European Journal of Medicinal Chemistry 110 (2016) 87–97

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

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

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ARTICLE INFO

*Article history:*
Received 25 May 2015
Received in revised form
8 November 2015
Accepted 14 December 2015
Available online 7 January 2016

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

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. HepG2 cells infected with dengue virus serotype 2 were used to test the activity against dengue virus replication.
*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 CID 54681617, CID 54692801 and CID 54715399 were strong inhibitors of NS2B/NS3, with $IC_{50}$ 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.


## 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

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http://dx.doi.org/10.1016/j.ejmech.2015.12.030
0223-5234/

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 SYBYL 8.1, 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 $IC_{50}$ 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