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Docking-Based Virtual Screening to Identify the Cysteine Protease Falcipain-2 Inhibitors of *Plasmodium falciparum*

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ABSTRACT. Malaria is an infectious disease caused by Plasmodium protozoan parasites that transmit via the female Anopheles sp. mosquito. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi* are the five Plasmodium species known to infect humans. *P. falciparum* is the most virulent species, causing the most deaths worldwide. The decrease in efficacy of most antimalarials suggests drug resistance. Therefore, the development of new effective antimalarials, particularly against novel targets, was still required. Cysteine protease falcipain-2 (CPF-2) plays a role in hemoglobin degradation; therefore, inhibiting the activity of this enzyme could be a viable antimalarial target. This study aimed to identify a potential inhibitor for the CPF-2 with PDB ID 3BPF from the ZINC15 database for treating malaria. This study employs a virtual screening workflow with HTVS, SP, and XP docking methods on the Glide Maestro Schrodinger. Based on the glide XP docking score, 10 hit compounds were identified, and their conformational interactions with CPF-2 were compared to the natural ligand (E-64). The binding energy values of hit compounds vary from -7.131 kcal/mol to -8.074 kcal/mol, which is more negative than the E-64 (-6.011 kcal/mol). The three best compounds identified from the ZINC15 database are ZINC000025691540, ZINC000096436101, and ZINC000097797430. All the hit compounds discovered similar interaction with E-64, specifically on the CPF-2 binding pocket residues with Gln36, Cys42, Gly83, Asn173, and His174. All hit compounds exhibit suitable Lipinski rule profiles and are potentially evaluated experimentally as CPF-2 enzyme inhibitor candidates.

Keywords: cysteine protease falcipain-2, malaria, molecular docking, *Plasmodium falciparum*, virtual screening

INTRODUCTION

Malaria is a worldwide infectious disease. This disease is the world's fifth major cause of infection-related death, trailing respiratory infections, HIV/AIDS, diarrhea, and tuberculosis (Perdana, 2021). According to the World Health Organization (WHO), malaria will continue to be a serious health concern in 107 nations until 2025 (Toloan et al., 2020). Indonesia contributes to 9% of all malaria cases in Southeast Asia and has become one of the risk countries, with around 15 million people infected yearly (Jiero & Pasaribu, 2021; Perdana, 2021).

Malaria is a disease caused by Plasmodium parasites that infect *Anopheles* sp. The parasite will degrade hemoglobin and amino acids from red blood cells for energy and protein synthesis, allowing it to replicate and proliferate (Tougan et al., 2020). *P. vivax*, *P. knowlesi*, *P. ovale*, *P. malariae*, and *P. falciparum* has been identified as the pathogens responsible for malaria in humans. *P. falciparum* is the most hazardous Plasmodium species, causing issues such as convulsions, coma, and even death (Belete, 2020).

Some antimalarial agents exhibit decreasing efficiency and resistance against Plasmodium species (Shibeshi et al., 2020). Artemisinin combination therapy (ACT) was recommended as the first-line medicine for uncomplicated malaria by the World Health Organization (Duru et al., 2016). Therefore, there are numerous prospects for discovering more effective antimalarial drugs that function against newly validated targets, particularly *P. falciparum* (Rosenthal, 2020).

Inhibiting enzyme activity is one strategy in drug development, particularly for antimalarials. Falcipain is an enzyme that plays a crucial part in Plasmodium's life cycle (Mishra et al., 2019; Munro & McMorran, 2022). Falcipain is a conventional protease enzyme from the papain family, with a cysteine residue on its catalytic site that distinguishes it from other malaria parasite proteases (Bekono et al., 2018; Lê et al., 2022; Pandey & Dixit, 2012). Plasmodium falciparum encodes four kinds of falcipain, including falcipain-1 (FP-1) on chromosome 14, falcipain-2 (FP-2), falcipain-2' (FP-2'), and falcipain-3 (FP-3) on chromosome 11 (Musyoka et al., 2019). Cysteine

protease falcipain-2 (CPF-2) is an essential enzyme in the *P. falciparum* life cycle because it degrades hemoglobin (Hb) and has been identified as a promising target for developing antimalarial drugs (Bekono et al., 2018; Pandey et al., 2005). Several in vitro investigations have established that CPF-2 inhibitors can decrease hemoglobin hydrolysis in parasites, preventing the formation of amino acids for parasite protein synthesis and terminating parasite development (Bekono et al., 2018; Caffrey et al., 2018).

Computer-aided drug design is one method for discovering and developing new drugs. Molecular docking is an approach in drug design that involves associating molecules with receptor targets. Virtual screening approaches can be used to select chemicals in large quantities from a database, one of which is the ZINC15 database (Arba et al., 2018). Molecular docking will optimize the drug development process by performing accelerated screening of specific chemical compounds, particularly those with unknown activity (Kasmawati et al., 2022; Torres et al., 2019). By applying a structure-based virtual screening technique to compounds from the ZINC15 database with a total of 17 million compounds, this study aims to reveal necessary knowledge for ongoing efforts to find new inhibitors with potential targets on cysteine protease falcipain-2 from *P. falciparum*.

EXPERIMENTAL SECTION

Protein Preparation

Protein preparation was carried out using the Protein Preparation wizard Maestro Schrödinger version 2016. The 3D structure of the CPF-2 enzyme (PDB ID: 3BPF) was obtained from the protein data bank (Kerr et al., 2009), which can be accessed at https://www.rcsb.org/. The protein structure was preprocessed by adding hydrogen atoms with the specified bond order and creating disulfide bonds. In addition to optimized H-bonds, protonation status was set at pH 7.0, and the water molecules were removed from the protein (Madhavi et al., 2013). Finally, the energy minimization method was performed using the OPLS3 force field (Harder et al., 2016). Receptor Grid Generation determined the active site in Maestro Schrödinger by following the position of the E-64 ligand with coordinates x = -56.9564, y = -1.2113, and z = -16.4668.

Ligand Preparation

The test ligand downloaded from the ZINC15 database was prepared using the Ligprep feature of Maestro Schrodinger by applying the default parameters. Furthermore, Epic is used to extend the degree of ionization, and the high energy ionization/ tautomer state was omitted for possible constraints under biological conditions (Madhavi et al., 2013).

Docking-based Virtual Screening

Protein-ligand complexes were prepared via Schrodinger's Virtual screening interface and filtered by Lipinski's rule and ADMET parameter assessment via QikProp (Patel et al., 2022). Compounds were docked into the receptor-generated grid using the OPLS3 force field, and their tethering scores were calculated using the High-Throughput Virtual Screening (HTVS), standard-precision (SP), and extra-precision (XP) scoring functions on the maestro's glide (Friesner et al., 2004, 2006; Halgren et al., 2004). The docking results were chosen from the top 10 hit compounds with lower XP docking scores than native ligands.

ADME Prediction

The SwissADME webserver was used to predict the ADME properties of the top 10 hit compounds based on the multilevel docking result (Daina et al., 2017). The Swiss Institute of Bioinformatics established this server, which is used to generate physiochemical, ADMET, and pharmacokinetic properties, and drug-like small molecule inhibitors to aid in drug discovery.

RESULTS AND DISCUSIONS

Docking-based Virual Screening

Molecular docking was done using the Maestro application. Maestro software was chosen in addition to its practical use; it also shows 2-dimensional interaction and 3-dimensional visualization. The molecular docking results can be assessed from the value of the binding free energy (ΔG). The lower the ΔG, the stronger the binding between the compound and the receptor. The first step is protein preparation, grid box determination, ligand preparation, and redocking with standard ligands. Redocking the E-64 obtained an RMSD value of 2.3 Å, slightly higher than the set value of < 2 Å (**Figure 1**). An RMSD range of 2.0 to 3.0 Å is acceptable, especially considering the high flexibility of the ligand, as long as it maintains the correct orientation (Ramírez & Caballero, 2018; Shoaib et al., 2023). However, RMSD values above 3.0 Å are not reliable in any aspect (Ramírez & Caballero, 2018). Furthermore, the E-64 ligand has a conformation that is not noticeably different from its x-ray orientation.

The virtual screening workflow consists of three docking stages, namely HTVS, SP and XP, where every 10% of the compounds with the best docking score will be used for the next docking stage. Finally, the top 10% of compounds that passed through Glide-XP were considered for further analysis based on the binding free energy. These compounds were also screened by ADMET parameter assessment via QikProp to eliminate non-drug molecules (Bhowmick et al., 2021). The ZINC15 Database was scanned for 17 million compounds, and 974 molecules were chosen based on conformation, 0-5 star code, XP score, and similar interactions and conformations with native ligands on receptors. **Table 1** shows native ligands E-64' 2D and 3D conformational structures and the 10 best compounds from the ZINC15 database. The results indicate that the conformation of the screened

compounds is similar to that of the native ligand, demonstrating a similar ability to mimic and occupy the active site of the CFP-2 enzyme. These findings suggest that the screened compounds have potential indications of binding affinity and structural compatibility relevant to the target enzyme.

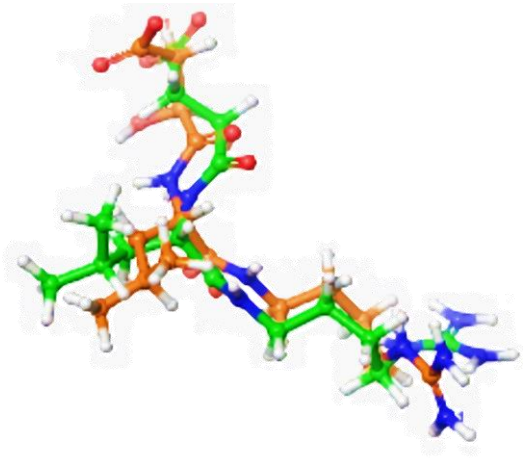
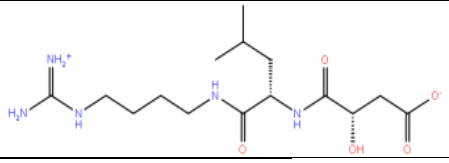
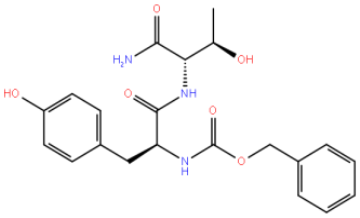
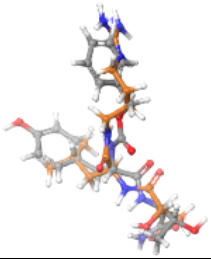
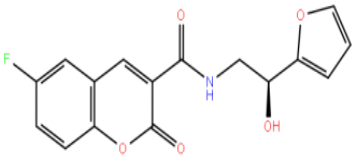
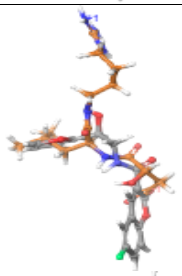
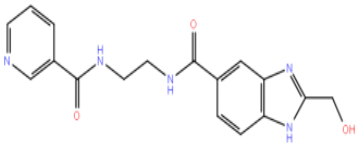
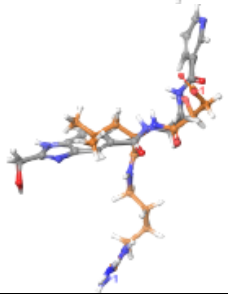
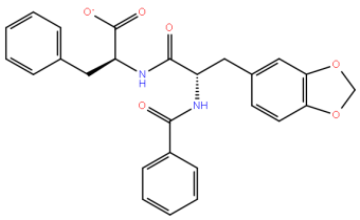
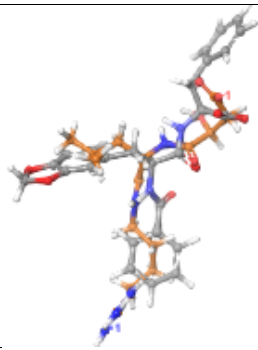
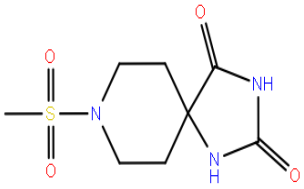
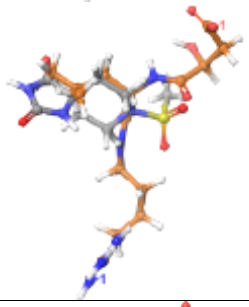
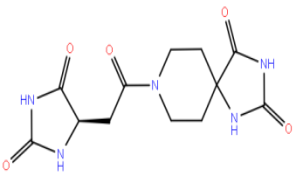
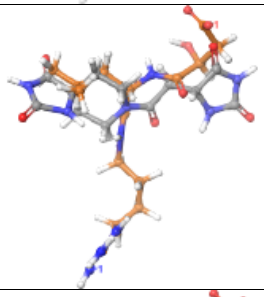
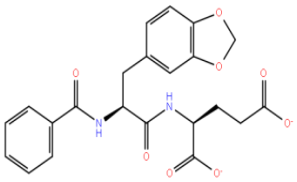
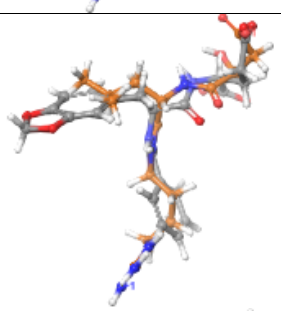
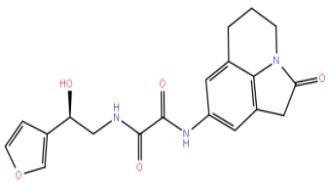
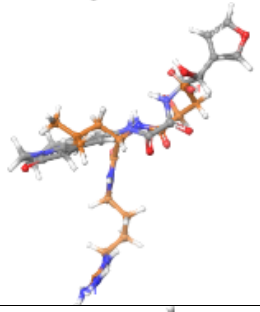
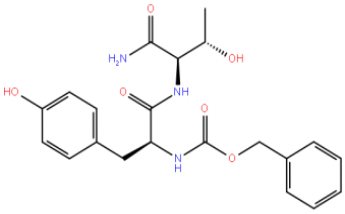
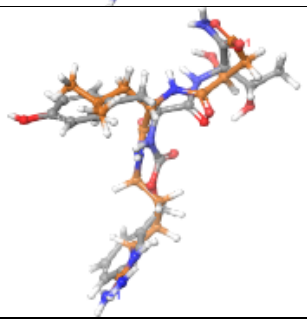
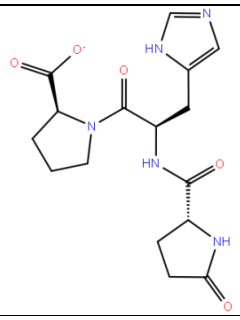
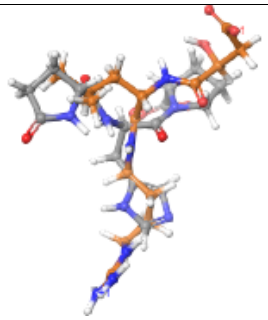


Figure 1. Visualization of the overlapping E-64 (green) with the redocked orientation (orange)

Table 1. 2D and 3D conformation of native ligand E-64 and top 10 compounds from the ZINC15 database.

No	Compounds	2D Structures	Comparison of the 3D structure of the compounds with E-64
1	Native ligand E-64		
2	ZINC000025691540		
3	ZINC000096436101		
4	ZINC000097797430		

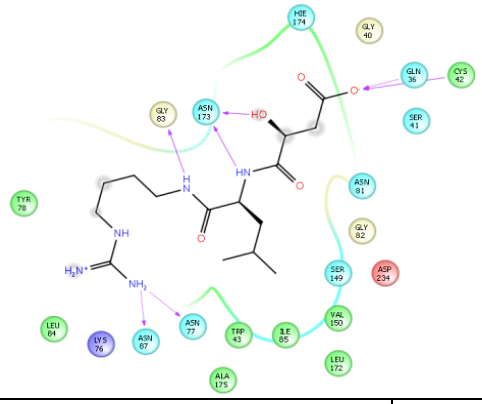
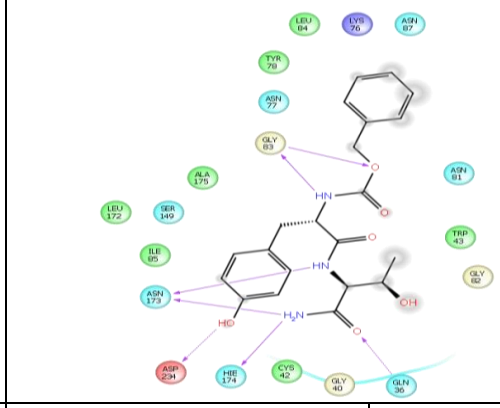
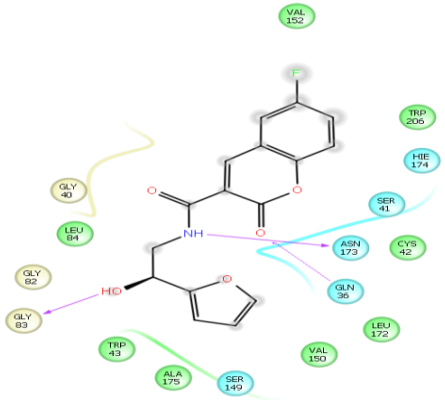
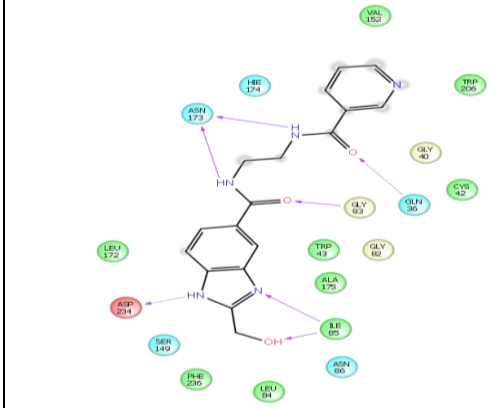
5	ZINC000002989004		
6	ZINC000046799780		
7	ZINC000071795603		
8	ZINC000002990725		
9	ZINC000077101353		
10	ZINC000299804235		

11	ZINC000033949581		

Each ligand docked to a macromolecule will produce a conformational ligand based on the binding free energy values ranking. This energy represents the strength of the interaction between the ligand and macromolecules or proteins; the lower the binding free energy value suggests a good level of stability between the ligand and the receptor, resulting in a stronger relationship (Arfan et al., 2024; Du et al., 2016). The binding results of the top 10 compounds with the CPF-2 enzyme can be seen in **Table 2.**

The simulation results obtained the top 10 hit compounds from the ZINC15 database, which have better binding energy than the E-64. Interestingly, 10 compounds have an energy range of -7.131 to -8.074 kcal/mol. The E-64, as a native ligand, has XP docking binding energies of -6.011 kcal/mol. Compounds in this group are ZINC000025691540, ZINC000096436101, ZINC000097797430, ZINC000002989004, ZINC000046799780, ZINC000071795603, ZINC000002990725, ZINC000077101353, ZINC000299804235, and ZINC000033949581 have a binding energy from XP docking of -8.074, -7.904, -7.891, -7.863, -7.717, -7.671, -7.666, -7.629, -7.628, and -7.131 kcal/mol, respectively.

Table 2. Binding energies and 2D visualization of docking results from the top 10 compounds in the ZINC15 Database with the CPF-2 enzyme.

Compounds	Binding Energy (Kcal/mol)	Compounds	Binding Energy (Kcal/mol)
Native ligand E-64	-6.011	ZINC000025691540	-8.074
			
ZINC000096436101	-7.904	ZINC000097797430	-7.891
			
ZINC000002989004	-7.863	ZINC000046799780	-7.717

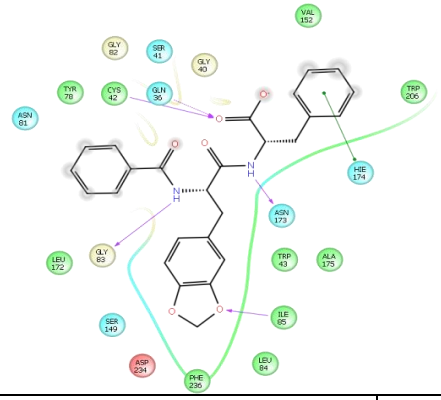
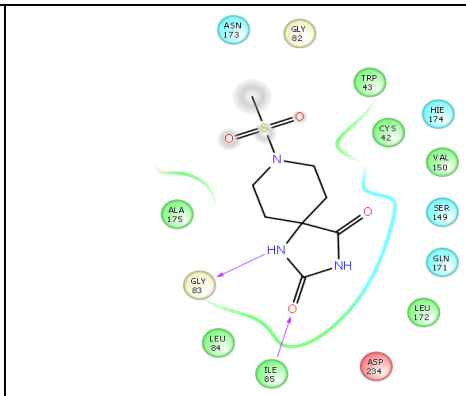
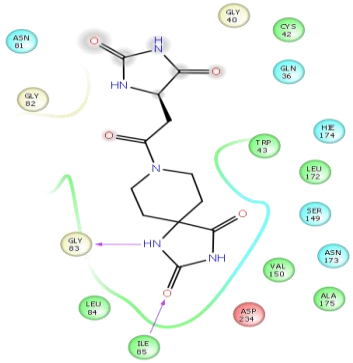
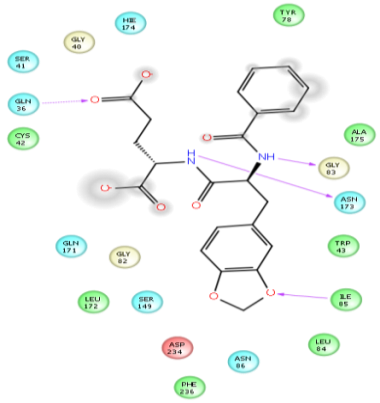
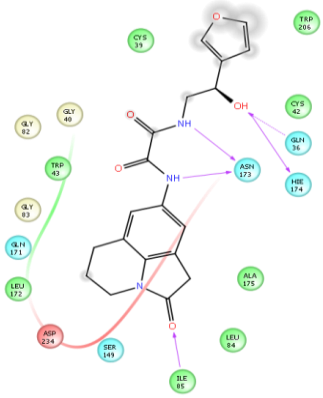
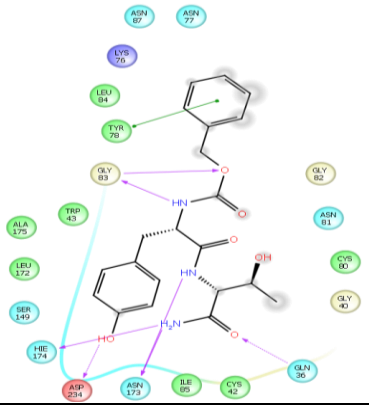
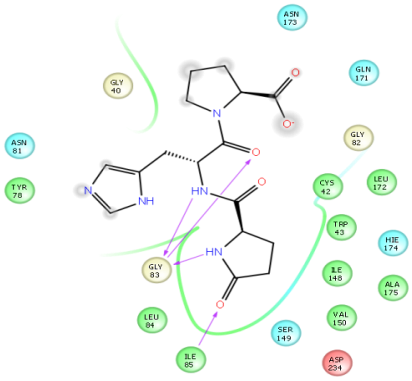
			
ZINC000071795603	-7.671	ZINC000002990725	-7.666
			
ZINC000077101353	-7.629	ZINC000299804235	-7.628
			
ZINC000033949581	-7.131		
			

Table 3. Hydrogen bonds summary from the top 10 compounds against CPF-2.

No	Compounds	Hydrogen Bonds	Distance (Å)
1	E-64	O (GLN36)	1.97 Å
		O (CYS42)	2.78 Å
		NH (GLY83)	2.03 Å
		NH (ASN173)	1.74 Å
		OH (ASN173)	1.89 Å
		NH_2 (ASN87)	2.19 Å
		NH_2 (ASN77)	2.48 Å
2	ZINC000025691540	O (GLN36)	2.45 Å
		O (GLY83)	2.40 Å
		NH (GLY83)	2.24 Å
		NH (ASN173)	2.01 Å
		NH_2 (ASN173)	1.93 Å
		NH_2 (HIE174)	2.24 Å
		OH (ASP234)	1.74 Å
3	ZINC000096436101	O (GLN36)	1.98 Å
		OH (GLY83)	1.73 Å
		NH (ASN173)	1.73 Å
4	ZINC000097797430	O (GLN36)	2.59 Å
		O (GLY83)	2.77 Å
		NH (ASN173)	1.93 Å
		NH (ASN173)	2.02 Å
		N (ILE85)	2.57 Å
		OH (ILE85)	2.38 Å
5	ZINC000002989004	O (GLN36)	2.79 Å
		O (CYS42)	2.79 Å
		NH (GLY83)	2.36 Å
		NH (ASN173)	1.72 Å
		O (ILE85)	2.23 Å
6	ZINC000046799780	NH (GLY83)	2.11 Å
		O (ILE85)	2.11 Å
7	ZINC000071795603	NH (GLY83)	1.93 Å
		O (ILE85)	2.16 Å
8	ZINC000002990725	NH (GLY83)	2.12 Å
		O (GLN36)	1.87 Å
		NH (ASN173)	1.60 Å
		O (ILE85)	1.78 Å
9	ZINC000077101353	OH (GLN36)	2.19 Å
		OH (HIE174)	2.37 Å
		NH (ASN173)	1.95 Å
		NH (ASN173)	1.97 Å
		O (ILE85)	2.15 Å
10	ZINC000299804235	NH (GLY83)	2.34 Å
		O (GLY83)	2.62 Å
		NH_2 (HIE174)	2.16 Å
		NH (ASN173)	1.87 Å
		NH_2 (ASN173)	1.99 Å
		OH (ASP234)	1.93 Å
11	ZINC000033949581	NH (GLY83)	2.17 Å
		NH (GLY83)	2.32 Å
		O (GLY83)	2.17 Å
		O (ILE85)	1.96 Å

Table 4. Summary of the ADME profile of the top 10 hit compounds from the ZINC15 database screening results

Compounds	GI absorption	BBB permeant	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4	Lipinksi rule
E-64	Low	No	No	No	No	No	No	Yes; 1 violation: NHorOH> 5
ZINC000025691540	Low	No	No	No	No	No	No	Yes; 0 violation
ZINC000096436101	High	No	No	No	No	No	No	Yes; 0 violation
ZINC000097797430	High	No	No	No	No	No	No	Yes; 0 violation
ZINC000002989004	High	No	No	Yes	Yes	Yes	Yes	Yes; 0 violation
ZINC000046799780	High	No	No	No	No	No	No	Yes; 0 violation
ZINC000071795603	Low	No	No	No	No	No	No	Yes; 0 violation
ZINC000002990725	Low	No	No	No	No	No	No	Yes; 0 violation
ZINC000077101353	High	No	No	No	No	No	No	Yes; 0 violation
ZINC000299804235	Low	No	No	No	No	No	No	Yes; 0 violation
ZINC000033949581	Low	No	No	No	No	No	No	Yes; 0 violation

The interaction between the ligand and the receptor was analyzed for binding to a residue that plays an important role in the active site area of CPF-2. Hydrogen bonds and hydrophobic interactions are observable interactions. Furthermore, the bond distance that occurs from each compound was observed. Research on the structure of CPF-2 (PDB code 3BPF) shows that the active site of CPF-2 has catalytic residues in the form of GLN36, CYS42, GLY83, HIS174, ASN204 and ASN81. Most of the top 10 compounds were able to interact with these catalytic residues. The native ligand E-64 forms six hydrogen bonds where the acetate group interacts with GLN36 and CYS42. Its amine group interacts with residues ASN77 and ASN87, the hydroxy group with residue ASN173, and the amide group interacts with residue GLY83. Experimental crystallographic studies on the structure of falcipain protease reveal that residues GLN36 and CYS42 are catalytic components. These residues play a crucial role in catalyzing proteolytic reactions essential for the life cycle of the malaria parasite. Binding to these residues can disrupt enzymatic activity, thereby indirectly inhibiting falcipain protease (Kerr et al., 2009).

Meanwhile, the best compound (ZINC000025691540) has five hydrogen bonds. The oxygen and amine atoms of the carbamic acid group interact with GLY83, and the oxygen atom and the amine group of the acetamide group interact with residues GLN36, HIE174, and ASN173, respectively. In addition, the hydroxy group on the phenol ring also interacts with ASP234. Interestingly, the compound with the second lowest energy (ZINC000096436101) has three hydrogen bonds, with the carbonyl group of the chromene ring interacting with GLN36. In addition, its amine and hydroxy groups interact with residues ASN173 and GLY83, respectively. In general, the top 10 compounds formed hydrogen bonds with residues GLN36, CYS42, GLY83, and ASN173, which were similar to native ligands. More details on these interactions are summarized in **Table 3**.

A hydrogen atom is connected to an atom with high electronegativities, such as flour (F), nitrogen (N), or oxygen (O). The number of hydrogen bonds in a substance as donors and acceptors can influence its activity and physicochemical attributes such as boiling and melting points, water solubility, and acidity. In general, receptor-drug binding is reversible, which means that as the drug concentration in the extracellular fluid decreases, the drug is released from the receptor instantaneously. Furthermore, the bond distance between one of the ligand atoms and the receptor atom influences the ligand-receptor bond strength (affinity). The ideal hydrogen bond distance between the ligand and the receptor is less than 2.8 Å (Nittinger et al., 2017); the less binding distance

between the ligand and the amino acid at the receptor, the stronger its binding interaction.

ADME Profiles Prediction

We analyzed the ADME profile of the top 10 compounds from ZINC15 database (**Table 4**). The ADME profile assessment was conducted using the SwissADME website (http://www.swissadme.ch/). SwissADME has advantages that can be utilized by various groups, especially in developing new drugs by predicting the properties of the compounds based on pharmacokinetic and pharmacodynamic aspects. The findings revealed that 5 of the 10 best compounds had high GI absorption profiles, including ZINC000096436101, ZINC000097797430, ZINC000002989004, ZINC000046799780, and ZINC000077101353.

Uniquely, all the best compounds are not expected to cross the blood-brain barrier (BBB). The permeability of the BBB protects neurons in the brain from exposure to toxic substances. In addition, the hit compounds also demonstrated favorable qualities by not inhibiting metabolizing enzymes except for compound ZINC000002989004. The CYP isoforms are enzymes that metabolize drugs and play an important role in drug elimination. Inhibiting this enzyme can result in toxicity and even unintended consequences (Hollenberg, 2002; Kirchmair et al., 2015). Interestingly, based on data analysis of drug characteristics' similarity to Lipinski's rule. All of the hit compounds have the potential to be drug candidates. Compounds that fulfill Lipinski's rules have the potential to be developed as oral drug candidates, perhaps enhancing their bioavailability (Lipinski, Lombardo, Dominy, & Feeney, 1997; Mermer & Vakal, 2021).

CONCLUSIONS

Based on the screening results, as many as 10 hit compounds were estimated to inhibit Cysteine Protease Falcipain-2. ZINC000025691540, ZINC000096436101, and ZINC000097797430 are the three best compounds identified from the ZINC15 database. In general, The top 10 hit compounds had similar binding interactions with GLN36, CYS42, GLY83, ASN173, and HIS174, with a bond-free energy range of -7.131 kcal/mol to -8.074 kcal/mol, which was more negative than the E-64, i.e., -6.011 kcal/mol. In addition, this top-hit compound also exhibits promising ADME and Lipinski profiles and is a potential candidate for the Cysteine Protease Falcipain-2 enzyme inhibitor.

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