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Original Article

IN SILICO STUDY OF THE SYNERGISTIC INTERACTION OF 5-FLUOROURACIL AND CURCUMIN ANALOGUES AS INHIBITORS OF B-CELL LYMPHOMA 2 PROTEIN

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ABSTRACT

Objective: The research objective is to explore the potential for synergistic inhibition of two curcumin analogs combined with 5-fluorouracil (5-FU) against the B-cell lymphoma 2 (BCL-2) protein.

Methods: We investigated the synergistic inhibition of two curcumin analogs, namely, (1*E*,4*E*)-1,5-bis(4-hydroxyphenyl)penta-1,4-dien-3-one (AC01) and (1*E*,4*E*)-1,5-bis(3,4-dihydroxyphenyl)penta-1,4-dien-3-one (AC02), each combined with 5-FU by calculating their binding free energies and binding stability. An *in silico* investigation of the synergistic interaction of ligand pairs was conducted using the multiple ligand simultaneous docking (MLSD) technique with the AutoDock Vina package. The stability of interactions and binding free energies of each BCL-2 and curcumin analogs were examined by applying molecular dynamics techniques with the Gromacs package and MMPBSA method.

Results: All ligand pairs had displayed strong binding affinity, as evidenced by highly negative free energy values, indicating a robust association with BCL-2. Molecular dynamics simulations were conducted over 100 ns, confirming good stability with controlled RMSD changes, suggesting that the ligand pairs had remained securely bound to the BCL-2 binding site. Additionally, RMSF analysis and energy decomposition had revealed that ligand interactions did not influence protein residue fluctuations during the simulation, and the protein-ligand complexes had maintained stability throughout the simulation. Furthermore, binding free energy calculations using the MMPBSA method had consistently shown negative values, signifying stable interactions with BCL-2 for all ligand pairs.

Conclusion: In conclusion, our study revealed that AC01 and AC02, when combined with 5-FU, had the ability to intercalate into the P2 and P4 sites of BCL-2. This suggested that AC01 and AC02 held promise for further study as candidates for anticancer drugs, individually or in combination with 5-FU.

Keywords: Synergistic, 5-fluorouracil, Lapatinib, Curcumin analog, BCL2

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INTRODUCTION

B-cell lymphoma 2 (BCL-2) is a crucial protein in the regulation of apoptosis, maintaining homeostasis and eliminating damaged cells [1]. BCL-2 can inhibit cysteine protease executioners that drive cell death, known as caspases [2]. Treatment with 5-fluorouracil (5-FU) has been found to trigger apoptosis in breast cancer cell lines, partly by its ability to disrupt the function of BCL-2 [3]. The use of 5-FU in cancer therapy has been extensively studied. Combining 5-FU with doxorubicin and paclitaxel has been shown to enhance caspase-dependent apoptosis in breast cancer cell lines [4]. Therefore, 5-FU therapy has the potential to disrupt BCL-2 function and induce cancer cell death [5]. In colorectal cancer cells, the stabilization of BCL-2 by paxillin has been found to confer resistance to 5-FU [6]. However, a study has shown that additional inhibition of BCL-XL, another anti-apoptotic member of the BCL-2 family, enhances the effectiveness of 5-FU in colorectal cancer [7]. In breast cancer cells exposed to low-dose α-ionizing particles, 5-FU has been shown to induce apoptosis by regulating the expression of Bax, Bcl-xL, and NF-κB [3].

Additionally, 5-FU has also been studied in combination with Lapatinib, known as a dual inhibitor of EGFR and HER2 [8]. Lapatinib, a dual EGFR and HER2 inhibitor, has been found to have a synergistic effect with 5-FU in esophageal carcinoma with HER2 amplification [9, 10]. The combination of Lapatinib and 5-FU has emerged as a promising treatment option for this type of cancer [11]. Lapatinib is a dual tyrosine kinase inhibitor that simultaneously inhibits EGFR and HER2 by reducing their phosphorylation, making it a promising choice for combination therapy with 5-FU [12].

Curcumin is a natural compound derived from turmeric that has potential anti-cancer properties. However, it is low bioavailability and limited stability have led to the development of analogs with improved characteristics [13]. Some examples of curcumin analogs and their potential anti-cancer effects are (i) bisdemethoxycurcumin, demethoxycurcumin, and dimethoxycurcumin. These are four different curcumin analogues that have shown anti-cancer effects on human glioma cells [14]; (ii) aonocarbonyl analogs. A study showed that the curcumin analog 1e is a promising agent against colorectal cancer, with improved stability and efficacy/safety [13]; (iii) PAC. This curcumin analogue has demonstrated potential as an anti-breast cancer agent [15]; (iv) Capsaicin, chlorogenic acid, ferulic acid. These are curcumin analogs with a higher bioavailability and wide-spectrum pharmacological activity that have shown potential as anti-breast cancer agents [16].

In this research, we intention was to explore the anti-cancer synergetic potential of (1*E*,*4E*)-1,5-bis(4-hydroxyphenyl)penta-1,4-dien-3-one (AC01) and (1*E*,4*E*)-1,5-bis(3,4-dihydroxyphenyl)penta-1,4-dien-3-one (AC02) in combination the 5-FU, a common chemotherapeutic drug. We will also individually compare their potential as anti-cancer agents using an *in silico* approach by utilizing multiple ligand simultaneous docking (MLSD) and molecular dynamics. This methodology entails the simultaneous inclusion of multiple substrates within the active site of BCL-2, thereby offering a more faithful depiction of biochemical processes. This approach allows us to comprehend how various ligands, including the combination of 5-FU pairs with curcumin analogs, collaborate when interacting with BCL-2. This approach can provide valuable insights into these compounds' potential mechanisms of action and efficacy.

MATERIALS AND METHODS

Data collection

The data collection process for this study involved retrieving the molecular structures of AC01, AC02, 5-FU, and lapatinib (collectively

referred to as FMM) from the chemical databases of the National Library of Medicine, specifically from https://pubchem.ncbi.nlm.nih.gov/. This encompassed both the 2D structures and molecular coordinates of the compounds' 3D molecular structures. Furthermore, the 3D structures of the BCL-2 protein were generated using the homology modeling technique, utilizing the Swiss Model tool, which was accessible at https://swissmodel.expasy.org/. We had selected a model based on various criteria, including a high degree of similarity, a lower Ramachandran outlier score, a favorable Ramachandran score, a lower clash score, and MolProbity [17].

Multiple ligand simultaneous docking

The molecular docking process commenced with the validation of the BCL-2 protein's binding site as the target. This validation involved the utilization of the comparison of binding poses technique, where the predicted binding pose of the re-docked ligand was juxtaposed with the experimentally determined binding pose identified within the crystal structure [18]. Subsequently, the alignment and similarity between the re-docked pose and the crystallographic pose were meticulously evaluated to verify the precision of the binding site [19, 20].

The ligand preparation was carried out using Open Babel [21] and AutoDockTools [22]. The preparation of the protein target, including the addition of hydrogen atoms and partial charges, was performed using Discovery Studio Visualizer 2022. The definition of the binding site or active site on the BCL-2 protein where the native ligand binds was also accomplished using Discovery Studio Visualizer 2022.

Molecular docking simulations were carried out using AutoDock Vina version 1.2.5 [23], encompassing both individual ligand docking and multiple ligand simultaneous docking (MLSD) within the binding site of the BCL-2 protein. Individual docking was performed for the native ligand of the BCL2 protein, AC01, AC02, 5-FU, and FMM ligands. In addition, the MLSD technique was employed for the ligand pairs 5-FU+AC01, 5-FU+AC02, and 5-FU+FMM following Vina's MLSD procedures [24]. The analysis of binding affinity and binding poses was executed using Discovery Studio Visualizer 2022 to elucidate the interactions between the protein and ligands. This involved the analysis of interaction patterns, such as hydrogen bonding, hydrophobic interactions, and π-π stacking [25].

Multiple ligand simultaneous molecular dynamics

The most promising protein-compound(s) complexes were identified from the docking results for subsequent molecular dynamics simulations. The molecular dynamics simulations were performed using the GROMACS software [26]. The analysis of the molecular dynamics outcomes included the assessment of complex stability, conformational changes, and binding free energy calculations using the MMPBSA and MMGBSA methods through the GMX-MMPBSA software [27].

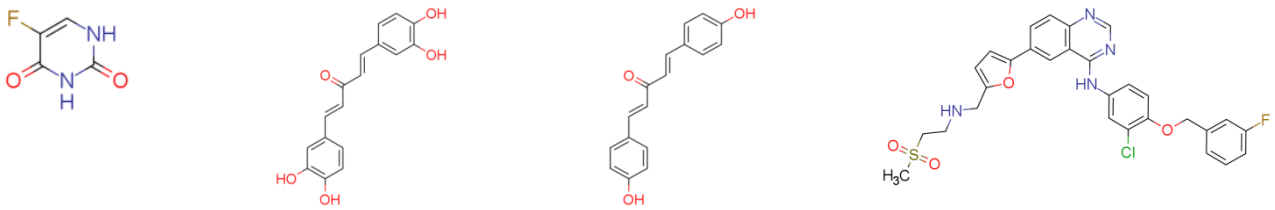
Synergistic effects analysis

Synergistic effects were investigated *in silico* by conducting docking of AC01 and AC02 in combination with 5-FU into the active site of the BCL-2 protein. The docking results, including binding affinities and interaction patterns, were compared both among the compounds in combination and also with the results obtained from individual docking simulations.

RESULTS

Data collection and preparation

The molecular structures of 5-FU, AC01, AC02, and FMM were obtained from the National Library of Medicine's databases, as shown in fig. 1. The 3D structure of the BCL-2 protein was successfully generated through homology modeling using the Swiss Model tool. The selected model used the BCL-XL structure as a template based on its high structural similarity to the target BCL2 protein, as demonstrated by the 97.85% sequence identity.



(a) **(b)** **(c)** **(d)**

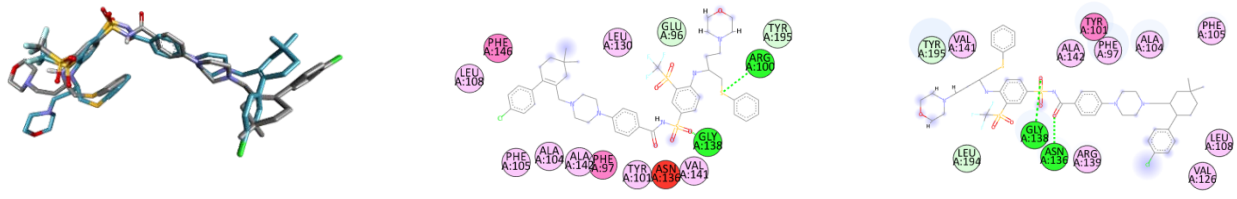
Fig. 1: The chemical structures of (a) 5-FU, (b) AC02, (c) AC01, and (d) FMM

The accurate representation of molecular structures was essential for the subsequent stages of this study, which involved molecular docking, binding affinity calculations, and molecular dynamics simulations. The selected structures laid the foundation for exploring the interactions between AC01, AC02, 5-FU, and Lapatinib with the BCL-2 protein.

Molecular docking

The overlay of 1XJ (native ligand) between the crystallographic pose and docking pose, as well as the comparison of BCL-2 residues in their interaction with 1XJ in molecular docking simulations in this study, was depicted in fig. 2. From this figure, it could be concluded that redocking 1XJ had effectively validated the docking method, thus demonstrating its applicability for docking test ligands.

The core objective of this study was to explore the potential synergistic interactions between active compound pairs employing molecular docking. The outcomes of the molecular docking simulations unveiled significant insights into the interplay between various ligands and the BCL-2 protein. These interactions were quantified through binding affinity values, providing a measurable gauge of the strength of binding between individual ligands and the protein.



(a) **(b)** **(c)**

Fig. 2: Overlay of the 1XJ chemical structure in crystallographic form (blue carbon) and from the redocking result (grey carbon) (a), the crystallographic (b) and the re-docked (c) pose of 1XJ in binding with the BCL-2 active site

Table 1: Outlines the binding affinity values acquired from the docking simulations

No.	Ligand	Binding Affinity (kcal/mol)
1	5-FU+AC01	-11.20
2	5-FU+AC02	-10.83
3	5-FU+Lapatinib	-11.38
4	5-FU	-4.84
5	AC01	-6.98
6	AC02	-7.09
7	Lapatinib	-8.97
8	1XJ (native ligand)	-10.62

From the presented data, a compelling pattern emerged; ligand pairs like 5-FU+AC01, 5-FU+AC02, and 5-FU+Lapatinib exhibited remarkable binding affinities of-11.20,-10.83, and-11.38 kcal/mol, respectively. These findings underscored robust interactions between these ligand pairs and the BCL-2 protein, potentially indicative of pronounced binding stability. In contrast, individual ligands, namely 5-FU, AC01, and AC02, displayed relatively modest binding affinities with values of-4.84,-6.98, and-7.09 kcal/mol, respectively. These less intense affinity values may have implied weaker and less stable associations with the protein.

Considered independently, lapatinib garnered a binding affinity of-8.97 kcal/mol, indicating intermediate interaction strength with the BCL-2 protein. Additionally, the reference native ligand, 1XJ, yielded a binding affinity of-10.62 kcal/mol, offering a basis for comparing the interactions of other ligands. The findings from these docking simulations provided a glimpse into the plausible interactions between assorted ligands and the BCL-2 protein. The quantified binding affinity values furnished insights into the vigor of these interactions.

In summary, 5-FU in combination with AC01 and AC02, in addition to showing hydrophobic interactions with P2, also formed hydrogen bonds with the ALA142 residue, one of the hot spot residues in P2 of BCL2. On the other hand, the hydroxyphenol groups of AC01 and AC02 formed hydrogen bonds with ALA93, in addition to hydrophobic and other interactions. Lapatinib, both individually and in combination with 5-FU, did not show direct interactions with the BCL-2 binding site (fig. 3).

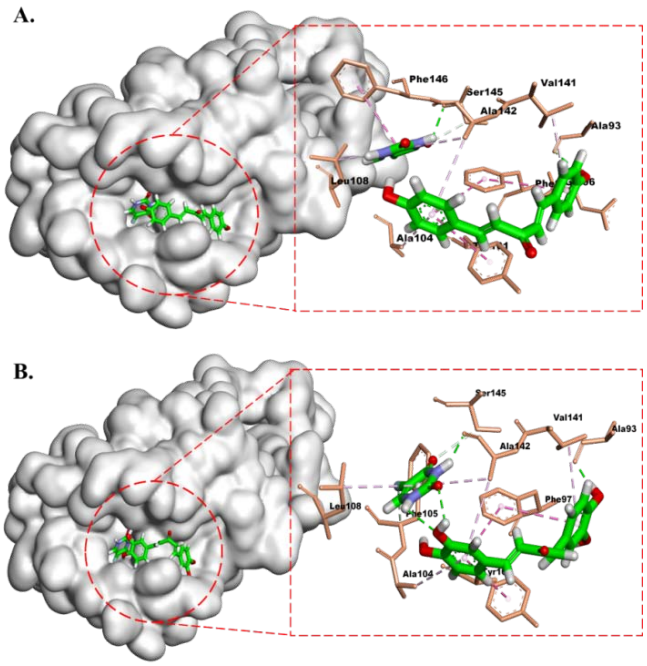


Fig. 3: Molecular interaction of (A) 5-FU+AC01 and (B) 5-FU+AC02 with BCL-2 active site

Simultaneous molecular docking simulations involving dual ligands showed how these ligands intercalated at the BCL-2 binding site. Combinations of 5-FU+AC01 and 5-FU+AC02 exhibited "shared binding site" behavior, similar to Navitoclax and its analogs, filling both P2 and P4 as single compounds. 5-FU occupied the P2 hot spot of BCL-2, meaning it "replaced" the position of the chloro-biphenyl group from Navitoclax, while one of the phenol groups from AC01 (and AC02) intercalated into P4, replacing the thio-phenyl group.

Lapatinib behaved differently in both single and combination docking. Lapatinib, by occupying the P2 site of BCL-2 with its sulfonamide group and the P4 site with a chloroaniline group, had the ability to fill the binding site. This was possibly due to the larger molecular size of Lapatinib compared to AC01, AC02, or 5-FU, allowing Lapatinib to reach both active sides of BCL-2. However, Lapatinib's ability to fill the binding site may not have been specific to BCL-2 due to the potential properties of the sulfonamide and chloroaniline groups not responding to BCL-2's biological function. This could have been illustrated by the visualization of the docking results (fig. 3), which showed that the sulfonamide and chloroaniline groups did not interact with BCL-2 residues, including hydrogen bonding, hydrophobic, or other non-covalent interactions.

Based on the docking results, it was also observed that the combination of 5-FU with AC01 and AC02 occupied the same binding site as FMM (fig. 4). This demonstrated the potential synergy between 5-FU and compounds AC01 and AC02 in suppressing cancer cells by binding more effectively to BCL-2.

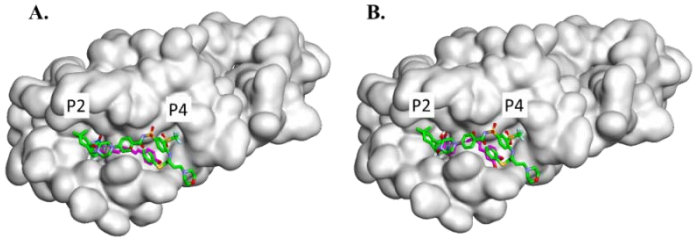


Fig. 4: Overlay conformation of (A) 5-FU+AC01 (blue-pink) with FMM (green) and (B) 5-FU+AC02 (blue-pink) with FMM (green) on BCL-2 active site

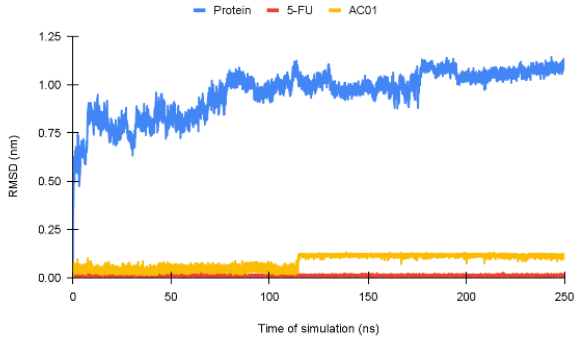
Molecular dynamics

In this study, we conducted a 250-nanosecond (ns) MD simulation to depict the dynamic behavior of a system involving a protein and two different ligand molecules. The system consisted of three complexes: BCL2 with the ligand pairs 5-FU+AC01, 5-FU+AC02, and 5-FU+FMM, using the best results from AutoDock Vina as the starting point for the MD simulation. Before the production phase, a comprehensive equilibration process was carried out, including system solvation in water and equilibration of temperature and pressure in NVT and NPT ensembles, each for 500 ps.

The results of the molecular dynamics simulations for the three receptor-ligand pairs, namely BCL-2 and 5-FU+AC01, BCL-2 and 5-FU+AC02, and BCL-2 and 5-FU+Lapatinib, were summarized in Root Mean Square Deviation (RMSD) (fig. 5a-c), Root Mean Square Fluctuation (RMSF) for protein fluctuation (fig. 5d), and the calculation of binding free energies using MMPBSA and MMGBSA methods (fig. 5e-f).

RMSD measured how much the molecular structure of a system changed over time compared to a reference structure. The initial or reference structure used in molecular dynamics analysis was the complex resulting from docking with the best binding affinity. The RMSD plots of protein and ligands overtime during the 250 ns simulation were summarized in fig. 5a-c. From fig. 5a-c, it was observed that the presence of ligands in the BCL-2 binding site remained stable in all three ligand pairs. This was indicated by RMSD values within a range below 1 nm. Only the movement of the protein molecule in the complex with the 5-FU+AC01 pair showed a slight deviation beyond 1 nm compared to the initial state (fig. 5a). Specifically, regarding ligand movements, in all three complexes, the three ligand pairs exhibited stability below 0.25 nm. Thus, the protein-ligand complexes studied in their interactions demonstrated a stable state.

To understand the fluctuations of atoms and protein residues during the simulation, we presented the results of RMSF calculations for the amino acid residues comprising BCL-2. RMSF indicated the behavior of atoms during the simulation, which could occur due to interactions with other atoms through forces such as Van der Waals forces, hydrogen bonding, and electrostatic forces, as these interactions could influence the positions of atoms and cause fluctuations. Fluctuations above 0.5 nm occurred in BCL-2 residues from sequences 25 to 85 (fig. 5d). When considering the decomposition analysis (fig. 5e), the fluctuations of BCL-2 residues were not related to their interaction with the ligands. The decomposition analysis results indicated that the dominant interaction energy was contributed by BCL-2 residues from sequence 90 upwards or those residues that did not experience significant fluctuations. This further confirmed the stability of BCL-2 interactions with the 5-FU+AC01, 5-FU+AC02, and 5-FU+Lapatinib pairs.



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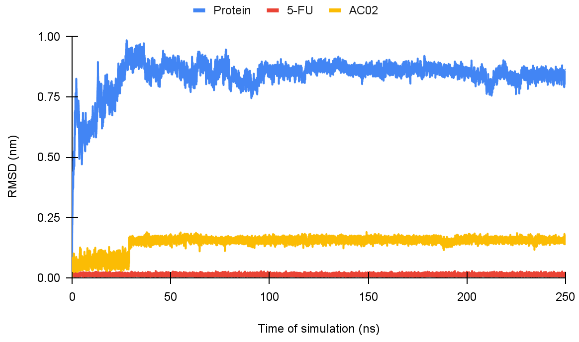

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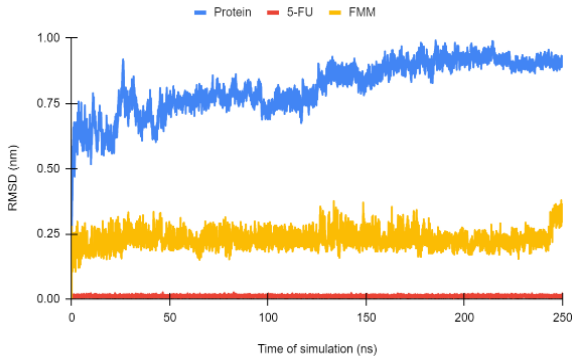

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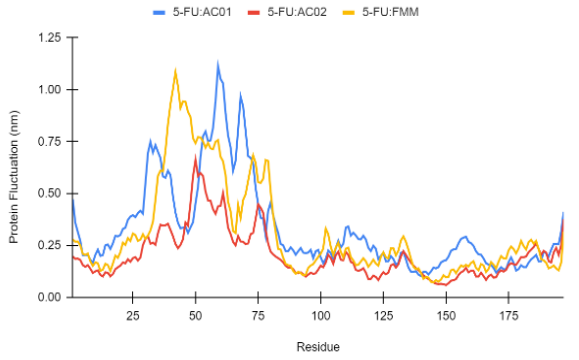

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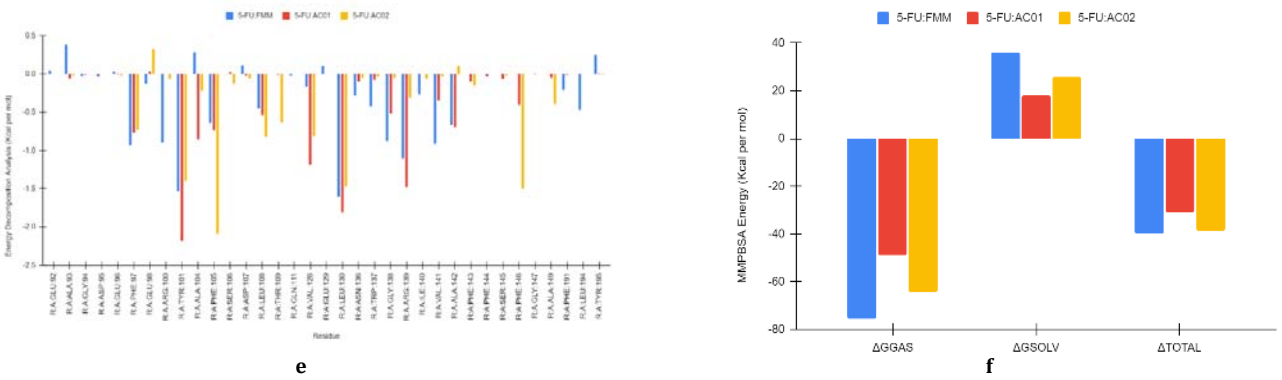

e f

Fig. 5: Stability and binding energy analysis from MD simulation include (a) RMSD values for the combination of 5-FU+AC01, (b) RMSD values for the combination of 5-FU+AC02, (c) RMSD values for the combination of 5-FU+FMM, (d) RMSF values for the 5-FU, AC01, AC02, and FMM complex, (e) energy contributions by each amino acid residue to binding affinity, and (f) binding free energy calculations using MMPBSA methods

Results of the binding free energy calculations using the MMPBSA method for the three complexes were summarized in fig. 5f. The binding free energies for 5-FU+AC01, 5-FU+AC02, and 5-FU+Lapatinib were-34.3,-38.7, and-39.67 kcal/mol, respectively. This indicated that based on the energy data, all three ligand pairs had the ability to bind stably to the BCL-2 binding site. More negative energy values were more likely to indicate stronger binding agents, which mean the 5-FU and Lapatinib pair had a higher binding affinity than the 5-FU+AC01 and 5-FU+AC02 pairs. However, based on prospective residual interaction analysis, 5-FU+AC01 and 5-FU+AC02 appeared promising.

DISCUSSION

5-FU was one of the chemotherapy agents used in the treatment of various types of cancer, including breast cancer. 5-FU, being a purine nucleoside analog, disrupted DNA replication and interfered with RNA synthesis, thereby inhibiting the growth of cancer cells and potentially causing cell death [28]. While 5-FU was not designed to directly target BCL2, it was often prescribed in cancer therapy along with other drugs that targeted BCL2. On the other hand, Lapatinib was a tyrosine kinase inhibitor that targeted ERBB2 (HER2) and EGFR (epidermal growth factor receptor), components of pathways associated with breast cancer cell growth. 5-FU and Lapatinib were frequently prescribed together in cancer treatment [29]. Therefore, it was important to study how 5-FU interacted with BCL-2, especially in the context of combination therapy.

BCL-2 was one member of the anti-apoptotic protein family with several binding sites that played a role in regulating the apoptosis (cell death) process. One of BCL-2's main binding sites was the hydrophobic pocket P2 and P4, which were used in strong interactions with proapoptotic proteins [30]. Some BCL-2 inhibitor compounds could produce electrostatic interactions with BCL-2, such as hydrogen bonding when an inhibitor intercalated into the BCL-2 binding pocket. Compounds like Navitoclax and its analogs, including ABT-737 and ABT-199, which were anticancer drugs, had interactions with BCL-2 at these hot spots P2 and P4 [31,32]. For instance, ABT-737 (a native ligand, 1XJ), when complexed with BCL-2, had a chloro-biphenyl group intercalating into pocket P2 of BCL-2, referred to as H2. The thio-phenyl group filled P4 of BCL-2, thus called H4. Ligand intercalation at the BCL-2 hot spot (and also BCL-X) was often followed by electrostatic bonds in addition to hydrophobic interactions. Tryptophan residue (Trp30), which intercalated with hot spot P4, formed a hydrogen bond with Asp103 through the nitrogen atom of Navitoclax indole [33,34].

In simultaneous docking simulations, 5-FU+AC01 and 5-FU+AC02 demonstrated behavior similar to Navitoclax. They shared the same binding site, occupying both P2 and P4. Specifically, 5-FU occupied the P2 hot spot, substituting Navitoclax's chloro-biphenyl group, while one of AC01's phenol groups intercalated into P4, replacing the thio-phenyl group. These results implied a promising potential for synergistic action between 5-FU and AC01/AC02, effectively hindering cancer cells by binding to BCL-2, reminiscent of the mechanisms employed by established BCL-2 inhibitors. Moreover, these combinations exhibited remarkable stability during a 250 ns dynamics study and displayed strong binding affinity to BCL2, approaching the affinity level of 5-FU+FMM. This result suggested that this combination could synergistically affect by targeting BCL2 and provide promising novel therapeutic strategies for breast cancer treatment.

CONCLUSION

The interaction between the BCL-2 protein and the 5-FU+AC01, 5-FU+AC02, and 5-FU+Lapatinib pairs was studied using molecular docking and dynamics methods. Docking validation had confirmed its reliability. All three ligand pairs had strongly bound to BCL-2 with negative binding energies, and molecular dynamics simulations had revealed stability. Protein fluctuations during the simulation had not affected ligand interactions, and MMPBSA calculations had shown a strong affinity to BCL-2. AC01 and AC02, which had intercalated into P2 and P4 sites of BCL-2, had been promising anticancer candidates for further research alone or in combination with 5-FU.

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AUTHORS CONTRIBUTIONS

All the authors have equally contributed to the current study.

CONFLICT OF INTERESTS

All the authors declare no conflicts of interest.

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