS3 proteins. Interaction of revaprazan hydrochloride with essential DENV-3 protein targets suggested the ability of this drug to inhibit the production of DENV-3 at different stages of its replication cycle. Further research is required to understand the detailed mechanism of action of the compound *in vivo* and for the development of therapeutic strategies against DENV infection. Revaprazan hydrochloride is, to the best of our knowledge, reported for strong antiviral efficacies against all four dengue serotypes.

Supplementary Materials: Supplementary materials are provided.

Author Contributions: Conceptualization: BR, CLP; Validation: SG; Formal analysis: SG, MM; Investigation: SG, MM; Resources: BR, CLP; Writing – Original draft: SG; Writing – Review & Editing: MFL, AV, MSR, SA, YSW; Visualization: SG; Supervision: BR, CLP, YSW; Funding acquisition: BR. All authors have read and approved the final version of the manuscript.

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