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Anticancer Activity of Pyrazoline A - D on MCF7 Breast Cancer Cell Line and Molecular Docking on Proliferation Proteins

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Abstract

Pyrazoline is a versatile heterocyclic compound with excellent biological activity, especially as a new therapeutic agent for anticancer. This study examined the binding of N-pyrazolines A - D in MCF7 cells to PKC, TRL7, and ERB/HER-2 proteins. N-phenyl-pyrazoline derivatives A, B, C, and D were evaluated for their anticancer activity against the MCF7 breast cancer cell line and their binding affinity to proliferation-related proteins PKC, TLR7, and ERB/HER-2, using *in vitro* and *in silico* methods. Pyrazoline C and D exhibited high anticancer activity with IC_{50} values of 0.43 and 1.21 µg/mL, respectively, while pyrazoline A showed moderate activity (IC_{50} = 86.74 µg/mL) and pyrazoline B was inactive (IC_{50} > 1,000 µg/mL). Molecular docking studies revealed that pyrazoline D had the best binding affinity to PKC (–9.532 kcal/mol), TLR7 (–5.964 kcal/mol), and ERB/HER-2 (–8.416 kcal/mol) compared to the other pyrazolines and native ligands. Pyrazoline C also showed better binding affinity than the native ligand for TLR7-A and similar binding affinity to PKC and ERB/HER-2. The binding interactions of pyrazolines C and D involved pi-sigma, pi-pi stacked, alkyl, pi-alkyl, and specific hydrogen-bonding interactions with key amino acid residues in the target proteins. These findings suggest that pyrazolines C and D have potential as anticancer agents with anti-proliferative activities.

Keywords: Anticancer, Breast cancer, N-phenyl-Pyrazoline, Molecular docking

Introduction

Breast cancer has a notably high prevalence among malignancies affecting females, ranking second in incidence alone to cervical cancer [1]. The development of breast cancer is caused by complex interactions between various factors, such as genetic, environmental, nutritional, hormonal, and hereditary factors; obesity; smoking; alcohol consumption; early

menarche; late menopause; inactive lifestyle; and hormone replacement therapy [2].

A study on breast cancer suggested that overexpression of several protein kinases C (PKC) has been reported in malignant human breast tissues and breast cancer cell lines [3]. PKC plays a pivotal role in the regulation of malignant cell proliferation, apoptosis, invasiveness, and migration [4]. It is particularly implicated in the progression of triple-negative breast cancer (TNBC) [5]. Toll-like receptors (TLRs) have been identified as key contributors to breast cancer development. TLR activation can cause pro-oncogenic effects such as triggering inflammation, activating proliferative signalling pathways, and suppressing antitumor immunity [6]. TLRs also contribute to hematopoietic malignancies [7]. ERB (also known as HER2) is another protein that is involved in cancer cell proliferation. Overexpression and/or mutation of these receptors results in increased cell proliferation, motility, angiogenic factors, tumor progression, and resistance to chemotherapy [8]. ERB-related genes are amplified or overexpressed in approximately 30% of human breast cancers, leading to increased metastasis [9]. The important roles of PKC, TRL7 and ERB/HER2 in cancer progression render them highly attractive targets for therapeutic intervention in breast cancer.

N-acetyl pyrazoline derivatives contain methoxy and chloro/hydroxyl substituents [10]. N-phenyl-pyrazoline was synthesized by reacting phenylhydrazine with chalcone derivatives [11,12]. Pyrazoline is a versatile heterocyclic compound with easy synthesis and structural modification, making it an excellent biological agent, especially new therapeutic agents for anticancer [13]. Previous studies have demonstrated that N-phenyl-pyrazolines possess several anticancer properties [14]. Pyrazoline A has moderate activity against breast cancer cell lines T47D and HeLa cells [10]. Pyrazoline 2 was active anticancer against the breast cancer cell line 4T1, cervical cancer cell line HeLa, and colorectal cancer line WiDr [11] and pyrazoline 2 and 5 were active against the HeLa cell line [12]. Pyrazoline 4d exhibits high toxicity toward HeLa and WiDr cells [13], and pyrazoline 4 is a potent compound in T47D and WiDr cell lines [15]. Pyrazoline 5 treatment significantly inhibited the proliferation rate of Hs578T and MDA MB 231 cells and reduced cell migration, tumor sphere size, and EGFR expression levels and activation in TNBC cells. Drug interaction analysis also showed that the combination of pyrazoline with paclitaxel and doxorubicin is safe and synergistically inhibits cancer cell proliferation [16,17].

Pyrazoline derivatives have also been studied using *in silico* molecular docking for many types of target proteins in cancer cells. Molecular docking of the epidermal growth factor (EGFR) receptor revealed that pyrazoline 2 [11] and pyrazoline 4 [15] had the lowest binding energies. EGFR appears to be the primary pathway targeted by pyrazoline derivatives, whereas ligands are likely to target the VEGFR1, VEGFR2, or P450 pathways when they successfully overcome binding barriers [18]. Pyrazoline 4d exhibits hydrogen bonding and low binding affinity for cyclooxygenase-2 (COX-2) [13]. Chlorinated thienyl-pyrazoline P1 and P11 exhibit the highest binding affinities for EGFR and COX-2 [19]. Pyrazoline C has potential as a breast cancer drug with COX-2 and HER-2 inhibitory activities, as well as docking analysis and molecular dynamics simulations [1,14]. The aim of this study was to investigate the efficacy of N-pyrazolines A, B, C and D in inhibiting cell growth in the breast cancer cell line MCF7 and to evaluate their binding affinity to PKC, TRL7, and ERB/HER-2 proteins, which are key players in cancer metastasis that have not been evaluated in previous studies.

Materials and methods

Cell culture and materials

MCF7 cells were maintained in DMEM (Thermo Fisher Scientific, Inc.) with 0.5% amphotericin B, 10% FBS (Gibco™, Thermo Fisher Scientific, Inc.), and 1% penicillin–streptomycin. Cell line culture was maintained in an incubator (CellExpert®, Eppendorf) at 37 °C with 5% CO_2. For maintenance, the cells were sub-cultured every 2 - 3 days and the number of viable cells was observed. N-phenyl-pyrazoline derivatives were acquired from the Chemistry Department of the Faculty of Mathematics and Natural Sciences at Universitas Gadjah Mada, specifically from the laboratory of Professor Tutik Dwi Wahyuningsih. The synthesis of N-phenyl pyrazoline derivatives was performed according to a previously published protocol [12]

Anticancer assay

MCF7 cells (5×10^3) were seeded in each well of a 96-well plate was seeded with 5×10^3 MCF7 cells. The cells were treated with N-phenyl pyrazoline compounds A, B, C and D, following incubation in basal DMEM. DMSO (Merck) was applied to the control groups for both cell types at a concentration equivalent to the maximum DMSO concentration (0.04%) used in the treatment groups. Following a 48-h incubation period, the medium was removed from each well. After adding 100 µL of 10% 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (Invitrogen), cells were incubated for 4 h at 37 °C in an incubator with 5% CO_2. The cells in each well were treated with 100 µL of 10% sodium dodecyl sulfate (SDS, Merck). Incubation was performed overnight at room temperature in the dark. Absorbance was quantified using a microplate reader (Bio-Rad) at a wavelength of 565 nm [16]. IC_{50} analyses were performed using the GraphPad Prism software (version 10.0.2).

***In-silico* molecular docking**

Finding the best shape for the PDB protein-ligand combination was the 1st stage in the *in-silico* test. Subsequently, the native ligand is eliminated to create a cavity or pocket in the ligand-binding region. Prior to docking with the ligand of the drug to be evaluated (Pyrazoline A, B, C and D), the molecular docking method was validated and the docking grid box was determined. Re-docking of native ligands on proteins whose native ligands were eliminated using the Autodock tool served as validation [20]. For docking validation, the Root Mean Square Deviation (RMSD) of the native ligand-analyzed protein complex was computed. If the RMSD value in the re-docking procedure was less than 2.0 angstroms (Å), the protein was used for further docking. An alternative conformation with a different PDB ID must be used instead of the specific conformation when the RMSD value is more than 2.0 angstroms (Å). After collecting the PKC, TRL7, and ERB/HER-2 proteins that were utilized for further docking, the grid box was determined. The grid, which is shown in the box coordinates in the AutoDockTools application, is the region where the interaction of a ligand and its protein or macromolecule should occur. Following re-docking of proteins linked to their corresponding native ligands, grids representing the binding regions of the proteins were produced. For all 3 proteins tested, the grids spacing were 0.375 Å, with the npts of 40, 40, 40 for the x, y, and z axis respectfully. The coordinates for PKC were 20.133, 11.009 and 15.742 for the x, y, and z axes, respectively; for TLR-7A, they were 10.045, –6.160 and 6.021 for the x, y, and z axes, respectively; and ERB/HER2 had grid coordinates of 8.413, –8.996 and –13.532 for the x, y, and z axes, respectively. The grid coordinates were used throughout all molecules docked with their respective protein. MolView, an online application based on a 2D structure, was used to prepare the pyrazoline 3D structure. PKC, TRL7, and ERB/HER-2 proteins, isolated from their natural ligands, were docked to this 3-dimensional structure. AutoDockTools were used to perform the docking operation for each protein. The ability of the ligand to bind to the related protein was demonstrated by a ligand-receptor complex with a binding energy of less than 0 kcal/mol. Then, binding energy of each pyrazoline reduced by binding energy of native ligand then was divided to each native ligand counterpart and presented as percentage of binding affinity (%). The Discovery Studio application was used to process 2D and 3D visualizations of amino acid residues interacting with the ligand [21].

Results and discussion

The results of this study showed that N-phenyl-pyrazoline A, B, C and D exhibited different anticancer activities in MCF7 breast cancer cells (**Figure 1**). Pyrazoline C showed high anticancer activity with an IC_{50} of 0.43 µg/mL, and pyrazoline D of 1.21 µg/mL. Moderate anticancer activity was shown by pyrazoline A with an IC_{50} of 86.74 ug/mL, whereas pyrazoline B did not show anticancer activity ($IC_{50} > 1{,}000$ µg/mL).

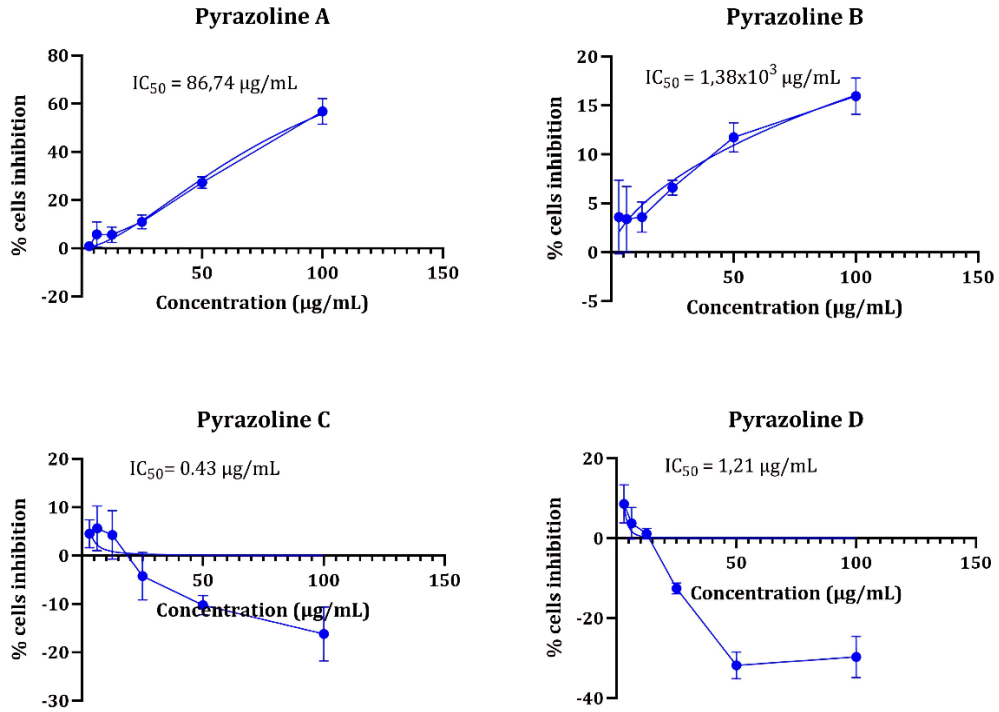

Figure 1 Anticancer activity of pyrazoline (A), (B), (C), and (D) on breast cancer cell line MCF7.

This result was in accordance with a previous study [10] showing that pyrazoline A shows moderate anticancer activity and has moderate inhibitory activity against all tested cancer cell lines, MCF7 (IC_{50} 40.47 µg/mL), T47D (IC_{50} 26.51 µg/mL), and HeLa (IC_{50} 31.19 µg/mL). Pyrazoline B did not exhibit anticancer activity (IC_{50} > 100 µg/mL) in any of the cell lines tested in this study. Different results were shown in a previous study that pyrazoline C has moderate activity in the inhibition of MCF7 (IC_{50} 94.02 µg/mL) and is inactive against T47D and HeLa cells. Pyrazoline was inactive in T47D and HeLa cell lines [10], whereas MCF7 showed high activity in this study (**Figure 1**). Similar results were obtained for Pyrazoline D, which was active in Hs578T, a human triple-negative breast cancer (TNBC) cell line. Pyrazoline D has an IC_{50} of 26.79 µM in Hs578T cells [16]. Thus, consistent with previous studies, N-pyrazolines C and D have high potential for use as anticancer candidates. Based on normal cell toxicity tests, pyrazoline C has good selectivity for normal cells, with an IC_{50} value of 44×10^3 µg/mL [10].

Based on this structure, there are structural differences between pyrazolines A, B, C, and D (**Figure 2**). The difference in their activities is due to the presence of functional groups attached to the molecule [10]. Pyrazoline has a heterocyclic structure and several hybrid compounds have been studied and reported as potent anticancer agents [22].

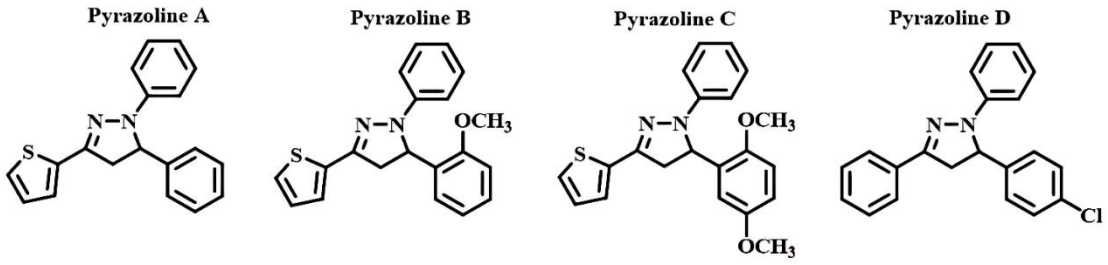

Figure 2 Pyrazoline (A), (B), (C), and (D) structure.

Several modes of action of pyrazolines have been reported in previous studies, including serine/threonine protein kinase (STPKs) inhibitors, kinesin spindle protein inhibitors, androgen or estrogen biosynthesis

inhibitors, carbonic anhydrase inhibitors, cell cycle arrest and/or induction of apoptosis, telomerase inhibitors, aminopeptidase N (APN) inhibitors, DNA binding and/or topoisomerase II inhibitors, heat shock protein 90 (Hsp90) inhibitors, tubulin polymerase inhibitors, and receptor tyrosine kinase inhibitors [23]. Another study reported the anticancer mechanism of lung cancer A549 using a compound containing a pyrazole ring with mitochondrial membrane potential (MMP) and EGFR inhibitory activity [24]. Pyrazoline derivatives have also been reported to have anticancer potential against human lung cancer (A549, IC_{50} = 12 - 14 μM) and fibrosarcoma cell lines (HT1080, IC_{50} = 11 - 23 μM) with low toxicity to human primary normal lung cells (HFL-1, IC_{50} = 116 - 152 μM) [25].

Table 1 The binding affinity of pyrazoline A, B, C and D on proliferation proteins of cancer cells.

Ligand	Binding affinity	RMSD	Binds to amino acids
PKC Protein (PDB ID 3TXO)			
2,6-Naphthyridines (NPT)	–8.341	0.383	Phe366, Ala496, Lys384, Glu403, Val369, Ala382, Val436, Phe644, Asp440, Leu486, Met433
Pyrazoline A	–8.286	0.557	Met433, Ala382, Leu486, Val436, Leu361, Val369, Phe366, Asp497, Ala496
Pyrazoline B	–8.149	0.618	Phe644, Leu486, Ala382, Leu361, Val436, Phe366, Lys384, Val369
Pyrazoline C	–8.364	0.366	Phe644, Leu486, Val436, Ala382, Leu361, Phe366, Lys384, Val369
Pyrazoline D	–9.532	0.172	Ala496, Phe366, Asp440, Phe644, Leu361, Val369, Leu486, Ala382, Val436
TLR7-A Protein (PDB ID 5GMF)			
Guanosin (GMPa)	–4.315	0.571	Ser490, Asp548, Tyr546, Cys491, Cys521, Asn523
Pyrazoline A	–5.360	0.649	Asn523, Cys491, Tyr492, Val459, Leu460, Arg378, Asp427
Pyrazoline B	–5.434	0.859	Asn523, Cys491, Tyr492, Asp499, Asp427, Ser429, Val459
Pyrazoline C	–5.327	0.975	Tyr492, Asn523, Cys491, Glu461, Val459, Leu460
Pyrazoline D	–5.964	0.571	Asn523, Tyr492, Cys491, Val459
ERB/HER2 Protein (PDB ID 7PCD)			
HER2 Inhibitor (70I)	–7.672	0.741	Ala751, Gln799, Leu852, Met801, Leu726, Gly804, Asp808, Cys805, Lys753, Val734, Leu796, Leu785, Ala771, Phe864, Ser783
Pyrazoline A	–7.605	0.435	Lys753, Val734, Asp863, Thr862, Leu852, Ala751, Thr798
Pyrazoline B	–7.841	0.336	Met801, Ala751, Leu852, Asp863, Val734, Lys753, Ser783, Thr798
Pyrazoline C	–7.459	0.281	Ala751, Leu785, Thr789, Met801, Leu852, Thr862, Asp863, Val734, Lys753
Pyrazoline D	–8.416	0.205	Leu852, Lys860, Met801, Thr798, Ala751, Lys753, Val734, Asp863

Affinity (Gibbs energy (kcal · mol^{-1})). RMSD = Root Mean Square Deviation. +

Based on the *in silico* molecular docking test, Pyrazoline D had the best binding affinity values for PKC (−9.532 kcal/mol), TRL7 (–5.964 kcal/mol), and ERB/HER-2 (–8.416 kcal/mol) proteins, compared to other pyrazolines, as well as native ligands 2,6-Naphthyridines (NPT), Guanosin (GMPa), and HER2 Inhibitor (70I) (**Table 1**). The percentage differences in the binding affinity of pyrazoline D compared to the native ligand were 14.28%, 38.22% and 9.70% higher than those of NPT, GMPa, and 70I, respectively. Pyrazoline C showed better binding affinity than the native ligand on TRL7-A but had similar binding to the native ligand on NPT and slightly lower affinity to the native ligand HER-2 inhibitor. This indicated that pyrazoline C has potency as a PKC, TRL7-A, and HER-2 inhibitor. This result was related to a previous study on molecular docking and molecular dynamics of COX-2 and HER-2 proteins. Pyrazoline C was the ligand with the highest affinity (–109.218 kcal/mol) compared to the native ligand 03Q (–170.697 kcal/mol) and neratinib (–83.416 kcal/mol). Pyrazoline C has the potential to be developed as a breast cancer drug that inhibits COX-2. Molecular dynamics simulations for 50 ns showed that RMSD, RMSF, and SASA were rigid and stable. Pyrazoline C has the potential to be developed as a breast cancer drug with HER-2 inhibitory activity [1,14].

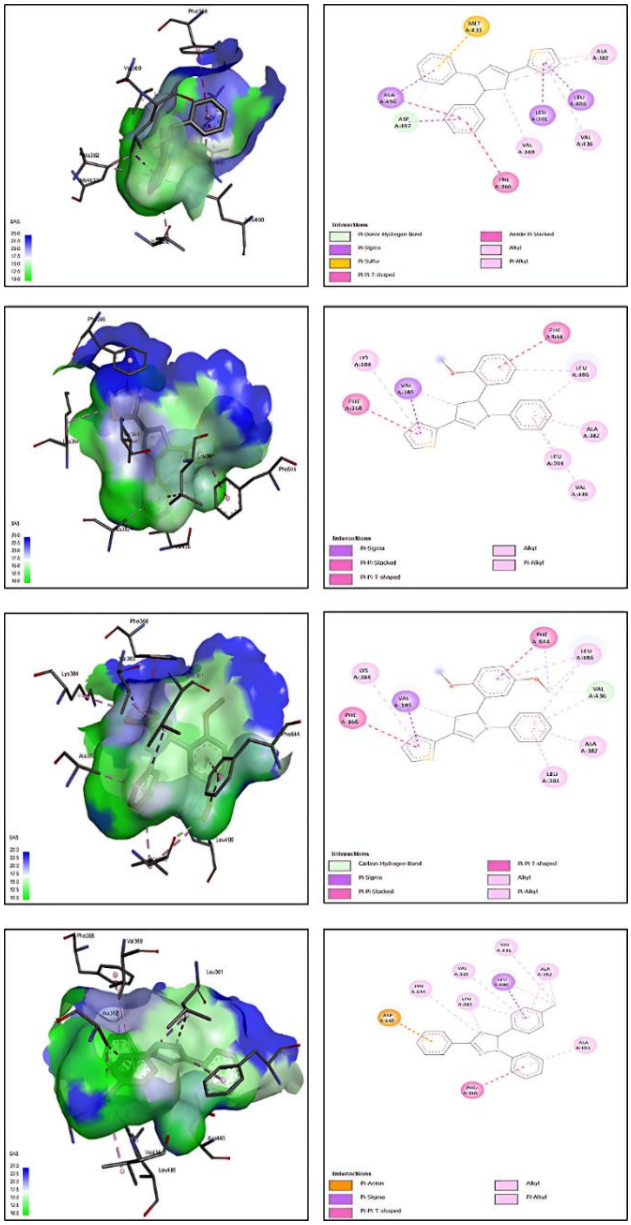

Figure 3 Three- and 2-dimensional docking visualization of pyrazoline (A) - (D) on PKC protein.

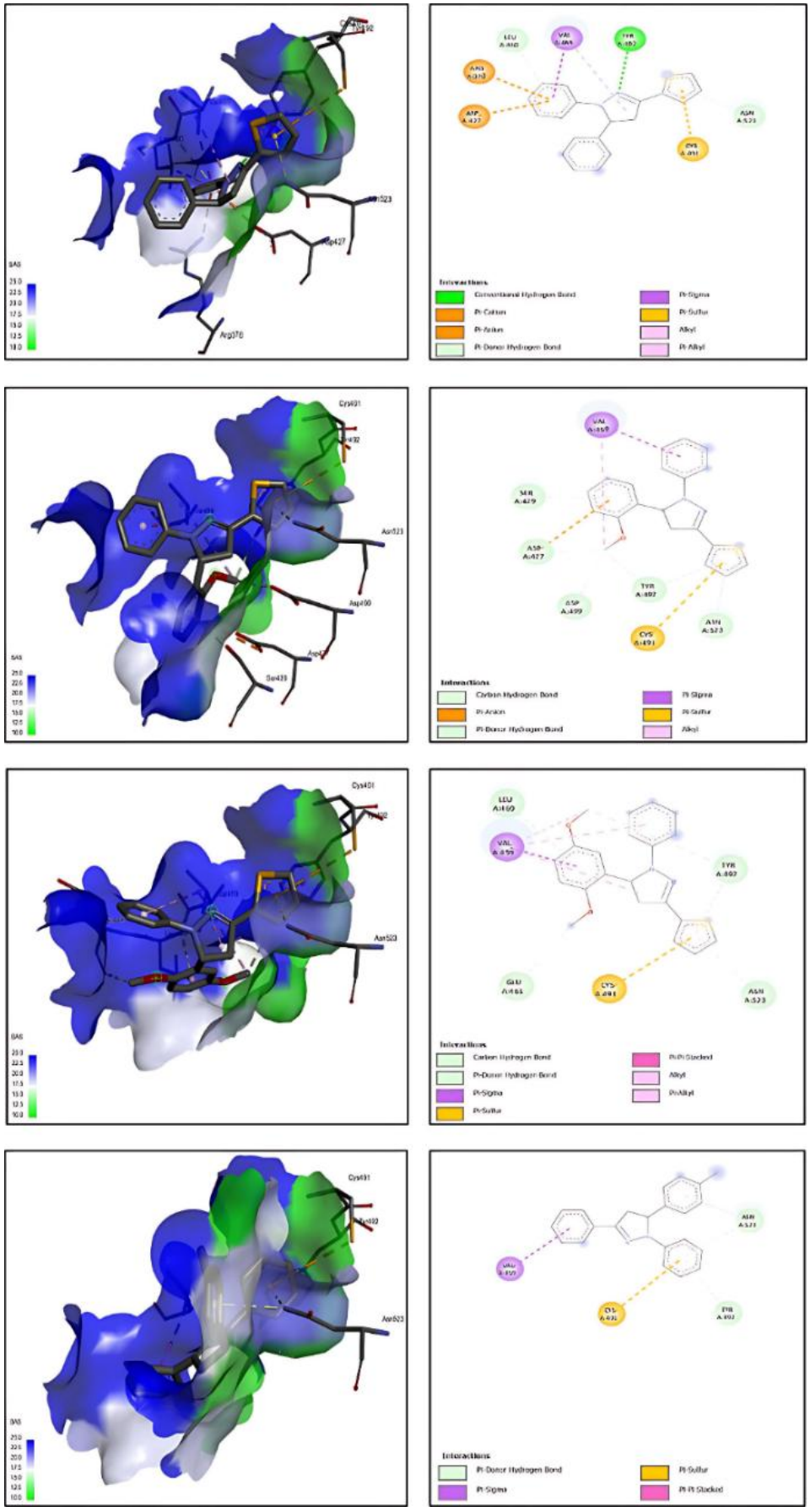

Figure 4 Three- and 2-dimensional docking visualization of pyrazoline (A) - (D) on TLR7-A protein.

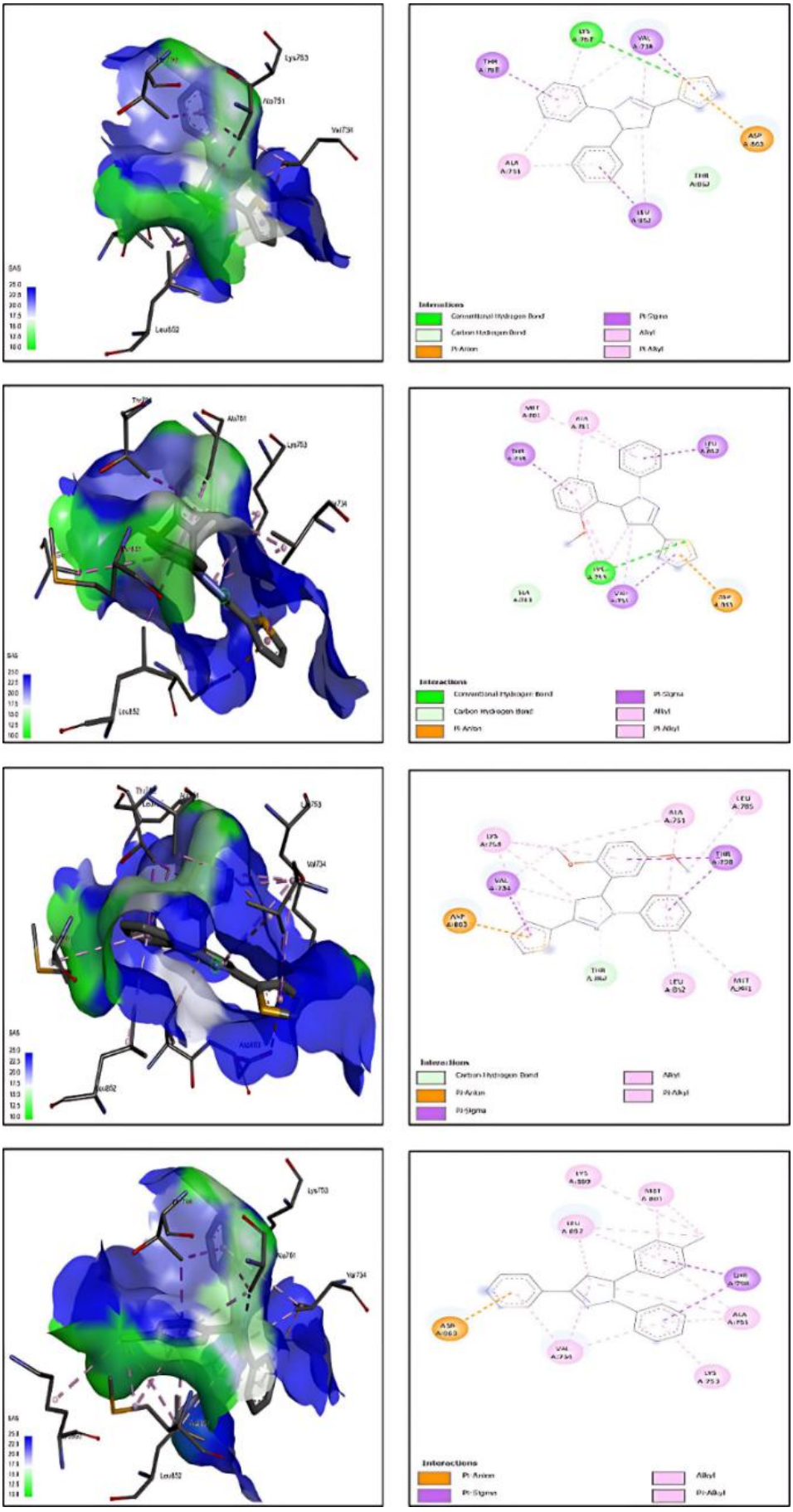

Figure 5 Three- and 2-dimensional docking visualization of pyrazoline (A) - (D) on ERB/HER-2 protein.

The binding affinity of pyrazoline to PKC, TRL7-A, and ERB/HER-2, proliferation-related proteins in cancer cells, was related to the pyrazoline hybrid in a previous study. The coumarin–pyrazoline hybrids exhibited excellent anti-proliferative activity. Further studies have shown that the functional group at N-1 of the pyrazoline ring exerts a strong influence on cytotoxicity in cancer cells [26]. This indicates the need for further research regarding the mode of action of pyrazoline as an anti-proliferative agent in this study. Pyrazolines D and C on PKC have the highest binding affinity. Pyrazolines D and C on PKC have Pi-sigma, Pi-pi T-shaped, Alkyl, and Pi-alkyl interactions. Pyrazoline D has a specific Pi-anion interaction, whereas Pyrazoline C has a specific carbon-hydrogen bond interaction, and Pi-Pi is stacked on the PKC protein (**Figure 3**). All pyrazolines in the TRL7-A protein showed better binding affinities than their native ligands. Pyrazolines D and C on the TRL7-A protein have Pi-sigma, Pi-sulfur and Pi-Pi stacking interactions. The specific interaction was the Pi-donor hydrogen bond on Pyrazoline D and the carbon-hydrogen bond, Alkyl and Pi-Alkyl interactions on Pyrazoline C (**Figure 4**). In the ERB/HER-2 protein, all pyrazolines showed better binding affinities than the native ligand, except for pyrazoline C, which was slightly lower than that of the native ligand. Pyrazolines D and C, with high binding affinity for the ERB/HER-2 protein, have Pi-Anion, Pi-Sigma, Alkyl, and Pi-Alkyl interactions, and a specific carbon-hydrogen bond in pyrazoline C (**Figure 5**).

The binding affinity of pyrazoline C and D in this study on PKC and HER-2 proteins was higher than the binding affinity on VEGFR1 (–5.1 and –6.0 kcal/mol) and VGEFR2 (–6.6 and –7.2 kcal/mol) [18]. In comparison to previous studies, the present research demonstrates that the binding affinity of pyrazoline D to the PKC protein is superior to that of pyrazolines 1 - 5 to the EGFR protein, with values ranging from –7.6 to –8.8 kcal/mol [11] and –7.85 to –8.04 kcal/mol [15]. With other target proteins on cancer cells, such as EGFR and COX-2, pyrazoline D also has a higher binding affinity than other novel thienyl pyrazoline derivatives (–7.8 to –8.7 kcal/mol for EGFR and –6.4 to –8.4 kcal/mol for COX-2) [19].

Overall, the findings of this study demonstrate a strong relationship between telomerase and anti-proliferative activity, suggesting that when the active substance inhibits telomerase, it also inhibits the proliferation of cancer cells. In the G0/G1 phase, the pyrazoline hybrid molecule induces cell cycle arrest, which inhibits cell proliferation [27]. Pyrazoline B showed affinity binding to Topoisomerase I and Topoisomerase II [17] but did not show anticancer activity in MCF7 cells in this study. Thus, the anti-proliferative mechanism of these pyrazolines involves other pathways.

In another study, pyrazole derivatives in the MTT assay showed anti-proliferative activity in 4 human cancer cell lines (MGC-803, HeLa, MCF-7, and Bel-7404) via a mechanism that induces G2/M cell cycle arrest and apoptosis in MGC-803 cells [28]. According to earlier research, the simultaneous targeting of EGFR and HER2 is a proven anticancer therapy. The most potent anti-proliferative derivatives were pyrazoline derivatives, and additional research has indicated that they are dual EGFR/HER2 inhibitors. According to a cell cycle study, this chemical causes 78.53% pre-G1 apoptosis and 5.28% necrosis during early apoptosis [29]. This indicated that pyrazolines C and D are potential anticancer agents with anti-proliferative effects. Further research, especially to validate the mechanism of action of pyrazolines D and C as anti-proliferative agents based on molecular and specific gene targets, is important.

Conclusions

N-phenyl-pyrazoline C and D exhibited high anticancer activity (IC_{50} = 0.43 and 1.21 µg/mL), while A showed moderate activity and B was inactive. Molecular docking revealed that pyrazoline D had the best binding affinity for the target proteins compared to other pyrazolines and native ligands. Pyrazoline C also showed better or similar binding affinity to native ligands. The binding interactions involved Pi-sigma, Pi-stacked, Alkyl, Pi-alkyl and hydrogen bonds with the key amino acid residues. These findings suggest that pyrazolines C and D have potential as anticancer agents with anti-proliferative activity, warranting further investigation of their mechanisms of action and molecular targets.

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