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In Silico Analgesic and Toxicity Analysis of Modified Paracetamol on COX-2 Receptor (PDB ID: 3LN1)

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Abstract

Background*: Paracetamol is often used as the main analgesic in Indonesia. The use of more than 4 g/day or a single dose above 10 g can cause hepatotoxicity. This can be overcome by modifying the structure through a computer-aided drug design (CADD) approach, particularly molecular docking, which aims to produce compounds with greater potency and fewer side effects.* ***Objective****: This study aimed to determine the analgesic activity and toxicity of paracetamol derivatives modified using the Topliss method.* ***Methods****: Analgesic activity was tested by molecular docking of the COX-2 receptor (PDB ID 3LN1) using AutoDock Tool 4.2 and toxicity testing using pkCSM and Protox Online Tool.* ***Results****: The results of docking showed that the free binding energy values for test compounds 1 to 5 are -10.59 kcal/mol, -10.17 kcal/mol, -8.79 kcal/mol, -10.01 kcal/mol, and -9.32 kcal/mol, respectively, with corresponding inhibition constants of 17.29 nM, 35.21 nM, 360.88 nM, 46.36 nM, and 146.65 nM. These values are lower than paracetamol, which has a free binding energy of -6.21 kcal/mol and an inhibition constant of 28,043 nM. The results showed that the test compound was more stable in ligand-receptor binding. Toxicity tests showed that all the test compounds and paracetamol belonged to toxicity class IV. The test compound had an LD_{50} value of 1551 mg/kg, which was higher than that of paracetamol (338 mg/kg), indicating better effectiveness.* ***Conclusions****: Compound 2 was predicted to have the best biological activity and potential as an alternative to paracetamol.*

Keywords*: analgesic, molecular docking, paracetamol, structure modification, toxicity*

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INTRODUCTION

Pain is a sensory feeling of discomfort due to tissue or potential damage (International Association Study of Pain [IASP], 2020). In the United States, 10-25% of people experience pain, with 50.2 million adults (20.5%) suffering from it daily (National Center for Health Statistics [NCHS], 2006; Jason et al., 2022). In Indonesia, 11.9% reported musculoskeletal pain based on diagnoses, and 24.7% reported musculoskeletal pain based on symptoms (Indonesian Ministry of Health, 2018). Pain is more common with age in women and among those doing heavy labor or with lower education (IASP, 2020).

Paracetamol is the most widely used pain reliever globally and is endorsed by the World Health Organization (WHO) as the first-choice treatment for pain (National Center for Biotechnology Information [NCBI], 2022). Although safe at appropriate doses, paracetamol can cause liver damage or hepatotoxicity in long-term use and excessive doses (NCBI, 2022; Rotundo & Pyrsopoulos, 2020). The Food and Drug Administration (FDA) states that paracetamol is safe for consumption up to a maximum dose of 4000 mg in 24 h. Paracetamol is the leading cause of acute liver failure worldwide, with studies showing 6% poisoning cases, 56% acute liver failure, and 0.4% fatal overdoses (Chidiac et al., 2023). In addition to its widespread use, paracetamol was chosen in this study because of its simple structure, easy modification, abundant available data, and broad-spectrum analgesic activity.

Previous studies have shown that modification of the chemical structure of paracetamol can increase its analgesic activity. For example, modification using the Schotten-Baumann acylation method produces compounds with higher analgesic activity (Lika, 2020). Another study using an acylation reaction between paracetamol and isoleucyl chloride produced compounds with activities comparable to that of paracetamol (Siswandono & Parwitha, 2020).

The toxic effects of paracetamol 'are due to one of its metabolites, N-acetyl-p-benzo-quinoneimine (NAPQI), which is produced during its metabolism in the liver (Rahayu & Solihat, 2018). Although specific data on paracetamol-induced hepatotoxicity in Indonesia are unavailable, liver damage from drug poisoning remains a global concern. This study aimed to perform in silico testing by modifying the hydroxyl group (-OH) of the chemical structure of paracetamol 'using the Topliss approach.

In silico test methods are often used because of their ability to make predictions with a high degree of accuracy, improve drug development efficiency, and reduce costs and time. Molecular docking, an in silico assay, was used to describe the interaction between compounds and receptors, such as COX-2 (PDB ID: 3LN1), using AutoDock Tool 4.2 program, which has an easy interface and good visualization of the results (Quan et al., 2022). Toxicity testing was performed using pkCSM and ProTox, both of which have good prediction performance and are extensive databases (Pires et al., 2015; Drwal et al., 2014). This study aimed to identify an alternative to paracetamol with minimal side effects and optimal analgesic activity through chemical structure modification and *in silico* testing.

MATERIALS AND METHODS

Materials

The two-dimensional (2D) structures of test compounds 1-5 modified paracetamol derivatives, namely 4-acetamidophenyl 4-chlorobenzoate, 4-acetamidophenyl 3,4-dichlorobenzoate, 4-acetamidophenyl 4-(trifluoromethyl)benzoate, 4-acetamidophenyl 2, 4-dichlorobenzoate, and 4-acetamidophenyl 4-nitrobenzoate, were drawn using Chem Draw Ultra 2D and the three-dimensional (3D) structure of COX-2 receptor with PDB ID code 3LN1 obtained from Protein Data Bank (PDB).

Tools

The tools used in this research were divided into two types: hardware and software. The hardware used includes an Asus laptop with Intel® Core™ i3-8145U processor specifications 4.00 GB RAM, Windows 10 operating system, and a 64-bit operating system. The software used is Chem Office 12.0 (Chem 2D & Chem 3D) (Agustina et al., 2018), SwissADME: www.swissadme.ch (Oner et al., 2022), AutoDock Tool 4.2 (Morris et al., 2009), BIOVIA Discovery Studio Visualizer (Khan & Lee, 2022), pkCSM: biosig.lab.uq.edu.au/pkcsm/ (Pires et al., 2015) and ProTox Online Tool: tox.charite.de/protox3/ (Drwal et al., 2014).

Method

The stages of researchs are (1) modification of the compound structure using the Topliss method, (2) 2D structure preparation: the compounds drawn using ChemDraw Ultra 12.0, (3) 2D to 3D structure conversion using Chem 3D Pro 12.0, (4) 3D structure preparation: receptor preparation by downloading the 3D structure of the COX-2 receptor (PDB ID: 3LN1) first on the RCSB PDB website (www.rcsb.org), then separation of receptors from solvents, native ligands, or unnecessary residues was performed using BIOVIA

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Discovery Studio Visualizer, (5) validation of receptors, (6) analgesic activity testing (docking) of candidate test compounds, (7) analysis of results to determine test compounds based on the Topliss method, (8) screening of test compounds (prediction of physicochemical properties & ADME), (9) preparation of 2D structure of test compounds, conversion to 3D, and preparation of 3D structure of test compounds, (10) analgesic activity test (docking) of test compounds with 3 replications, (11) analysis and visualization of docking results, (12) toxicity test, and (13) analysis of research results.

RESULTS AND DISCUSSION

2D & 3D structure of test compounds

Prior to *in silico* testing, 2D structures of the test compounds and paracetamol were drawn using ChemDraw Ultra 12.0. Subsequently, it was converted to 3D using Chem 3D Pro 12.0, as the docking stage must use a 3D structural model. The results for the 2D and 3D structures are shown in Figures 1 and 2.

The test compounds used in this study were identified using the Topliss method. The Topliss method is a structured approach used in medicinal chemistry to optimize lead compounds by systematically modifying their molecular structures and evaluating their impact on biological activity. This method involves substituting certain functional groups on a molecule selected based on its electronic, steric, and hydrophobic properties and testing the derivates of the compound to determine if their biological activity is higher, equal, or lower than that of the lead compound. The Topliss method was selected because of its systematic and efficient approach to evaluating the effect of substitution on the biological activity of compounds, thus supporting the optimization of the structure of paracetamol. The modification of a compound with the Topliss scheme requires an initial substitution with a Cl atom. Paracetamol has no Cl atoms in its structure, so it cannot bind directly to chloride atoms. Halogenated compounds that contain halogen atoms (F, Cl, Br), such as benzoyl chloride, can react with paracetamol. The addition of benzoyl chloride to paracetamol increases the activity and solubility of the compound and is advantageous in terms of reaction speed and stability (Wasilczyk et al., 2021; Wong et al., 2016). Benzoyl chloride reacts with hydroxyl groups (-OH) in the molecule, forming benzoate ester groups that change the physicochemical properties of the compound, such as the solubility and stability of chemical compounds, thereby increasing the effectiveness of drug development (Grinias et al., 2017). The replacement of hydroxyl groups with benzoate esters can improve the solubility in certain organic solvents and the stability of the compound against chemical degradation, expanding the use of such compounds in pharmaceutical formulations or the chemical industry (Wilhelms et al., 2023; Grinias et al., 2017).

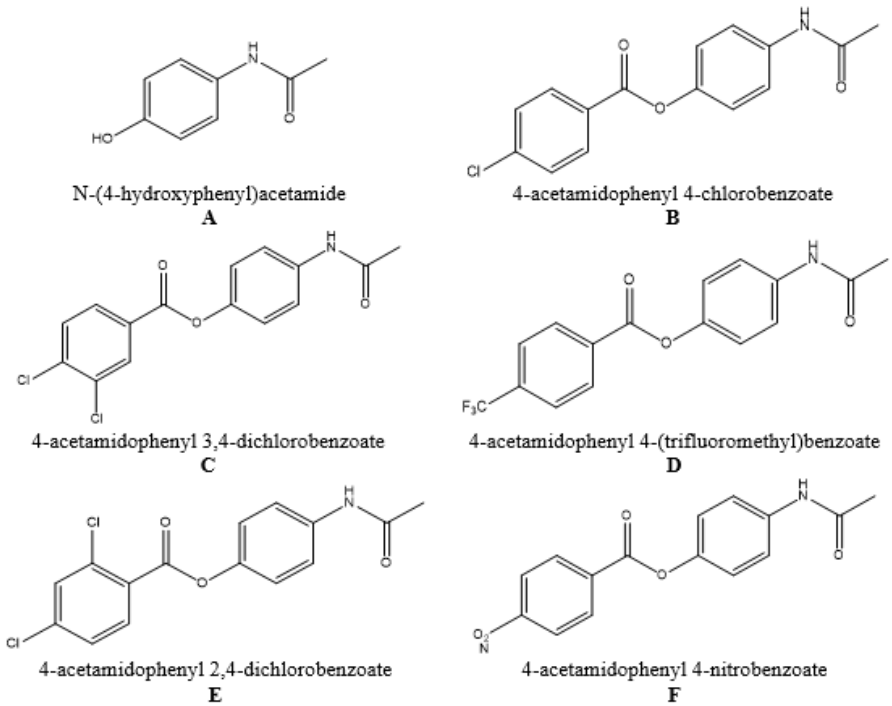

Figure 1. The 2D structure of comparator compound (paracetamol) (A) test compound 1 (B) test compound 2 (C) test compound 3 (D) test compound 4 (E) and test compound 5 (F) with respectively IUPAC name

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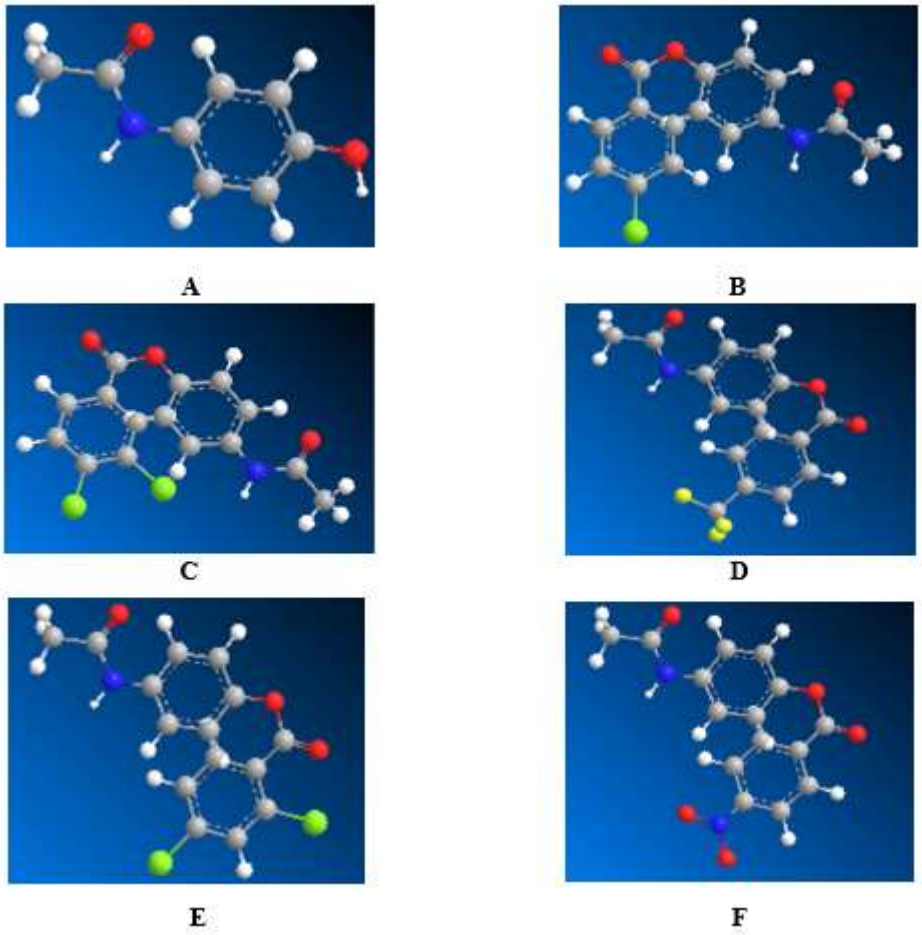

Figure 2. The 3D structure of comparator compound or paracetamol (A), test compound 1 (B), test compound 2 (C), test compound 3 (D), test compound 4 (E), and test compound 5 (F)

Table 1. Prediction Results of Physicochemical Properties

Compound	Lipinski & Veber Rules Parameters						Results
	MW ≤ 500	Log P ≤ 5	HBD ≤ 5	HBA ≤ 10	TPSA ≤ 140Å^2	T ≤ 10	
PCT	151.16	1,42	2	2	64.67	2	Yes
1	289.71	3,51	1	3	120.87	5	Yes
2	324.16	4,17	1	3	131.17	5	Yes
3	323.27	3,88	1	6	129.43	6	Yes
4	324.16	4,17	1	3	131.17	5	Yes
5	300.27	2,77	1	5	125.22	6	Yes

Note: MW = Molecule Weight, HBD = Hydrogen Bond Donor, HBA = Hydrogen Bond Acceptor, TPSA = Topological Polar Surface Area, T = Torsion

Prediction of physicochemical properties

Physicochemical property prediction is guided by Lipinski's Rule of Five and Veber's Rule, which require several key parameters. Lipinski's rule includes molecular weight, Log P, Hydrogen Bond Acceptors (HBA), Hydrogen Bond Donors (HBD), and Veber's rule, which includes the number of bonds between atoms that can rotate (torsion) and topological polar surface area (TPSA). The physicochemical properties were predicted by first drawing the 2D structure of the test compound and then converting it into SMILES code on the SwissADME website.

According to Lipinski et al. (2001), compounds with a molecular weight > 500, log P > 5, HBD > 5, and HBA > 10 are difficult to absorb and have low permeability. Based on Veber's Rule as well, the compound will be difficult to absorb if it has a torsion value of more than 10 and TPSA more than 140 Å^2. Lipinski's Rule and Veber's rule are complementary guidelines used to predict the oral bioavailability of drug candidates, with Lipinski focusing on molecular weight,

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lipophilicity, and hydrogen bonding capacity, while Veber emphasizes molecular flexibility (rotatable bonds) and polar surface area, addressing additional aspects of a molecule'smolecular' ability to cross biological membranes (Trossini et al., 2023). Drug molecules must be small enough to penetrate the cell membranes and be absorbed by the gastrointestinal system. Log P measures a compound's capability to enter the cell membrane; a lower value indicates that the compound is more lipophilic and easily distributed in the body. HBD and HBA indicate the hydrogen bonding ability, which is important for absorption. TPSA measures the total surface area of the polar atoms (oxygen and nitrogen) in a molecule, which helps predict drug absorption, membrane permeability, and molecular transport characteristics. Torsion measures the number of interatomic bonds that can rotate; torsion values < 10 indicate good permeability (Ivanovic et al., 2020; Truong et al., 2021; Veber et al., 2002).

Based on Table 1, test compounds 1 to 5 have molecular weight < 500, log P < 5, HBD < 5, HBA < 10, torsion < 10, TPSA < 140 Ă^2, thus fulfilling Lipinski's rules of five and Veber's rules. This indicated that the compounds were easily absorbed and had good permeability. Paracetamol, as a comparator compound, also fulfills Lipinski's rules and Veber's rule.

ADME prediction

Predictions of absorption, distribution, metabolism, and excretion (ADME) were made by entering the SMILES codes for the five test compounds on the pkCSM website. The ADME prediction results are listed in the following table.

As shown in Table 2, the best test compound in the absorption prediction results was test compound 1. Test compound 1 had the highest absorption value in the gut, and the predicted distribution results were test compound 2. Test compound 2 had the highest value in predicting the distribution of Vdss and CNS parameters, with values of -0.309 log L/kg and -1.910 log PS, respectively. In the prediction of metabolism, compounds 2, 3, 4, and 5 had the same results, which can be metabolized through enzymes CYP3A4, CYP1A2, CYP2C19, and CYP2C9. The best test compound for the prediction of excretion was test compound 5 because it had the highest total clearance value, which was 0.662 log ml/min/kg.

Receptor validation

Receptor validation aims to ensure that the method used meets the requirements and that the research results are as expected (Laksmiani et al., 2020). Receptors were regarded as accurate if the RMSD value was ≤ 2° (Hevener et al., 2009). Validation docking results usually show a consistent number of clusters from run to run, with one or two dominant clusters showing a better prediction of ligand conformation (Sotudian et al., 2021).

Table 2. Prediction results of ADME

	Prediction Category	Compound						Reference Value
		PCT	1	2	3	4	5	
A	Intestinal absorption (%)	92.527	92.895	92.338	91.195	91.646	91.335	>80%
	Skin permeability (Log Kp)	-2,583	-2,877	-2,930	-2,985	-2,953	-2.749	<-2.50
	$CaCO_2$ Permeability (Log Papp in 10^{-6} cm/s)	1.199	1.369	1.402	1.418	1.397	1.238	>0.90
	P-glycoprotein substrate	No	No	No	No	No	No	Yes
	P-glycoprotein I inhibitor	No	No	Yes	No	No	No	Yes
	P-glycoprotein II inhibitor	No	No	No	No	No	No	Yes
D	Vdss (log L/kg)	-0.134	-0.378	-0.309	-0.457	-0.339	-0.650	>0.45
	BBB Permeability (Log BB)	-0.234	-0.064	-0.094	-0.058	-0.114	-0.585	>0.3
	CNS Permeability (Log PS)	-2.851	-2.029	-1.910	-1.975	-1.912	-2.325	>-2
M	CYP2D6 substrate	No	No	No	No	No	No	Yes
	CYP3A4 substrate	No	Yes	Yes	Yes	Yes	Yes	Yes
	CYP1A2 inhibitor	No	Yes	Yes	Yes	Yes	Yes	Yes
	CYP2C19 inhibitor	No	Yes	Yes	Yes	Yes	Yes	Yes
	CYP2C9 inhibitor	No	No	Yes	Yes	Yes	Yes	Yes
	CYP2D6 inhibitor	No	No	No	No	No	No	Yes
	CYP3A4 inhibitor	No	No	No	No	No	No	Yes
E	Total Clearance (Log ml/min/kg)	0.517	-0.271	-0.086	0.508	-0.155	0.662	The higher, the better
	Renal OCT2 substrate	No	No	No	No	No	No	Yes

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Table 3. RMSD value of receptor validation results

Native Ligand	RMSD (Å)	Cluster
HEM	1,061	100
CEL	2,805	4
BOG	1,007	32
NAG	4,159	83

Based on Table 3, the valid native ligand used for the next docking process is the native ligand with the identifier name HEM because it has an RMSD value of 1.061 Ă, with the number of clusters 100 out of 100 runs. A lower RMSD value suggests high accuracy and reliability. HEM was selected over the others because its RMSD value was closer to the acceptable threshold and was supported by the largest cluster size (100 of 100), reflecting higher consistency and reproducibility in the docking results.

Docking of test compound and paracetamol

The molecular docking process yielded results in the form of free-binding energy values and inhibition constants. The determination of better activity of a test compound can be predicted by using a comparative drug compound as a control, namely paracetamol. Test compounds with lower values of free binding energy and inhibition constants than comparator compounds are expected to possess a more stable binding potential (Suhadi et al., 2019).

The determination of test compounds using the Topliss method is carried out by substituting a molecule that is then tested for biological activity to determine whether it is higher, the same, or lower than the activity of the lead compound or molecule. Based on the Topliss scheme, paracetamol, which has been added with benzoyl chloride, was substituted by a 4-Cl group. Next, the biological activity was tested using the docking method, and the results were analyzed based on free binding energy values and inhibition constants. The first compound exhibited a lower biological activity value than paracetamol, both the free binding energy value and inhibition constant value. This can be seen in Table 4. Due to its lower biological activity, a docking test was then conducted on the compound substituted with 3,4-Cl2. The results showed that the compound had an activity value similar to that reported previously. Therefore, based on the Topliss scheme, further substitution with 4-CF3, 2,4-Cl2, and 4-NO2 groups was performed.

Receptors and ligands bind selectively, as determined by the bond formation energy of the ligand and receptor. A compound (ligand) is considered to have biological activity against a particular receptor based on its binding free energy value. The binding free energy describes the stability of the ligand binding to the receptor. A lower value of the binding free energy indicates a more stable connection between the ligand and receptor (Frimayanti et al., 2021). Based on Table 4, the binding free energy value of the comparison compound or paracetamol is -6.21 kcal/mol. Test compound 1 has the lowest binding free energy among the compounds, with a value of -10.59 kcal/mol, so the compound has the highest level of bond stability between the ligand and receptor. Meanwhile, the test compound with the lowest level of stability was test compound 3, with a value of -8.79 kcal/mol.

Table 4. Docking results: free binding energy value

Compound	Free Binding Energy (kcal/mol)			Average (kcal/mol)
	Replication 1	Replication 2	Replication 3	
Paracetamol	-6.21	-6.21	-6.21	-6.21
Compound 1	-10.58	-10.60	-10.59	-10.59
Compound 2	-10.14	-10.18	-10.18	-10.17
Compound 3	-8.77	-8.77	-8.83	-8.79
Compound 4	-10.02	-10.02	-9.98	-10.01
Compound 5	-9.34	-9.30	-9.32	-9.32

Table 5. Docking results: inhibition constant value

Compound	Inhibition Constant (nM)			Average (nM)
	Replication 1	Replication 2	Replication 3	
Paracetamol	27,870	27,970	28,290	28,043
1	17.66	16.95	17.27	17.29
2	17.61	17.58	17.45	17.55
3	373.88	372.89	335.89	360.88
4	45.22	45.47	48.40	46.36
5	141.91	151.65	146.39	146.65

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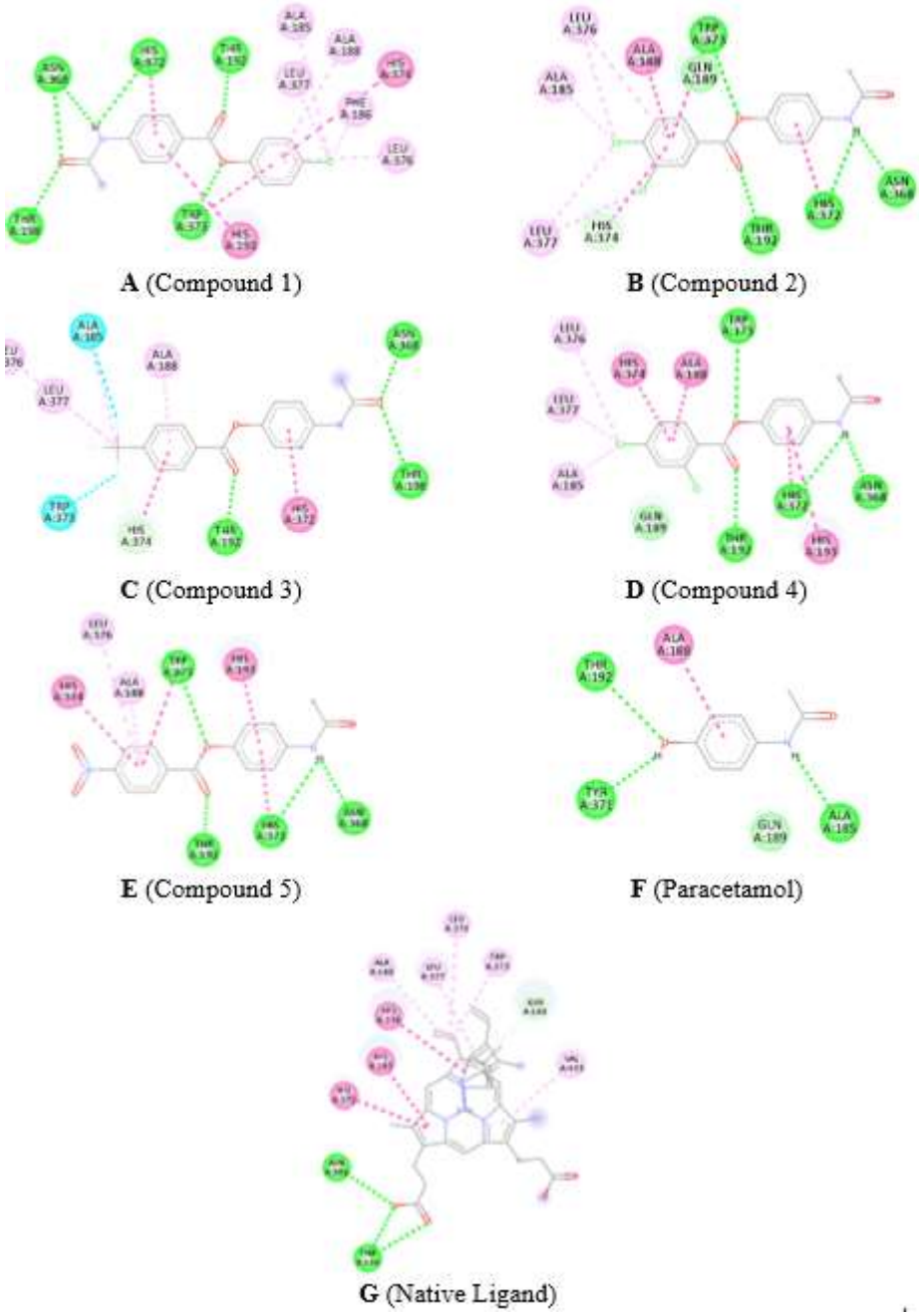

Figure 3. 2D visualization of amino acid residue interactions using BIOVIA *Discovery Studio Visualizer*: compound 1 (A), compound 2 (B), compound 3 (C), compound 4 (D), compound 5 (E), paracetamol as a comparator compound (F), and native ligand (G)

The inhibition constant (Ki) describes the capacity of a ligand to suppress the receptor activity. When the inhibition constant is low, a low ligand concentration is required to inhibit enzyme or receptor activity (Puspita et al., 2022). The stability of the ligand-receptor interaction is directly proportional to the binding potential of the compound; therefore, a reduced Ki value suggests a more robust bond between the molecule and its target (ligand-receptor) (Rena et al., 2021). As shown in Table 5, the test compound with the lowest Ki value is test compound 1, with a value of 17.29 nM; the compound has the strongest bond between the ligand and the receptor. The test compound with the lowest ligand-receptor bond was test compound 3, with a Ki value of 360.88 nM.

Analysis & visualization of docking results

Visualization results were analyzed using BIOVIA Discovery Studio Visualizer, which shows the results of ligand bonding with the nearest amino acid and

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produces amino acid residue bonds that form hydrogen bonds with their bond lengths.

As shown in Figure 3, the primary types of bonds that form are hydrogen and hydrophobic interactions. The drug binds to the receptor in a manner that can easily be undone, allowing it to not separate from the receptor as its external concentration decreases. These bonds should be relatively mild but strong enough to hold their own against other molecular interactions (Basuki & Melinda, 2017). Thus, covalent bonds are generally not observed in most docking results, as they are irreversible despite their high affinity and stable interactions (Prabowo, 2018). Hydrogen bonds are important for biological activity. Hydrogen bonds are bonds that can stabilize ligand-receptor interactions. Other interactions between ligands and receptors that can increase conformational stability are electrostatic and van der Waals interactions (Rachmania et al., 2018).

Table 6. Analysis results of amino acid residue interaction

Compound	Amino Acid Residue	
	Hydrogen Bond (distance in Å)	**Hydrophobic Interaction**
Paracetamol	Tyr371 (1.98) Ala185 (2.21) Thr192 (2.90) Gln189 (-)	Ala188
Native Ligand	Asn368 (2.87) Thr198 (3.01) Gln189 (3.23)	His372, His193, His374, Ala188, Leu376, Trp373, Val433, Leu377
Compound 1	His372 (2.26) Thr192 (2.90) Thr198 (3.06) Trp373 (3.09) Asn368 (3.09)	His372, His193, His374, Trp373, Ala185, Leu377, Phe186, Ala188, Leu376
Compound 2	Asn368 (2.60) His372 (3.01) Thr192 (3.06) Trp373 (3.27)	Ala188, His374, His372, His373, Ala185, Leu376, Leu377
Compound 3	Thr192 (2.54) Thr198 (3.09) Asn368 (3.06) His374 (3.45)	His372, His374, Ala185, Leu376, Leu377, Ala188
Compound 4	His372 (2.60) Asn368 (2.92) Thr192 (2.99) Trp373 (3.21)	His372, His193, His374, Ala188, Ala185, Leu376, Leu377
Compound 5	Asn368 (2.51) His372 (2.94) Thr192 (3.06) Trp373 (3.23)	His372, His193, His374, Trp373, Ala188, Leu376

Table 7. Similarity of amino acid residue interaction on hydrogen bond

Amino Acid Residue	PCT	Native Ligand	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5
Thr192	√	-	√	√	√	√	√
Thr198	-	√	√	-	√	-	-
Tyr371	√	-	-	-	-	-	-
Ala185	√	-	-	-	-	-	-
Asn368	-	√	√	√	√	√	√
His372	-	-	√	√	-	√	√
His374	-	-	-	-	√	-	-
Trp373	-	-	√	√	-	√	√
Gln189	√	√	-	-	-	-	-

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Table 8. Toxicity prediction results of test compounds and comparator compounds

Compound	Toxicity Parameters			
	Hepatotoxicity*	**LD_{50} (mg/kg)****	**Toxicity Class****	**Average Similarity****
Paracetamol	No	338	IV	100%
1	No	1551	IV	70.99%
2	No	1551	IV	66.97%
3	No	1551	IV	68.12%
4	No	1551	IV	65.96%
5	No	1551	IV	63.19%

Note: * Using pkCSM Online Tool ** Using Protox Online Tool

The findings of the ligand-receptor binding process showed that the test compound had amino acid residues similar to those of the original ligand and paracetamol. According to Gholam (2023), the more the amino acid residues formed, the more stable the compound interaction. Test compounds 1-5 had the same amino acid residues as the comparator compound or paracetamol, namely Thr192 and Asn38. Meanwhile, the residues of amino acids Tyr371, Ala185, and Gln189 were not formed in test compounds 1–5. The residue Thr198 was only formed in test compounds 1 and 3, as well as its native ligand. Meanwhile, the residues of amino acids His372 and Trp373 were only not formed in test compound 3, while His374 was only formed in test compound 3. Amino acids residues greatly affect functional groups. The His374 residue is formed as a type of carbon-hydrogen bond, as is illustrated in Figure 3(C). All test compounds have hydrogen bonds at varying distances, where the optimal distance is between 2.5-3.5 Å (Syahputra et al., 2014). An appropriate hydrogen bond distance increases the strength of the interaction, whereas too large a distance weakens the interaction (Prasetiawati et al., 2021). The distance between atoms in amino acid residues affects their ability to form hydrogen bonds and interact with other chemical groups (Naufa et al., 2021).

Toxicity prediction

The websites used to assess the toxicity of the test compounds and comparators were pkCSM and Protox Online Tool. The parameters tested in pkCSM were hepatotoxicity properties, while the parameters tested in Protox were LD50 values and toxicity classes. Toxicity classes were classified based on the Globally Harmonized System (GHS) and categorized by LD50 toxicity levels across a spectrum of six toxicity classes, with LD50 thresholds of 5, 50, 300, 2000, and 5000 mg/kg body weight with the following details. Class I (fatal if swallowed) had an LD50 value range of ≤ 5 mg/kg, class II (fatal if swallowed) had a value range of $5 < LD50 \leq 50$ mg/kg, class III (toxic if swallowed) had a value range of $50 < LD50 \leq 300$ mg/kg, class IV (dangerous if swallowed) had a value range of $300 < LD50 \leq 2000$ mg/kg, class V (possibly dangerous if swallowed) had a value range of $2000 < LD50 \leq 5000$ mg/kg, and class VI (non-toxic) had an LD50 value range > 5000 mg/kg (Nursanti *et al.*, 2022).

The predicted toxicity outcomes for the test compounds and comparators on both websites were obtained by entering the SMILES code for every substance, which was then analyzed from the results obtained.

As shown in Table 8, all test compounds were found, and the comparator compounds did not have hepatotoxicity predicted using the pkCSM Online Tool website. Hepatotoxicity refers to the ability of a compound or drug to cause liver damage. Drug-induced liver damage is a major cause of acute and chronic liver disease and is often the main reason for the removal of drugs from the market after approval (Francis & Navarro, 2022).

Other parameters in the toxicity prediction were the LD_{50} value and toxicity class. Although not hepatotoxic, all test compounds were classified as toxicity class IV with an LD50 value of 1551 mg/kg, which means they are dangerous if ingested but safer than paracetamol with an LD50 value of 338 mg/kg (Sulastra et al., 2020). The higher the LD50 value, the more secure the compound. Toxicity class IV is still considered safe, depending on the dose, duration, and context of use (Popiolek et al., 2021).

Ranking of test compound docking results

Ranking of test compounds from docking results was performed to establish the ranking of the top test compounds as a potential alternatives to paracetamol derivative analgesics. Ranking of the test compounds was performed by analyzing the best values for each parameter.

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Table 9. Ranking of test compounds based on predicted physicochemical properties

Ranking	Compound
1	Compound 1
2	Compound 2
3	Compound 4
4	Compound 5
5	Compound 3

Table 10. Ranking of test compounds based on ADME prediction results

Parameter	Ranking	Compound
A	1	Compound 1
	2	Compound 2
	3	Compound 4
	4	Compound 5
	5	Compound 3
D	1	Compound 2
	2	Compound 4
	3	Compound 3
	4	Compound 1
	5	Compound 5
M	1	Compound 2
	2	Compound 3
	3	Compound 4
	4	Compound 5
	5	Compound 1
E	1	Compound 5
	2	Compound 3
	3	Compound 2
	4	Compound 4
	5	Compound 1

Table 11. Ranking of test compounds docking results based on free binding energy value

Ranking	Compound	Free Binding Energy Value (kcal/mol)
-	Paracetamol	-6,21
1	Compound 1	-10,59
2	Compound 2	-10,17
3	Compound 4	-10,01
4	Compound 5	-9,32
5	Compound 3	-8,79

Table 12. Ranking of test compounds docking results based on inhibition constant value

Ranking	Compound	Inhibition Constant (nM)
-	Paracetamol	28.043
1	Compound 1	17,29
2	Compound 2	17,55
3	Compound 4	46,36
4	Compound 5	146,65
5	Compound 3	360,88

Table 13. Ranking of test compounds' amino acid residue interaction results

Ranking	Compound	Number of Amino Acid Residues
-	Paracetamol	5
1	Compound 1	14
2	Compound 4	11
3	Compound 2	11
4	Compound 5	10
5	Compound 3	10

Table 14. Ranking of test compounds toxicity prediction result

Ranking	Compound	Average Similarity (%)
-	Paracetamol	100
1	Compound 5	63.19
2	Compound 4	65.96
3	Compound 2	66.97
4	Compound 3	68.12
5	Compound 1	70.99

Based on the above tables, the test compound with the best ranking prediction was test compound 2. This is because the prediction results of test compound 2 have higher top ranks than the other compounds. Several other factors can influence this, such as the positions of atoms in the structure that distinguish test compounds from one another. Therefore, further preclinical research, such as in vitro and in vivo studies, is recommended to determine the validity of the results of this in silico research. In this method, all parameters are given equal importance, meaning that each parameter contributes the same amount to the ranking process without any special focus on specific factors. This approach ensures a balanced evaluation that considers the combined effects of all parameters involved.

CONCLUSION

Test compounds 1-5 showed superior pharmacological characteristics compared with paracetamol. Their free binding energy values ranged from -8.79 to -10.59 kcal/mol, significantly better than paracetamol's -6.21 kcal/mol. Inhibition constants varied from 17.29 to 360.88 nM, lower than paracetamol's 28,043 nM. Additionally, the compounds exhibited higher LD_{50} values (1551 mg/kg) than that of paracetamol ('338 mg/kg), suggesting improved safety. All test compounds were classified as toxicity class IV. Among the compounds tested, compound 2 showed the most promising biological activity and potential as a paracetamol alternative.

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AUTHOR CONTRIBUTIONS

Conceptualization: N.H., L.P., A.R.H., H.M.; Methodology, N.H., L.P., A.R.H., H.M.; Software, N.H.Validation: N.H., L.P., A.R.H., H.M.; Formal Analysis, N.H., L.P., A.R.H.; Investigation: N.H., L.P., A.R.H.; Resources, N.H.; Data Curration; N.H., L.P., A.R.H.; Writing - Original Draft, N.H.; Writing - Review and Editing, N.H., L.P., A.R.H.; Visualization: N.H., L.P., A.R.H., H.M.; Supervision: N.H., L.P., A.R.H., H.M.; Project Administration, N.H., L.P., A.R.H., H.M.; Funding Acquisition, N.H.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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