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Virtual screening of commercial cyclic peptides as NS2B-NS3 protease inhibitor of dengue virus serotype 2 through molecular docking simulation

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Abstract. A disease caused by dengue virus infection has become one of the major health problems in the world, particularly in Asia, Africa, and South America. This disease has become endemic in more than 100 countries, and approximately 100 million cases occur each year with 2.5 billion people or 40% of the world population at risk of having this virus infection. Therefore, we need an antiviral drug that can inhibit the activity of the enzymes that involved in the virus replication in the body. Lately, the peptide-based drug design has been developed and proved to have interesting pharmacological properties. This study uses commercially cyclic peptides that have already marketed. The purpose of this study is to screen the commercial cyclic peptides that can be used as an inhibitor of the NS2B-NS3 protease of dengue virus serotype 2 (DENV-2) through molecular docking simulations. Inhibition of NS3 protease enzyme can lead to enzymatic inhibition activity so the formed polyprotein from the translation of RNA cannot be cut into pieces and remain in the long strand form. Consequently, proteins that are vital for the sustainability of dengue virus replication cannot be formed. This research resulted in [alpha]-ANF (1-28), rat, Brain Natriuretic Peptide, porcine, Atrial Natriuretic Factor (3-28) (human) and Atrial Natriuretic Peptide (126-150) (rat) as the best drug candidate for inhibiting the NS2B-NS3 protease of DENV-2.

1. Introduction

Dengue virus infection has become one of the major health problems worldwide, especially in subtropical and tropical regions such as Asia, Africa, and South America. According to the latest data from World Health Organization (WHO), this disease has put approximately 3.9 billion people in 128 countries or roughly 40% of the world population at risk of infection, and the number of dengue incidence has been raised exponentially in latest decades [1-3]. Moreover, this disease infected more than 96 million people each year, resulting 20.000 deaths [4, 5]. Dengue virus infection may cause Dengue Hemorrhagic Fever (DHF), which leads to high body temperature (up to 41°C) and several symptoms such as a headache and muscle pain. In severe cases, the DHF can lead into Dengue Shock Syndrome (DSS), which significantly increase the mortality rate from 5% to 40% [6, 7]. Dengue virus infection is transmitted through female mosquito bites (*A. Aegypti* and *A. Albopictus*) [1]. Unfortunately, although several vaccines go through into clinical trial phase, there is no available commercial antiviral drug, nor vaccine to treat dengue fever efficiently. The development of dengue virus drugs and vaccines are difficult to conduct due to the pathogenicity of the virus itself [8]. Therefore, we need a new antiviral drug and vaccines that can combat the dengue fever by inhibiting its replication activity inside our body.

To date, there are five serotypes of dengue virus (DENV-1, DENV-2, DENV-3, DENV-4, and DENV-5), although only the former four serotype proved to be lethal to human [9]. These serotypes share almost identical genomes and morphology but show a different type of antigens. This unique property makes the body won't protect itself from another serotype's infection while increasing its pathogenicity [10]. Dengue virus is a positive-strand RNA virus that belongs to the *Flavivirus* genus of the *Flaviviridae* family [11]. It has a genome that encodes ten proteins; three structural protein (the capsid, envelope and membrane protein) and seven non-structural protein (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), all seven non-structural protein have an important role in the replication cycle of the virus [12, 13].

NS3 protease in DENV belongs to the serine protease family that consists of 184 amino acid residues at the N-terminal sequences. The NS3 protease is the second-largest viral protein (at 69 kDa) that has a crucial role in the life cycle of virus [14]. Recent studies indicated that the NS3 protease has the active site which consists of three functional amino acids (His51, Asp75, and Ser135), known as the catalytic triad [15]. The catalytic activity of NS3 protease is assisted by NS2B cofactors. They have a crucial role in cutting the polyprotein precursor to NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5, as well as the internal cutting of capsid protein, NS2A, NS3, and NS4A [16]. Therefore, targeting NS2B-NS3B protein for treating the dengue fever is viable and crucial for the development of a new antiviral drug for DENV.

Cyclic peptides are the polypeptide chains build from two or more amino acids that form a cyclic ring structure [17]. In general, the peptides have unique pharmacological properties, due to its selectivity and low toxicity. Moreover, the peptide-based drug has been widely distributed in the market and approved by FDA, such as Buserelin and Leuprorelin. However, because peptides consist of the amino acid, they are very vulnerable to hydrolysis and oxidation reaction. Additionally, the peptides also tend to have low oral bioavailability due to enormous molecular weight and huge Total Polar Surface Area (TPSA) value [18, 19]. Thus, reduce their potency to become the leading drug. To enhance the pharmacological properties of peptides, the cyclic peptides can be made to improve the chemical and physical stability of the peptides. In this research, we selected the commercial cyclic peptide from the vendors, to be simulated with the NS2B-NS3 protease of DENV via molecular docking simulation, in hope to get a leading compound to inhibit the protease, and later can be helpful in the dengue fever therapeutics.

2. Methods

This research was done by using the general research pipeline that has been utilized by this research group [20, 21]. Several kinds of software were used in this research, such as Molecular Operating Environment (MOE) 2008.10 [22], Vega ZZ 3.0.5.12, and PerkinElmer ChemBioDraw 13.0.

2.1. 3D structure preparation of the DENV NS2B-NS3 protease

Three-dimensional structure of DENV NS2B-NS3 protease was obtained from the Research Collaboratory for Structural Bioinformatics – Protein Data Bank (RCSB-PDB) database. The selected 3D structure was downloaded in PDB format file. After that, the visualization on the active site of NS2B-NS3 protease was observed by using MOE 2008.10 software. Afterward, the geometry optimization and energy minimization of DENV NS2B-NS3 protease was conducted by removing the water molecules. Then, the protonation of the DENV NS2B-NS3 protease was done through 'Protonate 3D' feature, followed by the optimization of the partial charge and energy minimization. The energy minimization was performed with an AMBER99 force field, gas phase solvation mode and RMS gradient of 0.05, while the other parameters were set at default setting. The optimized, minimized 3D structure of DENV NS2B-NS3 protease then was saved in the .moe format.

2.2. Commercial cyclic peptides preparation

The molecular structure of commercial cyclic peptides were collected from the chemical company's website, such as BaChem, Mimotopes, and American Peptide. First, the structures were drawn by using PerkinElmer ChemBioDraw 13.0 software. Then, the structure were optimized by using Vega ZZ 3.0.5.12 software. Afterward, the optimization of ligand geometry process was conducted by using MOE

2008.10 software, and it started by importing all ligands into MOE database viewer, followed by 'Wash' process. Furthermore, the optimization of peptide ligands were done by using MMFF94x force field and gas phase solvation. The RMS gradient of 0.001 kcal/Å was selected as well. The rest parameters were set at default setting.

2.3. Molecular docking simulation of DENV NS2B-NS3 protease and commercial cyclic peptides
The molecular docking simulation of DENV NS2B-NS3 protease and commercial cyclic peptides was done by using MOE 2008.10 software. At first, the docking simulation process began by selecting the 'Dock' feature from the 'Compute' panel. Then, we selected 'Triangle Matcher' placement method, with LondondG rescoring (1) function and retain value by 30. Additionally, the 'Forcefield' refine method was selected, followed by Generalized Born solvation model (GBVI/WSA) rescoring (2) function and retain value by 1. The rest parameters were set at default setting. The docking result can be saved in .mdb file format. The determination of the best ligand from docking simulation was decided on their molecular interaction (by observing the protein-ligand complex from the 'LigX' feature) and the Gibbs free binding energy that generated from the simulation.

3. Results and discussion

3.1. The preparation of commercial cyclic peptides and DENV NS2B-NS3 protease 3D structure
The three-dimensional protein structure of DENV NS2B-NS3 protease was taken from RCSB PDB (http://www.rscb.org/pdb/). This database contains the information about the structure of various proteins and nucleic acids. In this research, we downloaded the 3D structure of DENV NS2B-NS3B protease under the name of PDB ID: 2FOM [23]. This 3D structure itself was taken from the crystallized enzyme of DENV2 NS2B-NS3 protease, and it contains 247 amino acid residues, 62 amino acids from the NS2B, while the rest 185 amino acids from the NS3 protease. In this 3D structure, the NS2B is responsible as the cofactor of the NS3 protease. NS3 protease has three functional amino acids that act as the active site of the enzyme, namely His51, Asp75 and Ser135. These amino acids are commonly known as the catalytic triad of NS3 protease, and they have the polar and hydrophilic properties in their binding site area [24]. The visualization of theNS2B-NS3 protease was conducted with MOE 2008.10 software by using 'Gaussian Contact' feature from 'Surface and Maps' menu. This visualization was done to observe the important residues in the enzyme that will be used later in the docking process, as we can see the figure 1.

Before the geometry optimization and energy minimization process was conducted, first we removed the chlorine ions, water molecules and glycerol that exist in the 3D structure. Then, the process was started by protonated the NS2B-NS3 protease structure and added the hydrogen atom through 'Protonate 3D' protocol, since the 3D structure generated from the X-ray crystallography doesn't include the hydrogen atom. After the enzyme had been protonated, we added the partial charge via 'Partial Charge' protocol with the current method forcefield parameters. Finally, we minimized the structure, through 'Energy Minimization' menu. The gas phase solvation mode and AMBER99 forcefield was selected for this simulation, and the selected forcefield was used due to its reliability in optimizing the protein geometry than other types of forcefield, such as MMFF94x. The energy minimization process was carried out at RMS value of 0.05 kcal/mol.Å. In general, this process aims to create the most stable protein structure, by eliminating the bad contact or undesirable interactions and generate the lowest energy conformation of the protein structure. Hence, the protein structure will be ready later for the molecular docking simulation process.

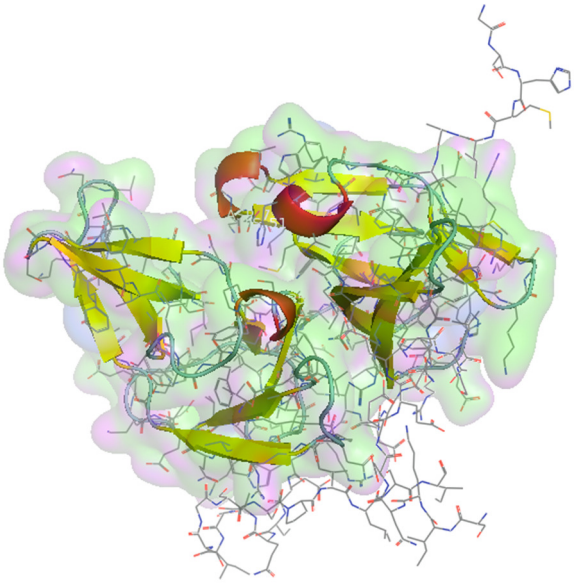

Figure 1. The three-dimensional protein structure of DENV NS2B-NS3 protease (PDB ID: 2FOM).

The next step of this research is the preparation of commercial cyclic peptides. We drawn all the cyclic peptides based on their structure in several online databases and vendors, such as American Peptide, Mimotopes, and BaChem. The molecular structure of cyclic peptides was drawn by using PerkinElmer ChemBioDraw 13 software, and the structure was later optimized by using Vega ZZ 3.0.5.12 software, resulting 308 commercial cyclic peptide structures in the process. As the comparison of these ligands, the linear peptide Bz-Nle-Lys-Arg-H was chosen as the standard ligand in this experiment, as this ligand activity and anti-dengue potency have been studied before by Yin *et al.* in 2005 [25]. The ligands that have been designed were saved in Molfile MDL format (.mol). Afterward, the geometry optimization and energy minimization of commercial cyclic peptides was done by using MOE 2008.10 software through "Database Viewer" menu. First, the 'Wash' protocol was conducted to improve the position of the hydrogen atom in the ligand. Then, the 'Partial Charge' protocol was done, prior to the 'Energy Minimization' process. The latter step was done by using MMFF94x forcefield, Gas Phase solvation mode and RMS gradient of 0.001 kcal/Å. The output file was stored in a.mdb format, and it will be ready for the molecular docking simulation.

3.2. Molecular docking simulation of DENV NS2B-NS3 protease and commercial cyclic peptides

The docking process between 308 commercial cyclic peptides as ligands and NS2B-NS3 protease was done by using MOE 2008.10 software. The docking scoring was done to determine the binding affinity between the ligand and receptor, as well as the refinement that was done to adjust the ligand-receptor complex with their respective forcefield [24]. After the ligand interacts with the receptor, the binding affinity of the ligand was evaluated by estimating the Gibbs free binding energy [26]. In this research, we used Triangle Matcher as the placement method and London dG as the scoring function, which are the default parameters of molecular docking simulation on MOE 2008.10. The docking process was done twice, each with 'Retain' value of 30 and 100, respectively. The aim of this treatment is to regulate the amount of 'Retain' pose interactions between the ligands and the receptor in molecular docking process. Out of 308 ligands that screened during the initial screening, 100 ligands, with the lowest Gibbs free binding energy value, were selected and taken into the secondary screening. The screening process was conducted twice to obtain the best conformation and interaction between the each ligand with NS2B-NS3 protease. In the end, we acquire 18 best ligands that have the lowest Gibbs free binding energy among all. The interaction between the ligand and the NS2B-NS3 protease can be seen in figure

2 below, while the results of 18 best ligands during the molecular docking simulation process is shown in table 1.

The best 18 ligands were selected not only because they have the lower Gibbs free binding energy ($\Delta G_{binding}$) value, but also good interaction with the catalytic triad of NS3 protease. The calculation of the $\Delta G_{binding}$ value is based on the laws of thermodynamics, which involves the equilibrium constant (K) value. The lower (negative) $\Delta G_{binding}$ value means the ligand-enzyme complex interaction is stronger and tends to be more stable. This is due to the stability and strength of non-covalent interactions (e.g. hydrogen bonds) on ligand-enzyme complex, which affects the molecular interaction of the complex

Table 1. The result of molecular docking simulation phase two (retain value at 100)

No.	Ligand Name	Gibbs free binding energy ($\Delta G_{binding}$) (kcal/mol)	Inhibition Constant (pKi)
1	[alpha]-ANF (1-28), rat	-53,469	38,952
2	[Tyr8]-Atrial Natriuretic Peptide (5-27), rat; [Tyr8]-Atriopeptin II, rat	-46,883	34,153
3	gp38	-46,696	34,017
4	[Tyr0] Calcitonin Gene Related Peptide II, human	-45,868	33,414
5	Vasonatrin Peptide (VNP)	-44,931	32,732
6	[CysCys21] Atrial Natriuretic Factor (3-28), Rat	-40,597	29,574
7	[beta]-Calcitonin Gene Related Peptide, rat	-39,878	29,051
8	[Tyr1] - Somatostatin 14	-39,812	29,002
9	Urodilatin (95-126)	-39,567	28,824
10	Calcitonin, eel	-52.613	38,327
11	Calcitonin Gene Related Peptide, human	-50,846	37,041
12	Calcitonin	-49,347	35,948
13	Brain Natriuretic Peptide, porcine	-47,732	34,772
14	Atrial Natriuretic Factor (3-28) (human)	-44,615	32,501
15	Atrial Natriuretic Peptide (126-150) (rat)	-44,206	32,203
16	Neuron Specific Peptide	-43,288	31,534
17	Amylin,human	-40,467	29,479
18	ANP (11-30), frog	-39,599	28,847
19	Bz-Nle-Lys-Arg-H (standard ligand)	-10,467	7,940

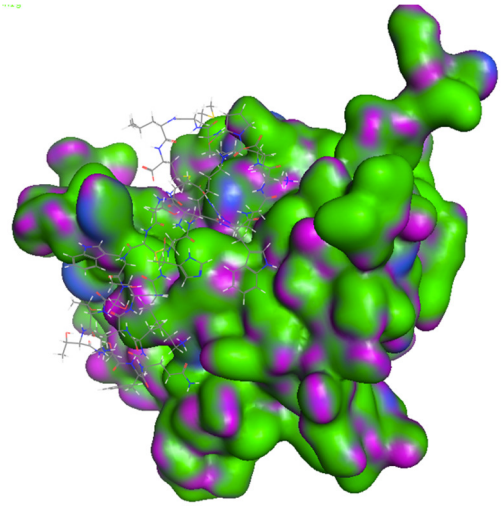

Figure 2. The visualization of molecular docking simulation between the cyclic peptide ligand and the NS2-NS3B protease.

and lowering the free binding energy value. Furthermore, the lower $\Delta G_{binding}$ value also inversely proportional to inhibitory constant (Ki) value, the smaller Ki value, the greater the concentration of the ligand-enzyme complex, which means the complex is more stable and the ligand also has a higher binding affinity for the enzyme [27]. Therefore, the smaller the Ki value or the greater the pKi value of a ligand-enzyme complex means the ligand has a higher potential to interact with the enzyme. Thus, the dissociation reaction between the ligand-enzyme complex tends to be more difficult to happen. From the table 1, we can see that the [alpha]-ANF (1-28), rat ligand has the best results by having the lowest Gibbs free binding energy at -53,469 kcal/mol, far below the second-most low Gibbs free binding energy, [Tyr8]-Atrial Natriuretic Peptide (5-27), rat; [Tyr8]-Atriopeptin II, rat, at -46,883 kcal/mol. Moreover, the standard ligand, Bz-Nle-Lys-Arg-H, surprisingly has -10,467 kcal/mol, higher than the highest Gibbs free binding energy ligand, ANP (11-30), frog, at -39,599 kcal/mol. Therefore, based on the current Gibbs free binding energy results, we can consider that the [alpha]-ANF (1-28), rat ligand can be selected as the potential inhibitor of DENV NS2B-NS3 protease.

After we observed the $\Delta G_{binding}$ and the inhibition constant value from the previous result, we continue to view the visualization between the selected cyclic peptides and the NS2B-NS3 protease after the docking simulation was conducted. This interaction can be seen via 'LigX' feature in MOE 2008.10. The interaction between the cyclic peptides and the NS2B-NS3 protease can happen not only in the hydrogen bond form, but also with other non-covalent interaction, such as Van der Waals interaction, which can affect the binding affinity between them. The hydrogen bond itself can be defined as the intermolecular force that occurs between high electronegative atoms with hydrogen atoms that covalently bonded to an electronegative atom [28]. In general, the interactions that involved in the ligand-enzyme complex are weak, compared to covalent bond [27]. The visualization between selected cyclic peptides and the NS2B-NS3 protease can be seen in figure 3, while the list of the binding interaction between them is shown in table 2.

From the figure 3, we notice that the Calcitonin, Brain Natriuretic Peptide, porcine, and Atrial Natriuretic Factor (3-28) (human) ligand may form at least eight, eleven, and fourteen hydrogen bonds with the NS2-NS3 protein, respectively. The hydrogen bonds itself between the ligand and the receptor are essential in inhibiting the receptor's activity, although the other non-covalent bonds between them are important as well.

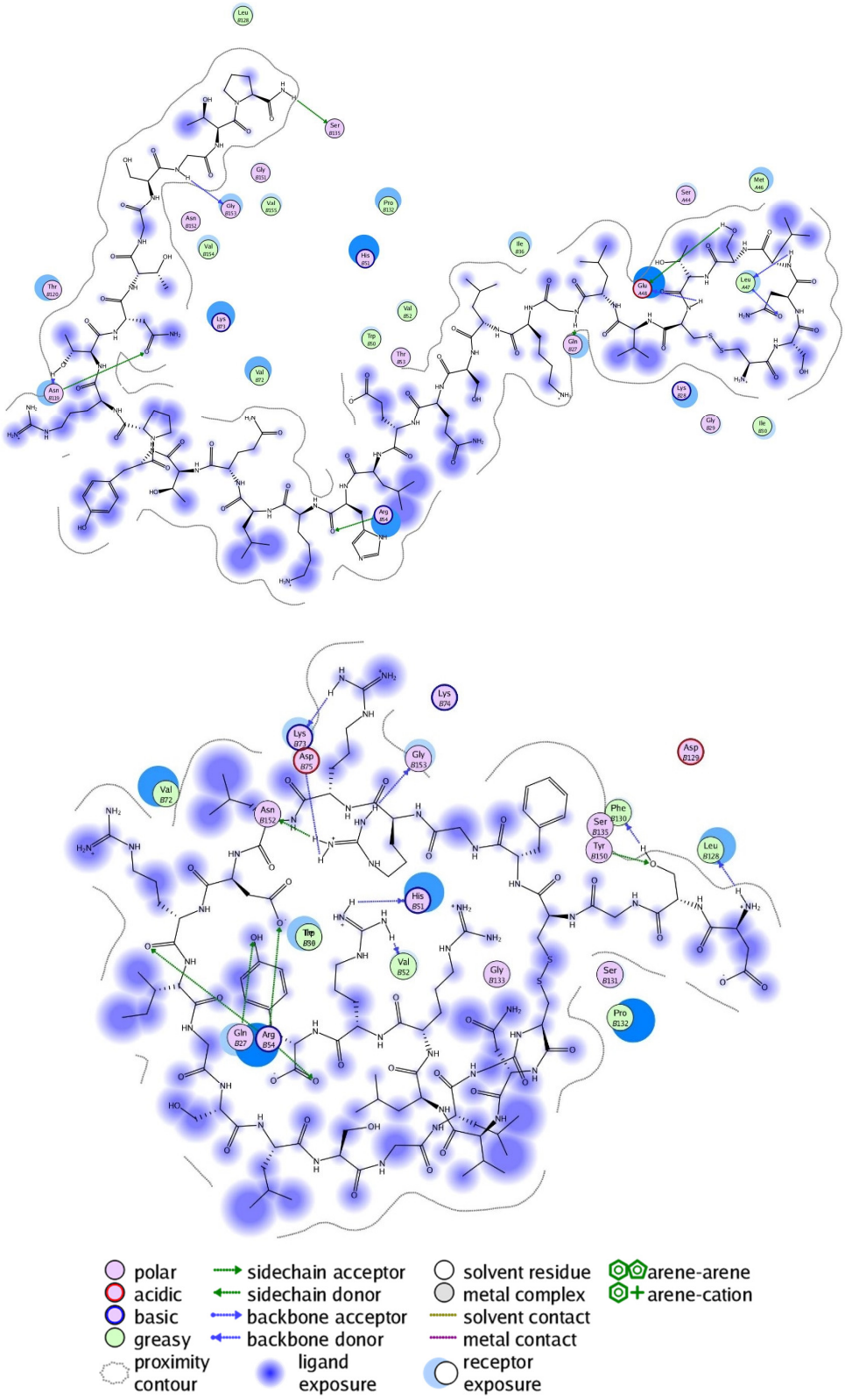

Figure 3. The 2D visualization of molecular docking simulation between the NS2-NS3B protease and calcitonin (top) and brain natriuretic peptide, porcine (bottom)

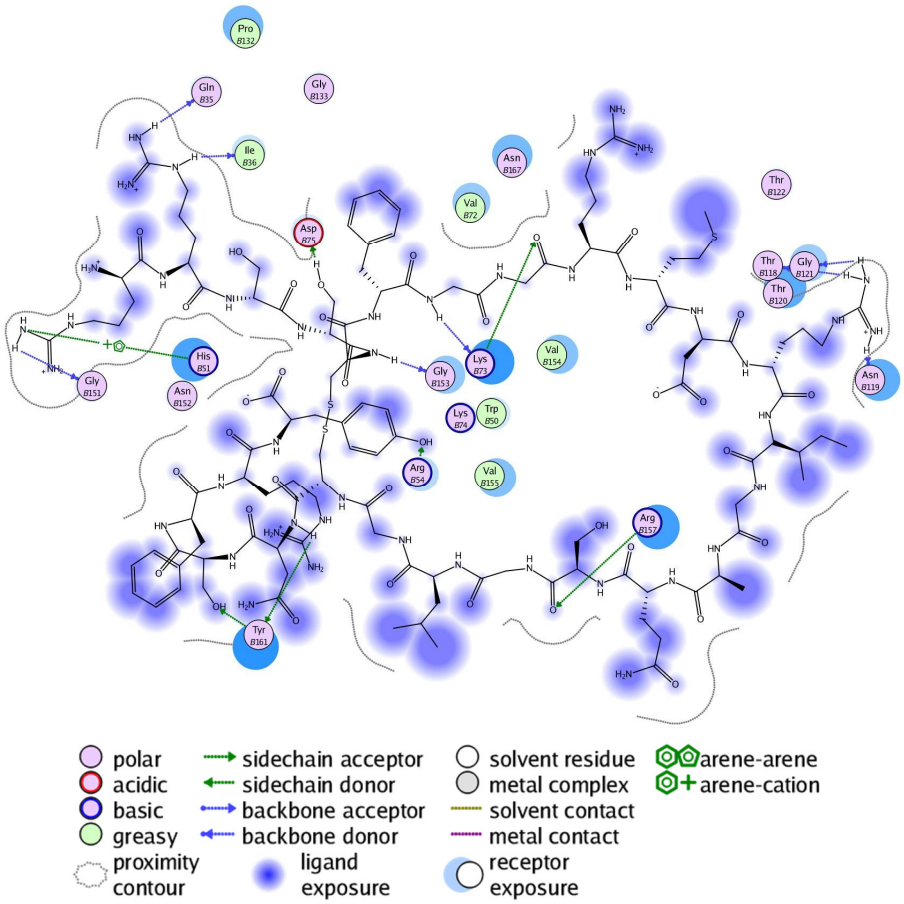

Figure 3 (cont.). The 2D visualization of molecular docking simulation between the NS2-NS3B protease and atrial natriuretic factor (3-28) (human)

As we can see in table 2, the catalytic triad of NS2B-NS3 protease plays an important role in the binding interaction between the cyclic peptide and the NS2B-NS3 protease, with at least ten, eleven and three interactions between the best 18 ligands that involve His51, Asp75, and Ser135, respectively. Moreover, Brain Natriuretic Peptide, porcine, Atrial Natriuretic Factor (3-28) (human), and Atrial Natriuretic Peptide (126-150) (rat) ligand can interact with two catalytic sites of NS2B-NS3 protease, means that they are more likely to bind well with the NS2B-NS3 protease, thus interrupting its function and may disturb the life cycle of the DENV. After we observed the molecular interaction among all of the best 18 cyclic peptides ligands, we concluded that the [alpha]-ANF (1-28), rat-, Brain Natriuretic Peptide, porcine, Atrial Natriuretic Factor (3-28) (human) and Atrial Natriuretic Peptide (126-150) (rat) ligand are the best ligands from this research to inhibit the NS2B-NS3 protease.

4. Conclusions

Cyclic peptides are one of the most fascinating molecules to become a leading compound due to their unique properties and exhibit various bioactivities, such as antiviral. In this research, we screened about 308 commercial cyclic peptides with the NS2B-NS3 protease to obtain the best ligands for inhibiting that protein, as well as treating the dengue fever. The screening was done by using molecular docking simulation, which resulted about 18 ligands that have lower Gibbs free binding energy than the standards. These ligands tend to interact with the NS2B-NS3 protease active site as well. In the end, we concluded that the [alpha]-ANF (1-28), rat-, Brain Natriuretic Peptide, porcine, Atrial Natriuretic Factor (3-28) (human) and Atrial Natriuretic Peptide (126-150) (rat) ligand are the best ligands to interact with

Table 2. Contact residue of the best 18 ligands with NS2B-NS3 protease after docking, red highlight shows the catalytic site of the NS3 protease

No.	Ligand Name	Binding Residue
1	[alpha]-ANF (1-28), rat	Glu 92, Lys 104, Asp 129, Ser 131, Pro 132, Thr 53, Val 52, Ile 36, Arg 54, His B51, Asp 75, Gly 153, Gly 151, Asn 152
2	[Tyr8]-Atrial Natriuretic Peptide (5-27), rat; [Tyr8]-Atriopeptin II, rat	Pro 132, Arg 54, Ile 36, Thr 53, Gln 27, Val 52, Gly 153, Arg 54, Glu 48, Asp 75
3	gp38	Leu 128, Glu 92, Ser 131, His 51, Arg 54, Lys 73, Gly 153, Asn 152
4	[Tyr0] Calcitonin Gene Related Peptide II, human	Glu 92, Gln 27, Gly 32, Ser 131, Val 155, Asn 152, Lys 73, Arg 54, His 51, Val 52
5	Vasonatrin Peptide (VNP)	Glu 48, Gln 27, Asn 152, His 51, Arg 54, Gly 153, Gly 151, Leu 128, Trp 50
6	[CysCys21] Atrial Natriuretic Factor (3-28), Rat	Arg 157, His 51, Arg 54, Thr 120, Val 155, Asn 119, Lys 73, Gly 153
7	[beta]-Calcitonin Gene Related Peptide, rat	Glu 88, Gly 87, Val 147, Ile 165, Lys 74, Trp 83, Asp 71, Lys 73, Asn 152, Asp 75, Gly 153, Tyr 161, Arg 54, Glu 92
8	[Tyr1] - Somatostatin 14	Arg 54, Gln 27, Asp 75, Val 72, Gly 153, Asn 152
9	Urodilatin (95-126)	Glu 92, Ile 36, Gln 27, Val 52, Lys 73, Asp 75, Gly 153, Asn 152
10	Calcitonin, eel	Lys 74, Asp 71, Lys 73, Asp 75, Asn 152, Gly 153, Ile 36,
11	Calcitonin Gene Related Peptide, human	Gln 35, His 51, Ile 36, Asn 167, Lys 75, Thr 118, Ala 70, Arg 54
12	Calcitonin	Ser 135, Gly 153, Asn 119, Arg 54, Glu 48, Gln 27, Leu 47,
13	Brain Natriuretic Peptide, porcine	Lys 73, Asp 75, Gly 153, Asn 152, His 51, Val 52, Gln 27, Arg 54, Tyr 150, Phe 130, Leu 128
14	Atrial Natriuretic Factor (3-28) (human)	Gln 35, Ile 36, Asp 75, His 51, Gly 151, Tyr 161, Arg 54, Gly 153, Lys B73, Thr 118, Gly 121, Asn 119, Arg B157
15	Atrial Natriuretic Peptide (126-150) (rat)	Arg 54, Lys 73, Asp 75, Thr 12, Ser 135, His 151, Val 155, Asn 119, Arg 54
16	Neuron Specific Peptide	Thr 120, Lys 73, His 51, Ser 34
17	Amylin,human	Gln 35, Glu 92, His 51, Arg 54, Gly 153, Asn 152, Asp 71, Val 72
18	ANP (11-30), frog	Lys 73, Val 22, Asp 71, Asn 152, Asp 75, Asn 119, Arg 54, Val 52, Gln 27, Glu 48, Val 57
19	Bz-Nle-Lys-Arg-H (standard ligand)	Arg 54, Asp 75, Val 52, Ile 36,His 51, Gly 133, Gln 27, Ser 135, Pro 132, Gly 153, Asn 152

the NS2B-NS3 protease. Therefore we suggest to continue this study by observing the ligand-protease stability through molecular dynamics simulation, or go into the in vitro test, to validate these results and determine their potency to become the leading compound for treating dengue fever.

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