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Research Article

Radiolabeling and In-Silico Study of ^{131}I-(4-fluorobenzoyl-3-methylthiourea) as Radiopharmaceuticals for Breast Cancer Theranostics

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Abstract

The chemicals produced from thiourea are actively being studied as anticancer possibilities. In complexes with radionuclides like Iodine-131, the 1-(4-Fluorobenzoyl)-3-methyl thiourea is a promising ligand for theragnostic applications. This study aimed to label 1-(4-fluorobenzoyl-3-methylthiourea) with iodine-131 and observe its interaction with breast cancer receptors. The radiolabeling of ^{131}I-(4-fluorobenzoyl-3-methylthiourea) uses the radioiodination method with Chloramine-T, and an in-silico investigation of breast cancer receptors was conducted. According to the results of molecular docking using AutoDockTools, this radiopharmaceutical molecule has the best activity on the HER2 receptor (PDB ID: 3PP0) compared to the native ligand and control positive, with a binding affinity of -6.13 kcal/mol and a Ki value of 32.05 mM. According to the molecular dynamics data using Desmond, the radiopharmaceutical molecule ^{131}I-(4-Fluorobenzoyl-3-methylthiourea) displays good stability starting from the 50ns range. The indirect radioiodination method has successfully labeled 1-(4-Fluorobenzoyl-3-methylthiourea) with iodine-131.

Keywords: HER2; iodine-131; molecular dynamics; radioiodination; thiourea

1. INTRODUCTION

Iodine-131 (^{131}I) is a gamma (γ) and beta (β) ray emitting radionuclide. ^{131}I produces γ energy of 364 keV and β of 0.67 MeV to emit γ and β rays and has a half-life of 8 days. The ^{131}I is an ideal radionuclide for cancer diagnosis and nuclear medicine therapy which is often used by labeling compounds that have pharmacological activity against cancer cells [1]. Iodine-131 has advantages such as its therapeutic applications, selective delivery, and availability, but it also has disadvantages including radiation emission, low specificity, and low molar activity that need to be carefully considered in the development and use of radiopharmaceuticals for diagnosis and therapy [2–4].

Radiopharmaceuticals are drugs containing radioisotopes that are safe to administer to humans for diagnosis or therapy. The use of radiopharmaceuticals for imaging organ function and disease states is a unique capability in nuclear medicine [5]. Radionuclides or radioisotopes are atoms that have excess nuclear energy, making them unstable. This excessive energy can be utilized in one of three ways: emitted from the nucleus as gamma (γ) radiation; transferred to one of its electrons to release it as a conversion electron; or used to create and emit new particles (alpha (α) particles or beta (β) particles) from the nucleus. Radiopharmaceuticals used for diagnostic purposes are those with gamma emitters, while radioactive atoms emit particles or are used for internal radiotherapy [6].

Tiourea is one compound that is widely used in new drug discovery research. Tiourea compounds have pharmacological activity as an anticancer [7]. Some of the advantages of thiourea derivatives in cancer treatment include their potent anticancer properties, high selectivity, and low toxicity. The mechanism of interaction that occurs with cancer cells involves the

ability of thiourea derivatives to favor the formation of hydrogen bonding NH moieties, increasing their solubility in an aqueous medium and allowing better cellular uptake [8–10]. Thiourea is an organic compound containing carbon, hydrogen, nitrogen, and sulfur atoms. The structure of thiourea is similar to urea, except that the S atom in thiourea is replaced by an O atom in urea [11]. Derivates of thiourea compounds have good cytotoxic effects on breast cancer cells [12]. One of the derivatives of thiourea compounds is 1-(4-fluorobenzoyl-3-methylthiourea), where It had an IC-50 value of 251 μg/mL[13]. This compound is synthesized from N-methyl thiourea and 4-fluorobenzoyl chloride and has been shown to have anticancer activity [14]. The radiolabeling of ^{131}I-(4-fluorobenzoyl-(3-methylthiourea) is a promising approach for the development of a new radiopharmaceutical candidate for cancer diagnosis and therapy. The use of molecular docking and molecular dynamic simulation is essential to study the interaction of this candidate with its target protein, as it provides insights into the binding mode, affinity, and stability of the compound, which are critical factors in the development of effective radiopharmaceuticals.

2. RESEARCH METHODS

Instruments and Materials

The tools used in this research include several software. For molecular docking, AutodockTools 1.5.6 was utilized, MarvinSketch 21.17.0 for ligand preparation, Molegro and Pymol for protein preparation, Discovery Studio 20.1 for visualization, Desmond software (academic license, D.E. Shaw Research, New York) for molecular dynamic simulation, and web-based programs including Protein Data Bank and PubChem for download the protein and ligand, pkCSM for toxicity prediction. Meanwhile, the hardware used in this research was a portable computer with specifications AMD A4-9125 Radeon R3 2.30 GHz, 8 GB Ram 64-bit Operating System of Windows 10 and Intel i7 9700k GTX 1070 Ti 8GB DDR5 Ram 32GB LPX DDR4 2666MHz OS Linux Ubuntu. The instruments used for work in the PRTRRB ORTN lab included microtubes, erlenmeyer, beaker, measuring cup, stirring rod, micropipette (*Eppendorf*), 0.2 and 0.5 mL microcentrifuge tubes, a set of paper chromatography and paper electrophoresis equipment, analytical balance (*Metler Toledo*), oven (*Memmert*), digital shaking dry bath (*Thermo Scientific*), laminar airflow cabinet for radioactivity (*Comecer*), refrigerator -20°C (*Samsung*), dose calibrator (*Biodex*), RadioTLC Scanner (*Bioscan*), H-NMR spectrophotometer (*JEOL*).

The materials used in this study are ligands 1-(4-Fluorobenzoil-3-methylthiourea), ^{131}I-(4-fluorobenzoil-3-methylthiourea), Doxorubicin and breast cancer estrogen receptor (PDB ID 3ERT), HER2 (PDB ID 3PP0) and NUDT5 (PDB ID 5NQR). In addition, N-methylthiourea p.a, 4-fluorobenzoyl chloride p.a, tetrahydrofuran p.a, triethylamine p.a, ethyl acetate p.a, Na^{131}I, chloramine T, sodium metabisulphite, sodium bicarbonate p.a, benzene, ethanol p.a, chloroform p.a, n-hexane p. a, acetone p.a, silica gel GF254, sodium hydrogen phosphate, sodium dihydrogen phosphate, acetonitrile, methanol p.a, demineralized water, universal pH paper, hydrochloric acid, sodium hydroxide, Whatman paper 1, Whatman paper 3 MM.

Preparation and Analysis of Receptors

The three receptors were obtained from the Protein Data Bank (*https://www.rcsb.org/pdb*). The downloaded receptors were analyzed using a web-based server (*https://www.ebi.ac.uk/pdbsum*). The results of the analysis were seen based on the fit with the Ramachandran plot parameters. The receptors were prepared by removing water residues and adding hydrogen atoms and separating the receptors with their native ligands [15].

Ligand Structure Preparation

The ligand was drawn and prepared using MarvinSketch software (https://chemaxon.com/marvin) by protonation at blood pH (7.4) and confirmed. Then the ligands were saved in the '*.mol2*' file format [16].

Docking Validation

Validation of the docking method is done by re-docking each of the native ligands to the receptor (PDB ID 3ERT), HER2 (PDB ID 3PP0), and NUDT5 (PDB ID 5NQR). The parameter for this validation result is based on the Root Mean Square Deviation (RMSD) value with a good value of <2 Å [17].

Molecular Docking

Molecular docking uses AutodockTools 1.5.6 software to see the interaction between ligands and proteins. The prepared ligand must first be converted into PDBQT format in order to do docking. Then adjust the grid box the same as during docking validation. Ligand analysis was performed with the default docking system in this application and using LGA for 100 runs. The parameter used in docking is binding energy [17].

Drug Scan and Toxicity Profile Prediction

Drug scan analysis was performed according to Lipinski's parameters of medicine (Lipinski's Rule of Five). In these parameters, it is considered that a good drug must have a lipophility <5, molecular weight <500 g/mol, hydrogen acceptor <10, hydrogen donor <5, and molar refractory between 40-130 [14]. Pharmacokinetic and toxicity profiling of compounds carried out by the open-sourced pkCSM website (*https://structure.bioc.cam.ac.uk./pkcsm*) [17].

Molecular Dynamic Simulation

Molecular dynamics simulations were performed by the Desmond application to get the stability of the compounds. This simulation was carried out on TIP3P (Transferable Intermolecular Potential with 3 Points) type water and 0.15 M NaCl to mimic a physiological ionic concentration. The TIP3P is favored for its simplicity and efficiency, making it suitable for simulations that require a large number of water molecules and where computational resources may be limited. It provides a good approximation of water's physical properties that are relevant for a wide range of biological and chemical systems[18]. System energy was minimized to obtain the lowest energy configuration and followed by equilibration for 100 ps before a production run of frames at temperature 300 K and standard pressure (1.01325 bar) in 200ns using the orthorhombic box (10 Å x 10 Å x 10 Å) and NPT ensembles [19]. NPT stands for the isothermal-isobaric ensemble, where: N stands for the number of particles in the system, which is kept constant; P stands for the pressure of the system, which is kept constant; and T stands for the temperature of the system, which is also kept constant. Neutralization of the protein-ligand complex was performed by adding Na^+ and Cl^- ions. Noose-Hoover and Martyna-Tobias_klein algorithms were used [20].

Synthesis of 1-(4-fluorobenzoyl-3-methylthiourea)

N-methylthiourea (0.032 mol) was mixed with 20 mL of tetrahydrofuran solvent in a 250 mL flat-bottom flask. Triethylamine was added to the catalyst. Next, 0.016 mol of 4-fluorobenzoyl chloride in 15 mL of tetrahydrofuran was dripped into the mixture in the flat bottom flask using a separatory funnel while stirring using a magnetic stirrer. The mixture was refluxed for 8 hours and analyzed every hour by Thin Layer Chromatography (TLC) using an eluent of methanol: chloroform 9:1. Reflux was stopped when a single spot was obtained on the TLC plate and then evaporated with a rotary evaporator until the solvent disappeared due to evaporation. The results obtained were added to saturated sodium bicarbonate solution while stirring until there was no foam. The synthesis product was washed with 2 x 100 mL of distilled water, and then filtered with a Buchner funnel [7].

Radioiodination ^{131}I-(4-fluorobenzoyl-3-methylthiourea)

Labeling of 1-(4-fluorobenzoyl-3-methylthiourea) with iodine can be done directly or indirectly. In direct labeling, the compound 1-(4-fluorobenzoyl-3-methylthiourea) is labeled via a direct reaction method with $Na^{131}I$ using chloramine T (CAT). In a 0.2 mL micro-tube, 25 µL of 1-(4-fluorobenzoyl-3-methylthiourea) (2 mg/mL in ethanol), and then 10 µL of CAT (1 mg/mL) was added. The micro-tube was placed into the Pb container, and then 5-10 µL of $Na^{131}I$ solution with radioactivity between 100-300 µCi was added. The solution was shaken until homogeneous using a vortex stirrer for 1-15 minutes at room temperature. Sodium metabisulfite solution (10-100 µL, 2.5 mg/mL) was added to stop the reaction [21].

This study uses indirect labeling to label histamine (concentration 2.2 mg/mL) with $Na^{131}I$ (radioactivity 100-300 µCi). Chloramine T (5 mg/mL) is added to the solution, and the reaction is stopped by adding sodium metabisulphite (300 mg/mL). 4-fluorobenzoyl-3-methylthiourea is activated by dissolving it in dioxane (20 µg/µL), and then added to an iodination tube containing ^{131}I-histamine. The conjugation is tested by electrophoresis. Purification of ^{131}I-(4-fluorobenzoyl-3-methylthiourea) iodohistamine is done using toluene extraction, and the radiochemical purity is tested by electrophoresis [21].

Selection of Chromatographic System for Determination of Radiochemical Purity of ^{131}I-(4-fluorobenzoyl-3-methylthiourea)

The purity of ^{131}I-(4-fluorobenzoyl-3-methylthiourea) radiochemical was determined by ascending paper chromatography and electrophoresis method. Paper chromatography used six stationary phase variations namely TLC SG, Whatman 1 and Whatman 3 MM with various mobile phases; chloroform: ethanol (90:10), (70:30) and (1:1); methanol: water (70:30) and (80:20). This method was chosen to separate ^{131}I-(4-fluorobenzoyl-3-methylthiourea) from impurities $^{131}I_2$ and free iodine ($^{131}I^-$). For comparison, the assay was performed by paper electrophoresis. Cellulose acetate paper was used as the stationary phase and Whatman 1 used phosphate buffer solution (0.05 M, pH 7.4) as the electrolyte. Electrophoresis was performed for 60 minutes at 300 V. The paper for paper chromatography and TLC was dried for 5 minutes hanging with a lamp. Then, these papers were enumerated using a RadioTLC Scanner. The data were processed using Excel to calculate the percentage of radiochemical purity of ^{131}I-(4-fluorobenzoyl-3-methylthiourea) in solution. The ^{131}I-(4-fluorobenzoyl-3-methylthiourea) labeled compound meets the radiochemical purity requirement if the percentage is $\geq$ 95% [21].

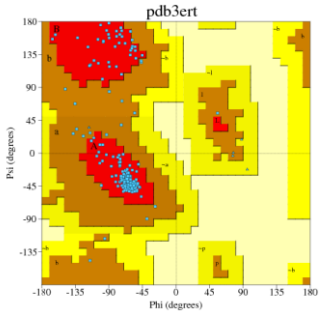
Radiolabelling Optimization of ^{131}I-(4-fluorobenzoyl-3-methylthiourea)

Effect of Conjugation Time

The optimum conjugation time was determined by varying the conjugation period at 2, 22, and 24 hours at 4-8°C. The radiochemical purity of ^{131}I-(4-fluorobenzoyl-3-methylthiourea) was determined by paper chromatography using Whatman 1 and chloroform: ethanol (90:10) as the mobile phase, which was then enumerated by TLC-Scanner [21].

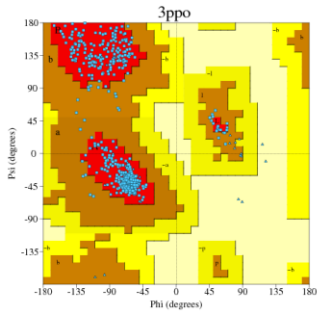
Effect of pH

The initial optimization stage was carried out by varying the pH from 7-10 with a fixed conjugation time of 2 hours. The radiochemical purity of ^{131}I-(4-



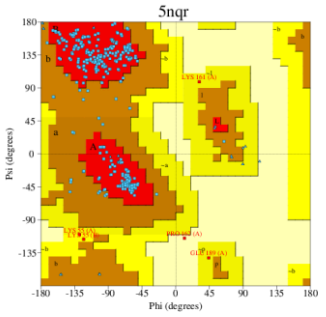
		No. of residues	%-tage
Most favoured regions	[A,B,L]	207	91.2%
Additional allowed regions	[a,b,l,p]	20	8.8%
Generously allowed regions	[~a,~b,~l,~p]	0	0.0%
Disallowed regions	[XX]	0	0.0%
Non-glycine and non-proline residues		227	100.0%
End-residues (excl. Gly and Pro)		1	
Glycine residues		10	
Proline residues		9	
Total number of residues		247	

(a)



		No. of residues	%-tage
Most favoured regions	[A,B,L]	455	93.6%
Additional allowed regions	[a,b,l,p]	31	6.4%
Generously allowed regions	[~a,~b,~l,~p]	0	0.0%
Disallowed regions	[XX]	0	0.0%
Non-glycine and non-proline residues		486	100.0%
End-residues (excl. Gly and Pro)		4	
Glycine residues		38	
Proline residues		16	
Total number of residues		544	

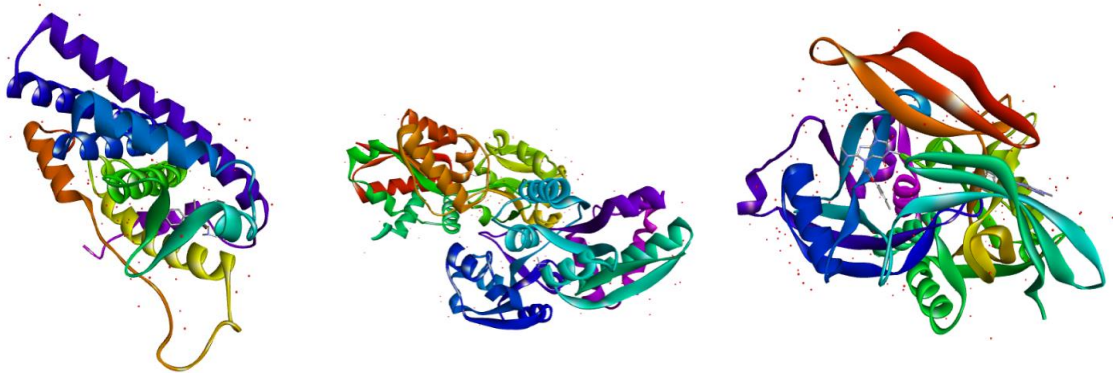
(b)



		No. of residues	%-tage
Most favoured regions	[A,B,L]	306	91.1%
Additional allowed regions	[a,b,l,p]	26	7.7%
Generously allowed regions	[~a,~b,~l,~p]	4	1.2%
Disallowed regions	[XX]	0	0.0%
Non-glycine and non-proline residues		336	100.0%
End-residues (excl. Gly and Pro)		4	
Glycine residues		26	
Proline residues		24	
Total number of residues		390	

(c)

Figure 1. Ramachandran plots (a) 3ERT (b) 3PP0 (c) 5NQR



(a) (b) (c)

Figure 2. Protein Structure (a) 3ERT (b) 3PP0 (c) 5NQR

fluorobenzoyl-3-methylthiourea) was determined by paper chromatography using Whatman 1 and a mobile phase of chloroform: ethanol (90:10), which was then counted by TLC-Scanner [21].

Effect Amount of 1-(4-fluorobenzoyl-3-methylthiourea)

1-(4-fluorobenzoyl-3-methylthiourea) was varied from 10, 15, 25, 50, and 100 µg with the optimum amount of CAT and conjugation time of 2 hours. The radiochemical purity of ^{131}I-(4-fluorobenzoyl-3-methylthiourea) was determined by paper chromatography using Whatman 1 and mobile phase chloroform: ethanol (90:10), which was then quantified by TLC-Scanner [21].

3. RESULTS AND DISCUSSION

Receptor Quality Analysis and Preparation

Receptors analysis was performed by viewing the Ramachandran plot profiles of the proteins available on the website www.ebi.ac.uk/pdbsum by entering the PDB IDs of the breast receptors used, namely estrogen (3ERT), HER2 (3PP0), and NUDT5 (5NQR). Ramachandran plots aim to assess the stereochemical quality of the protein structure by showing the phi-psi dihedral angle distribution for all residues in the structure (except at the chain ends) and visualizing it in three dimensions. Therefore, each amino acid residue is specified as a region in this Ramachandran plot. [12].

In this Ramachandran plot, the most favored regions value parameter is used, which indicates the most favored core regions with an ideal value of >90% and disallowed regions with a recommended value of <15% [14]. In Figure 1, it can be seen that the proteins 3ERT, 3PP0, and 5NQR have the most favored regions of 91.2%, 93.6%, and 91.1% respectively and all three have the same disallowed regions value of 0.0%. These results indicate that the three receptors are of good quality and stable because they fulfill the requirements of the Ramachandran plot. The red, brown, and yellow regions represent the favored, allowed, and "generously allowed" regions.

The three proteins used in this study were pretreated by removing solvents and other residues such as water that can interfere with ligand-protein interactions and ease the work of hardware and adding hydrogen bonds to the protein to make it polar[22]. The structures of the three proteins can be seen in Figure 2.

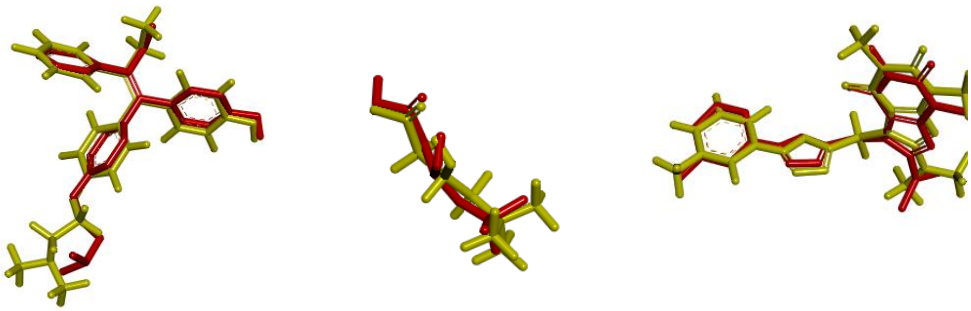
Docking Validation

The validation method is conducted via determination of Root Mean Square Deviation (RMSD) value. The parameter shows the magnitude of the change in the interaction between the protein with the crystallographic natural ligand compared with the redocking result. The docking method can be trusted or declared valid if the RMSD value ≤ 2 Å [22]. In Table 1, it can be seen that the RMSD values of the three proteins are below 2 Å so that the method can be declared valid and can perform testing on the test compound.

In Figure 3, it can be seen the overlay comparison between the natural ligand crystallography with redocking results with a very small difference distance because the RMSD value of the redocking results of the three proteins is below 2 Å

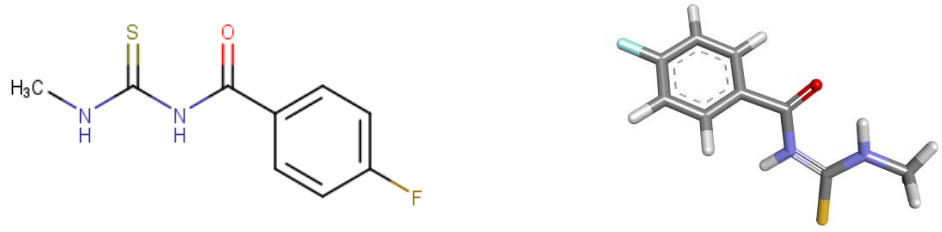
Table 1. Docking method validation results

PDB ID	Grid Box Dimension			Center			Spacing	RMSD (Å)
	X	Y	Z	X	Y	Z		
3ERT	30.134	-1.884	25.180					0.69
3PP0	-1.963	32.388	9.313		40		0.375	0.50
5NQR	84.185	15.358	108.760					0.80

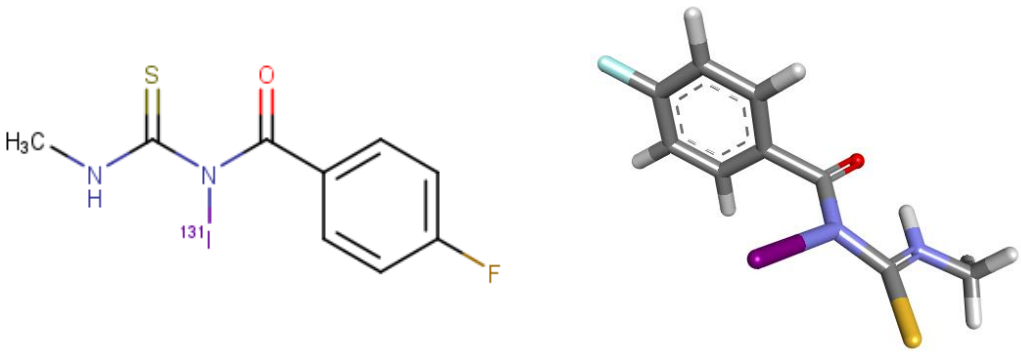


(a) (b) (c)

Figure 3. Overlay of crystallographic natural ligands (yellow) and redocking results (red) on proteins (a) 3ERT (b) 3PP0 (c) 5NQR.



(a)



(b)

Figure 4. The 2D and 3D structures of (a) 1-(4-fluorobenzoyl-3-methylthiourea) and (b) ^{131}I-(4-fluorobenzoyl-3-methylthiourea)

Table 2. Binding Affinity and Inhibition Constant Values of Docking Results Against Receptors 3ERT, 3PP0, and 5NQR

Compounds	ΔG (kcal/mol)			Ki (uM)		
	3ERT	3PP0	5NQR	3ERT	3PP0	5NQR
Native Ligand	-11.17	-4.11	-6.68	0.006	977	12.59
4F	-4.96	-6.33	-4.60	231.82	23.10	428.13
^{131}I-4F	-6.31	-6.13	-6.01	23.54	32.05	39.20
Doxorubicin	-8.16	-5.02	-9.42	1.05	209.25	0.00125

Note: ^{131}I-4F: ^{131}I-(4-Fluorobenzoyl-3-methylthiourea); 4F: 1-(4-fluorobenzoyl-3-methylthiourea)

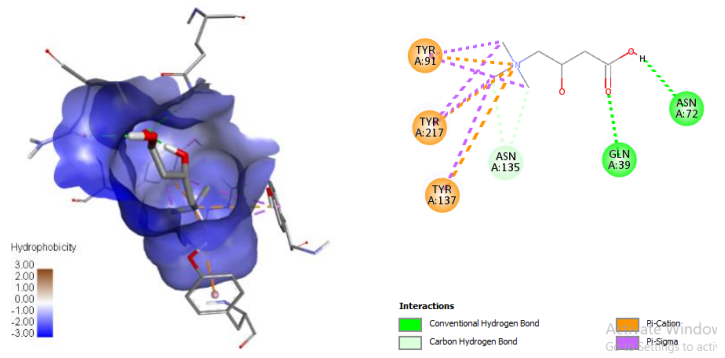
Ligand Structure Preparation

The ligand was drawn and pretreated using MarvinSketch software with protonation done by equalizing the pH to the blood pH (7.4). The best conformation of the protonated ligand was used. The stability of the ligand and protein interaction will be greatly influenced by the conformation of the ligand itself [23]. Visualization of ligand and radioligand can be seen in Figure 4.

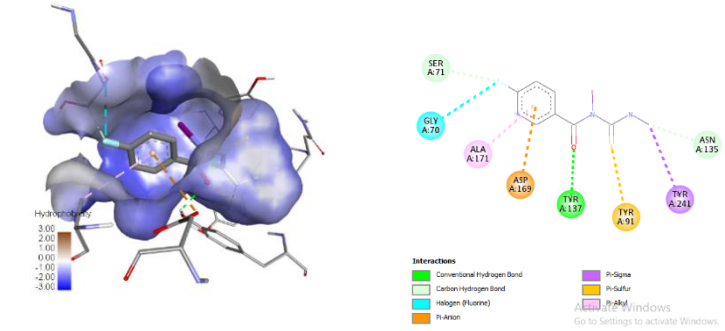
Molecular Docking

Molecular docking simulation was performed by docking the test ligand, ^{131}I-(4-fluorobenzoyl-3-methylthiourea) complex, to the three receptors, 3ERT, 3PP0, and 5NQR, with the original ligand, 1-(4-fluorobenzoyl-3-methylthiourea) and breast cancer drug, doxorubicin. The grid box used in this docking test simulation is the grid box used during the method validation of each receptor. The value of Gibbs binding affinity (ΔG) and inhibition constant (Ki) contained in the file with '.dlg' format indicate show the stability of the bond between ligand and receptor. This simulation uses the Lamarckian genetic algorithm (LGA) with 100 times run conformation. The advantages of using the Lamarckian Genetic Algorithm in AutoDock compared to other methods, such as pure genetic algorithms or simulated annealing, include efficiency, balance between exploration and exploitation, avoidance of local minima, better for complex landscapes, and adaptability.

Control PositiveTo visualize the interaction between ligand and amino acid residue, it can be seen using Discovery Studio software from the .pdb file of the



(a)



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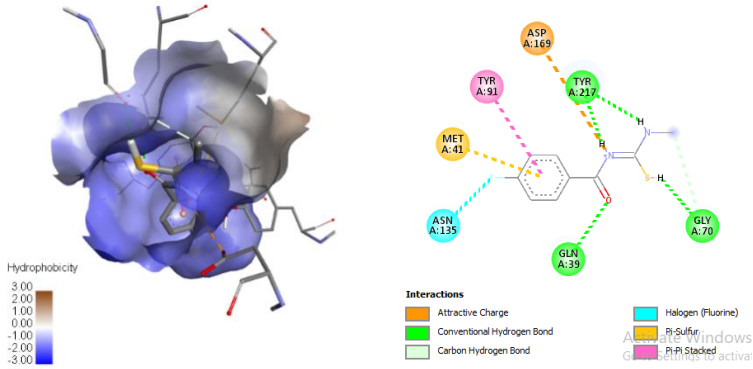
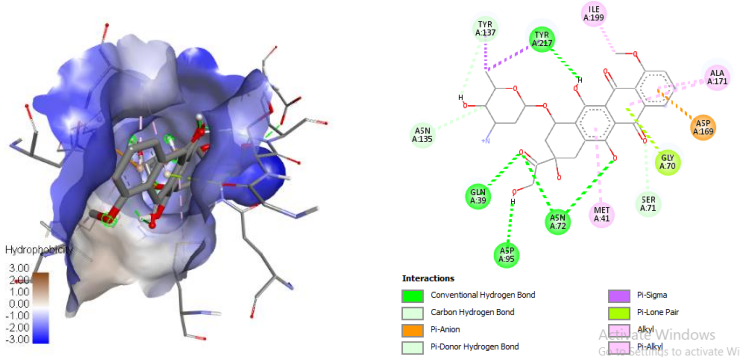


(c)



(d)

Figure 5. The 3D and 2D Visualisation on 3PP0 Receptor of (a) Natural Ligand (b) ^{131}I-(4-fluorobenzoyl-3-methylthiourea) (c) 1-(4-fluorobenzoyl-3-methylthiourea) (d) doxorubicin

Table 3. Ligand-Residue Amino Acid Bonds on the 3PP0 Receptor

Compounds	Hydrogen Bonds		Hydrophobic Bond	
	Total	Bond Type	Total	Bond Type
Native Ligand	2	ASN A:72, GLN A:39	4	ASN A:135; TYR A:137, TYR A:217, TYR A:91
^{131}I-4F	1	TRY A:137	7	GLY A:70, SER A:71, ALA A:171, ASP A:169; **ASN A:135**; **TYR A:91**, TYR A:241
4F	3	TYR A:217, GLY A:70, GLN A:39	11	ILE A:199, **TYR A:137**, SER A:71, ASN A:72, THR A:94, TYR A:241, THR A:136; **ASN A:135**; ASP A:169; MET A:41; **TYR A:91**
doxorubicin	4	TYR A:217, GLN A:39, ASP A:95, ASN A:72	15	GLN A:198, ASN A:170, ARG A:175, THR A:89, ARG A:90, TRP A:127, GLN A:75, **TYR A:91**, THR A:94, TYR A:241; SER A:71; **ASN A:135**, **TYR A:137**; ASP A:169; GLY A:70; ILE A:199, ALA A:171, MET A:41

best docking result that has been simulated before. Interactions observed include hydrogen bonds and hydrophobic bonds. 3D and 2D visualization of the best receptor can be seen in Figure 5 and the amino acid residues can be seen in Table 3.

In receptor 3PP0, the test ligand forms one hydrogen bond (TRY A:137) and 7 hydrophobic bonds, some matching the natural ligand (ASN A:135 and TYR A:91). The comparator ligand has 3 hydrogen bonds (TYR A:217, GLY A:70, GLN A:39) and 11 hydrophobic bonds, with 3 matching the natural ligand (TYR A:137, ASN A:135, TYR A:91). Doxorubicin, used for comparison, forms 4 hydrogen bonds (TYR A:217, GLN A:39, ASP A:95, ASN A:72) and 18 hydrophobic bonds, with 3 matching the natural ligand (TYR A:91, ASN A:135, TYR A:137). Hydrogen and hydrophobic bonds are crucial for the biological activity of drugs, affecting their properties and stability [24].

Drug Scan, Pharmacokinetic and Toxicity Profile Prediction

Drug scans are conducted based on similarity to existing drugs using Lipinski's rules of five parameters. These scan aim to characterize the physicochemical properties including water solubility, intestinal permeability, and oral bioavailability. Table 4 shows that the radiopharmaceutical compound passed the test of all Lipinski's rules of five parameters compared to its control positive(doxorubicin), which only matched the LogP parameter.

Overall, based on Table 4, the 4F and ^{131}I-4F appear to fit within the typical profile of drug-like molecules more closely than Doxorubicin, at least regarding the criteria presented here. Doxorubicin, however, is an established chemotherapy medication, indicating that despite its divergence from the typical "drug-like" range in molar refractivity and LogP, it is still a therapeutically effective compound. This illustrates that while guidelines like the "Rule of Five" are helpful, there are successful drugs that do not meet all the criteria, emphasizing the importance of considering the full context of each compound's pharmacodynamics and pharmacokinetics. Molecular weight in physico-chemical properties can affect the absorption and distribution of the compound, the compound will be easier to diffuse through the cell membrane and distributed throughout the cell to bind to the receptor if the molecular weight of the compound is lower. The LogP value affects the ability of the compound to dissolve in solvents such as oils, fats, and also other non-polar solvents. Meanwhile, hydrogen donor and acceptor values affect the biological activity of the compound. Molar refractivity relates to the total polarisability of the compound [23].

In drug development, predicting pharmacokinetic and toxicity profiles is key to assessing a drug candidate's effectiveness and side effects. Key parameters include CaCO2, intestinal absorption, VDss, BBB permeability, CYP3A4 and OCT2 interactions, AMES toxicity, hepatotoxicity, and LD50. Radioligand shows promising results: a high CaCO2 value (0.975), strong intestinal absorption (over 80%), a low volume of distribution (below -0.15), and adequate BBB penetration, as seen in Table 5. It's neither a substrate nor an inhibitor of CYP3A4 and OCT2. Toxicity-wise, it's non-mutagenic, non-carcinogenic, and not hepatotoxic [25].

Table 4. Drug Scan Analysis

Compounds	Parameters				
	Mass	**Hydrogen Bond Donors**	**Hydrogen Bond Acceptors**	**Logp**	**Molar Refractivity**
	<500 g/mol	**<5**	**<10**	**<5**	**40-130**
4F	212.24	2	2	1.74	56.48
^{131}I-4F	342.14	1	2	2.63	69.02
Doxorubicin	544.53	6	11	-0.77	145.89

Table 5. Predicted Pharmacokinetic and Toxicity Profile

Parameters			Compounds		
			^{131}I-4F	**4F**	**Doxo**
CaCO2		Log cm/s	1.011	0.975	0.853
Intestinal Absorption		%	92.663	81.369	47.218
VDss		Log L/Kg	-0.232	-0.301	0.704
BBB		LogBB	0.138	-0.117	-1.626
CYP3A4	substrate	Yes/No	No	No	No
	inhibitor				
Renal OCT2	substrate				
AMES toxicity					Yes
Hepatotoxicity					No

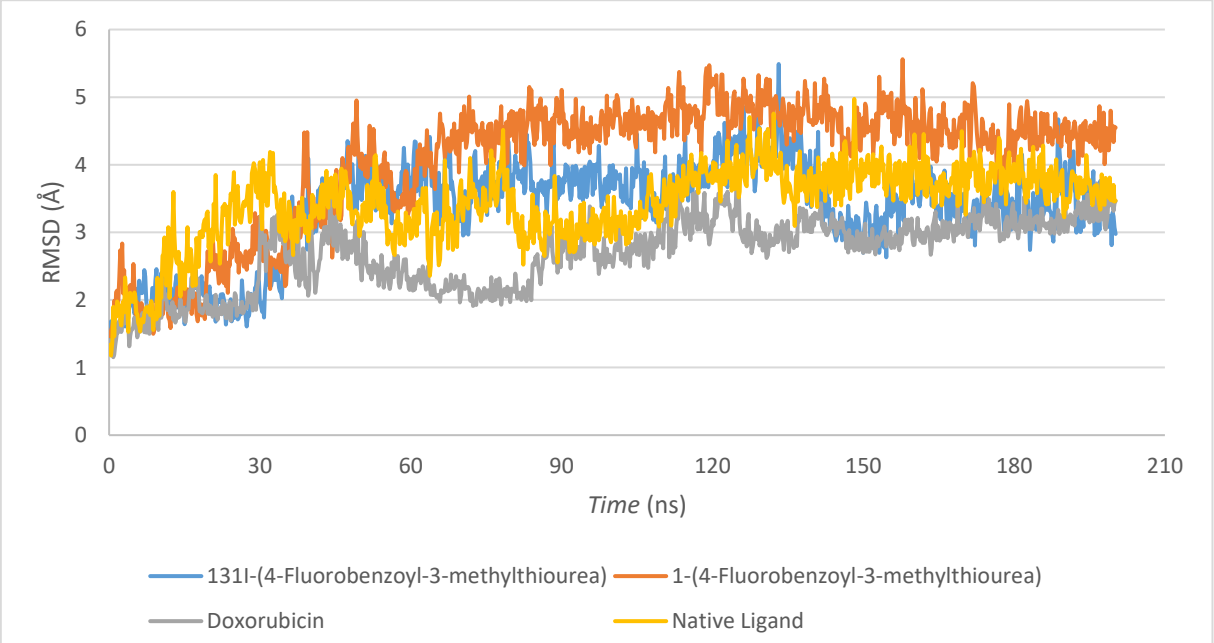


Figure 6 RMSD chart of protein complex 3PP0

Table 6. Mean, Minimum and Maximum RMSD Values of Complex 3PP0

Compounds	Average	Min	Max
Native Ligand	3.416	1.177	4.975
^{131}I-4F	3.355	1.271	5.490
4F	4.051	1.460	5.561
Doxorubicin	2.684	1.153	3.735

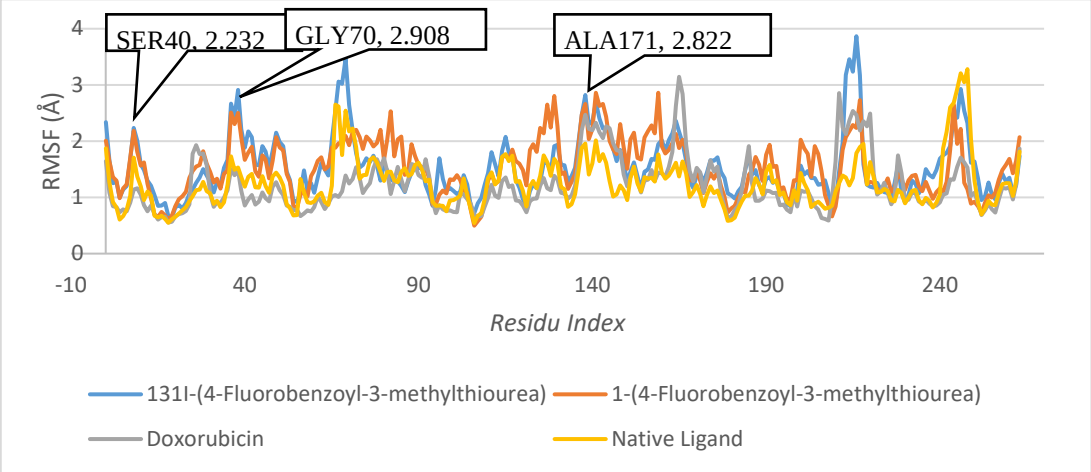
Molecular Dynamic Simulation

Molecular dynamics simulation in this study uses Desmond software to see the stability of the tested radioligand by looking at the RMSD (Root Mean Square Deviation) and RMSF (Root Mean Square Fluctuation) graphs. Molecular dynamics itself is an application to see information from the interactions that occur between ligands and proteins in a flexible state involving atomic and molecular interactions in a certain time span so this stage can be called an advanced stage of molecular docking. In this stage, we can also see the stability of the protein enzyme, protein structure, protein folding, conformational changes, and ion transport [26].

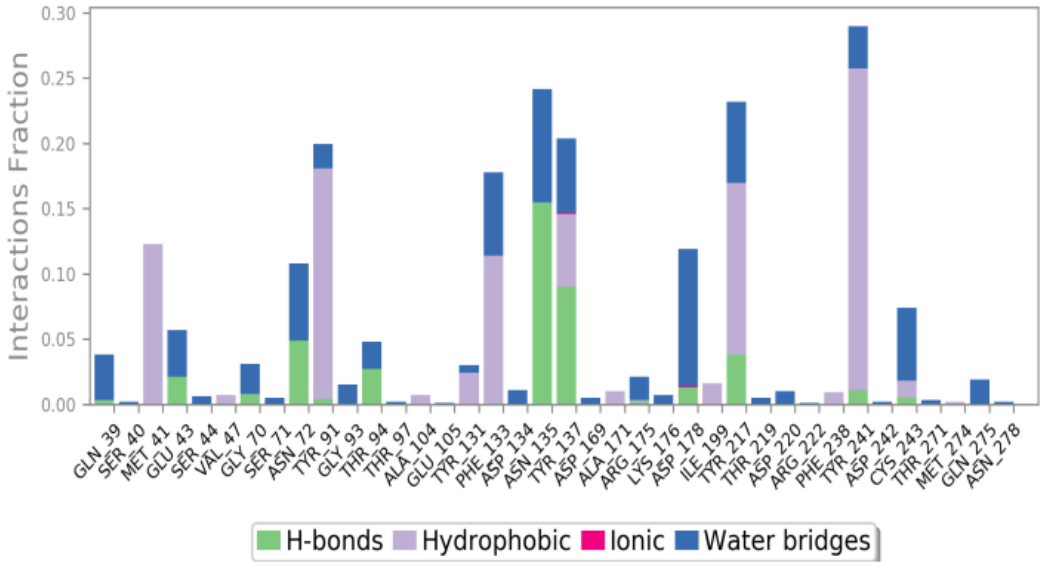
Compounds that are continued at the molecular dynamics stage are compounds that are tested by molecular docking with proteins or receptors with better binding affinity values than their natural ligands, namely radiopharmaceutical compounds on 3PP0 receptors. Graphs of RMSD and RMSF values can be seen in Figures 6 and 7.

Table 7. Average, Minimum and Maximum RMSF Values of Complex 3PP0

Compounds	Average	Min	Max
Native Ligand	1.247	0.541	3.278
^{131}I-4F	1.531	0.631	3.862
4F	1.543	0.497	2.859
Doxorubicin	1.216	0.555	3.144



(a)



(b)

Figure 7. (a) RMSF Graph of Protein Complex 3PP0 (b) Interaction of Amino Acid Residues with Radioligand

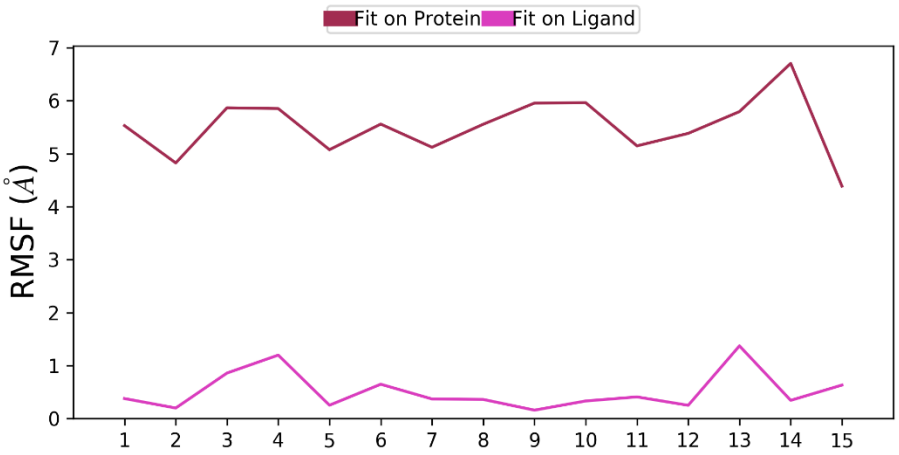


Figure 8. RMSF of Radioligand ^{131}I-(4-Fluorobenzoyl-3-methylthiourea)

From the RMSD graph above, it can be seen the stability of each ligand analyzed against protein 3PP0. For radioligand as a test ligand, it can be seen that it starts to stabilize from 50ns onwards. There is a slight fluctuation in a certain time range but can return to its stable area. The RMSD values can be seen in Table 6.

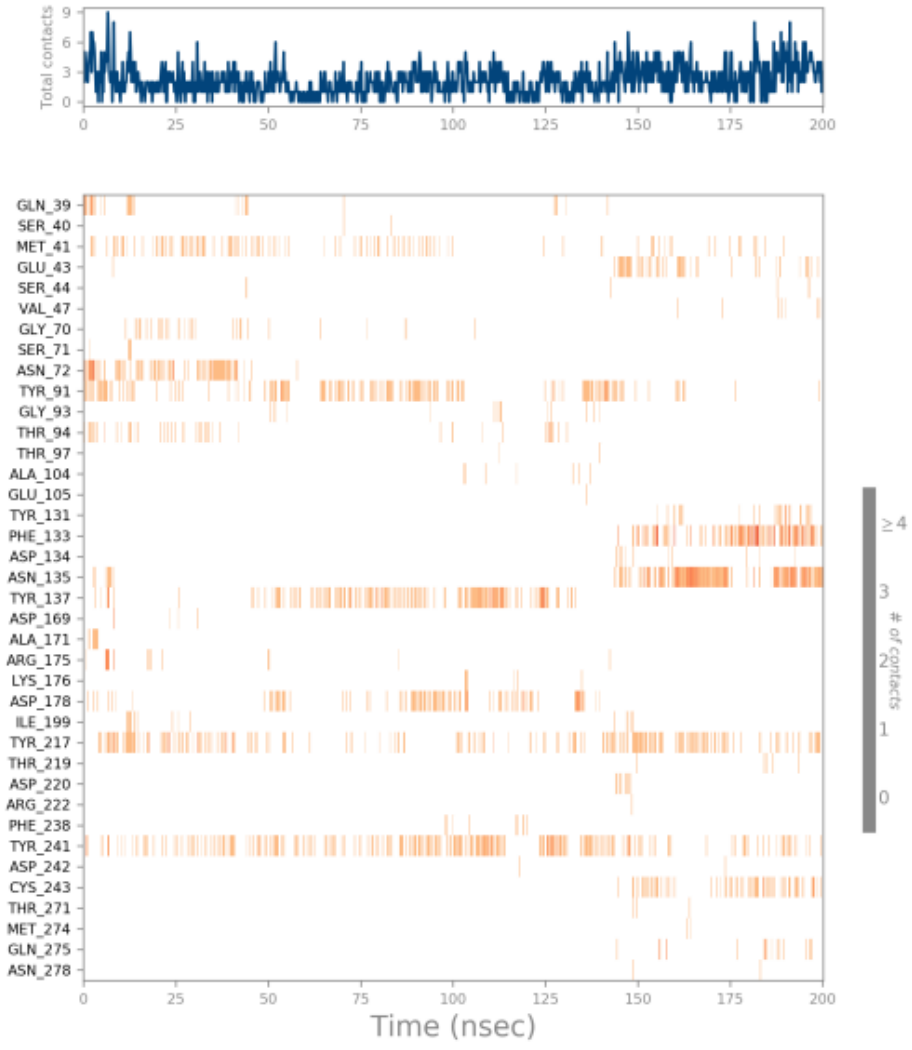
The RMSF values can be seen in Table 7. In addition to seeing the stability, RMSF can also see the interaction of the radioligand with the residue. The interaction of the radioligand with amino acid residues can be seen in Figure 7, including interaction with SER_40 (RMSD 2.232), GLY_70 (RMSD 2.908), and ALA_171 (RMSD 2.822

Protein RMSF can see the flexibility of the ligand and local residues that can serve for evaluation and see the atomic fluctuations of the ligand. Figure 8 shows the RMSF of the tested radioligand ^{131}I-(4-Fluorobenzoyl-3-methylthiourea). Meanwhile, Figure 9 shows the amino acid residues interacting with the radioligand with a darker orange color indicating more than one specific contact between the residue and the radioligand during 200ns. Protein structure fluctuations in molecular dynamics are influenced by factors such as protein architecture and fold, temperature and environmental conditions, atomic fluctuations and protein flexibility, solvent interactions, and residue-level fluctuations [27,28]. In general, protein structure fluctuations in molecular dynamics are influenced by both internal and external factors. Internally, the amino acid composition, protein fold and secondary structures, intramolecular interactions, and conformational states dictate flexibility and stability. Externally, environmental conditions like temperature, pH, ionic strength, solvent properties, and pressure play significant roles. Additionally, ligand binding, post-translational modifications, and external mechanical forces or biomolecular interactions can considerably affect protein dynamics. These factors collectively determine how a protein behaves and responds to its environment.

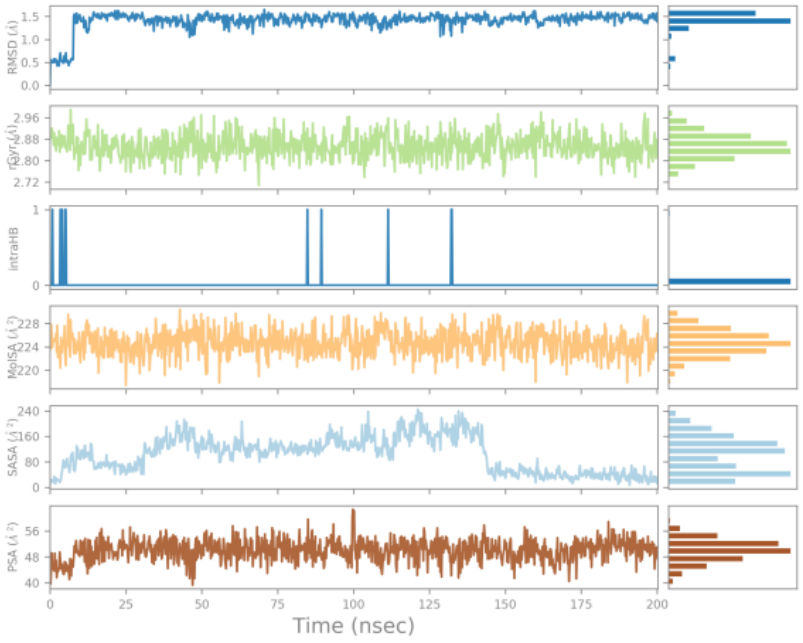
The parameters used in addition to RMSD and RMSF in molecular dynamics also include the radius of gyration (rGyr), NS34, Molecular Surface Area (MolSA), Solvent Accessible Surface Area (SASA), Polar Surface Area (PSA) which can be seen in Figure 9 It can be seen that the RMSD and rGyr of the tested radioligands are stable with no excessive fluctuations during 200ns, there is only an increase at the beginning for RMSD. In the IntraHB plot, there is an interaction characterized by fluctuating graphs at the beginning and middle of the time. For the MolSA plot, it is stable throughout the time, while in the SASA plot, there are fluctuations at 30ns and 140ns. Meanwhile, the PSA plot is stable with slight fluctuations including at times 45ns and 100ns.

Synthesis of 4-fluorobenzoyl-3-methylthiourea

This method synthesizes amides, reacting amine nucleophiles with benzoyl chloride in a basic atmosphere. Specifically, 4-Fluorobenzoyl chloride is used, conditioned with triethylamine to achieve pH 8, and heated at 100°C for about 7 hours. The reaction employs 0.016 mol of 4-Fluorobenzoyl chloride and 0.032 mol of N-Methylthiourea. HCl, a by-product, reacts with excess N-Methylthiourea to produce a soluble thiourea salt. Post-reaction, the product is recrystallized to purify and remove impurities. The resulting crystal is yellowish-white, odorless, and soluble in DMSO, with a weight of 2.2736 grams and a recovery rate of 78.81% from the theoretical yield of 2.885 grams.



(a)



(b)

Figure 9. (a) Representation of Time in Contact of 3PP0 Amino Acid Residue with Radioligand (b) Properties of Radioligands

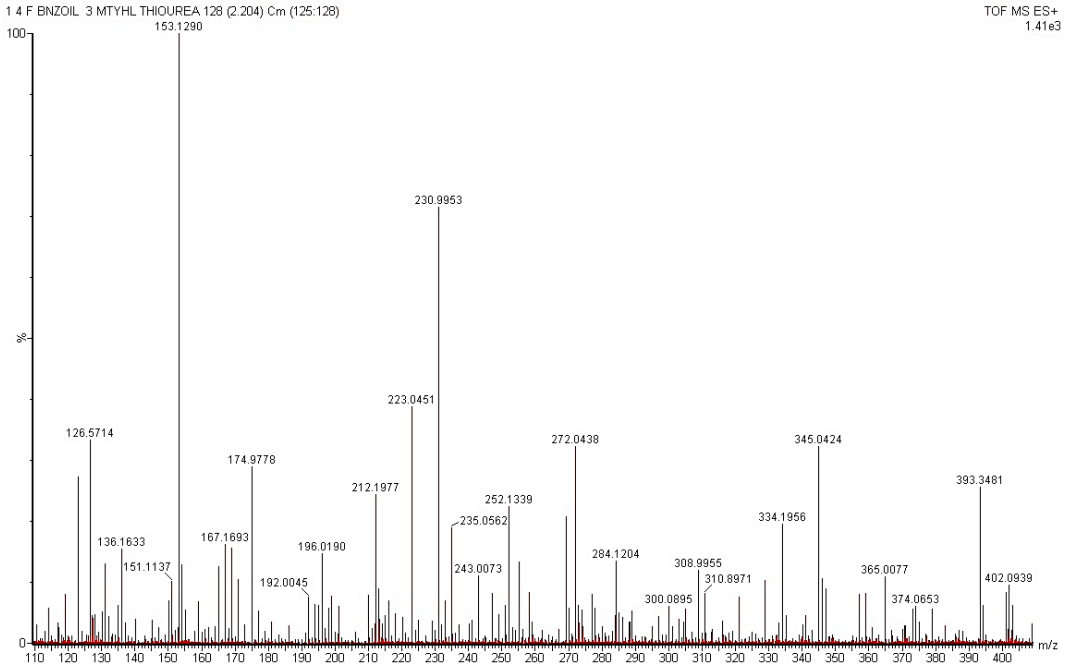


Figure 10. Data from Mass Spectrophotometry Analysis of 1-(4-Fluorobenzoyl-3-methylthiourea) Ligand

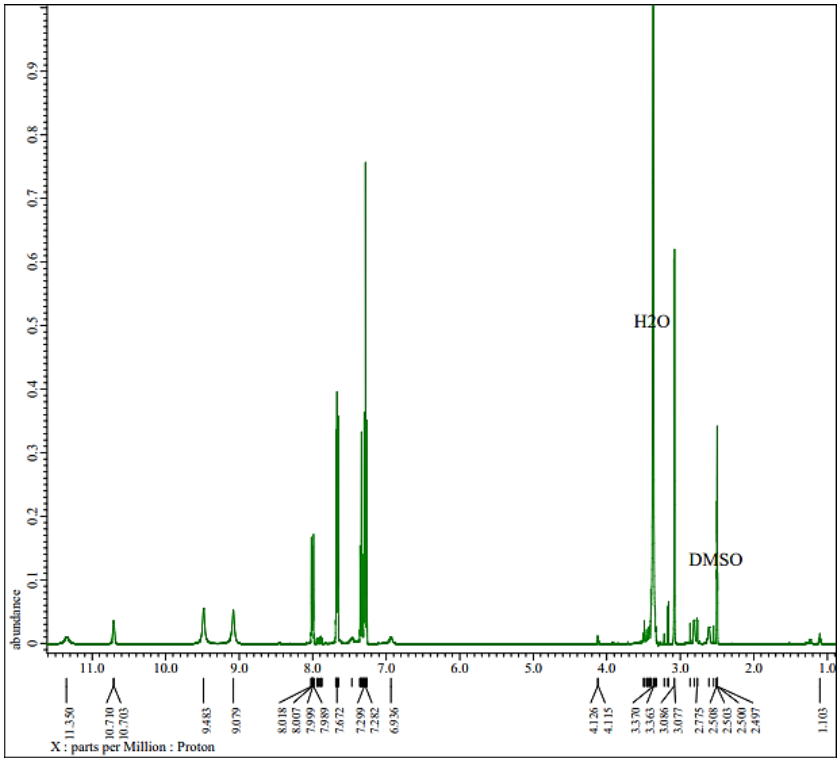


Figure 11. The H-NMR spectrum of 1-(4-Fluorobenzoyl-3-methylthiourea)

Based on Figure 10, it is known that the results of characterization using mass spectrometry of the 1-(4-Fluorobenzoyl-3-methylthiourea) compound obtained a molecular weight of 212.1977 g/mol. The results of the analysis are not much different from the prediction results of the compound using MarvinSketch software of 212.24 g/mol. Meanwhile, from the analysis data using 1H-NMR (CHCl3-DD2, 500 MHz) in Figure 11, there are 9 hydrogen atoms seen in the spectrum as follows: 3.086-3.077 (3H, d, J = 4.5 Hz, -CH3); 7.682-7.653 (2H, d, J = 14.5 Hz, H-ar); 8.018-7.989 (2H, d, J = 14.5 Hz, H-ar); 9.079 (1H, s, NH); 9.483 (1H, s, NH).

Radioiodination ^{131}I-(4-fluorobenzoyl-3-methylthiourea)

Radioiodination is a method for labeling substances using γ-emitting iodine radioisotopes, producing high-specific compounds with low concentrations. It involves oxidizing Sodium Iodide to I^+, which forms hydrated iodonium ions or hypoiodic

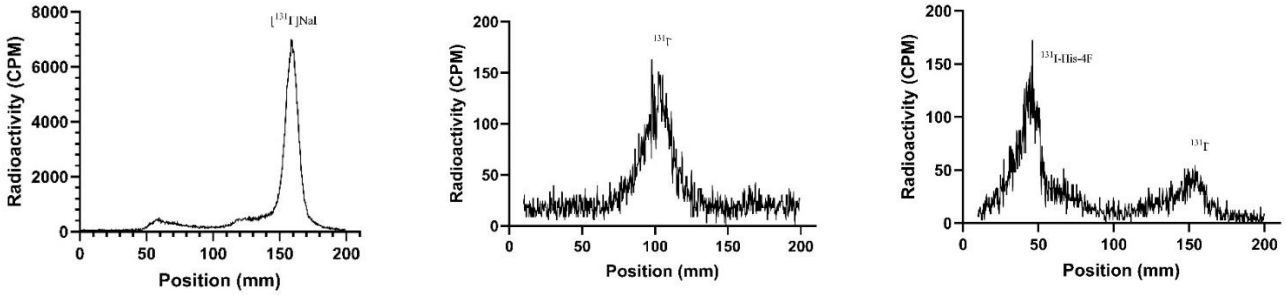
acids through electrophilic substitution processes [29,30]. The compound 1-(4-Fluorobenzoyl-3-methylthiourea) was not labeled directly by iodine-131 due to the same chromatogram profile. Instead, it was labeled indirectly using conjugation with histamine. This indirect labeling was achieved through radioiodination, where histamine was labeled with Iodine-131 to ^{131}I-histamine. The compounds were tested for radiochemical purity.

This is different from the in-silico results where the 1-(4-Fluorobenzoyl-3-methylthiourea) compound can be characterized directly by iodine-131. This is because in the in-silico study, the conditions used are optimal conditions where iodine-131 can bind to the 1-(4-Fluorobenzoyl-3-methylthiourea) compound. In practice, the optimal conditions that have been adjusted in silico cannot be achieved due to the limitations of non-optimal conditions as in the in-silico setting. Ligand conditions that still have impurities can also affect the direct labeling results.

Selection of Chromatographic System for Determination of Radiochemical Purity of ^{131}I-(4-fluorobenzoyl-3-methylthiourea)

The initial step in radiolabeling 131I-(4-Fluorobenzoyl-3-methylthiourea) involves selecting suitable mobile and stationary phases to separate the compound from its impurities, mainly free Iodine-131 and reduced Iodine ($^{131}I_2$). The effectiveness of this separation is assessed using paper chromatography. Trials with various eluents revealed that a chloroform-ethanol mixture can distinguish $^{131}I_2$ from the labeled ^{131}I-(4-Fluorobenzoyl-3-methylthiourea), but it's ineffective in separating free Iodine-131. These findings are summarized in Table 8.

The electrophoresis method was used to determine the radiochemical purity of ^{131}I-(4-Fluorobenzoyl-3-methylthiourea) based on its chemical properties and the compounds to be separated. The results showed that free Iodine-131 with a negative charge was at the anode (right peak), while ^{131}I-(4-Fluorobenzoyl-3-methylthiourea) was at the cathode (left peak), and $^{131}I_2$ as a neutral charge was at the center peak, as shown in Figure 13 and 14.



(a) (b) (c)

Figure 12. (a) Radiochromatogram profiles of bulk Iodine-131 (b), direct radiolabelling (c), and indirect radiolabelling

Table 8. Chromatography System Optimisation

Chromatography System		*Retention Factor* (RF)	
Stationary Phase	**Mobile Phase**	**^{131}I**	**^{131}I-4F**
Direct Radioiodination			
Whatman 1	Methanol: Water (80:20)	0.8-0.9	0.8-0.9
Indirect Radioiodination			
Whatman 3	Methanol: Water (70:30)	0.8-0.9	0.8-0.9
TLC SG GF^{254}	Chloroform: Ethanol (90:10)	0.8-0.9	0.0-0.2
	Chloroform: Ethanol (70:30)	0.8-0.9	0.8-0.9
	Chloroform: Ethanol (50:50)	0.8-0.9	0.8-0.9

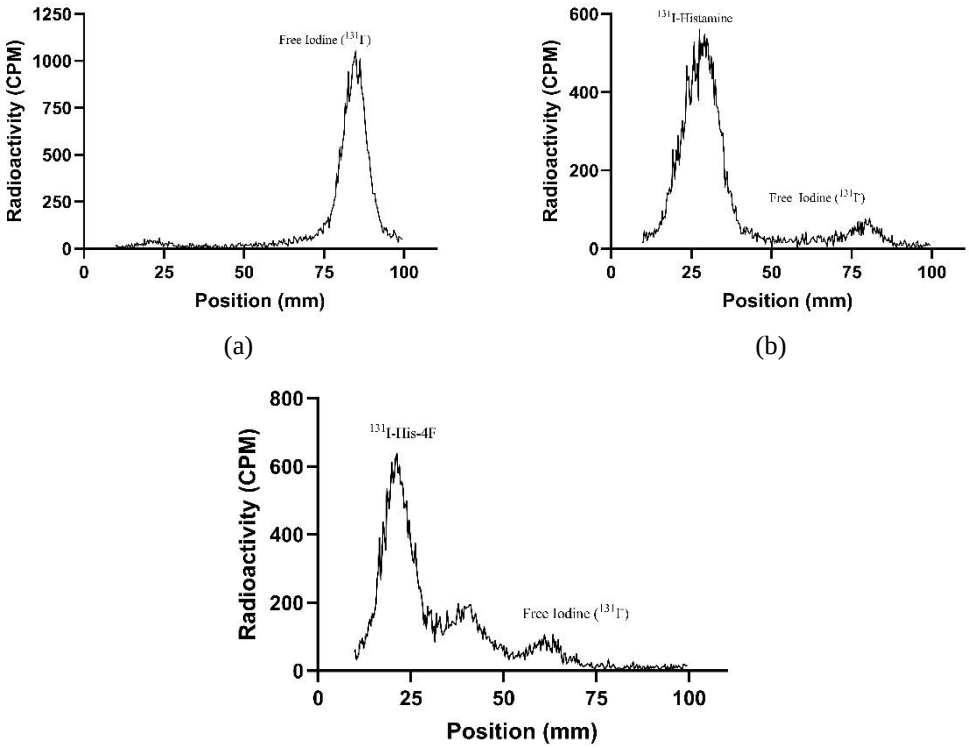


Figure 13. Radiochromatogram profiles of Iodine-131 (a), ^{131}I-Histamine (b) and ^{131}I-His-(4-Fluorobenzoyl-3-methylthiourea) (c) TLC SG as stationary phase and Chloroform: Ethanol (90:10) as mobile phase

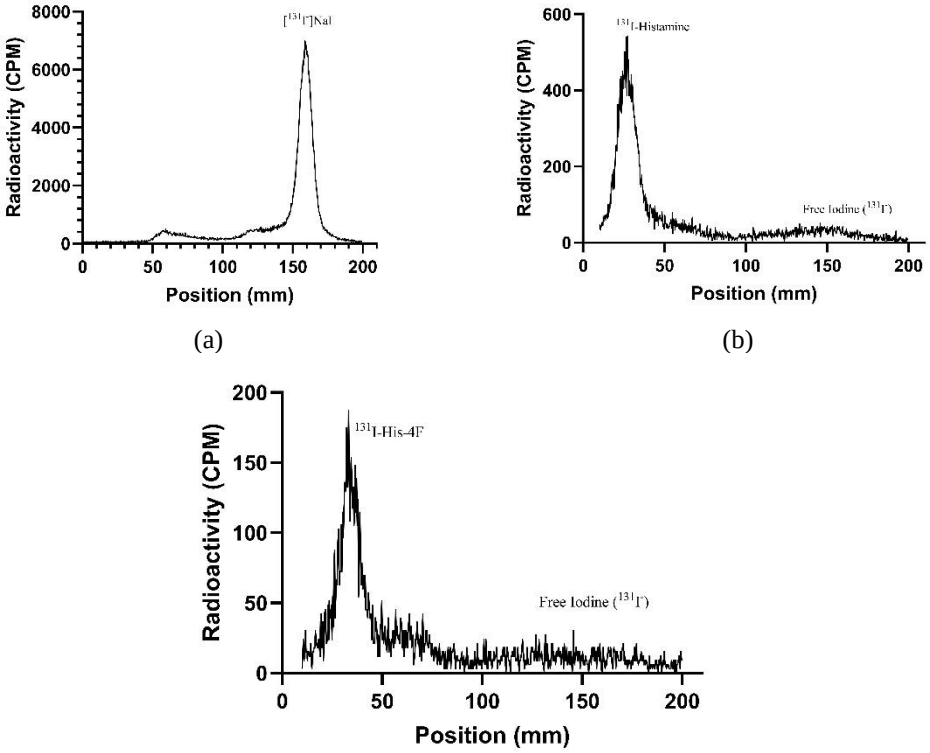


Figure 14. Radiochromatogram profiles of Iodine-131 (a), ^{131}I-Histamine (b) and ^{131}I-His-(4-Fluorobenzoyl-3-methylthiourea) (c) Electrophoresis using Whatmann 1 as the stationary phase and Phosphate Buffer (0.5 M, pH 7.4) as the electrolyte

Radiolabelling Optimisation of ^{131}I-(4-fluorobenzoyl-3-methylthiourea)

Effect of Conjugation Time

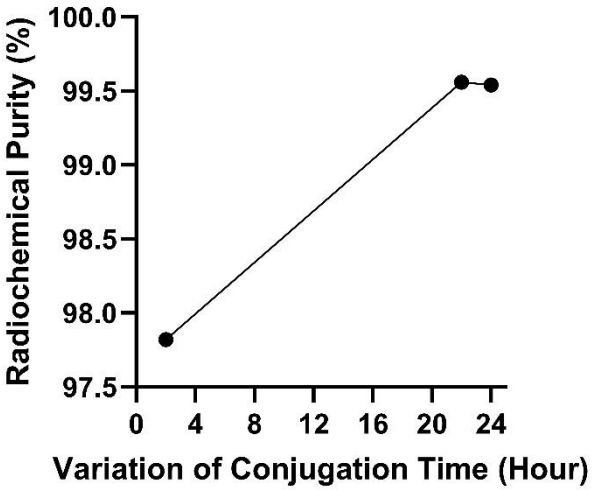


Figure 15. Variation of Radiochemical Purity versus Conjugation Time

Conjugation time is an important parameter in labeling optimization to see how long it takes for ^{131}I-Histamine and 1-(4-Fluorobenzoyl-3-methylthiourea) to conjugate optimally. The variation of conjugation time was carried out at 2, 22, and 24 hours and the radiochemical purity obtained was 97.82%, 99.56%, and 99.54%, respectively. According to Figure 15, the most optimal conjugation time was at the 22nd hour with the highest radiochemical purity.

Effect of pH

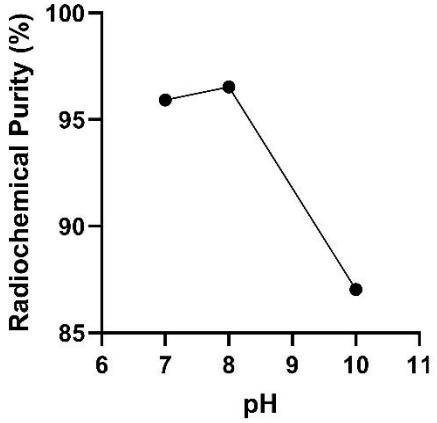


Figure 16. Variation of Radiochemical Purity against pH Condition

To ensure stability and integrity, radiopharmaceuticals must be formulated and maintained at an appropriate pH. Ideally, the pH of parenteral radiopharmaceuticals should be the same as that of blood, i.e. pH = 7.4; however, due to the high buffering capacity of the blood, the pH can vary between 2 and 9 [31].

The pH variation was carried out to see the radiochemical purity obtained from various pH. As can be seen from Figure 16, pH variations of 7, 8, and 10 were used with the respective radiochemical purity of 95.92%, 96.53%, and 87.03%. The optimal pH range used in the labeling of 1-(4-Fluorobenzoyl-3-methylthiourea) compounds by iodine-131 is pH 7-8 with the most optimal pH being at pH 8. The pH of the labeled compound should ideally be around the pH of blood (7.4) intended for intravenous use [32].

Effect Amount of 1-(4-fluorobenzoyl-3-methylthiourea)

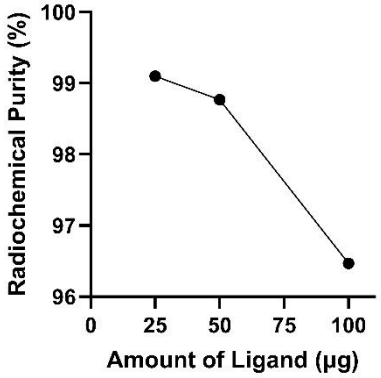


Figure 17. Variation of Radiochemical Purity against the Number of Ligands.

To determine the optimal yield, the amount of ligand was varied to 25, 50, and 100 µg. The resulting radiochemical purity was 99.10%, 98.77%, and 96.47%, respectively. Based on the data in Figure 17, the optimum amount of 1-(4-Fluorobenzoyl-3-methylthiourea) for labeling is 25 µg. The fewer ligands used, the higher the purity of the labeling results achieved. This can happen because the amount of ligand used in the reaction will affect the mole ratio between the ligand and the radioisotope. In addition, the use of fewer ligands can also help reduce the possibility of unwanted radioactive contamination in the labeled product.

4. CONCLUSIONS

The ^{131}I-(4-Fluorobenzoyl-3-methylthiourea) based on the results of drug scan parameter Lipinski's Rule of Five has good drug similarity and also good pharmacokinetic and toxicity profiles. From the molecular docking results, this compound has the best activity on the HER2 receptor (PDB ID 3PP0) with a binding affinity (ΔG) -6.13 and Ki value (uM) 32.05. In the molecular dynamics simulation results, the ^{131}I-(4-Fluorobenzoyl-3-methylthiourea) compound has good stability starting from the 45ns range. This 1-(4-Fluorobenzoyl-3-methylthiourea) compound has been successfully synthesized and labelled with iodine-131 using indirect radioiodination method

ACKNOWLEDGMENTS

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