

IN SILICO DESIGN OF THE M2 PROTON CHANNEL INHIBITORS OF H1N1 VIRUS

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ABSTRACT

M2 proton channel of H1N1 Influenza A virus is the target protein anti-flu drugs amantadine and rimantadine. However, the two once powerful adamantane-based drugs lost their 90% bioactivity because of mutations of virus in recent twenty years. The resistance of influenza A virus to amantadine need to develop more effective adamantane-based drugs. Several research by molecular docking method have been conducted to design and discover ligands which become potential inhibitors for the M2 channel protein of influenza virus in order to inhibit the replication of influenza virus. In this research are studied and evaluated the interaction of ligands towards the protein in the hydrated state using molecular dynamics simulations at two different temperatures. Analysis of ligand interaction yields that AM-L6-R6 ligand has best affinity towards the protein than the T-R6-L6, T-L6-R12 and the standard ligand. It is shown by the ligand interaction on the enzyme active site which remains to be formed during the simulation performed. At the end of simulation temperature of 300 K, AM-L6-R6 ligand has a residue contact with the Arg45 and formed hydrogen bond with Asp44. Then at the end of simulation temperature of 312 K, AM-L6-R6 ligands also could form a hydrogen bond with Asp44. Conformational changes of protein which occur during simulation showed the dynamicization of an protein in the presence of solvent and inhibitor.

Keywords: Amantadine, M2 Channel, H1N1 Virus, Molecular Docking, Molecular Dynamics

1. INTRODUCTION

Recently, the outbreak of H1N1 influenza A virus is a pandemic of a new strain of influenza virus identified in April 2009 (Trifonov *et al.*, 2009), commonly to as "swine flu". Within only four months, the pandemic has caused many deaths from the first detected country Mexico to almost all countries of the world (<http://www.who.int/csr/disease/swineflu/>). The H1N1 influenza virus is quite familiar to us because it had caused the 1918-1919 Spain pandemic that had infected 5% of the world population and resulted in 20-50 million deaths worldwide (Trifonov *et al.*, 2009). In July 2009 the World Health Organization (WHO) enhanced the warning phase 6, meaning that the spread of H1N1

influenza virus has become a serious global pandemic. The even worse news is that cases were reported that several strains of H1N1 influenza a viruses were resistant to oseltamivir (Tamiflu) (Du *et al.*, 2009).

The M2 channel of the influenza A virus is a homotetrameric protein that contains three different kinds of Transmembrane (TM) four-helix channel with 97 residues per subunit, each of which comprises an intracellular C-terminal domain with a length of 54 residues, a transmembrane domain with a length of 19 residues and an extracellular N-terminal domain with a length of 24 residues (Holsinger and Lamb, 1991; Sugrue and Hay, 1991; Huang *et al.*, 2008; Du *et al.*, 2009; Intharathep *et al.*, 2008). This protein has a major function as a proton-selective channel that is controlled

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by the endosomal pH values (Shimbo *et al.*, 1996; Huang *et al.*, 2008; Du *et al.*, 2009; Intharathep *et al.*, 2008). In conducting endosomal protons into the virion, the M2 channel is considered to play a crucial role in the viral life cycle (Bright *et al.*, 2006; Wang *et al.*, 2009). Once protons flux into the virion, the uncoating process of the viral nucleic acid in endosomes will be activated, which is essential for the virion to invade the host cells. The pH regulation of the influenza virus M2 channel is believed to have strong relationship with the TM four-helix bundle (Chou *et al.*, 1988; 1992) that is experimentally demonstrated to have proton conductive activity (Duff and Ashley, 1992; Chen *et al.*, 2007). Additionally, this channel has another function to preserve the pre-fusion state of HA during viral maturation (Wang *et al.*, 2009; Chen *et al.*, 2007; Grambas and Hay, 1992; Takeuchi and Lamb, 1994).

Influenza A virus has the ability to undergo changes by the mechanisms of antigenic drift and shift, resulting new evolving virus strains, which may be extremely toxic and drug resistant (Du *et al.*, 2010; Deyde *et al.*, 2007; Smith *et al.*, 2002). Given that influenza shift may occur every 20-30 years, the danger of future influenza a pandemics highlights the need to develop more effective drugs. The threat of an impending influenza pandemic, possibly through the mutations of the present avian strain H5N1 or swine strain H1N1, has triggered a global effort to develop more effective antiviral drugs. However, during the past several decades many efforts in developing anti influenza drugs have almost been futile due to the rapid mutations of the influenza virus, resulting in the persistent resistance to the existing drugs (Du *et al.*, 2010).

Although vaccination is the ideal way to prevent influenza virus infection, the preparation of a new vaccine requires more than 6 months (Couzin-Frankel, 2009). Thus, antiviral drugs are the most effective for short-term defense against influenza. The only known anti-influenza A drugs are M2 (matrix-2-protein) inhibitors (amantadine and its derivative rimantadine) and NA (neuraminidase) inhibitors (zanamivir and oseltamivir) (Hayden and Hay, 1992; Schmidtke *et al.*, 2006). The mechanism of M2 inhibitors is to block the ion channel activity of the M2 protein of most influenza A viruses. This action inhibits viral replication by blocking proton flow. The amino group in amantadine is likely the pharmacophore and is necessary to block proton transport (Hatta *et al.*, 2004; Wang *et al.*, 1993; Betakova, 2007; Cady and Hong, 2008; Tran *et al.*, 2011). Both amantadine and rimantadine are limited in their use in the U.S because of the rapid development of resistance. In addition, there is growing concern that

antineuraminidase-resistant viruses may emerge if these drugs are widely used (Lipatov *et al.*, 2004). Thus, there is an urgent need to discover new types of M2 inhibitors for the development of new antiinfluenza drugs. The present study was initiated in an attempt to solve drug resistant problem, to design and screen a small primary amine library of scaffold-hops based on amantadine to generate new hits in the M2 inhibitors class by conducting molecular docking and dynamics method using MOE software (Vilar *et al.*, 2008).

2. MATERIALS AND METHODS

The following steps were conducted using Microsoft Windows 7 based PC. Search and choose the sequences (Veits *et al.*, 2012). M2 proton channel sequences were downloaded from Influenza Virus Resource database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov.html>).

2.1. Multiple Sequence Alignment and Homology Modeling

This step was conducted by using ClustalW online program (www.ebi.ac.uk/Tools/clustalw2/index.html). Moreover, Homology modeling was performed using the SWISS MODEL which can be accessed through <http://swissmodel.expasy.org/SWISS-MODEL.html>. The SWISS MODEL service was utilized to build 3D models for H1N1 virus protein, by finding the exact templates (Abdussamad and Aris-Brosou, 2011). The PDB files were exposed by using Molecular Operating Environment (MOE) (Vilar *et al.*, 2008).

2.2. Molecular Docking

The found 3D structure was docked with its ligand by using molecular docking software. Before the docking, preparation steps must be done. This was done by removing water molecules, addition of hydrogen atoms and charges. Further minimization was done using MMFF94x (Vilar *et al.*, 2008). The utilized parameters for analyzing the complex between protein and ligand are ΔG° binding and inhibition constant.

2.3. Molecular Dynamics

The simulation on protein/ligand complex was done after molecular docking steps with MOE 2008.10. Before molecular dynamics was computed preparation steps were done for molecular docking, followed by inserting the ligand in order to form the protein-ligand complex. Then, the complex was minimized by force field MMFF94x and solvable in Born form. The parameters were utilized in accordance with MOE default, which is ensemble NVT (N,

total atom; V, Volume; T, Temperature) by using the NPA algorithm. We employed two different temperatures level in this simulation, 300 K as default MOE temperature and 312 K for fever temperature. The system was heated up to 312 K through a constant-volume MD simulation lasting 20 ps. The enzyme-inhibitor binding interactions and stability of enzyme-ligand complex were calculated after 5 ns of simulation for all ligands (Tambunan *et al.*, 2010).

3. RESULTS

3.1. M2 Channel Sequence

M2 channel sequences downloaded from the Influenza Virus Resource database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov.html>). The sequences in NCBI database were downloaded between June 2009 to June 2010. The result give 1203 full-length sequences and 67 protein sequences after collapsing.

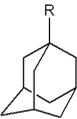
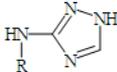
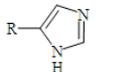
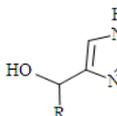
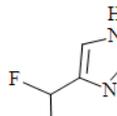
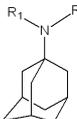
3.2. Multiple Sequence Alignment

Sequence alignments performed using ClustalW2 program that can be accessed through European Bioinformatics Institute (EBI) (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The downloaded M2 channel FASTA sequences were loaded into ClustalW2 program for multiple alignment process. Sequence alignments were used to see differences of protein sequences and to determine a single representative of M2 channel that will be targeted. M2 channel sequences was selected >gi|294862152|gb|ACU00946.2| matrix protein 2 [Influenza A virus (A/Denmark/524/2009 (H1N1))] that isolated from human, male sex and 22 years old from Denmark. These sequences submitted on April 19, 2010 by the WHO Collaborating Centre for Reference and Research on Influenza, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK (Tambunan *et al.*, 2010; Le and Leluk, 2011).

3.3. Homology Modeling

The sequence alignment was performed to determine template for docking simulation. Swiss-model (<http://swissmodel.expasy.org/>) was employed for template identification for M2 channel (Du *et al.*, 2010; Arnold *et al.*, 2006). The process was conducted by giving sequence file input for browsing the crystal structure in FASTA format. The chosen mode is automated. Based on template identification result, crystal structure of the M2 channel (PDB entry code: 2rlfD) were utilized.

Table 1. Structure of functional groups

R	
	1 H_2N-Cl
	2 $H_2N-Cl^+-CH_3$ $HN=C(NH_2)NH$
	3 $R-NH$
	4 
	5 
	6 
	7 
	8 $R-C(=O)-NH_2$
	9 $R-C(=O)-CH_3$
	10 $R-C(=O)-O-CH_3$
	11 $R-C(=O)-H$
	12 $R-C(=O)-OH$

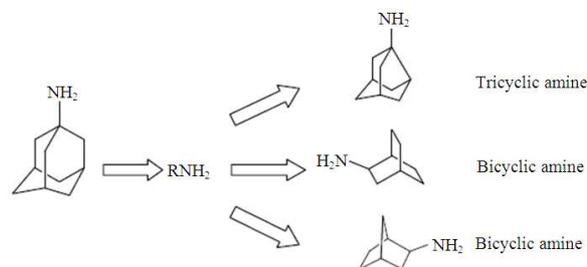


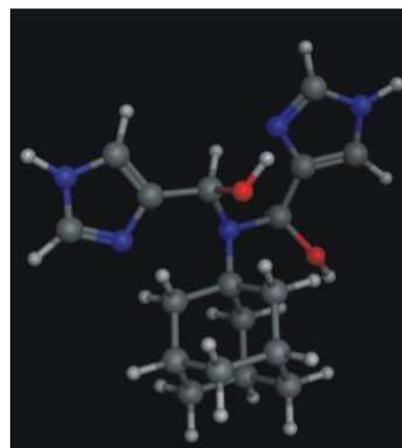
Fig. 1. Design strategy used for the primary amine

3.4. Building Ligand Structure

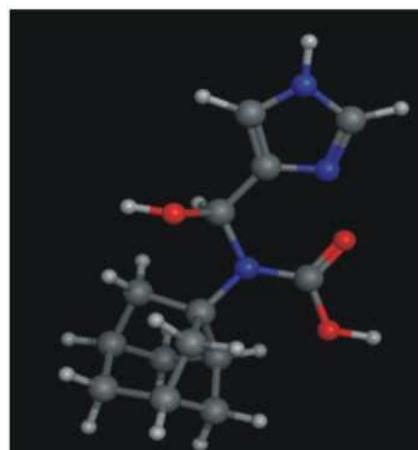
In this study, 1447 amantadine-based drugs inhibitors have already designed. We designed and screened a small primary amine library of scaffold-hops based on amantadine to generate new hits in the M2 inhibitors class. Among these 1447 compounds, [hydroxy(1H-imidazol-4-yl)methyl]tricyclo[3.3.1.0^{3,7}]non-3-ylcarbamic acid (AML6R6), (tricyclo[3.3.1.1^{3,7}]dec-1-ylimino)bis(1H-imidazol-4-ylmethanol) (TL6R12), (tricyclo[3.3.1.0^{3,7}]non-3-ylimino)bis(1H-imidazol-4-ylmethanol) (TR6L6) were identified to significantly inhibit M2 channel. The amino group in amantadine is likely the pharmacophore and is necessary to block proton transport (Du *et al.*, 2009). As shown in **Fig. 1**, the strategy for our library design was based on diversity and the structure-activity relationships of amantadine. The scaffold covers different molecular properties, with an emphasis on steric effect. Herein, we used bicyclic and tricyclic amines. The structures of functional groups are shown in **Table 1**, to substitutes pharmacopore group of adamantane-based drugs. The ligand structures were built and minimized optimization by using ACD/PhysChem Suite 12.0. The output is MDL Molfile and then, the output format was converted to MDL Mol format with VEGA ZZ software (Matsubara *et al.*, 2010; Hu *et al.*, 2010).

3.5. Molecular Docking

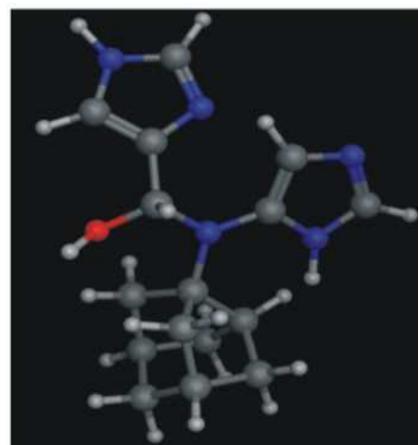
The protein structure was prepared for docking (Pyrkov *et al.*, 2010). This included the addition of missing hydrogens (Gordon *et al.*, 2005). During docking, series of poses (ligand-protein complexes) were generated for each molecule. The quality of each pose was further assessed by the London dG (LdG) Scoring Function (SF) which estimated the binding free energy of the ligand and a set of the highest score poses were chosen for each molecule (pose) docked (Mazur *et al.*, 2009; Vilar *et al.*, 2008). In this study, MMFF94 and MMFF94x were used for forcefield minimization. MMFF94x was reported as the efficient forcefield for minimizing ligand-protein complexes (Mazur *et al.*, 2009; Kaminski and Jorgensen, 1996; Halgren, 1990). Triangle Matcher (Wildman and Crippen, 2001; 2003; Cook *et al.*, 2010) was applied to orient the ligands in the active site based on charge groups and spatial fit. Triangle Matcher performs a random walk with the ligand in the active site to define the optimal binding orientations. Based on docking simulation result, there are three ligand, namely AML6R6, TR6L6 and TL6R12, could be proposed as a potential inhibitor to the interaction of M2 channel in A/2009(H1N1) virus (**Fig. 2**).



(a)



(b)



(c)

Fig. 2. The structure of ligands, (a) AML6R6, (b) TL6R12, (c) TR6L6

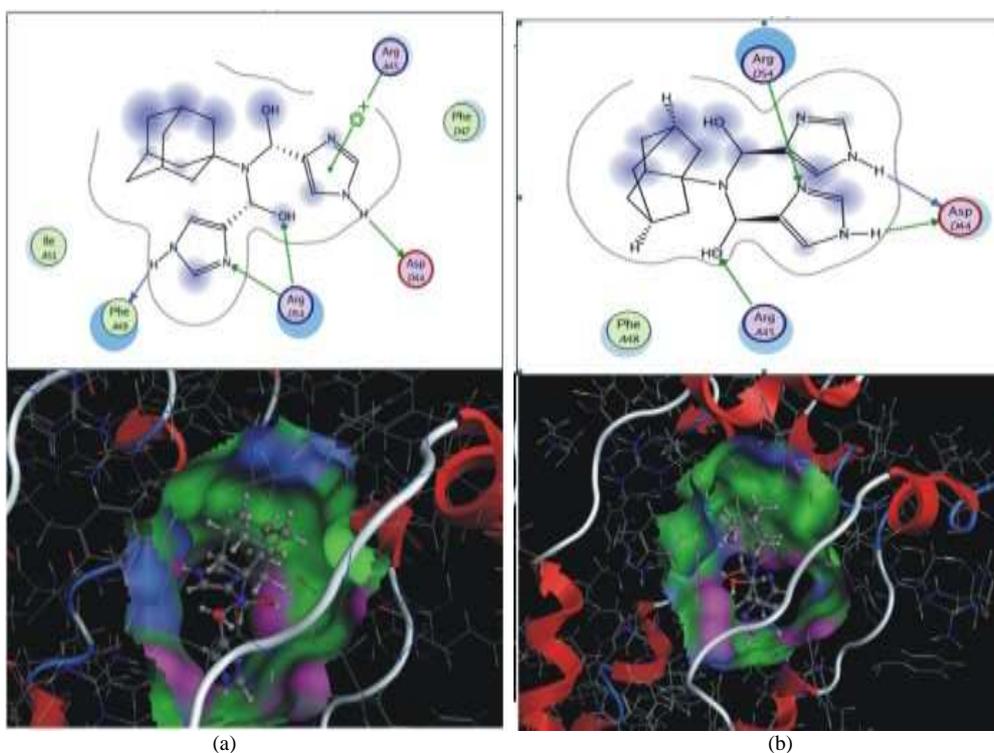
In **Table 2**, it shows the calculated free binding energy (ΔG_{bind}) and hydrogen bond of flexible-ligand docking simulation from the best ligand (Frimurer *et al.*, 2003). Free energy of binding for all the designed ligands were lower than the comparative ligand (amantadine and rimantadine) (Du *et al.*, 2010). The free energy of AML6R6, TR6L6, TL6R12, rimantadine and amantadine ligands were negative value, respectively, -6.6766, -5.7709, -4.9738, -4.1198 and -3.9574 kcal/mol. The negative and low value of ΔG_{bind} indicated the strong favorable bond between enzyme and ligand. In **Table 2**, we also presented hydrogen bond of AML6R6, TR6L6, TL6R12, rimantadine and amantadine ligands. Bold letters indicate residues of the active site of the M2 channel. The results showed that AML6R6, TR6L6 and TL6R12 binds at channel lock Asp44. Illustrated in **Fig. 3-5**, ligand T-R6-L6 and L6-R12-T form two hydrogen bonds with functional residues Asp44. The Asp44 residue is not only the channel lock, but also the protein exit of the M2 channel. Therefore, drug binding at this position not only can hold the channel in the closed conformation but also can simply block the proton exits (Pielak *et al.*, 2009). While the standard ligand amantadine and rimantadine do not form hydrogen bonds with the active site of the M2 channel. Interactions that occur will be validated with the state of the hydrated molecules using molecular

dynamics simulation method (Du *et al.*, 2010; Kumar, 2011; Jing *et al.*, 2008; Zhou and Schulten, 1996).

3.6. Molecular Dynamics Simulation

Molecular dynamics simulations are one of the most versatile and widely applied computational techniques for the study of biological macromolecules. They are very valuable for understanding the dynamic behavior of proteins at different timescales, from fast internal motions to slow conformational changes or even protein folding processes (Snow *et al.*, 2005; Alonso *et al.*, 2006). It is also possible to study the effect of explicit solvent molecules on protein structure.

In **Table 3**, we presented comparison hydrogen bond of AML6R6, TR6L6, TL6R12, rimantadine and amantadine ligands after molecular docking and dynamics simulation. Molecular docking process has resulted all shown ligand to form hydrogen bonds with the functional residues Asp44 (Du *et al.*, 2010). After the simulation conducted, the three ligands are still forming hydrogen bonds with Asp44. AML6R6 and TL6R12 ligands form two hydrogen bonds with Asp44 residue after molecular dynamics simulations (Huang *et al.*, 2008). However TR6L6 ligand losing one hydrogen bond with Asp44 after molecular dynamics simulation process.



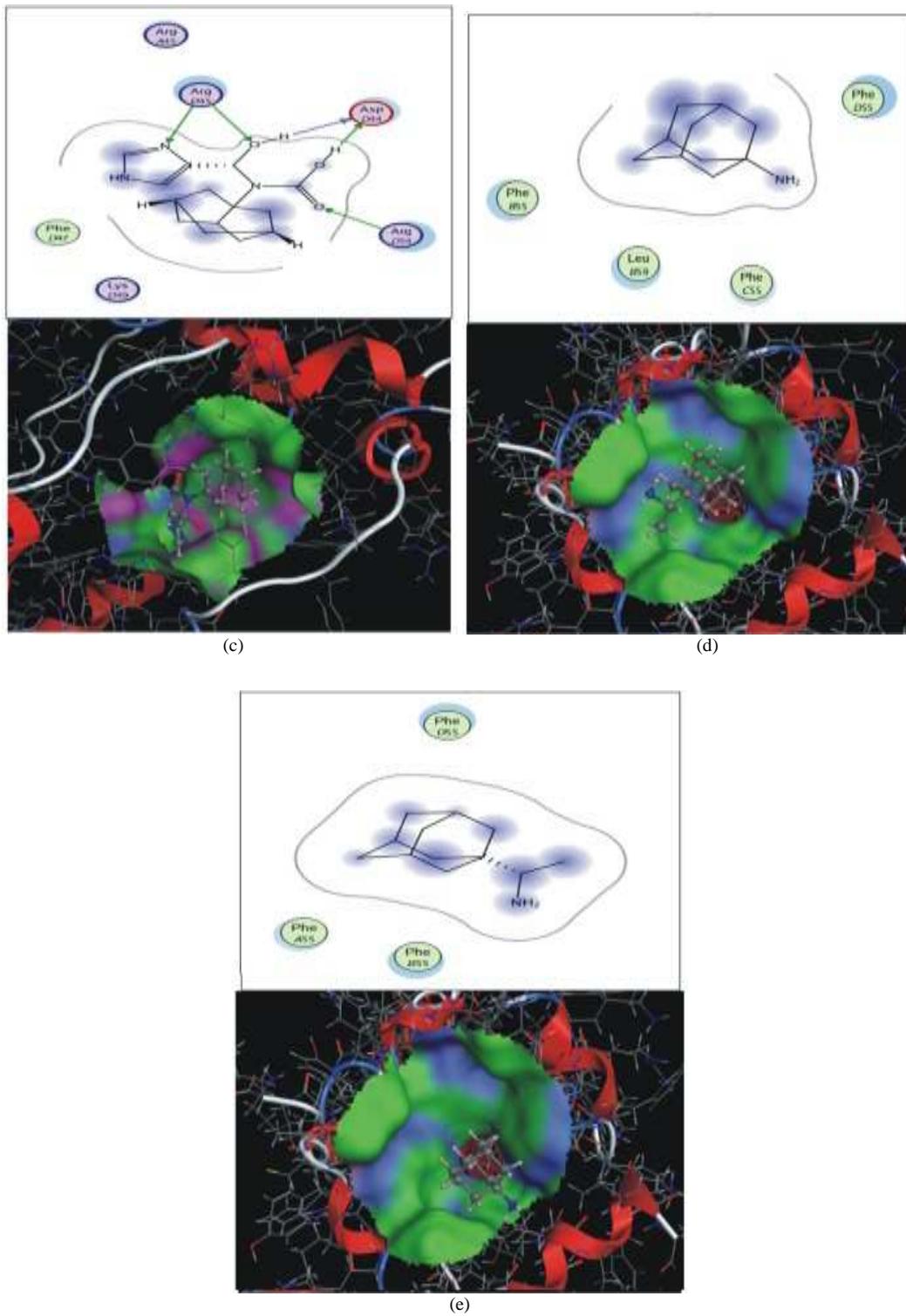


Fig. 3. Interaction between the M2 channel and ligands (a) AM-L6-R6 (b) T-R6-L6 (c) T-L6-R12 (d) amantadineand (e) rimantadine

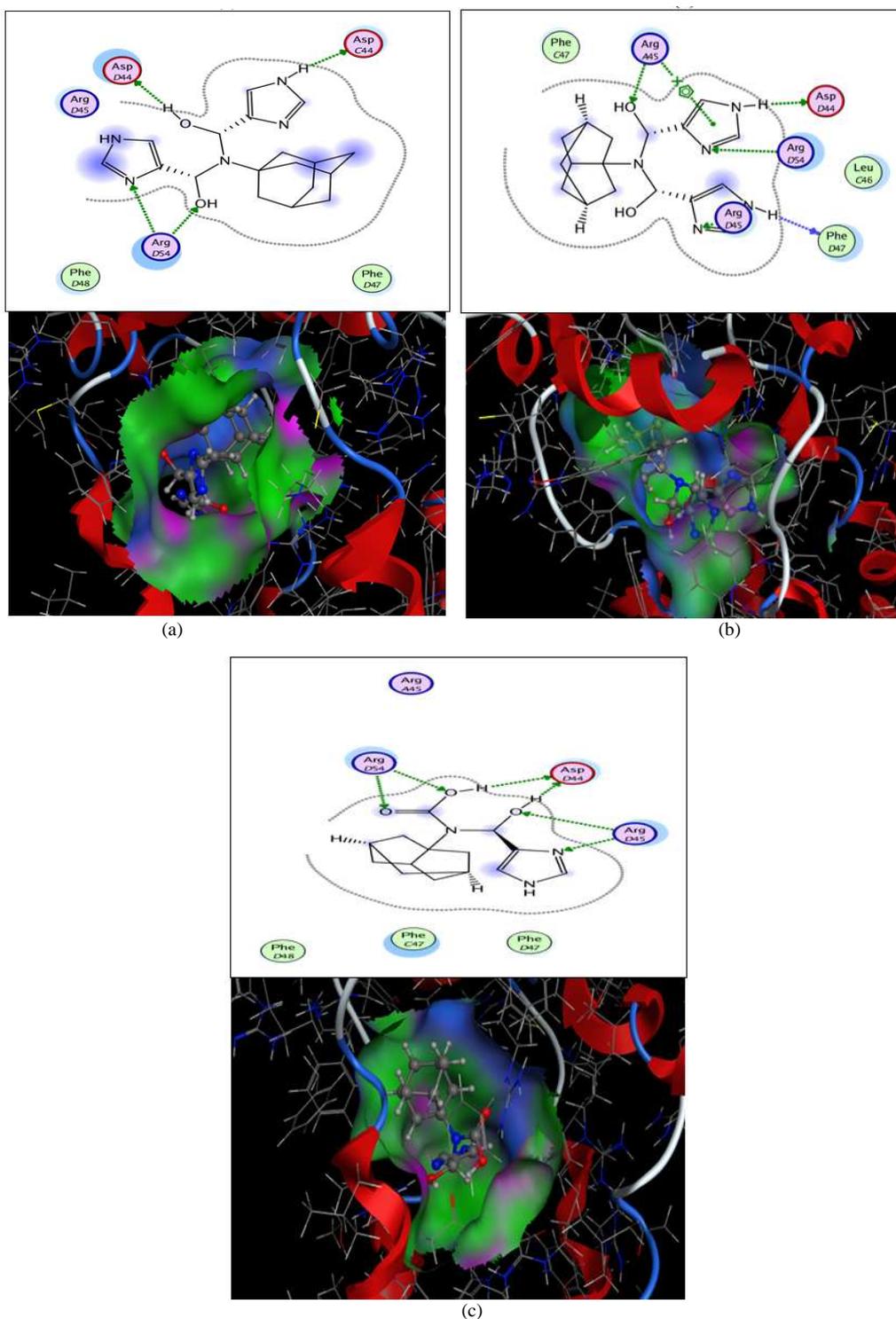


Fig. 4. Interaction between ligand and the respective amino acid residues in molecular dynamics simulation at 300 K. (a) AML6R6, (b) TR6L6, (c) TL6R12

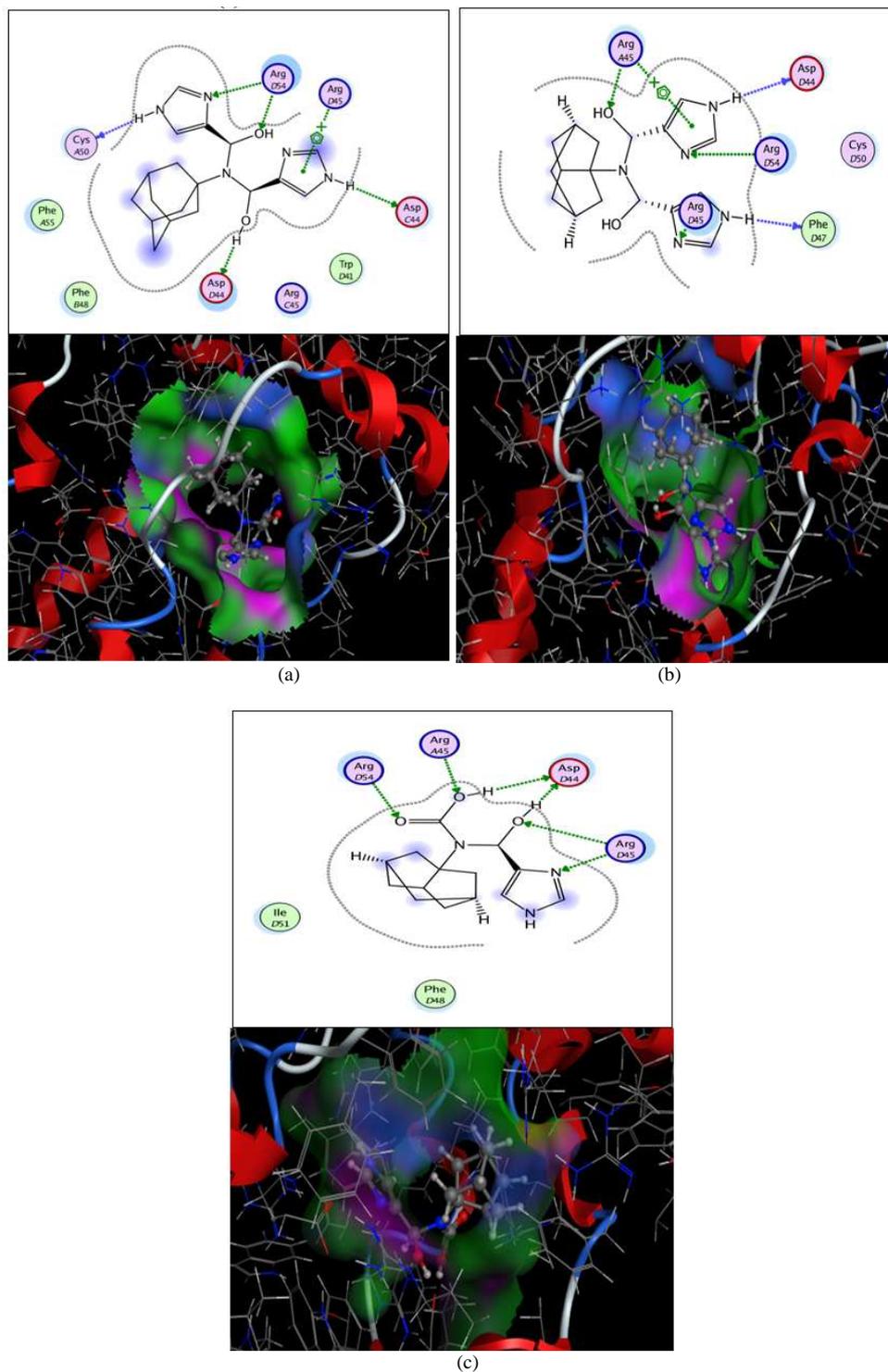


Fig. 5. Interaction between ligand and the respective amino acid residues in molecular dynamics simulation at 312 K. (a) AML6R6, (b) TR6L6, (c) TL6R12

Table 2. Binding free energies (ΔG_{bind}) and hydrogen bond of the best of three ligand

Ligand	$\Delta G_{\text{binding}}$ (Kcal/mol)	Hydrogen bond
AM-L6-R6	-6.6766	Asp44, Phe48, Arg54, Arg54
T-R6-L6	-5.7709	Asp44, Asp44, Arg45, Arg54
T-L6-R12	-4.9738	Asp44, Asp44, Arg45, Arg45, Arg54
Rimantadine	-4.1198	-
Amantadine	-3.9574	-

Table 3. Comparison hydrogen bond of the best of three ligand at docking and after dynamics simulation

Ligan	Docking	Dynamics at 300 K	Dynamics at 312 K
AM-L6-R6	Ile51, Phe47, Asp44, Phe48, Arg54, Arg54	Arg45, Phe47, Phe48, Asp44, Asp44, Arg54, Arg54	Trp41, Arg45, Phe48, Phe55, Asp44, Cys50, Arg54, Arg54
T-R6-L6	Phe48, Asp44, Asp44, Arg45, Arg54	Leu46, Phe47, Asp44, Arg45, Arg45, Phe47, Arg54	Cys50, Asp44, Arg45, Arg45, Phe47, Arg54
T-L6-R12	Arg45, Phe47, Lys49, Asp44, Asp44, Arg45, Arg45, Arg54	Arg45, Phe47, Phe47, Phe48, Asp44, Asp44, Arg45, Arg45, Arg54, Arg54	Phe48, Ile51, Asp44, Asp44, Arg45, Arg45, Arg45, Arg54

Residue contact is interaction of M2 channel residue between ligand (inhibitor) (Du *et al.*, 2010). Interaction between complex ligand-M2 channels is formed with hydrogen bond, but also it occurs non-covalent interactions which influence the stability of proteins (Ball and Maechling, 2009). Hydrophobic interactions that cause the protein to maintain its integration because of folding and aromatic interactions that affect the stability of tertiary structure. In **Table 3** shown AML6R6, TR6L6 and TL6R12 have more residue contacts (italic letter) with M2 channel after molecular dynamics simulations. TL6R12 ligand has more residue contact (Arg45, Phe47, Phe47, Phe48) than two other ligands.

4. DISCUSSION

The development of molecular docking and dynamics method has eventually making the drug design workable. The advances of the utilized software are sufficient for generating the necessary ligands and then redirect them for the molecular docking and dynamics computation. Moreover, the pipeline is sufficient for handling this huge amount of data. The time and space complexity of this pipeline are handled by the efficacy of the available graphic subsystem of existing hardware.

Our pipeline could cope with more than 1400 lead compounds, which are much more numerous than what we have before. The screening pipeline was resulted in only three potential inhibitors that perform much better than the standards. Here, the scaling up of our developed pipeline to cope with bigger amount of data seemed to be feasible, provided that the existing hardware and software architecture will support it.

Hydrogen bond, as one of the important chemical bond, is definitely a focal point of this study due its important role for maintaining the interaction between ligand and the protein. The calculation of binding free energy and the related binding sites have eventually exposed important clues on the reactivity of the drugs candidate.

The determination of the molecular dynamics at 300 and 312 K are indeed useful for simulating the normal and fever human body temperature. The generated data has already sufficient for predicting the stability of the drugs candidate in both temperatures. In this end, a better predictability of the drug mechanism could be achieved by the improved simulation of the temperature.

5. CONCLUSION

There are 1447 amantadine-based drugs already designed in this study. The docking of these inhibitors to determine their interaction activities with M2 channel was carried out. The free energy binding (ΔG_{bind}) values for all the inhibitors were small, indicating the molecules bound considerably well to the binding site. From docking process there are three ligands that have smaller free binding energy (ΔG_{bind}) than ligand standard (amantadine and rimantadine) and the best interaction with M2 channel, there are AML6R6, TR6L6 and T-L6-L6-R12. Docking result shown that all ligands form hydrogen bond with functional residue Asp44. After dynamics simulation at 300 K and 312 K, all ligands still form hydrogen bond with Asp44 residue. To ensure the absorption, distribution, metabolism, excretion and toxicity, ADMET calculations on the ligands are recommended. Thus, from this study, the AML6R6, TR6L6 and TL6R12 could be proposed as a potential inhibitor to inhibit interaction of M2 channel in H1N1 virus.

5.1. Competing Interest

The researchers declare that they have no competing interests.

6. ACKNOWLEDGEMENT

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