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Article

Therapeutic Options for COVID-19: Drug Repurposing of Serine Protease Inhibitor against TMPRSS2

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Abstract: The SARS-Coronavirus 2 (SARS-CoV-2) outbreak is a serious global public health threat. Researchers around the world are conducting mass research to control this epidemic, starting from the discovery of vaccines, to new drugs that have specific activities as antivirals. Drug repurposing is a potential method of using drugs with known activity for reuse as COVID-19 therapy. This method has the advantage that it can reduce costs and also the duration in the development of potential drugs. The initial step in drug repurposing can be done computationally to determine the effectiveness and specificity of the drug on the target protein. Molecular docking analysis can see the specific interactions of potential compounds with target proteins by analyzing the energy of the bonds formed. The spike protein of SARS-CoV-2 is a major target in the design and discovery of new drugs for the treatment of Covid-19 disease. In addition, transmembrane protein serine protease (TMPRSS2) from host cells has been shown to have an important role in the proteolytic cleavage of viral spike protein to the ACE2 receptor present in human cells. Based on screening studies, it is known that there are several drugs that have been established that have the potential to inhibit the SARS-CoV-2 transfection mechanism into host cells. 10 potential drug candidates used in this study namely Arbecacin, Bromhexine hydrochloride, Hydroxychloroquine, Camostat mesylate, Darunavir, Dequalinium, Fleroxacin, Lopinavir, Remdesivir, and Octopamine were used in molecular docking. Docking analysis revealed that there were three potential compounds, namely Bromhexine hydrochloride, Camostat mesylate and Octopamine with low binding affinity and inhibition constants. Based on the docking result, Camostat mesylate as the best candidate has a high specific binding affinity for the Ser441 and Asp435 residues present in the TMPRSS2 catalytic triad. Thus, these results reveal the mechanism of inhibition of TMPRSS2 by the known inhibitor Camostat mesylate in detail at the molecular level. Where, Camostat mesylate has a strong bond. This structural information could also be useful for designing and discovering new inhibitors of TMPRSS2, which may be useful for preventing the entry of SARS-CoV 2 into human cells.

Keywords: SARS-CoV-2; TMPRSS2; Molecular docking; Drug Repurposing

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1. Introduction

SARS-CoV-2 (Severe Acute Respiratory Syndrome Corona Virus 2) which started from Wuhan, Hubei Province, China in December 2019 is an infectious disease that attacks the respiratory tract and is very easy to spread throughout the world [1]. Based on data on the covid19.who.int site, the COVID-19 outbreak has spread widely in 224 countries with the number of positive cases worldwide reaching 263 million cases and the number of patients dying reaching 5.32 million. Meanwhile in Indonesia, in early December 2021, 4.26 million cases were confirmed positive with the number of patients dying reaching

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155,000 people [2]. This causes the COVID-19 outbreak to be categorized as an emergency condition thus therapy and efforts to overcome this outbreak are urgently needed.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belongs to the *Coronaviridae* family, ordo of *Nodovirales* and the genus of *Betacoronavirus* [1,3,4]. Based on research conducted by Harrison et al., 2020 it was found that SARS-CoV-2 enters the host cell by interacting with receptors located on epithelial cells, namely Angiotensin Converting Enzyme-2 (ACE-2) [5,6,7]. Most of the deaths of COVID patients were caused by damage to the lungs, this could be because one of the locations for the highest expression of ACE-2 is located in the lungs. Currently, researchers are working on developing an effective vaccine to control the SARS-CoV-2 pandemic, but this is proving to be time consuming and may require more clinical trials to hit the market [8,9,10].

Besides of vaccine development, researchers around the world are working on developing appropriate therapies and drugs for COVID-19 patients. One type of drug being developed is an antiviral that can suppress the effects or symptoms suffered by COVID-19 patients. An appropriate antiviral with high specificity to treat SARS-CoV-2 is still not available [11,12]. Thus, other alternative option is by tracing the reuse of existing drugs (drug repurposing), which has been proven as a therapy for other diseases and has potential activity that can inhibit and against SARS-CoV-2 activity. This effort is considered as the right choice in tackling this epidemic due to the shorter development process, and lower costs in carrying out drug reuse research [13]. Efforts to repurposing existing potential drugs provide benefits in cutting research and development costs, reducing drug development time, due to the existing drugs have shown their safety in humans. Thus, phase 1 clinical trials is not necessary to be done [14].

In determining the potential drug substance for therapy and prevention of SARS-CoV-2 infection, it is necessary to know the exact target site. Several candidates include the major coronavirus protease-3CLpro/Mpro [15], papain-like protease-Plpro [16], RNA-dependent RNA polymerase-RdRp [17], angiotensin-converting enzyme 2-ACE2[18], viral spike glycoprotein S, and transmembrane serine protease 2-TMPRSS2[19] has been used as a therapeutic target. In a study conducted by Hoffman et al., 2020, it was proved that SARS-CoV-2 infection can be controlled by targeting the transmembrane serine protease enzyme (TMPRSS2). TMPRSS2 is a proteolytic enzyme that functions to cleave the spike protein 'S' which is useful for integrating with the ACE2 receptor on the host cell[20]. Targeting TMPRSS2 could be a promising therapy to prevent the entry of SARS-CoV-2 into human cells [21–23].

To inhibit TMPRSS2 activity, several inhibitor proteases were screened for further analysis regarding the potential to against SARS-CoV-2 infection. Nafamostat as a drug that has been reported to have serine protease inhibitor activity and is a native ligand that has been published in the protein data bank can be used as a reference in finding other potential drug candidates that may have comparable or even better activity than Nafamostat [24–26]. Several drug candidates that have similar characteristics to Nafamostat and are known to have the potential to have serine protease inhibitor activity can be used to confirm their ability as COVID-19 drug candidates [26].

Previously, TMPRSS2 was known to be responsible for cell membrane fusion. TMPRSS2 has also been a target in treatment against MERS-CoV and SARS-CoV [27]. Serine protease inhibitor agents have been known to have activity in reducing symptoms and inhibiting the spread of the virus to other cells. Camostat Mesylate, Nafamostat and other serine protease inhibitors exert positive effects in several treatments targeting TMPRSS2 [28,29]. So, by studying and conducting in-depth analyzes down to the molecular level in search of other serine protease inhibitors that have the potential to TMPRSS are necessary.

Therefore, in this study, the search for potential drug candidates that can inhibit the proteolytic activity of the TMPRSS2 protein will be carried out using molecular docking analysis to inhibit the transfection mechanism of SARS-CoV-2 into host cells. This research is expected to help in finding the right therapeutic drug to overcome this COVID-19 outbreak. And can be used as a new reference in determining potential candidates for anti-SARS-CoV-2 compound.

2. Results

In this study, screening ligand for TMPRSS2 inhibitor candidates was conducted on 10 drug candidates based on the results of the screening from the database on the drug-bank.com website. The 10 selected candidates are established drug that potential to bind with TMPRSS2 protein from host cell or the spike protein from SARS-CoV-2 so that they have the potential to inhibit the SARS-CoV-2 transfection mechanism into host cells. The 10 ligand candidates that were successfully screened include Arbekacin [6,30], Bromhexine hydrochloride [8,31], Hydroxychloroquine [32,33], Camostat mesylate [20,23,34], Darunavir [35], Dequalinium [6,36], Fleroxacin [37], Lopinavir [38,39], Remdesivir [38,40], and Octopamine [41]. The candidate ligands used in this study are drugs that have received marketing authorization with other indications, so they can be proposed and have the potential as repurposing drugs for COVID-19 patients.

The SARS-CoV-2 virus can enter host cells through its receptor, namely ACE2 (Angiotensin converting enzyme-2), which is found in various organs, especially the liver, lungs, kidneys, and digestive tract of target cells. The entry mechanism of SARS-CoV-2 into the host cell begins through the interaction of glycoprotein S with the ACE2 receptor on the host cell. Then, the virus enters and binding occurs followed by fusion of the viral and host cell membranes [3,42]. After fusion, type II transmembrane serine protease (TMPRSS2) on the surface of the host cell clears ACE2 and activates protein S. Activation of protein S causes a conformational change and allows the virus to enter the cell. The two proteins (TMPRSS2 and ACE2) are the main determinants of the entry of this virus [43,44]. Furthermore, the incoming SARS-CoV-2 will release its genetic material in the cytoplasm. The genetic material released by this virus is mRNA which is ready to be translated into protein.

2.1. Molecular docking analysis of 10 candidate ligands to the target protein TMPRSS2

Due to its activity involved in the transfection mechanism of SARS-CoV-2 into host cells especially in the activation of glycoprotein S from the virus, inhibition of TMPRSS2 has great potential in targeting candidate drugs to prevent infection. The 3D conformational structure of the serine protease-2 transmembrane protein has been reported in the protein databank as a protein having hydrolase activity. The reported 3D structure is the result of X-ray diffraction, with a resolution of 1.95 A. Based on the data obtained, this protein has a native ligand, namely Nafamostat as a serine protease inhibitor that can inhibit the proteolytic activity of TMPRSS2. The complete structure of the TMPRSS2 protein can be seen in Figure 1 [45].

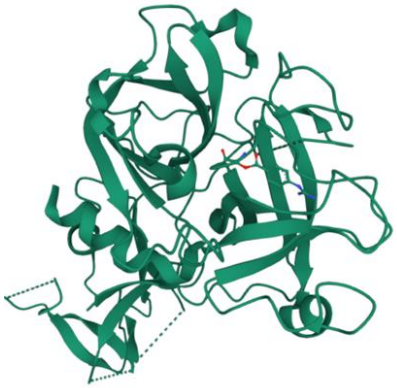

Figure 1. 3D Conformational structure of Transmembrane serine protease-2 (TMPRSS2) in complex with Nafamostat (PDB ID:7MEQ)[45].

Prior to the docking analysis, it is necessary to pre-process TMPRSS2 as a target macromolecule and also to 10 drug candidates as ligands. Preparations were carried out using the Autodock application to separate native ligands (Nafamostat), adding polar hydrogen

groups, adding gasteiger charges and minimizing energy. This preparation was done to help facilitate the binding between ligands and macromolecules with good affinity.

In molecular docking analysis, it is necessary to know the binding potential position by optimizing and validating the Grid box on macromolecules. By validating the Grid box, it is hoped that it can facilitate drug candidates in the right binding pocket so as to produce specific interactions with the active sites owned by macromolecules with the functional groups of each potential ligand candidate. Grid box validation can be done by using the binding position of the native ligand as a reference, and optimizing the grid box by determining the optimum coordinates that have the lowest Grid center affinity, RMSD value, and inhibition constant.

Table 1. Grid box optimization and validation using autodock. Based on the output, a comparison is made on the parameters of the grid center, RMSD, and the inhibition constant.

Validation Parameter	Grid Box		
	40x40x40	50x50x50	60x60x60
Grid Center	-6.28 kcal/mol	-6.26 kcal/mol	-6.26 kcal/mol
RMSD	0.990 Å	1.023 Å	0.996 Å
Inhibition constant	24.99 µM	25.66 µM	25.68 µM

In this study, optimization and validation of grid boxes were carried out with parameters 40x40x40, 50x50x50, and 60x60x60. Based on further analysis, it is known that a grid box with a parameter of 40x40x40 has the potential for tethering because it has the lowest grid center of -6.28 kcal/mol, a small RMSD distance of 0.990 and a low inhibition constant of 24.99 M. So the boss grid is selected at 40x40x40 with coordinates x = 11,174; y = 9.472; z = 22,995. After that, 10 drug candidates were tethered to TMPRSS2 using Autodock. The results of the docking were then compared to obtain three drug candidates which had the lowest binding affinity and also the lowest inhibition constant.

Table 2. The results of the molecular binding of 10 drug candidates to the TMPRSS2 protein. 3 out of 10 drug candidates have low binding affinity.

Ligand Candidates	Binding Affinity (kcal/mol)	Constant Inhibition (µM)
Nafamostat*	-7.76	8.45
Arbekacin	-2.17	25.78
Bromhexine hydrochloride	-7.01	6.60
Camostat mesylate	-8.45	0.63
Darunavir	-2.48	15.32
Dequalinium	-2.03	32.53
Fleroxacin	2.54	46.44
Hydrochloroquine	0.13	28.21
Lopinavir	-5.01	211.57
Octopamine	-6.06	6.22
Remdesivir	-1.45	41.26

*Native ligand

Based on the results of the molecular docking analysis of 10 drug candidates against the TMPRSS2 target, the compound Camostat mesylate, Bromhexine hydrochloride, and octopamine are three drug candidates that have higher potency than other ligand candidates. This is because the three candidates have low binding affinity energy for

TMPRSS2 which indicates that in the formation of the interaction complex between the TMPRSS2 target and the ligand, low binding energy is required. So that complex formation is easier and ligand bonds have a high affinity for TMPRSS2. In addition, these three candidates have low inhibitory constants so that it is easier to inhibit TMPRSS2 activity in low drug concentrations.

The binding affinity values for these three potential candidates are -7.01 kcal/mol, -8.45 kcal/mol, and -6.06 kcal/mol for Bromhexine hydrochloride, Camostat mesylate, and octopamine, respectively. The constant inhibition values of 6.60 M, 0.63 M, and 6.22 M were owned by Bromhexine hydrochloride, Camostat mesylate, and octopamine, respectively. In addition, according to Table 2. Camostat mesylate has a much lower binding affinity energy and inhibition constant than Nafamostat as a native ligand of TMPRSS2. This can confirm that Camostat mesylate has a higher potential as a serine protease inhibitor than TMPRSS2 which can inhibit the proteolytic activity of TMPRSS2 in activating the spike glycoprotein conformation of SARS-CoV-2 in the transfection mechanism against host cells.

Specific interactions between the three potential drug candidates against TMPRSS2 need to be further confirmed to determine the binding site of the functional group of the ligand compound to the amino acid of the target protein. The binding site on this protein-ligand complex can be confirmed by 2D and 3D visualization of the complex with further analysis.

2.2. *Analysis and visualization of 2D and 3D bonding of Camostat Mesylate, Bromhexine Hydrochloride and Optamine with TMPRSS2*

In computational molecular docking, it is necessary to know the binding position between the ligand and the target protein in order to see and evaluate at what amino acid position results in the interaction and binding of the ligand. The interaction between the ligand and protein is influenced by the functional groups contained in the ligand and also the amino acids that construct the protein that produce specific interactions. The interaction of each functional group can be formed based on the hydrophobicity, charge, hydrogen bond donor, hydrogen bond acceptor, and conformational characteristics of each compound that is exposed to each other. This interaction can produce specific interaction such as hydrogen bonds, electrostatic interactions, van der Walls interactions, hydrophobic interactions, and others [46].

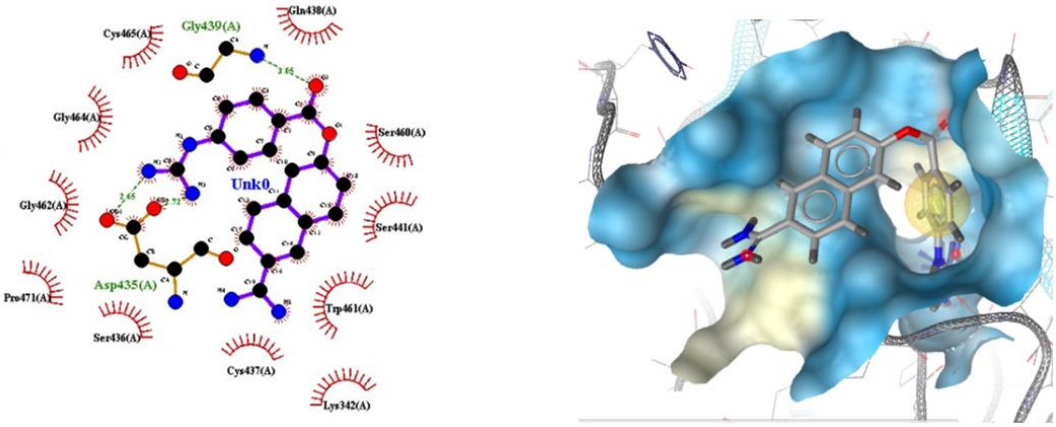

Figure 2. Visualization of the interaction of Nafamostat (native ligand) with TMPRSS2 in 2 dimensions (left) and 3 dimensions (right)

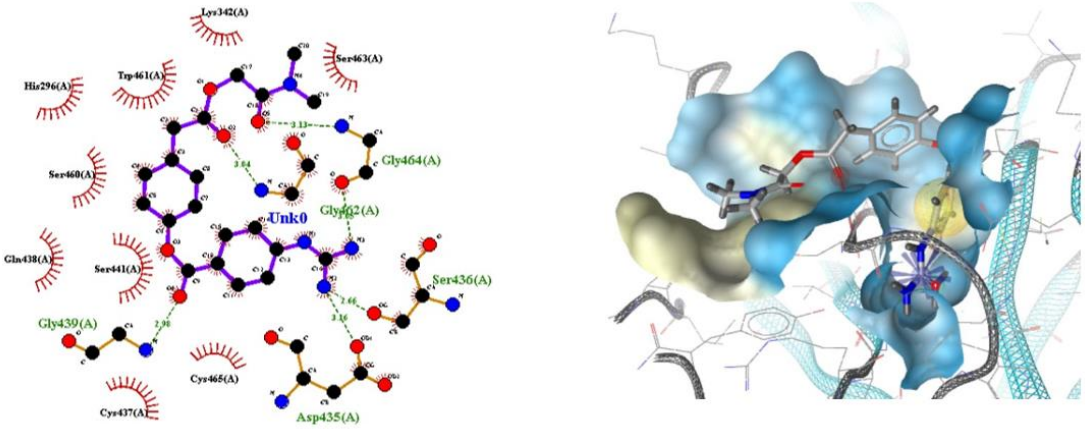

Figure 3.Visualization of the interaction of Camostat Mesylate (Best 1) with TMPRSS2 in 2 dimensions (left) and 3 dimensions (right)

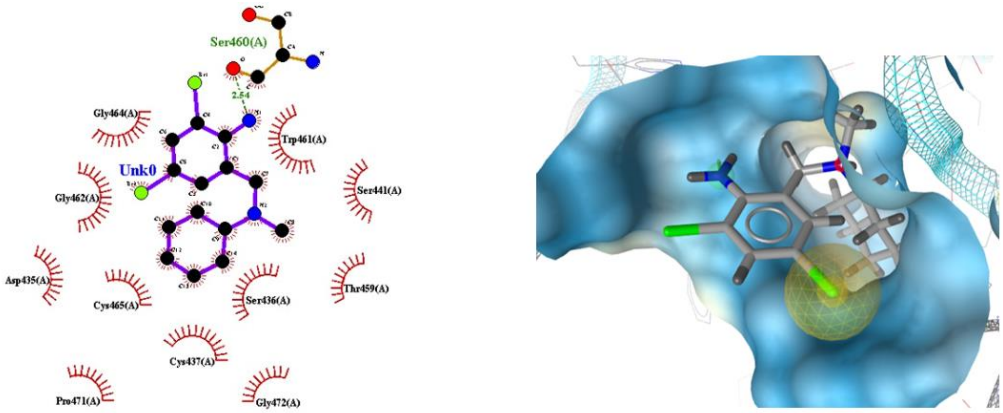

Figure 4. Visualization of the interaction of Bromhexine hydrochloride (Best 2) with TMPRSS2 in 2 dimensions (left) and 3 dimensions (right)

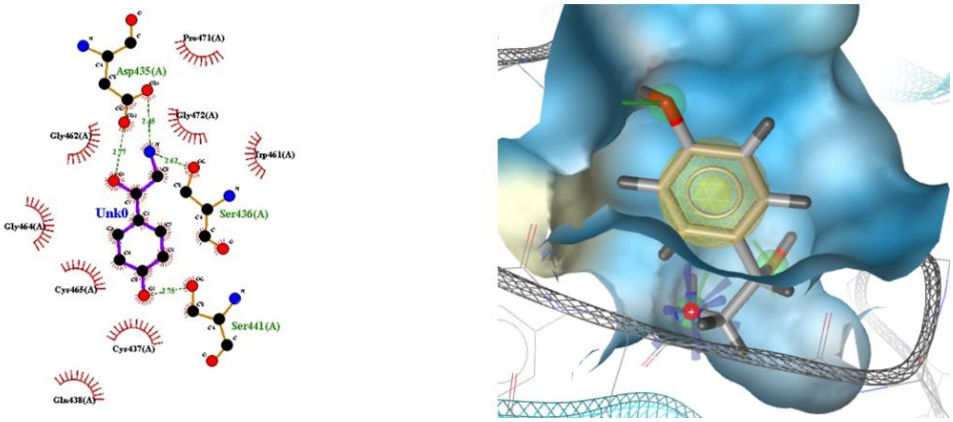

Figure 5. Visualization of the interaction of Bromhexine hydrochloride (Best 2) with TMPRSS2 in 2 dimensions (left) and 3 dimensions (right)

Two-dimensional visualization of ligand and protein complexes can be performed using LigPlot, and three-dimensional visualization can be performed using Ligand Scout. From this visualization, the specific position of the ligand and the target protein can be known. Based on Figure 3. It is known that Camostat mesylate as a drug candidate has 5 hydrogen bond interactions. This actually exceeds the hydrogen bonding of Nafamostat (Figure 2) which only has one hydrogen bond. Based on the literature, in inhibiting the proteolytic activity of serine protease proteins, the presence of a large number of hydrogen bonds in the ligand will increase the binding affinity and decrease the inhibitory constant of the ligand to the protein, this can increase the inhibitory activity by Camostat mesylate against TMPRSS2.

Table 3. The binding position of Camostat mesylate, Bromhexine hydrochlaride, and Octopamine to TMPRSS2 based on 2D and 3D visualization of the complex.

Amino Acid	Native Ligand	Ligand Candidate		
	Nafamostat	*Camostat Mesylate*	*Bromhexine hydrochloride*	*Octopamine*
Gly 439	H (3.65 Å)	H (2.96 Å)	-	-
Asp 435	H (2.45 Å)	H	HB	H (2.45 Å)
Cys 437	HB	HB	HB	HB
Ser 463	HB	-	HB	HB
Ser 441	HB	HB	-	HB (2.75 Å)
Gly 464	HB	H (3.15 Å)	-	HB
Trp 461	-	HB	-	-
Gly 462	-	H (3.65 Å)	-	-
Lys 342	-	HB (3.16 Å)	-	-
Ser 436	-	H (2.66 Å)	-	H (2.62 Å)

H: Hydrogen bond, HB: Hydrophobic bond

Based on the visualization results of the protein-ligand complex which have been summarized in Table 3, the specific interactions between the three drug candidates with TMPRSS2 show a specific binding pattern found in the native ligand and also the three drug candidates, especially in the amino acids ASP435, CYS437, and SER463. Camostat mesylate is a candidate ligand that has the most hydrogen bonds with TMPRSS2 compared to other candidates. The chemical interaction between camostat mesylate and TMPRSS2 confirmed that the more chemical interactions the ligand had with the protein, the stronger the inhibitory activity was indicated by a small constant inhibition. Based on the research of Sonawane et al [23], TMPRSS2 has three binding pockets and has a catalytic triad of SER, ASP, and HIS amino acids. The interaction of camostat mesylate with SER463, SER441, and ASP435 confirmed that the interaction between camostat mesylate and TMPRSS2 in the catalytic triad of TMPRSS2. This specific binding has the potential to produce specific inhibition of the proteolytic activity of TMPRSS2.

2.3. Physicochemical characteristic prediction from ADME

Prediction of ADME characteristics (absorption, distribution, metabolism, and excretion) can help determine the physicochemical properties and further potential of drug candidates. This analysis can assist in further analysis in conducting drug discovery and development, such as providing guidance in the synthesis of derivative compounds, developing dosages and formulation materials, and others. ADME analysis can be done using swissADME and ADMEstar by uploading the canonical smile of the drug candidate on the site. The machine learning algorithm will process the specific canonical smile of each compound so that it can predict the absorption, distribution, metabolism, and excretion characteristics of Bromhexine hydrocholride, Camostat mesylate, and Octopamine.

Table 4. Admetox analysis of the four ligands (Nafamostat, Camostat mesylate, Bromhexine hydrochloride, and Octapamine

Ligands	Druglikeness				Pharmacokinetics						Toxicities		
	MW (g/mol)	HBA	HBD	LogP	GI Abs	Inhibitor CYP					AMES	Carcino-genesis	AOT
						CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4			
Nafamostat	347,37	4	4	2,14	Low	no	no	no	no	no	Non	Non	III
Camostat Mesylate	494,52	9	3	1,30	Low	yes	no	no	no	no	Non	Non	III
Bromhexine Hydrochloride	412,59	1	1	3,61	High	Yes	no	no	no	yes	Non	Non	III
Optamine	153,18	3	3	0,20	High	no	no	no	no	no	Non	Non	II

According to Table 4. that summarized the characteristics of druglikeness, pharmacokinetics, and toxicity of each ligand, it is known that the four ligands are in accordance with the Lipinski five of rules [47]. Which has a molecular weight below 500 Da, has a total hydrogen bound acceptor (HBA) below 10, and has less than 5 total hydrogen bound donors (HBD) [48,49]. Thus, it can confirm that these three compounds are compounds that have the potential to be developed as candidates for therapeutic compounds against protein targets. The four ligands also have low LogP which represents hydrophilic characteristics of each compound. Camostat mesylate and Nafamostat have low gastrointestinal absorption (GI Abs) constant. Low gastrointestinal absorption constant is a challenge in the development of oral drugs for these compounds, it is necessary to carry out further preparation in the formulation to make these compounds as oral preparations.

Based on the toxicity analysis, the four candidates do not have the potential as AMES and carcinogenesis so they are quite safe. Nafamostat, Camostat mesylate, and Bromhexine hydrochloride have an acute oral toxicities potential (AOT) in class III which is categorized as safe, however, Octopamine has an AOT in class II so that it becomes a challenge in further drug development

4. Materials and Methods

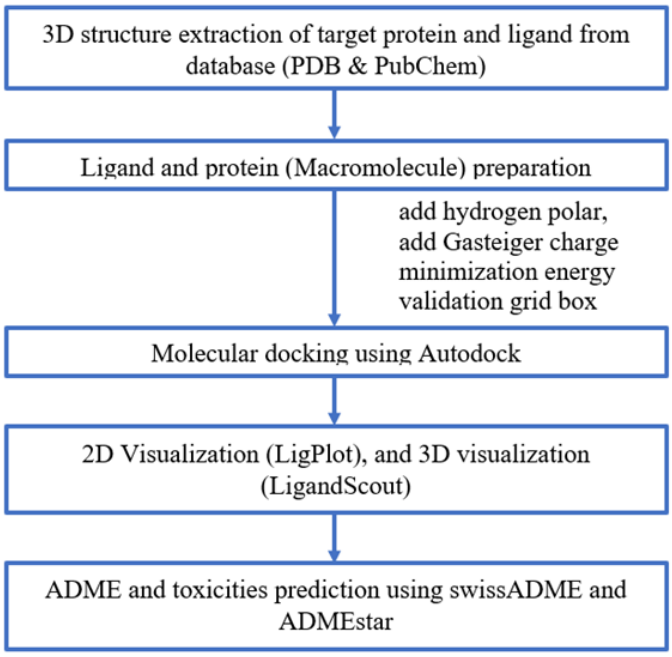

Figure 6. Work flow of molecular docking analysis 10 candidates serine protease inhibitor of TMPRSS2.

The software used consists of the application Marvin Sketch version 20.6, LigandScout 4.4.7, AutoDock ver 4.2.6, PyMOL, and LigPlus. The 3-dimensional structure of the transmembrane protein serine protease-2 (TMPRSS2) with protein code of 7MEQ is downloaded from the protein data bank at https://www.rcsb.org/ in pdb format. In addition, the three-dimensional structure of the 10 drug candidates (Table 5) were extracted on the PubChem database in sdf format https://pubchem.ncbi.nlm.nih.gov page. Then the files were converted into pdb format.

4.1. Preparation of Macromolecule TMPRSS2

The 3-dimensional crystal structure of TMPRSS2 is complex with Nafamostat as a native ligand. Thus, the native ligands and ions that are not amino acids need to be separated from the 3-dimensional structure of the 7MEQ, then the water molecules are removed from the macromolecules. The separated protein was optimized by adding polar hydrogen atoms, removing non-polar hydrogen atoms, energy minimization, and adding gasteiger charges. The number of gasteiger for molecules that can be used for docking is less than 32.

4.2. Preparation of ligand candidates

The ligand candidates were screening on www.drugbank.com using LigandScout (4.4.7) to evaluate the similarity structure by analyze the structure and fingerprint. Based on screening result, 10 ligands that have a similar structure compound with native ligand and has a potential activity as serine protease inhibitor were found. 10 ligand candidates were collected from PubChem database. All candidates were prepared using AutoDock by adding polar hydrogen atoms, removing non-polar hydrogen atoms, energy minimization, and adding gasteiger charges. All prepared ligand structure was saved in pdbqt format.

Table 5. List of 10 ligand candidates and native ligand (Nafamostat)

Ligand Candidate	CID Number
Nafamostat*	4413
Arbekacin	68682
Bromhexine hydrochloride	5702220
Hydroxychloroquine	3652
Camostat mesylate	5284360
Darunavir	213039
Dequalinium	2993
Fleroxacin	3357
Remdesivir	121304016
Octopamine	4581
Lopinavir	92727

**Native Ligand*

4.3. Analysis and Visualization of Molecular Docking

Docking validation was carried out by optimizing the grid box docking approach using native ligands for their binding sites on grid boxes 40x40x40, 50x50x50, 60x60x60 with AutoDock4. The optimum grid box was selected based on a low Root Mean Square Deviation (RMSD) rating. Grid box which produces a fairly low bond energy and has the smallest RMSD value, is used for docking candidate ligands. After that, 10 candidate ligands were pre-prepared against the TMPRSS2 target protein using AutoDock4 on a validated grid box. The docking results of the protein-ligand complex were evaluated by analyzing binding affinity and inhibition constants. Three candidate ligands with the lowest binding affinity, and lowest inhibition constants were further analyzed for visualization using LigandScout and LigPlus.

4.4. Physicochemical characteristic prediction from ADME

ADME (absorption, distribution, metabolism, and excretion) analysis was performed to predict the physicochemical characteristics, druglikeness, pharmacokinetics, and toxicity of the ligand compounds. This analysis was carried out using a website based on the ADMETsar and swissADME web based by uploading canonical smiles from candidate ligands extracted from the PubChem databases

5. Conclusions

TMPRSS2 as a regulatory protein in the transfection mechanism of SARS-CoV-2 has potential importance in the search for drug candidates against COVID-19. Based on research that has been carried out in the search for anti-SARS-CoV-2 drug candidates by targeting TMPRSS2 as a functional protein involved in the transfection mechanism of SARS-CoV-2 into host cells, three potential drug candidates were found, namely Camostat mesylate, Bromhexine hydrochloride and octopamine. These three candidates have low binding energies and low inhibition constants. In fact, Camostat mesylate has much lower binding energy and inhibition constants than Nafamostat as a native ligand. Based on the visualization of the interaction of the ligand-protein complex, Camostat mesylate has hydrogen bonds and interactions in the catalytic triad of TMPRSS2, namely SER441, ASP435, and Gly464 residues. Admetox analysis is useful for determining drug potency candidates based on the similar drug characteristics, pharmacokinetics, and toxicity. These results can be used as guidelines in the formulation development.

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