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Discovery of antiviral molecules for dengue: *In silico* search and biological evaluation

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Title**DISCOVERY OF ANTIVIRAL MOLECULES FOR DENGUE: *IN SILICO* SEARCH AND BIOLOGICAL EVALUATION****Author Names**

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ABSTRACT

Background: Dengue disease is a global disease that has no effective treatment. The dengue virus (DENV) NS2B/NS3 protease complex is a target for designing specific antivirals due to its importance in viral replication and its high degree of conservation. Methods: NS2B/NS3 protease complex structural information was employed to find small molecules that are capable of inhibiting the activity of the enzyme complex. This inhibitory activity was probed with *in vitro* assays using a fluorescent substrate and the complex NS2B/NS3 obtained by recombinant DNA techniques, for testing the activity against dengue virus replication, HepG2 cells infected with dengue virus serotype 2 were used. Results: A total of 210,903 small molecules from PubChem were docked *in silico* to the NS2B/NS3 structure (PDB: 2FOM) to find molecules that were capable of inhibiting this protein complex. Five of the best 500 leading compounds, according to their affinity values (-11.6 and -13.5 kcal/mol), were purchased. The inhibitory protease activity on the recombinant protein and antiviral assays was tested. Conclusions: Chemicals CID54681617, CID54692801 and CID54715399 were strong inhibitors of NS2B/NS3, with IC₅₀ values (μM) and percentages of viral titer reductions of 19.9, 79.9%; 17.5, 69.8%; and 9.1, 73.9 %, respectively. Multivariate methods applied to the molecular descriptors showed two compounds that were structurally different from other DENV inhibitors. General significance: This discovery opens new possibilities for obtaining drug candidates against Dengue virus.

Keywords: Dengue protease inhibitor; NS2B/NS3 complex; Molecular docking; *In vitro* inhibition; Inhibitory activity.

1. INTRODUCTION

Dengue is the fastest spreading viral disease that is transmitted by mosquitoes (*Aedes aegypti*, mainly) worldwide [1], and two-fifths of the world's population is at risk for becoming infected [2]. In tropical and sub-tropical countries, dengue is a major public health problem due to multiple factors, such as reemergence and intense transmission, the behavior of epidemic cycles every two or three years, the increase in the frequency of severe dengue outbreaks and the simultaneous circulation of different serotypes. Consequently, the incidence rate has shown a growing trend over time. Approximately 50,000 people suffer from dengue annually, and relatively 10% of the total cases involved dengue hemorrhagic fever [3, 4]. Despite its global spread, currently there is no drug that is available for the disease.

The etiologic agent for dengue is a virus that belongs to the genus *Flavivirus*, which has five known serotypes. Dengue virus (DENV) co-circulates as a complex of five closely related but antigenically distinct serotypes (DENV-1–DENV-5), [5] all of which are etiologic agents of dengue fever, life-threatening dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). DENV-2 and DENV-3 have historically been the most prevalent agents that have caused severe disease [6]. This disease is characterized by high fever, headache, joint pain, fatigue, swollen lymph nodes, skin rashes, and in some cases, bleeding and shock (DHF), which can cause death [3].

The dengue virus genome consists of a single-stranded, positive sense RNA of approximately 11 kb that is organized as 5'NCR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-

NS4B-NS5-3'NCR, where NCR is the noncoding region, C is the capsid, prM is the pre-membrane, and E is the envelope, while NS corresponds to nonstructural proteins. Among these proteins, envelope glycoprotein, NS3 protease, NS3 helicase, NS5 methyltransferase and NS5 RNA-dependent RNA polymerase have been suggested to be therapeutic targets for specific antiviral searches against dengue [7].

Currently, studies to find antiviral molecules have focused on targeting essential viral enzymes in the infection process by the direct or indirect inhibition of their biological functions or by blocking the viral replication machinery [8, 9]. One of the most used targets that is employed for this purpose is the NS2B/NS3 protease, as shown by several authors [10-12]. It is clear that flavivirus proteases, including NS2B/NS3, are essential for viral replication and infectivity. For example, it has been shown that dengue virus infectiousness decreases by 80% in cells that are treated with peptide inhibitors of this enzyme [13]. In addition, the inhibition of viral proteases is a well-established route for preventing viral infection in clinical settings. In fact, a large number of HIV protease inhibitors are clinically used to treat HIV infection and AIDS [14]. Similarly, two hepatitis C virus protease inhibitors, Simeprevir [15] and Sofosbuvir [16], which were recently approved for therapeutic use, have become the standard of care. Thus, the use of compounds that target the NS2B/NS3 protease becomes a prominent strategy for dengue virus therapy.

The use of bioinformatics tools, molecular modeling programs and high performance computing has been leading the process of designing and *in silico* searching for

therapeutically useful molecules. This same approach has been applied to the discovery or development of compounds that could target several diseases [17, 18]. One of the strategies at the forefront of drug discovery is the virtual screening of databases [19], which can identify powerful new protein ligands [20, 21]. Using the structural information on the dengue virus protease and molecules published in PubChem (<http://pubchem.ncbi.nlm.nih.gov>), virtual screening was performed to search for low molecular weight molecules that could inhibit this protease and that could be potentially employed in the treatment of dengue infection. Therefore, the main aim of this research was to find non-peptide molecules that have the *in vitro* ability to inhibit the NS2B/NS3 protease activity of the dengue virus. The discovery of molecules that interfere with the proteolytic activity of the DENV NS2B/NS3 protease could become a potential alternative for the treatment and control of the disease.

For this purpose, after determining the best leading compounds, the complex NS2B/NS3 was obtained using recombinant DNA techniques. The inhibitory capacity against the proteolytic activity of the isolated complex was determined using the synthetic peptide Boc-Gly-Arg-Arg-AMC as a fluorescent substrate. Finally, the inhibitory effect of these compounds was investigated on dengue virus replication *in vitro* using HepG2 cells that were infected with dengue virus serotype 2.

2. MATERIALS AND METHODS

The methodology used in this study comprised the steps detailed below:

2.1. *In silico* studies

The dengue virus NS2B/NS3 protease crystal structure was obtained from the Protein Data Bank (PDB: 2FOM). This crystallographic structure has been used recently by several authors for *in silico* molecular docking approaches [22-24]. The 3D structure of the protein was prepared using the SYBYL 8.1 software package (Tripos, Inc., 2008) by removing all of the water molecules and substructures that were present. Furthermore, the side chain amide groups were fixed for each amino acid. The resulting structure was minimized using the Powell method of molecular mechanics, with the combined force fields Kollman united/Kollman All Atom, applying Amber charges and a gradient convergence criterion of 0.005 kcal/mol. The final structure was saved in PDB format. The program MGLTools 1.5.0 [25] was utilized to convert the PDB file to the PDBQT format, adding polar hydrogens for all of the docking calculations.

Three-dimensional structures of ligands that were uploaded into the NCBI PubChem Database from March/2005 to December/2011, were downloaded from this database (<http://pubchem.ncbi.nlm.nih.gov/>) in SDF format. Structures were optimized with the SYBYL 8.1.1 package [26] with molecular mechanics methods, employing a Tripos force field, Gasteiger charges, gradient convergence of 0.01 kcal/mol and a maximum number of optimization iterations set to 1000. Optimized structures were saved in MOL2 format, and converted to PDBQT files with MGLTools 1.5.0 [27] for molecular docking procedures.

The AutoDock Vina 1.1 program [28] was employed for all of the fittings that were required for the virtual screening. The protein was maintained rigid throughout the docking process, while the ligands were allowed to be flexible. The molecular parameters used were the following: a grid spacing of 1.000 Å; box size dimensions of 22 × 30 × 32 (x, y, z); coordinates of x = 9.790 Å, y = 10.355 Å, and z = 11.150 Å at the center of the box; number of modes = 20; energy range = 1.5; and exhaustiveness = 20. The substrate-based inhibitor binding pocket shown in the crystallographic structure (PDB: 2FOM) was chosen for placing the grid box to cover the entire enzyme binding site and accommodate ligands to move freely. The interactions between the molecules and dengue virus protease were checked with LigandScout 2.0 [29]. This software extracts and interprets ligands and their macromolecular environment from a PDB file, which was previously prepared in SYBYL8.1, displaying tridimensional and 2D images of the interactions.

Docking validation with biological data. The docking protocol was validated using a test set of 40 molecules, including active and inactive reported inhibitors against dengue virus, as well as reported inhibitors for proteases from other viruses, for comparative purposes [30]. For dengue inhibitors, molecules with IC₅₀ values less than 15 µM were chosen. For some molecules, the three-dimensional structures were downloaded from the NCBI PubChem Database, and processing was similar to that described above. Such is the case of the compounds CID_3010818, CID_53487990, CID_10324367, CID_35370, CID_5362440, CID_60825, CID_441243, CID_54682461, CID_44246257, CID_4101471, CID_60194816, and CID_23350827. For remaining ligands, 3D structure

was drawn and optimized using DFT at the B3LYP/6-31G level. Calculations were carried out with Gaussian 09 package program [31]. SYBYL 8.1.1 package was used to convert files to MOL2 format and MGLTools 1.5.0 to PDBQT format.

Molecules with the best binding affinities were compared to other chemicals that were published as active and inactive inhibitors of dengue protease. This comparison was based on their molecular characteristics using hierarchical agglomerative clustering. Cluster analysis was performed using the Tanimoto coefficient method with a measure of similarity and an average linkage [32].

The web-based software tool FAFDrugs3 [33], which is hosted on the public domain of The Ressource Parisienne en Bioinformatique Structurale (<http://fafdrugs2.mti.univ-paris-diderot.fr/references.html>), was used to filter for Pan Assay Interference Compounds (PAINS). The canonical Simplified Molecular Input Line Entry System (SMILES) strings were used as Input data, which were obtained from the PubChem database.

2.2. *In vitro* studies

2.2.1. Protein expression

A soluble His-tagged recombinant DEN-2 NS2B/NS3 protease was constructed according to the sequence published by Yang et al. [34]. In this study, the vector pET28c was used for the inducible expression of the hexahistidine-tagged recombinant protein. The DEN-2 NS2B/NS3 protease sequence was inserted between the NdeI and

XhoI restriction sites with a flexible linker (Gly4) between NS2B and NS3. The vector was then transformed into the *Escherichia coli* strain BL21 star (DE3) for protein expression. Cultures of this *E. coli* strain were grown in 5 L of LB medium that contained 30 µg/mL kanamycin at 37 °C until the absorbance at 600 nm (A₆₀₀) reached 0.6. The cells were induced for expression by the addition of isopropyl-β-D-thiogalactopyranose to a final concentration of 0.4 mM and were incubated for an additional 4 h at 37°C. The cells were then harvested by centrifugation (8900 rpm, 20 min, 4 °C), and the pellets were stored at –80 °C until used. For protein purification, the cells were thawed and re-suspended in BugBuster® Protein Extraction Reagent. The re-suspended cells were stirred at 300 rpm for 20 minutes at room temperature. Then, they were centrifuged 30 min at 5,000 g and 4 °C. The supernatant was purified immediately by passage through a Ni²⁺ column and pre-equilibrated with 50 mM Na₂HPO₄ and 300 mM NaCl, pH 7.0. The column was extensively washed with buffer that contained 10 mM and 150 mM imidazole, and protein was then eluted from the column in buffer that contained 300 mM imidazole. Finally, the elution buffer was changed for storage buffer (Tris 125 mM, pH 7.5, NaCl 0,6 M, glycerol 25%). The elution fractions were analyzed by 12% SDS-PAGE. Samples of pre- and post-induced cells as well as soluble and insoluble fractions following lysis were collected and also analyzed by 12% SDS-PAGE.

2.2.2. Fluorimetric enzyme activity assay

The bioassay used here is a modification of that reported by Tomlinson and Watowich [35]. The molecules used were those that were commercially available, with the best binding affinities obtained with the *in silico* studies: C₂₉H₂₅N₃O₄S (PubChem ID:

54710332, STK841825, Purity > 90%), $C_{25}H_{14}O_6$ (PubChem ID: 54692153, STK523898, Purity > 90%), $C_{35}H_{27}NO_9$ (PubChem ID: 54681617, STK530661, Purity > 90%), $C_{30}H_{25}NO_5$ (PubChem ID: 54692801, STK332077, Purity > 90%), and $C_{34}H_{23}NO_7S_2$ (PubChem ID: 54715399, STK529754, Purity 95%), as well as aprotinin, a competitive serine protease inhibitor. The assay was performed with 96-well plates, with a final volume of 100 μ L. The NS2B/NS3 dengue virus protease (0.1 μ M) was pre-incubated in storage buffer (200 mM Tris, pH 9.5, 20% glycerol) at room temperature for 30 min. Small molecules were tested at 1, 2 and 10 μ M. The reaction was initiated by the addition of the substrate Boc-Gly-Arg-Arg-AMC at 100 μ M. The reaction progress was monitored continuously for 60 minutes. Fluorescence readings were recorded at 355 nm excitation and 460 nm emission. Triplicate measurements were conducted for each data point. The data are reported as the means $\pm$ S.E. The 50% inhibitory concentration (IC_{50}) values were estimated graphically from the plots of percent inhibition versus inhibitor concentration [36]. IC_{50} was defined as the inhibitor concentration at which 50% of the control protease activity was inhibited. All of the protease inhibitor and cytotoxicity assays were performed in triplicate.

2.2.3. Cell and Virus

The human hepatocellular liver carcinoma cell line, HepG-2 (from American Type Culture Collection [ATCC] HB-8065), was grown in DMEM/F-12 medium (GIBCO® Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) supplemented with 10% (v/v) fetal calf serum and maintained in a humidified 5% (v/v) CO_2 incubator at 37°C. The DENV-2 New Guinea C strain (NGC) provided by the Center for Disease Control (CDC,

Fort Collins, CO) was used in this study. Stock virus was prepared in *Ae. albopictus* (C6/36) cells, and the virus titer was determined by the conventional Reed & Muench method [37].

2.2.4. Cell proliferation assay

An MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-di-phenyl tetrazolium bromide] cell proliferation assay was performed to estimate the potential cytotoxicity of the compounds on HepG-2 cells using the protocol described by Meneses et al. [38]. Cells were seeded into 96-well plates at 1×10^4 cells per well and incubated for 24 h at 37°C; then, the culture medium was removed, and fresh medium with each compound at final concentrations of 200, 100, 50, 25 and 12.5 μ M was added. After 72 h of incubation at 37°C, MTT solution (20 μ L; 5 mg/mL. Sigma Co.) was added, and the cells were incubated for an additional 4 h. The reduction of MTT to a formazan product was quantified by measuring the optical density (OD) at 580 nm using a microplate reader (Thermo Scientific). Data are expressed as the concentration of compound that reduces the OD₅₈₀ of the treated cells to 50% with respect to the untreated cells (50% Cytotoxic Concentration, CC₅₀).

2.2.5. Virus yield reduction assay

Viral protein NS1 reduction assays were performed to examine the antiviral activity of the tested compounds. Secreted NS1 in the culture medium of virus-infected cells correlates with viral replication [39]. Approximately 1×10^4 HepG-2 cells per well were seeded in a 96-well plate, and after incubating for 24 h at 37°C, these cells were

infected with DENV-2 at a multiplicity of infection (MOI) of 11.0. One hour after virus adsorption, the cells were washed and replenished with medium that contained concentrations of the compound below the Maximum Non-Toxic Concentration (MNTC). Samples of culture medium were collected at 72 h post infection, stored at -20°C, and later tested for the presence of DENV-2/NS1 protein. Assays were performed in duplicate in at least two independent experiments. Virus-infected cells, non-drug treated or treated with ribavirin, were run in parallel as negative and positive controls, respectively. The differences in the NS1 levels between the treated and untreated cells were tested for significance by Student's t-test. The IC_{50} and selectivity index ($SI = CC_{50}/CI_{50}$) were calculated.

2.2.6. NS1 protein capture assay

The Panbio® Dengue Early kit (PanBio Diagnostic Brisbane, Australia) is a sandwich format microplate enzyme immunoassay for the quantitative detection of dengue NS1 protein. Samples of culture medium from DENV-2-infected cells treated and untreated with compound or ribavirin were tested according to the manufacturer's instructions. The presence of NS1 was demonstrated by a color development. The optical density (OD) reading was taken at 450 nm, and the amount of NS1 was determined by comparing the OD of the sample with the OD of the cut-off control cell medium. The results are expressed as "Panbio units: PbU," with 11.0 defined as the minimum concentration that was detected [40].

2.2.7. Data Analysis

Mean comparisons between groups were performed using the Kruskal-Wallis test. Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). The p-value that was considered to be significant is indicated in each Figure. Error bars are expressed as $\pm$ SD.

3. RESULTS

This report describes the discovery of compounds that can inhibit NS2B/NS3 dengue virus protease, an essential enzyme for viral replication, which could provide pharmacological benefits for the medical treatment of this disease. It has been found that this enzyme has an essential role in dengue virus replication through the cleavage of viral polyprotein [41]. Therefore, disturbing the activity of dengue protease will contribute to blocking the viral replication in host cells. The NS2B/NS3 two-component protease of dengue virus is currently being investigated with the purpose of discovering small molecule inhibitors that are suitable as treatment options for the disease with the same name [42].

3.1. Screening with the PubChem Database

The use of the dengue virus protease structural information for *in silico* approaches allowed the identification of different compounds with good inhibitory activity for this protein. From the PubChem Database, 210,903 compounds (Table S1) were docked into the DENV protease model (PDB: 2FOM). The scoring of the *in silico* binding affinities ranged from -15.1 to 165.0 kcal/mol. Theoretically, a lower Gibbs free energy of a protein–ligand complex indicates more stability, and in addition, binding requires a

negative Gibbs free energy, which is similar to any other spontaneous process [43]. Of the total number of examined molecules, 113,849 showed equal or better binding affinity values than the average (-7.4 kcal/mol); these are molecules that are less than 2000 Da, and most have more H bond acceptors than donors.

3.2. Selecting the inhibitors

Among the molecules with the best docking scores, five were commercially available, purchased, and tested *in vitro* as dengue protease inhibitors (Table 1). All of them have molecular weights between 410.38 and 621.68 Da. For these molecules, the potential drug-like properties were calculated using the OSIRIS property calculator (<http://www.organic-chemistry.org/prog/peo>). This software suggests that except for C₃₀H₂₅NO₅ (CID 54692801), these small molecules do not contain the pharmacophores that are associated with increased risk of adverse mutagenic, tumorigenic or irritant effects. For C₃₀H₂₅NO₅ (CID 54692801), naphtalen-1-amine is considered to be a high risk fragment that indicates tumorigenicity and mutagenicity.

Table 1. Purchased and tested compounds from the PubChem database with high *in silico* affinity values (kcal/mol) for the DENV protease model (PDB: 2FOM).

PubChem ID	Structure	Molecular formula	Docking affinity scores (kcal/mol)	Molecular weight (g/mol)
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54692801		$C_{30}H_{25}NO_5$	-13.5	478.52
54715399		$C_{34}H_{23}NO_7S_2$	-11.4	621.68
54710332		$C_{29}H_{25}N_3O_4S$	-11.7	511.59
54692153		$C_{25}H_{14}O_6$	-11.4	410.38
54681617		$C_{35}H_{27}NO_9$	-11.6	605.59

According to Bael and Holloway [44], PAINS appear as frequent hits in high throughput screening (HTS). Some of the molecules with the best docking scores obtained from this type of assay involve false positives as a result of their high protein reactivity. The substructural features identified in promiscuous inhibitors were searched by FAFDRUGS3 [33] (<http://fafdrugs2.mti.univ-paris-diderot.fr/mobyle.html>). None of the five hits reported in this paper were described as PAINS (Figure S2), which corroborates that these compounds are promising starting points for further exploration in current dengue research.

Protein-ligand complexes for selected compounds are presented in Figure 1. Most of the interactions were hydrophobic and hydrogen bonding interactions, van der Waals forces, and a few aromatic ring interactions. Comparing the conformation adopted for these molecules in the binding site of the dengue protease, it is evidenced that certain residues interact with most of these molecules, in particular, Ile65, Trp69, Lys74, Leu76, Thr120, Ile123, Val154, Ala164, Ile165, and Ala166; thus, it is likely that these residues share the same binding site.

Figure 1. Tridimensional view of the docking and interacting residues in the binding site cavity of Dengue virus protease (PDB: 2FOM) in complex with A. C₃₀H₂₅NO₅, CID 54692801. B. C₃₄H₂₃NO₇S₂, CID 54715399. C. C₂₉H₂₅N₃O₄S, CID 54710332. D. C₂₅H₁₄O₆, CID 54692153. E. C₃₅H₂₇NO₉, CID 54681617.

Given the high amino acid sequence homology between the NS3 proteases of the Flaviviridae family [45], it is feasible that the inhibitors reported for other viral proteases might have an important inhibitory activity for this enzyme in dengue virus. For this reason, the crystal structure of the dengue NS2B/NS3 protease (PDB: 2FOM) was docked with some inhibitors that were reported for proteases from viruses, such as Hepatitis C (HCV), West Nile (WNV), Dengue and Human Immunodeficiency Virus (HIV) (Table 2). For dengue inhibitors, molecules with IC_{50} values of less than 15 μ M were chosen. The affinity values obtained for these inhibitors were in the range from -10,1 kcal/mol to -5.5 kcal/mol. From these, the best values were those obtained for dengue virus protease inhibitors, as expected. However, the binding affinity values obtained with these molecules were not as good as those calculated for the molecules suggested in this paper. The PDB crystal structure 2FOM was also used for docking with some molecules that were reported as not being active inhibitors for dengue protease [46-48]. The molecular structure of inhibitors reported for other proteases (HCV, WNV, Dengue and HIV), as well as those known as not active inhibitors for dengue protease, that were docked onto the crystal structure of the dengue NS2B/NS3 complex (PDB: 2FOM), are shown in Table S3. The affinity values range varied from -9,0 kcal/mol to -6,2 kcal/mol (Table 2), which shows significant differences ($P < 0.05$) from the values reported in inhibitors (Figure S4).

Table 2. Affinity binding values for viral protease inhibitors and molecules reported as not active dengue virus protease inhibitors, docked with the DEN2V protease crystal structure (PDB: 2FOM).

Code	Compound	Inhibited virus	Replicates of docking affinity with DEN2V (kcal/mol)			Mean	SD	Reference
			R1	R2	R3			
C1	CID_3010818	HCV ^a	-7,0	-7,0	-7,1	-7,0	0.1	[49]
C2	CID_53487990	HCV	-6,7	-6,7	-6,7	-6,7	0.3	
C3	CID_10324367	HCV	-7,2	-7,3	-7,1	-7,2	0.1	
C4	CID_35370	HIV ^b	-6,3	-6,3	-6,3	-6,3	0.0	[50]
C5	CID_5362440	HIV	-7,8	-7,9	-8	-7,9	0.2	
C6	CID_60825	HIV	-5,6	-5,6	-5,4	-5,5	0.1	
C7	CID_441243	HIV	-8,1	-8,1	-8	-8,1	0.4	[51]
C8	CID_54682461	HIV	-7,1	-7	-8,8	-7,6	0.7	[52]
C9	CID_44246257	WNV ^c	-8,2	-8,1	-7,7	-8,0	0.2	[53]
C10	N1,N4-bis(4-(4-methyl-4,5-dihydro-1H-imidazol-2-yl)phenyl)phthalazine-1,4-diamine	DENV	-9,4	-9,4	-9,3	-9,4	0.1	[54]
C11	CID 4101471	DENV	-9,1	-8,6	-9,1	-8,9	0.2	[35]
C12	ARDP0006	DENV	-8	-8	-8	-8,0	0.0	
C14	Chemdiv K286-0036	DENV	-8,6	-8,6	-8,6	-8,6	0,0	[55]
C15	CID 60194816	DENV	-8,4	-7,8	-7,9	-8,0	0,3	[56]
C16	CID 60194819	DENV	-8,9	-9,4	-8,3	-8,9	0,6	
C17	Compound 7b	DENV	-9,6	-9,7	-8,8	-9,4	0,5	[57]
C18	Compound 7c	DENV	-9,8	-9,8	-9,1	-9,6	0,4	
C19	Compound 7e	DENV	-9,6	-9,5	-8,7	-9,3	0,5	
C20	Compound 8a	DENV	-9,4	-9,8	-9	-9,4	0,4	
C21	Compound 8b	DENV	-10,2	-10,2	-9,9	-10,1	0,2	

C22	Compound 8e	DENV	-9,3	-9,8	-9,3	-9,5	0,3	
C23	Compound 7a		-9,2	-9,0	-8,9	-9,0	0,2	[47]
C24	Compound 8f		-9,8	-9,9	-10	-9,9	0,1	
C25	Compound 2		-6,3	-6,4	-6,2	-6,3	0,1	[46]
C26	Compound 4a		-6,7	-6,7	-7,6	-7,0	0,5	
C27	Compound 4b		-8,0	-8,7	-8,8	-8,5	0,4	
C28	Compound 4c		-7,7	-8,6	-7,9	-8,1	0,5	
C29	Compound 4d	NOT	-7,5	-7,5	-7,2	-7,4	0,2	
C30	Compound 4e	ACTIVE	-8,6	-8,2	-7,9	-8,2	0,4	
C31	Compound 4f	DENGUE	-8,1	-7,9	-7,8	-7,9	0,2	
C32	Compound 4g	VIRUS	-8,2	-7,9	-7,3	-7,8	0,5	
C33	Compound 4h	PROTEASE	-7,8	-7,8	-7,5	-7,7	0,2	[48]
C34	Compound 4i	INHIBITORS	-7,7	-7,8	-7,5	-7,7	0,2	
C35	Compound 4j		-8,1	-8,4	-8,5	-8,3	0,2	
C36	Compound 4k		-8,8	-7,9	-8,8	-8,5	0,5	
C37	Compound 4m		-8,4	-8,4	-9,0	-8,6	0,3	
C38	Compound 4n		-8,8	-8,3	-8,7	-8,6	0,3	
C39	Compound 4o		-7,3	-7,5	-7,4	-7,4	0,1	
C40	Compound 4p		-7,2	-7,0	-7,0	-7,1	0,1	

Cluster analysis allowed finding similarities between the molecular structures in Table 1 and the dengue inhibitors reported and shown in Table 2 (Figure 2). Four of the five compounds proposed for *in silico* results as dengue protease inhibitor candidates were grouped together, where the last cluster encompasses two of our identified molecules ($C_{34}H_{23}NO_7S_2$, CID 54715399 and $C_{35}H_{27}NO_9$, CID 54681617), which were the ones with the largest structure-based difference when they were compared to some of the reported inhibitory molecules. They both share the structure bis(4-hydroxy-2-oxo-2H-chromen-3-yl)(2-methoxyphenyl)methyl.

Figure 2. Dendrogram that shows the clustering between the structures of the *in vitro* tested molecules and some of the reported dengue virus NS2B/NS3 protease inhibitors. The CIDs of the tested molecules are in blue.

3.3. *In vitro* studies

The commercially available molecules that had a high theoretical binding affinity to NS2B/NS3 protease were analyzed *in vitro* for their inhibition capacity (Figure 3). The calculation of the percent inhibition was obtained by subtracting the experimental value (with inhibitor) from the control (without inhibitor), which results in a value that is divided by the control and multiplied by one hundred [58]. For all of the tested molecules, the higher the concentration, the higher the percentage of inhibition, this demonstrates a dose-dependent response (Figure 3). The IC_{50} values suggested activity against dengue virus protease for four of the tested compounds, with $C_{30}H_{25}NO_5$ (CID: 54692801) and $C_{34}H_{23}NO_7S_2$ (CID: 54715399) showing significant differences ($P < 0.05$) compared to the negative control. The lowest activity against dengue virus protease was observed for $C_{29}H_{25}N_3O_4S$ (CID 54710332), which was apparently inactive, with an $IC_{50} > 100 \mu M$. The IC_{50} values for the others were between $9.09 \mu M$ and $19.87 \mu M$, with $C_{34}H_{23}NO_7S_2$ (CID 54715399) having the highest inhibitory activity.

Figure 3. Percentage of inhibition of dengue virus protease activity for the *in vitro* tested compounds. A. $C_{29}H_{25}N_3O_4S$ (CID: 54710332). B. $C_{35}H_{27}NO_9$ (CID: 54681617). C. $C_{25}H_{14}O_6$ (CID: 54692153). D. $C_{30}H_{25}NO_5$ (CID: 54692801). E. $C_{34}H_{23}NO_7S_2$ (CID: 54715399). * Significantly different (P<0.05) when compared to the negative control.

3.4. Evaluation of antiviral activity by using cell-based assays that include the viral inhibitory effect and cell toxicity

To exclude the possibility that the antiviral activities were due to compound-mediated cytotoxicity, uninfected HepG-2 cells were incubated with different concentrations of each compound for 72 h, and the cell viability was measured by the MTT method. Significant cell viability reduction was not observed at concentrations of up to 100.0, 85.7, 19.5, 13.5 and 12.5 μM for $\text{C}_{29}\text{H}_{25}\text{N}_3\text{O}_4\text{S}$ (CID 54710332), $\text{C}_{35}\text{H}_{27}\text{NO}_9$ (CID 54681617), $\text{C}_{30}\text{H}_{25}\text{NO}_5$ (CID 54692801), $\text{C}_{34}\text{H}_{23}\text{NO}_7\text{S}_2$ (CID 54715399) and $\text{C}_{25}\text{H}_{14}\text{O}_6$ (CID 54692153), respectively. Thus, these concentrations were considered to be non-toxic to the cells.

To verify the antiviral activity, the cells were infected with DENV-2 and treated with compounds at non-toxic concentrations. The NS1 viral titer reduction assay was performed in culture medium at 72 h p.i. The level of NS1 in the medium from infected cells that were untreated and treated with ribavirin were 44.5 ± 3.2 PbU and 8.3 ± 2.5 PbU, respectively. Only three compounds were active (Figure 4), two of them with a good inhibitory activity against dengue protease, as was also observed in the fluorometric enzyme assay. The $\text{C}_{30}\text{H}_{25}\text{NO}_5$, (CID 54692801) compound reduced NS1 by 69.8% (13.3 ± 3.3 PbU) at 19.5 μM and 15.5% (37.6 ± 3.7 PbU) at 9.8 μM ; $\text{C}_{34}\text{H}_{23}\text{NO}_7\text{S}_2$ (CID 54715399) reduced NS1 by 73.9% (11.6 ± 0.8 PbU) at 13.5 μM with no reduction observed at lower concentrations; and the $\text{C}_{35}\text{H}_{27}\text{NO}_9$ (CID 54681617) compound reduced NS1 by 79.9% (8.9 ± 2.1 PbU) at 85.7 μM and 37.0% (28.2 ± 7.9 PbU) at 49.2 μM . In contrast, the $\text{C}_{25}\text{H}_{14}\text{O}_6$ (CID 54692153) and $\text{C}_{29}\text{H}_{25}\text{N}_3\text{O}_4\text{S}$ (CID

54710332) compounds did not reduce NS1 production at the highest non-toxic tested concentrations (12.5 μM and 100.0 μM , respectively) under the experimental conditions.

The relevant activity of synthetic compounds is generally expressed by the IC_{50} and SI toward the supporting host (Table 3). An important parameter for the interpretation of the antiviral of a compound tested by using cell-based assays is the SI ($\text{SI} = \text{CC}_{50}/\text{IC}_{50}$). The $\text{C}_{30}\text{H}_{25}\text{NO}_5$ (CID 54692801) was the most active, followed by $\text{C}_{34}\text{H}_{23}\text{NO}_7\text{S}_2$ (CID 54715399), with both compounds having an IC_{50} of less than 20 μM and an SI of more than 3.0. The $\text{C}_{35}\text{H}_{27}\text{NO}_9$ (CID 54681617) compound was active at an IC_{50} of 61.5 ± 4.6 μM with an SI of 2.6. The results suggest that $\text{C}_{30}\text{H}_{25}\text{NO}_5$ (CID 54692801) and $\text{C}_{34}\text{H}_{23}\text{NO}_7\text{S}_2$ (CID 54715399) are compounds that exert promising anti-dengue activity through significant inhibition of DENV-2 replication *in vitro* in accordance with the values of IC_{50} and SI.

Table 3. Relevant antiviral activities of active compounds on DENV-2 replication *in vitro*.

Compound	CC_{50} : μM	IC_{50} : μM	SI
$\text{C}_{30}\text{H}_{25}\text{NO}_5$ (CID: 54692801)	58.6 ± 3.0	14.9 ± 2.9	3.9
$\text{C}_{34}\text{H}_{23}\text{NO}_7\text{S}_2$ (CID: 54715399)	42.1 ± 1.6	11.8 ± 0.2	3.6
$\text{C}_{35}\text{H}_{27}\text{NO}_9$ (CID: 54681617)	162.4 ± 0.9	61.5 ± 4.6	2.6

Figure 4. Inhibition of DENV-2 and cytotoxicity of the compounds. A. NS1 protein secretion monitoring (%PbU) from virus-infected HepG-2 cells, 72 h after non-toxic concentrations of compounds. The values indicate the percentages of PbU from the compound-treated virus-infected compared with those from untreated infected-cells. *,**. Significantly different $P < 0.01$ and $P < 0.05$, respectively, when compared to non-exposure. B. Compound-mediated cytotoxicity (cell viability percentage) derived from the compound-treated cells compared with that from the untreated cells.

4. DISCUSSION

Several efforts have been made to find non-peptidic inhibitors for dengue virus protease. However, most of them do not possess the desired properties for drug candidate or the antiviral activity required. Until now, some of the potent known dengue protease inhibitors reported in literature (listed in Table 2 and Table S3) include some compounds with specific mode of inhibition for different dengue serotypes like C10 [54]; others, that interact with conserved residues of the catalytic triad and the active site, decreasing the likelihood of drug-resistant mutations, like CID 4101471 and ARDP0006 [35] or Chemdiv K286-0036 [55]; or forming hydrogen bonds with the P4 region, a specificity determinant of dengue virus NS3 protease [59], like CID 60194816 and CID 60194819 [56] or finally compounds 7b, 7c, 7e, 8a, 8b and 8e with inhibitory rates over 75% and IC₅₀ values lower than 5 μ M [57].

4.1. *In silico* Studies

The results show that the molecules have promising antiviral features against dengue. On the one hand, they have low molecular weights, between 410.38 and 621.68; numbers close to 500 Da, value recommended for small molecule drug development [60], and on the other hand, they do not have a risk of causing adverse effects, at least in theory for four of them, as shown by the OSIRIS calculator. Even for C₃₀H₂₅NO₅ (CID 54692801), because naphtalen-1-amine, which was the only fragment described as a non-drug-like molecular fragment because of its high toxicity risks of undesired effects, such as tumorigenicity and mutagenicity, could be replaced for drug-like molecular groups.

None of the examined compounds has any report as a dengue antiviral. Of these molecules, $C_{25}H_{14}O_6$ (CID 54692153) was reported by Nolan et al. [61] as a human NAD(P)H quinone oxidoreductase-1 (NQO1) inhibitor that was useful for cancer therapeutics. However, no biological information was found for the other four compounds.

As shown for the protein-ligand complexes (Figure 1), these molecules do not interact directly with residues of the catalytic triad (His51, Asp75, Ser135), although those involved in the interactions are at positions close to them and also near to the residues that are reported as being located inside of the S1, S2 and S3 pockets [62]. Asn152 (identified as an interacting residue for $C_{34}H_{23}NO_7S_2$, CID 54715399 and $C_{29}H_{25}N_3O_4S$, CID 54710332; Figure 1) and Ile165 were also reported by Salaemae et al. [63] as determinants of binding with the substrate in the active site of the dengue virus NS3 protease, as was also noted by other authors [35, 64, 65]. In addition, some of the interacting residues between dengue virus protease and these molecules, such as Lys 74, Gly 87, Leu 149, Asn 152, Ala 164, and Asn 167, were described by Senthilvel et al. [66] as key residues for the binding affinity of the flavonoid quercetin. This compound is one of the seven phenolic molecules that are found in the leaves of *Carica papaya*, which have inhibitory activity on NS2B/NS3 protease.

When comparing the affinity values obtained between the *Flaviviridae* family protease inhibitors and dengue virus protease, none showed significant inhibitory

activity for this enzyme. The low affinity registered for the docking between the dengue protease structure and the protease inhibitors reported for other viruses could be explained by the altered specificity relative to other trypsin-like serine proteases. One of the reasons for this behavior could be related to the flatness of the dengue virus protease active site [34]. This flatness substantially hampers drug development against this target because conformational changes are required to allow the protein-ligand interaction.

The obtained results when comparing affinity binding values from molecules reported as dengue protease inhibitors [35, 54-57], and for those reported as not active inhibitors validate the parameters used in the *in silico* experiments, which is supported by the statistically significant differences that are found between these groups of molecules.

Cluster analysis grouped four of the five compounds that were proposed for *in silico* results as dengue protease inhibitor candidates (Figure 2), with $C_{34}H_{23}NO_7S_2$ (CID 54715399) and $C_{35}H_{27}NO_9$ (CID 54681617) as the compounds that had the largest structure-based differences when they were compared with some of the reported inhibitory molecules. They both share the structure bis(4-hydroxy-2-oxo-2H-chromen-3-yl)(2-methoxyphenyl)methyl. This molecule is an analogue of bis-coumarine, which is a known inhibitor of urease [67, 68], as well as nucleotide pyrophosphatase phosphodiesterase-1 [69]. As proteases, ureases and pyrophosphatases are hydrolase enzymes. Ureases act on carbon-nitrogen bonds, but in linear amides other than peptide bonds that are involved in the nitrogen cycle

[70]. Pyrophosphatase hydrolyses inorganic pyrophosphate and is produced in a very large number of biochemical reactions in the cell, such as in DNA or protein biosynthesis [71].

4.2. *In vitro studies*

A dose-dependent response was shown by all of the tested molecules, which was demonstrated by an increase in the percentage of inhibition as the concentration increases. The inhibitory activity of the compound $C_{34}H_{23}NO_7S_2$ should be noted, which at the highest tested concentration (10 μ M) showed an inhibition of more than 50% of the dengue virus protease. These IC_{50} values are better than those reported for other inhibitors [13, 34], which is evidence that the proposed methodology is useful for the identification of compounds that have dengue virus protease inhibitory activity.

Using concentrations that are non-toxic to the cells of each tested molecule, three compounds showed inhibitory activity. The relevant antiviral activity of the active compounds was defined according to the IC_{50} and SI, as shown in Table 3. The selectivity index (SI) enables to distinguish between the antiviral activity of the evaluated compounds and the possible toxic effects on the cells [72, 73]. According to the SI value, it is established if the antiviral activity is not a consequence of the cytotoxic effect on the cells, indicating the selectivity of the tested compounds on the assessed cell lines [72]. According to Senthil [74], SI values greater than 3.0 reflect some selectivity of the samples for the tested cell lines. In fact, very well-known antiviral molecules have SI values around 5, such as

amantadine against influenza A(H1N1)pdm09 virus (SI = 4) [75] and ribavirin towards DENV2 (SI = 4.7) [76]. However, while searching for promising antiviral molecules, SI values should be greater than 10. Therefore, for the two molecules with the highest SI values that were discovered in this work, it can be inferred that those have a modest anti-dengue protease activity. Additionally, it is also established that the antiviral activity is relevant when the IC₅₀ values are below 100 mg/mL [77]. Taken together, the C₃₀H₂₅NO₅ (CID: 54692801) and C₃₄H₂₃NO₇S₂ (CID: 54715399) compounds present a moderate anti-DENV-2 activity, which affects the functional activity of the NS2B/NS3 protease complex. C₃₅H₂₇NO₉ (CID: 54681617) was active, but it does not appear to be a promising chemical in accordance with the IC₅₀ (61.5 µM) and IS (2.6) values. C₂₅H₁₄O₆ (CID: 54692153) appears to be very toxic to human hepatocytes (maximum non-toxic concentration of 12.5 µM), and therefore, concentrations of below 25 µM had to be used for antiviral assays to not have an inhibitory effect. In contrast, C₂₉H₂₅N₃O₄S (CID: 54710332) exhibited low cytotoxic effects, although at a high concentration (100 µM), it was inactive.

Studies demonstrate that *in vitro* cytotoxicity is commonly cell-type specific and that cytotoxicity in one cell

does not necessarily suggest cytotoxicity in another [78]. In addition, the inability of compounds to inhibit dengue can depend on the type of cell, and discrepancies could be attributed to differences in the membrane permeability or the molecule uptake into the host cell. In this study, hepatic cells were used because they are target cells for virus infection in dengue patients, which contributes to the reliability

of the assay. On the other hand, the primary screening that uses DENV-2 could lead to an inaccurate assessment of the anti-dengue activity due to the possibility that compounds that do not inhibit this serotype could exhibit activity of varying degrees against other infections [79].

5. CONCLUSIONS

In this study, the anti-dengue activity of the tested compounds under particular experimental conditions was examined. Further *in vitro* assays could be conducted to verify whether these compounds indeed have antiviral activity on virus serotypes while they infect other cell types. Our findings revealed that among the 210,903 compounds screened, at least three ($C_{35}H_{27}NO_9$, $C_{30}H_{25}NO_5$ and $C_{34}H_{23}NO_7S_2$) had good inhibitory activity against NS2B/NS3 serine protease. Along with the results of the comparison of multivariate methods, the bis (4-hydroxy-2-oxo-2H-chromen-3-yl) (2-methoxyphenyl) methyl structure can be used as a starting point molecule for developing new drug candidates that are effective against the dengue virus.

AUTHOR CONTRIBUTIONS

This manuscript was written according to the equal contributions of all of the authors, and all of the authors have given approval to the final version.

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HIGHLIGHTS

- *In silico-in vitro* assays were performed to find Dengue virus protease inhibitors.
- Three molecules showed promising inhibitory activity against DENV2 protease.
- Bis(4hydroxy2oxo2Hchromen3yl)(2methoxyphenyl)methyl is an anti-dengue motif.