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Molecular Dynamic Simulation Study of Newly Cytotoxic 2-(Aminomethyl)Benzimidazole Derivatives as Tyrosine Kinase Inhibitors

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Abstract: Receptor tyrosine kinases (RTK) are considered one of the main targets in cancer therapy due to their high expression. Unfortunately, multiple molecular mechanisms of resistance have been identified, leading to drug resistance and toxicity, which increases the need to discover new structural tyrosine kinase inhibitors. Instead of the rigid molecular docking method, molecular dynamic simulations can treat both the ligand and the protein flexibility. This lets the receptor-binding site fit around the new ligand. Also, the effect of explicit water molecules can be studied directly, and very accurate binding free energies can be obtained. The cytotoxic study does not explain the mechanism by which the tested compound could act, so further costly biological studies are needed. So, molecular dynamics is used as a computational technique that simulates the dynamic behavior of molecular systems as a function of time. Using Maestro v 13.0.135 interface (Schrodinger, New York, NY, 2021), molecular dynamic simulation was done with two proteins (EGFR and HER2) that are co-crystallized with the same ligand of a dual EGFR/HER2 inhibitor (TAK-285), and then they were tested with compounds of highly cytotoxic activity (2g and 4g) of suspected dual TKI activity from our previous study. According to the resulted data that were shown in the simulation interactions diagram reports, we recognize that the 2g compound showed a good interaction complex with both 3POZ (EGFR) and 3RCD (HRE2) proteins, in contrast, only the 4g-3RCD complex showed a good interaction report. So, 2g could be considered a dual EGFR/HER2 inhibitor and 4g as a HER2 inhibitor, for further investigation, both compounds could be tested further with other biological studies, especially enzyme inhibitory assays with suspected promising results.

Keywords: tyrosine kinase inhibitors, TAK-285, 2-(aminomethyl)benzimidazole derivatives, molecular dynamic simulation.

1. Introduction

Receptor Tyrosine Kinases (RTKs) are expressed at a high level in cancer cells, making their targeting a justifiable strategy. [1] EGFR is frequently mutated and/or overexpressed in different types of human cancers, (mostly lung and breast cancer and glioblastoma), and it is the target of multiple cancer therapies currently adopted in clinical practice. [2] Overexpression of the human epidermal growth factor receptor (EGFR/HER-1/ERBb1) and epidermal growth factor-2 (HER-2/HER2/neu/ERBb2) has been associated with a more aggressive course of disease in human breast carcinomas. Therefore, these two receptor tyrosine kinases represent one of the most interesting targets for breast cancer-specific therapy. [3] Tyrosine kinase inhibitors (TKIs) are target-specific inhibitors of abnormal PTKs, and they act as homologs of adenosine triphosphate (ATP) that inhibit its binding competitively. [4]

Molecular dynamics (MD) is a computational technique that simulates the dynamic behavior of molecular systems as a function of time. Unlike the rigid molecular docking method, MD simulations

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can flexibly treat both ligand and protein, allowing for an induced fit of the receptor-binding site around the newly introduced ligand. Also, the effect of explicit water molecules can be studied directly, and very accurate binding free energies can be obtained. However, the combination of the two techniques in a protocol where docking is used for the fast screening of large libraries and MD simulations are then applied to explore conformations of the protein receptor, optimize the structures of the final complexes, and calculate accurate energies is a logical approach to improving the drug-design process. [5,6]

According to our previous study, new 2-(aminomethyl)benzimidazole derivatives were designed, synthesized, and tested virtually by docking with two downloaded proteins (EGFR and HER2) downloaded from the protein data bank (PDB code; 3POZ and 3RCD, respectively) and the ADME study by the SwissADME web site, as well as biological cytotoxic evaluation using two cancer cell lines. Upon analysis of the resulting data, the highly cytotoxic compounds were making a complex explanation about their pharmacological activity against cancer cell lines. For more investigation, it has been suggested that many other studies should be done for more confirmation of their activity, especially those concerning the mechanism of action. [7]

In this study, molecular dynamic simulation was done with two proteins (EGFR and HER2) that are co-crystallized with the same ligand of a dual EGFR/HER2 inhibitor (TAK-285), [8,9] as shown in **Figure 1**, and then they were tested with compounds of the highly cytotoxic activity (**2g** and **4g**) with suspected dual TKI activity (**Figure 2)**. So, this study is considered a complementary study and as a stability assessment to our previous investigations as well as an insight into future biological tests.

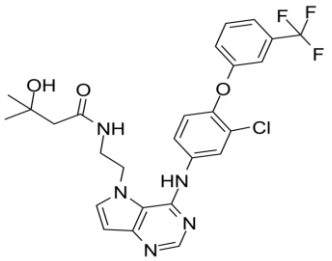
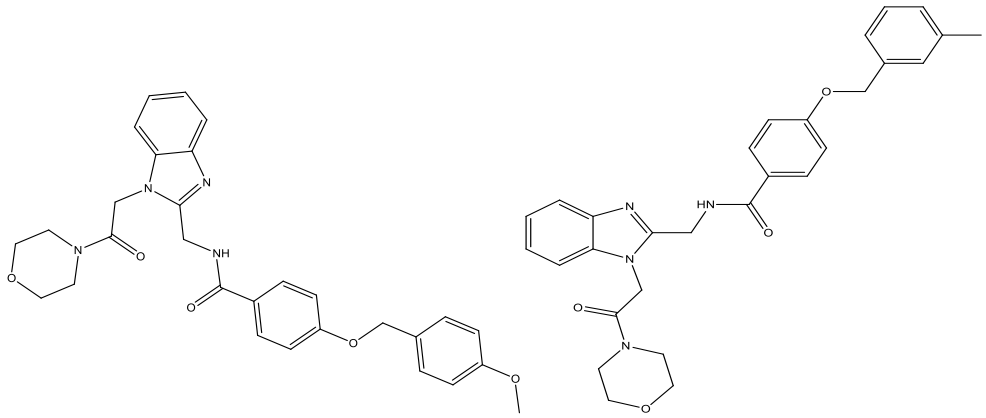


Figure 1. Chemical structure of TAK-285



2g **4g**

Figure 2. Chemical structures of **2g** and **4g** tested compounds [7]

2. Computational Method

2.1. Molecular docking:

According to the previous docking study using the Molecular Operating Environment (MOE), (which was explained with details in a supporting file), [10,11] as well as the cytotoxic study of six tested compounds with two cancerous cell lines, two compounds (**2g** and **4g**) were chosen with high docking scores, represented by lower binding energy and an RMSD of 1.8 for each compound, supported by their highly cytotoxic activities against breast cancer cell line compared with gefitinib as a reference standard in both docking and biological tests. [7]

2.2. Molecular dynamic simulation (MDS):

MDS was done with the Maestro v 13.0.135 interface (Schrodinger, New York, NY, 2021). So,

to get a more realistic picture of the protein and ligand interactions, we chose and exposed four optimal complexes for MD simulation using the Desmond module version 2.0 (academic version) according to both docking and biological studies. The system was designed by inserting a TIP3P water model in an orthorhombic periodic box of dimension 10 A^0 with an OPLS4 force field, then neutralizing it with counterions (Na^{+1} and Cl^{-1}) at neutral pH. In various constrained steps, the protein-ligand complex with the solvent system was maintained for energy minimization and pre-equilibration. MD simulations were inspected for 20 ns at a constant temperature of 300 K with a relaxation time of 2 ps in the NPT ensemble with the Nose-Hoover thermostat. Electrostatic interactions were treated using the Particle Mesh Ewald method for long and short ranges (cut-off distance of 9.0 Å), with a 10–9 tolerance limit. [12,13]

3. Results and discussion

Monitoring the RMSD of the protein can give insights into its structural conformation throughout the simulation. Changes of the order of 1-3 Å are perfectly acceptable for small, globular proteins, as well as the RMSD values that stabilize around a fixed value along the simulation time, i.e., a well equilibrated-system [14].

Unlike the more static molecular docking method, MD modeling does not ignore the fact that proteins change over time [15], so for more confirmation about the pharmacological activity of the most cytotoxic compounds (**2g** and **4g**) MDS was done with both 3POZ and 3RCD proteins that represent EGFR and HER2, respectively.

MDS uses a broad model of the physics behind interatomic interactions to predict the motion of each atom in a protein or other molecular system across time. Additionally, at extremely fine temporal resolution and in complete atomic detail, these simulations illustrate the behavior of proteins and other biomolecules. [16].

The Simulation Interactions Diagram Reports cover analysis of protein-ligand Root Mean Square Deviation (RMSD), protein Root Mean Square Fluctuation (RMSF), protein secondary structure, ligand RMSF, protein-ligand contacts, and ligand properties, which are all illustrated in Figures: 3,4,5 and 6. [17]

The protein conformational change was explained by the difference in the position of the Cα atoms of the protein backbone and was reported in Å and plotted as a function of simulation time, as in **figures (3a-4a) and (5a-6a)** related to compounds **2g** and **4g** respectively. The RMSD of the **3POZ**-protein was **2** and **2.5** with both **2g** and **4g** respectively, which are considered acceptable results. While the **3RCD**-protein give RMSD approximately equal to 2 with both compound, which indicates little change in the binding site concerning the reference frame. [18]

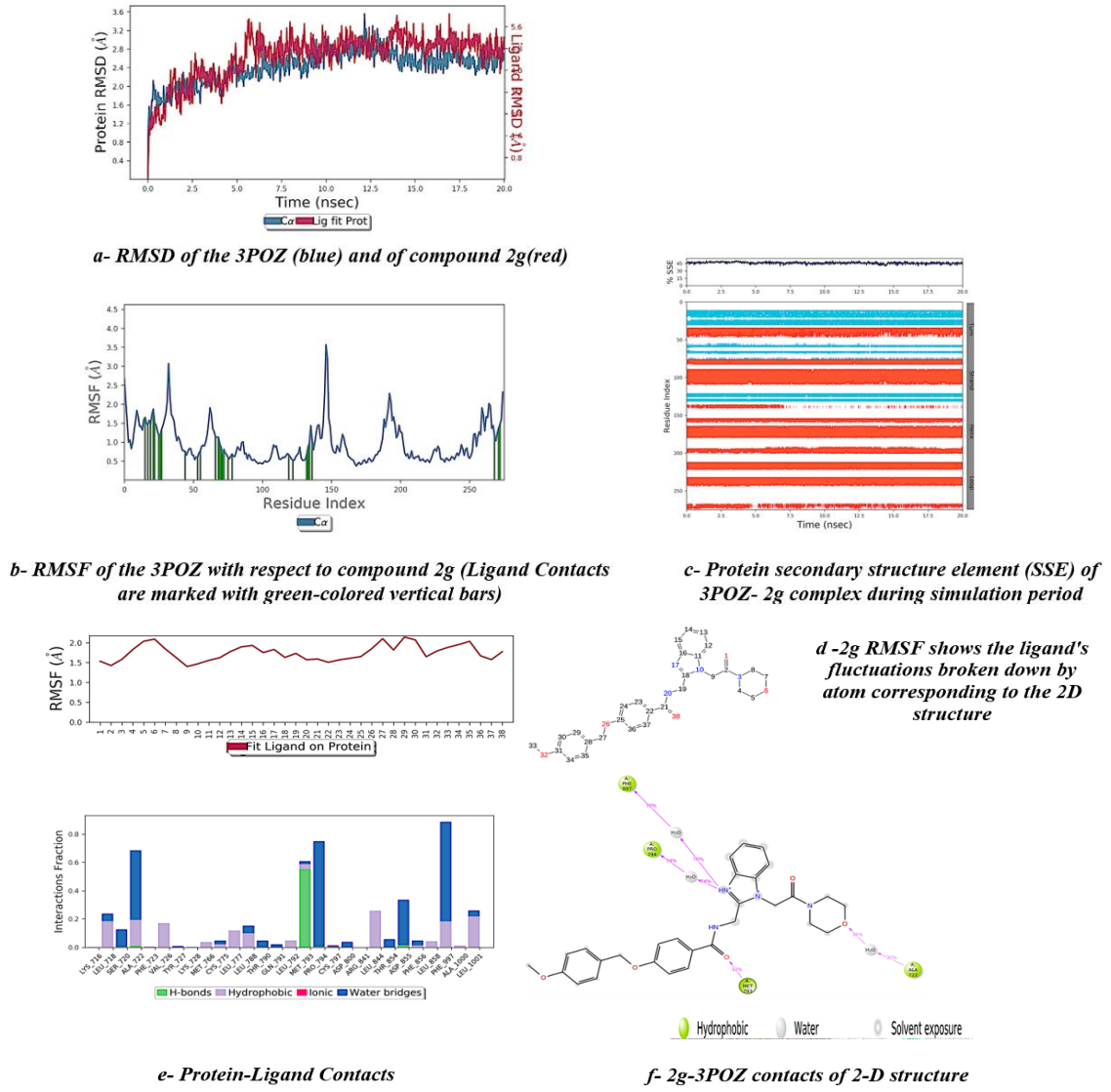
Ligand RMSD (right Y-axis of **a-Figures**) indicates how stable the ligand is with respect to the protein and its binding pocket. In these plots, 'Lig fit Prot' showed the RMSD of a ligand when the protein-ligand complex is first aligned on the protein backbone of the reference and then the RMSD of the ligand heavy atoms is measured. If the values observed are significantly larger than the RMSD of the protein, then it is likely that the ligand has diffused away from its initial binding site. [17] Our tested compounds ligand-RMSD showed little changes with respect to protein-RMSD.

The Root Mean Square Fluctuation (RMSF) is useful for characterizing local changes along the protein chain. [19] Secondary structure elements like alpha helices and beta strands are usually more rigid than the unstructured part of the protein, and thus fluctuate less than the loop regions, while the tails (N- and C-terminal) fluctuate more than any other part of the protein, as seen in **Figures (3b-4b) and (5b-6b)** related to compounds **2g** and **4g** respectively.

A narrow range of RMSF values of the active site residues of the complexes, marked with green-colored vertical bars, demonstrates that these compounds are capable of forming stable interactions with the protein during the MDS trajectory.

The best diagrams were represented by the **2g-3POZ and 4g-3RCD** since ligand-protein contact did not exceed 2 Å along each trajectory frame throughout the simulation.

Protein secondary structure elements (SSE) like alpha-helices and beta-strands are monitored throughout the simulation, and it is used to determine the changes that occur in the 3D structure of the protein during the simulation period for each frame in the trajectory. [20]



a- RMSD of the 3POZ (blue) and of compound 2g(red)

b- RMSF of the 3POZ with respect to compound 2g (Ligand Contacts are marked with green-colored vertical bars)

c- Protein secondary structure element (SSE) of 3POZ- 2g complex during simulation period

d -2g RMSF shows the ligand's fluctuations broken down by atom corresponding to the 2D structure

e- Protein-Ligand Contacts

f- 2g-3POZ contacts of 2-D structure

Figure 3. Simulation Interactions Diagram Report of 2g-3POZ

Figures (3c-4c) and (5c-6c) showed 2 plots, the top plots summarize the SSE composition for each trajectory frame over the course of the simulation, while the below monitor each residue and its SSE assignment over time.

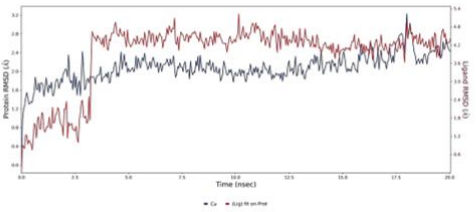
The stable interaction is validated by the SSE, which remains relatively constant throughout the simulation. [21]

The Ligand Root Mean Square Fluctuation (L-RMSF) is useful for characterizing changes in the ligand atom positions. [22] The ligand RMSF may give us insights into how ligand fragments interact with the protein and their entropic role in the binding event. Ligand RMSF shows the ligand's fluctuations broken down by atom, the more the stable ligand the less fluctuation will be observed, as seen in (3d,4d) and (6d) related to compounds 2g and 4g respectively. In the case of the 4g-3POZ complex, L-RMSF showed a high fluctuation during the simulation trajectory, as seen in Figure (5d).

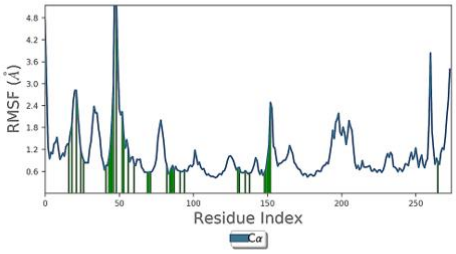
Protein interactions with the ligand can be monitored throughout the simulation. These interactions can be categorized by type and summarized in **Figures (3e&f-4e&f) and (5e&f -6e&f)** related to compounds **2g** and **4g** respectively. These figures explained the types of interactions that occurred in more than 30% of the simulation time in the selected trajectory.

In the **2g-3RCD** complex, ASP 863 interacts by a hydrogen bond in approximately 81% of the simulation time. LYS 753 also interacts by both a

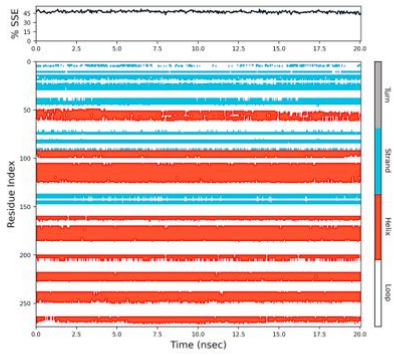
hydrogen bond and pi-cation interaction in about 55% and 39%, respectively, while in the **2g-3POZ** complex, only hydrophobic interaction and water bridges are seen along the simulation time.



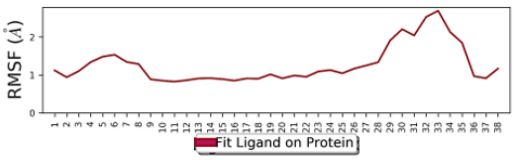
a- RMSD of the 3RCD(blue) and of compound 2g (red)

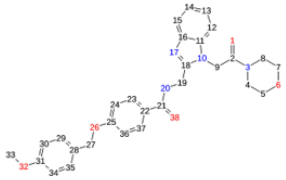


b- RMSF of the 3RCD with respect to compound 2g (Ligand Contacts are marked with green-colored vertical bars)

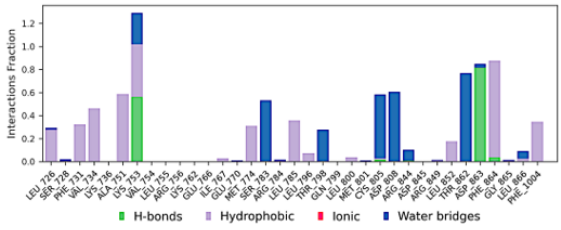


c- Protein secondary structure element (SSE) of 3RCD- 2g complex during simulation period

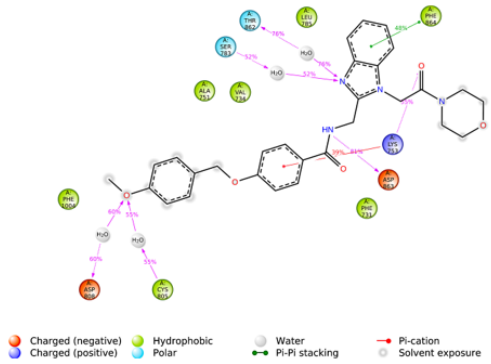




d -2g RMSF shows the ligand's fluctuations broken down by atom corresponding to the 2D structure



e- Protein-Ligand Contacts



f- 2g-3RCD contacts of 2-D structure

Figure 4. Simulation Interactions Diagram Report of **2g-3RCD**

In both **4g-3RCD** and **4g-3POZ** complexes, hydrophobic interactions are dominant with little hydrogen bonding. It is worth noting that there is intramolecular hydrogen bonding during the simulation trajectory in **4g-3RCD** that could stabilize the compound at the binding site.[23]

The protein conformational change was explained by the difference in the position of the Cα atoms of the protein backbone and was reported in Å and plotted as a function of simulation time, as in **figures (3a-4a) and (5a-6a)** related to compounds **2g** and **4g** respectively. The RMSD of the **3POZ**-protein was **2** and **2.5** with both **2g** and **4g**

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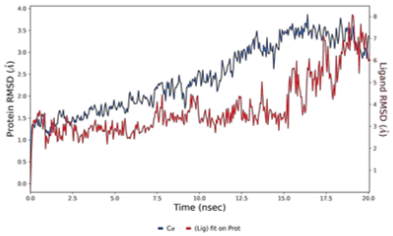
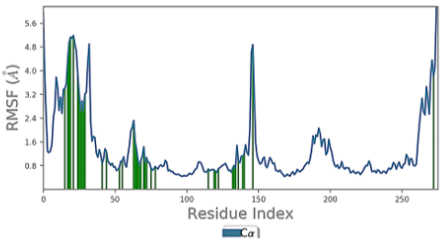
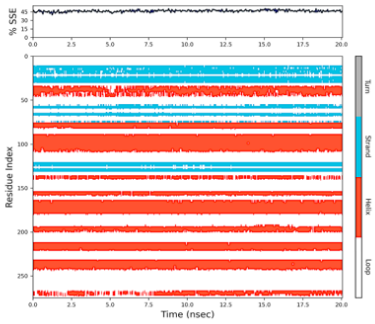


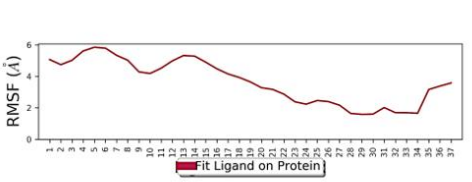
a- RMSD of the 3POZ (blue) and of compound 4g (red)

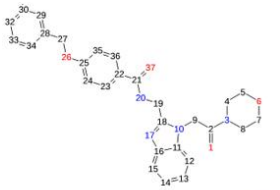


b- RMSF of the 3POZ with respect to compound 4g (Ligand Contacts are marked with green-colored vertical bars)



c- Protein secondary structure element (SSE) of 3POZ- 4g complex during simulation period





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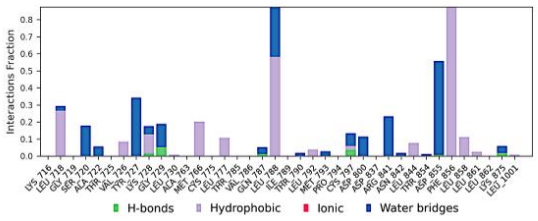

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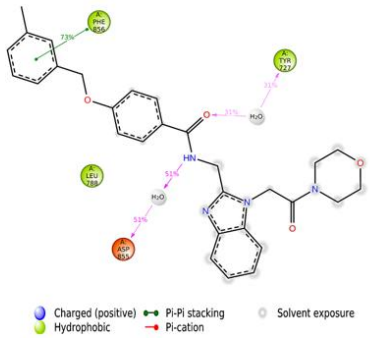

f- 4g-3POZ contacts of 2-D structure

Figure 5. Simulation Interactions Diagram Report of **4g-3POZ**

Ligand RMSD (right Y-axis of **a-Figures**) indicates how stable the ligand is with respect to the protein and its binding pocket. In these plots, 'Lig fit Prot' showed the RMSD of a ligand when the protein-ligand complex is first aligned on the protein backbone of the reference and then the RMSD of the ligand heavy atoms is measured. If the values observed are significantly larger than the RMSD of the protein, then it is likely that the ligand has diffused away from its initial binding site. [17] Our tested compounds ligand-RMSD showed little changes with respect to protein-RMSD.

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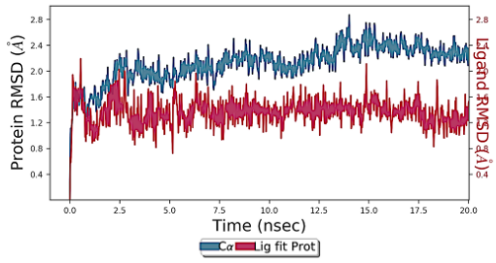

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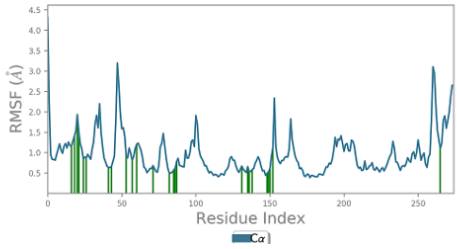
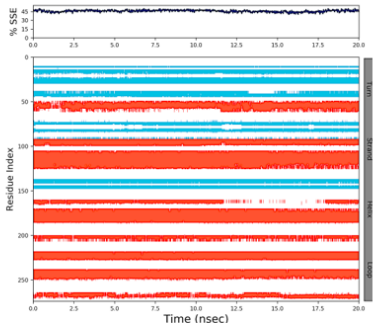
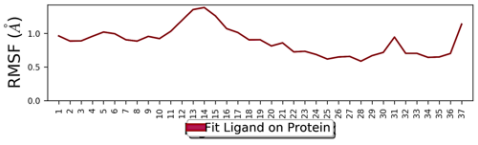
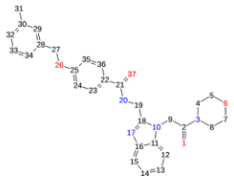


b- RMSF of the 3RCD with respect to compound 4g (Ligand Contacts are marked with green-colored vertical bars)



c- Protein secondary structure element (SSE) of 3RCD- 4g complex during simulation period





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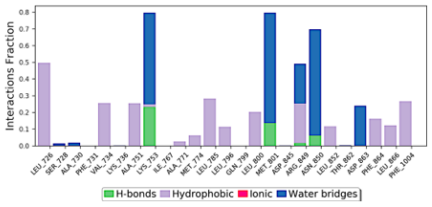

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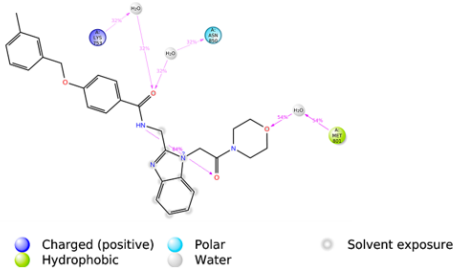

e- 4g-3RCD contacts of 2-D structure

Figure 6. Simulation Interactions Diagram Report of 4g-3RCD

The best diagrams were represented by the **2g-3POZ and 4g-3RCD** since ligand-protein contact did not exceed 2 Å along each trajectory frame throughout the simulation.

Protein secondary structure elements (SSE) like alpha-helices and beta-strands are monitored throughout the simulation, and it is used to determine the changes that occur in the 3D structure of the protein during the simulation period for each frame in the trajectory. [20]

Figures (3c-4c) and (5c-6c) showed 2 plots, the top plots summarize the SSE composition for each trajectory frame over the course of the simulation, while the below monitor each residue and its SSE assignment over time.

The stable interaction is validated by the SSE, which remains relatively constant throughout the simulation. [21]

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ligand atom positions. [22] The ligand RMSF may give us insights into how ligand fragments interact with the protein and their entropic role in the binding event. Ligand RMSF shows the ligand's fluctuations broken down by atom, the more the stable ligand the less fluctuation will be observed, as seen in **(3d,4d) and (6d)** related to compounds **2g** and **4g** respectively. In the case of the **4g-3POZ** complex, L-RMSF showed a high fluctuation during the simulation trajectory, as seen in **Figure (5d).**

Protein interactions with the ligand can be monitored throughout the simulation. These interactions can be categorized by type and summarized in **Figures (3e&f-4e&f) and (5e&f - 6e&f)** related to compounds **2g** and **4g** respectively. These figures explained the types of interactions that occurred in more than 30% of the simulation time in the selected trajectory.

In the **2g-3RCD** complex, ASP 863 interacts by a hydrogen bond in approximately 81% of the simulation time. LYS 753 also interacts by both a hydrogen bond and pi-cation interaction in about 55% and 39%, respectively, while in the **2g-3POZ** complex, only hydrophobic interaction and water bridges are seen along the simulation time.

In both **4g-3RCD** and **4g-3POZ** complexes, hydrophobic interactions are dominant with little hydrogen bonding. It is worth noting that there is intramolecular hydrogen bonding during the simulation trajectory in **4g-3RCD** that could stabilize the compound at the binding site.[23]

4. Conclusions

From the resulting data that were shown in the simulation interactions diagram reports of the tested compounds with two selected proteins, it is clear that both tested compounds had different affinity for interaction with the selected proteins. The **2g** compound showed a good interaction complex with both the **3POZ (EGFR)** and **3RCD (HRE2)** proteins, while only **the 4g-3RCD** complex showed a good interaction report. Comparing with a previous docking study, both compounds displayed good interactions with binding sites and good RMSD. Thus, it is possible to classify **2g** as a dual EGFR/HER2 inhibitor and **4g** as a HER2 inhibitor. Both compounds might then be examined in further detail using additional biological research, particularly enzyme inhibitory experiments that have the potential to yield positive results.

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Supporting File

This Molecular Docking study is related to the previous in-press article: S. Al-Sultan, M. Mohammed, , W. Talib, Newly Designed 2- (amino methyl) Benzimidazole Derivatives as possible Tyrosine Kinase Inhibitors: Synthesis, Characterization, Preliminary Cytotoxic Evaluation and *In Silico* Studies. *Chemical Review and Letters*, in-press (2024). doi: 10.22034/crl.2024.463505.1360

The method: The chemical structures of the designed compounds were drawn using the Molecular Operating Environment (MOE) 2014 builder, then their energies were minimized and all compounds were docked with 2 proteins (3RCD; HER2 and 3POZ; EGFR downloaded from PDB) after many processing starting from protein preparation to pharmacophore adjustment. After completion of the docking runs, the scores of enzyme-ligand free energies of binding were obtained. The best pose of ligand binding is indicated by the lowest free energy (ΔG) and RMSD less than 2. The Ki (inhibitory constant of each compound with lower energy was calculated using the following equation:

$Ki = e^{\Delta G}/RT$

where ΔG is the binding free energy (kcal. Mol^{-1}), R is the gas constant (1.987 cal. Mol^{-1}. K^{-1}), and T is the absolute temperature (298.15 K).

The results: In order to get a preliminary confirmation about the enzyme inhibition mode of our synthesized compounds, all compounds were docked with two selected proteins of RTKs (EGFR, 3POZ and HER2, 3RCD), which are co-crystallized with same ligand (dual EGFR and HER2 TKI) and downloaded from PDB (www.rcsb.org). The docking scores represented by binding free energy with calculated Ki as well as RMSD are summarized in **table 1**, and the 2D and 3D interaction with binding sites of highly cytotoxic compounds (**2g** and **4g)** are shown in **figure 1**.

As it is known, the small (ΔG) value, the good interaction between the protein and the ligand. And the RMSD near 1 and less than 2 means that the ligand good fits with reference co-crystallized ligand with in the binding pocket.

According to the data mentioned in **table 1**, all the docking scores represented by binding energy (ΔG) as well as the RMSD values showed good results in comparing with gefitinib as a clinically approved reference. This indicates in good binding interaction mode especially with **4g** and **2g** compounds.

Figure 1, below, illustrates the 2D and 3D interaction of the highly cytotoxic ligands **4g** and **2g** with proteins binding pockets. In this figure all compounds show a good accommodation with binding pocket extending to the back hydrophobic pocket. Also, these compounds are interacted with binding site residues as shown in **table 3.**

2g and **4g** compounds were candidates for more investigation into molecular dynamic simulation.

Table 2. docking results of the designed compounds with both EGFR and HER2

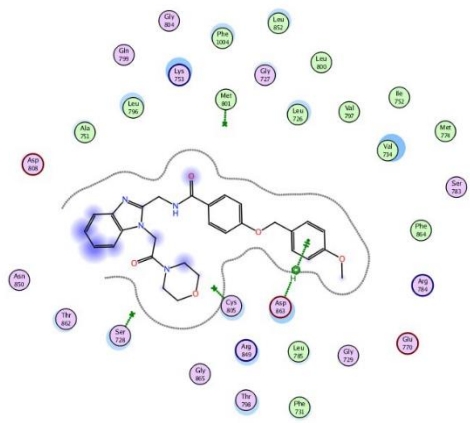
Compound	EGFR, 3POZ			HER2, 3RCD		
	$\Delta G_{(kcal/mol)}$	RMSD*	$Ki^{\#}$	$\Delta G_{(kcal/mol)}$	RMSD	Ki
1g	-9.2	2.1	0.17	-8.1	1.8	0.51
2g	-9.3	1.8	0.12	-8.1	1.1	0.51
3g	-9.1	2.5	0.18	-8.5	2.2	0.34
4g	-9	1.8	0.2	-8.1	1.1	0.51
1h	-9.6	1.9	0.11	-8.8	1.5	0.25
2h	-9.6	1.1	0.11	-8.6	2	0.31
3h	-9.8	3.8	0.09	-7.8	2	0.69

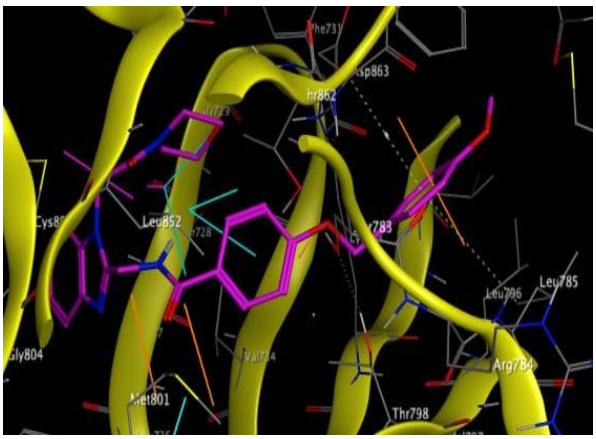
4h	-7.6	1.7	0.84	-7.6	2.1	0.84
Gefitinib	-7.7	2	0.76	-8	2	0.56

** RMSD, root mean square deviation of a ligand with respect to the reference conformation, #µM*

Table 3. Ligand- receptor interaction reports of the tested compounds

Compound	EGFR, 3POZ				HER2, 3RCD			
	Receptor	***Interaction***	***Distance***	***E kcal/mol***	***Receptor***	***Interaction***	***Distance***	***E kcal/mol***
2g	LYS 745 pi-H CYS 797	pi-H pi-H	3.69 3.98	-0.7 -1.7	ASP 863	pi-H	4.08	-0.6
4g	LEU 718 LYS 745 CYS 797	pi-H pi-H pi-H pi-H	4.38 4.18 3.71 4.23	-0.6 -0.7 -0.6 -0.9	GLY 727 LEU 785	pi-H pi-H	4.17 4.2	-0.8 -0.6





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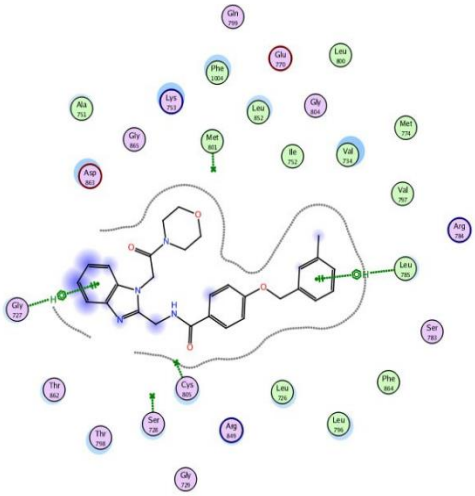
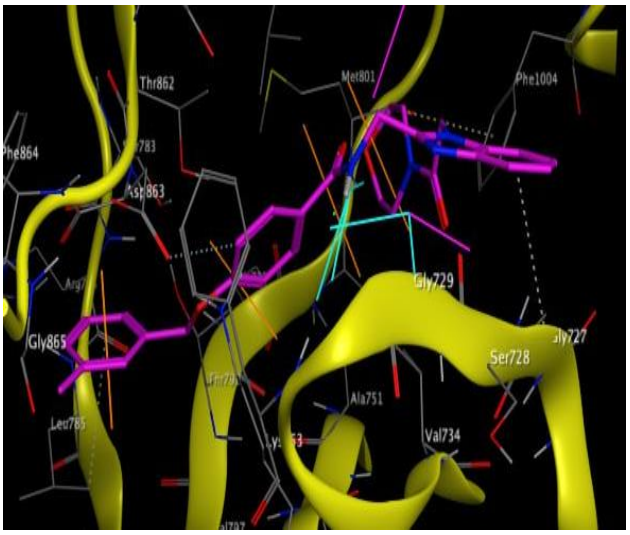
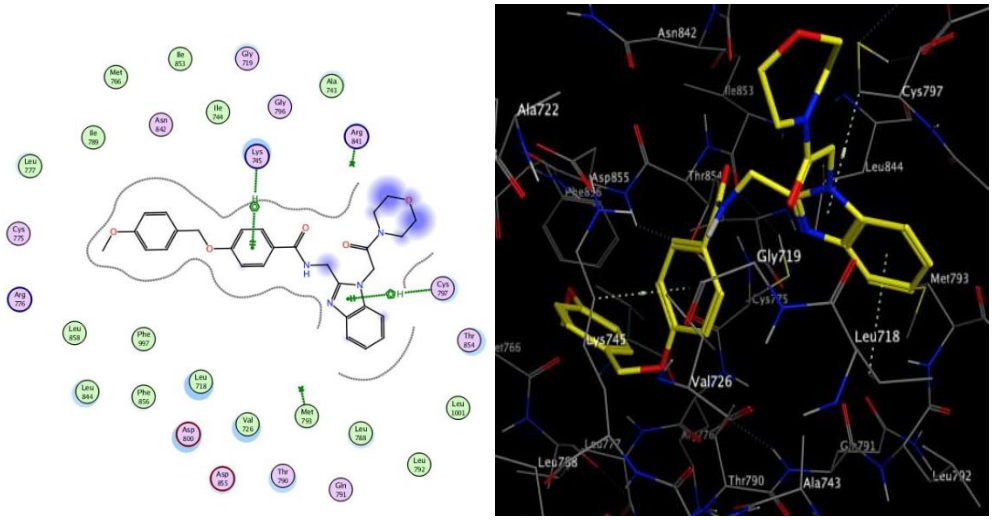
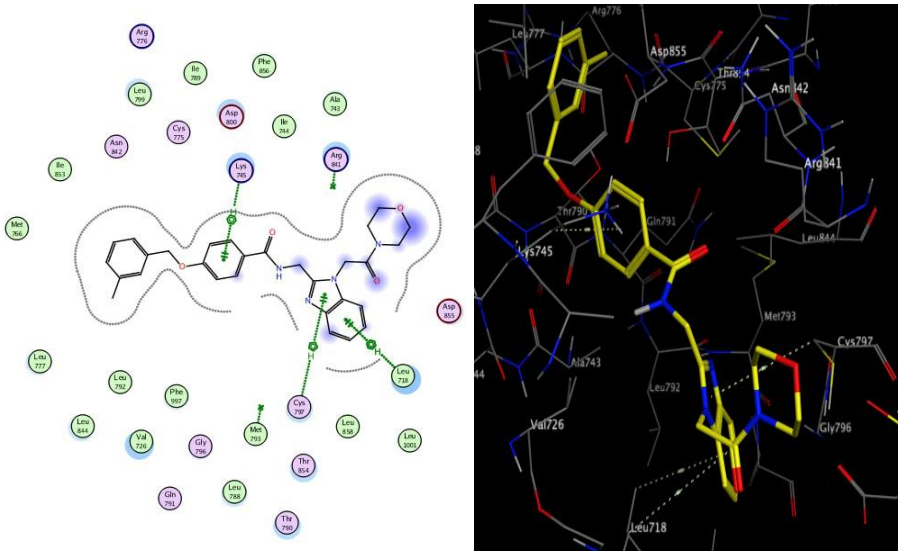




4g-3RCD



2g-3POZ



4g-3POZ

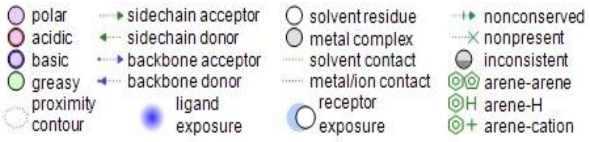


Figure 1: 2D and 3D ligand interaction with the binding pocket