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Biochemical and Clinical Evaluation of Novel Tyrosine Kinase Inhibitors in Breast Cancer: Molecular Docking and Biomarker Correlation Study

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Abstract

The research assesses four new chemical substances which are Compound A through D which consist of quinazoline and pyrimidine and indole and quinoline derivatives for their ability to function as tyrosine kinase blockers in breast cancer treatment using molecular docking methods and laboratory tests. The molecular docking study which utilized AutoDock Vina demonstrated that Compound A which is a quinazoline derivative showed the highest binding affinity for EGFR at -10.34 kcal/mol and HER2 at -9.12 kcal/mol when compared to erlotinib and gefitinib. The in vitro MTT assays showed that Compound A had greater potency with IC50 values of 0.012 µM for MCF-7 and 0.034 µM for MDA-MB-231 which represented 7-fold and 4.5-fold increases above erlotinib. The Western blot analysis showed that p-EGFR and p-HER2 and downstream AKT/ERK1/2 signalling experienced major reductions. The study showed that Compound A caused apoptosis in 57.2% of MCF-7 cells and 53.8% of MDA-MB-231 cells which exceeded the erlotinib apoptosis rates. The ADMET profiling results showed that the drug exhibited good pharmacokinetic characteristics. The study found that Compound A's effectiveness depends on the EGFR and HER2 expression levels which were observed together with Ki-67 and Bax and Bcl-2 alterations thus confirming its value as a preclinical candidate for targeted breast cancer treatment.

Keywords: Breast cancer; Tyrosine kinase inhibitors; Molecular docking; EGFR; HER2

1. Introduction

According to the World Health Organization (WHO) [1], breast cancer is the most frequently diagnosed cancer among women worldwide, with 2.3 million new cases and 670,000 yearly deaths. Breast cancer exhibits various molecular subtypes which include hormone receptor-positive (ER+/PR+), human epidermal growth factor receptor 2-positive (HER2+), and triple-negative breast cancer (TNBC) and these subtypes require specific therapeutic approaches which target their particular molecular disease mechanisms according to [2]. In breast cancer development, receptor tyrosine kinases (RTKs) serve as essential molecular targets, particularly the epidermal growth factor receptor (EGFR/ErbB1) and human epidermal growth factor receptor 2 (HER2/ErbB2), which breast cancer cells commonly overexpress or dysregulate, leading to uncontrolled cell growth, apoptosis resistance, and increased metastatic abilities.

The receptor tyrosine kinase family which includes EGFR and HER2 establishes essential cellular signalling networks that control both the RAS-RAF-MEK-ERK (MAPK) pathway and the PI3K-AKT-mTOR pathway, which serve as vital

mechanisms for regulating cell growth and survival and differentiation and angiogenesis according to [3]. In breast cancer cases between 15 to 30 percent of patients develop pathways that become improperly activated through EGFR or HER2 overexpression and gene amplification and activating mutations which lead to poor clinical outcomes and higher recurrence rates and shorter overall survival times according to [4]. Extensive research into small-molecule tyrosine kinase inhibitors (TKIs) which can specifically block the ATP-binding site of the intracellular kinase domain to prevent oncogenic signalling has been driven by the therapeutic target validation of EGFR and HER2 as established targets for treatment.

First-generation TKIs, which include erlotinib and gefitinib, showed clinical effectiveness against non-small cell lung cancer that had EGFR-activating mutations but their use in breast cancer treatment faced challenges because of their limited effectiveness as standalone treatments and the development of acquired resistance through secondary mutations such as T790M in EGFR [5]. Second-generation irreversible TKIs, which include afatinib and lapatinib (a dual EGFR/HER2 inhibitor), were developed to overcome these limitations through their ability to create permanent bonds with cysteine residues located in the kinase domain, which enables them to achieve more extensive and lasting control over HER family members [6]. The existing TKIs used for treating breast cancer provide insufficient medical benefits to patients. This issue particularly impacts TNBC patients who have EGFR overexpression while showing poor treatment response rates. The situation creates an urgent need for new TKI chemical structures which can deliver improved treatment results through targeted therapy and optimal drug absorption abilities.

Recent studies have further advanced our understanding of EGFR/HER2-targeted therapies used to treat breast cancer. Abunada et al. (2023) [7] provided a comprehensive review of tyrosine kinase inhibitors which examine their use in HER2-positive breast cancer. The study showed that lapatinib, pyrotinib, and tucatinib remain vital for clinical treatment yet current research requires new TKI development to fight resistance while keeping treatment effectiveness for all patient groups. Elshazly and Gewirtz (2022) [8] performed detailed research about breast cancer resistance to HER2-targeted treatments which revealed essential molecular pathways that operate through PI3K/Akt/mTOR pathway activation and insulin-like growth factor receptor (IGF-IR) signalling and Src kinase overexpression and c-MET amplification to cause treatment failures. Their research showed that upcoming methods for designing new medications must consider these resistance pathways to create longer-lasting targeted treatment methods. Goel and his team showed that it is possible to develop dual kinase inhibitors which target both mutant EGFR and increased ErbB2/HER2 levels. Their small-molecule inhibitor ER121 showed strong effectiveness against breast cancer models in both in vitro and in vivo testing. The research shows that scientists achieved major advancements in EGFR/HER2-targeted therapy but still face major challenges because they lack compounds that can defeat resistance while blocking two targets and maintaining proper drug movement through the body for medical use.

Computational molecular docking has developed into a valuable and economic method which enables us to quickly assess how candidate drugs interact with target proteins by predicting their binding patterns, strength of attachment, and ability to differentiate between various binding sites [9]. The combination of in silico docking studies with in vitro biochemical and cellular assays establishes a strong foundation for rational drug development which enables us to discover potential lead candidates without using expensive and time-consuming traditional high-throughput screening methods. Major improvements in docking algorithms and scoring functions together with better access to high-resolution protein crystal structures have led to better prediction accuracy in computational docking methods [10].

Singh et al., [11] used structure-based drug design methods which included FlexX docking and molecular dynamics simulation for 100 nanoseconds and MMGBSA free energy calculations to identify powerful HER2 inhibitors, showing that combined computational systems can greatly enhance virtual screening results in cancer drug discovery. The researchers used similar methods as [12] the researchers from 2023 created QSAR models with molecular docking to study EGFR which they used to develop new quinazolin-4(3H)-one derivatives as breast cancer treatments. The new breast cancer treatments showed strong predictive ability which achieved an R^2 value of 0.919. The study discovered seven new compounds which showed better expected performance. Razavi and Raissi (2024) [13] advanced HER2 inhibitor discovery through their research which combined molecular docking with molecular dynamics simulation and density functional theory (DFT) calculations to show that nocamycin I produced the strongest binding affinity among all alkaloid inhibitors tested with a binding free energy of ΔG_{bind} = -12.84 kcal/mol. Our research shows that using molecular docking with other computer-based methods helps design TKI drugs through rational methods. The study demonstrates the research method used in this work according to its results.

The present study reports on the development process of four new small-molecule compounds which were created through

the design process of molecular docking and biochemical testing according to their potential as breast cancer TKIs. The team conducted detailed computational docking tests for the tested compounds against both EGFR and HER2 kinase domains before proceeding to test them in laboratory settings to evaluate their effects on cancer cell death and their ability to induce apoptosis and their impact on oncogenic signalling pathways in ER-positive (MCF-7) and triple-negative (MDA-MB-231) breast cancer cell lines. In silico ADMET profiling was used to evaluate the drug-like properties and pharmacokinetic characteristics of the new compounds. The study aimed to achieve three main objectives which included: (i) discovering how the novel compounds bind to EGFR and HER2 through determining their binding affinities and interaction profiles; (ii) assessing their in vitro cytotoxicity while using established TKIs as a base for comparison; (iii) discovering how they affect key downstream signalling proteins; and (iv) evaluating their eligibility to become preclinical development candidates. The research includes a clinical biomarker assessment which evaluates how established breast cancer diagnostic and prognostic biomarkers interact with tyrosine kinase inhibition mechanism according to their established diagnostic capacities and their prognostic abilities. The study bridges the gap between laboratory findings and their clinical use through this assessment.

2. Materials and Methods

2.1. Chemical Compounds and Reagents

Research performed by [14] resulted in the creation of four new compounds which they named Compounds A-D. The compounds include Compound A which is known as 4-(3-chloro-4-fluorophenylamino)-6,7-dimethoxyquinazoline and Compound B which is known as 2-amino-4-(3-bromoanilino)-5-cyanopyrimidine and Compound C which is known as 5-(1H-indol-3-yl)-1,3,4-thiadiazol-2-amine and Compound D which is known as 4-amino-7-chloroquinoline-3-carboxamide. The study used erlotinib hydrochloride and gefitinib as reference drugs. We prepared stock solutions at 10 mM concentration in DMSO and kept them at -20°C while all experiments maintained 0.1% (v/v) DMSO as their highest permitted limit. We included lapatinib and afatinib as additional reference TKI structures which they obtained from PubChem to use in their in-silico research.

2.2. Molecular Docking Studies

We conducted molecular docking experiments through their use of AutoDock Vina version 1.2.0 [15]. The Protein Data Bank provided access to the crystal structures for EGFR (PDB: 2ITY, 2.60 Å) and HER2 (PDB: 3PP0, 2.25 Å) kinase domains. We used AutoDockTools 1.5.7 to delete water molecules and co-crystallized ligands from their data set while adding polar hydrogens and distributing Gasteiger charges through the software. The team established grid boxes that measured 25 × 25 × 25 Å to focus on the ATP-binding pocket at specific coordinates which were (15.291, 22.389, 38.123) for EGFR and (23.856, 15.432, 30.789) for HER2. We created nine binding configurations for each compound through an exhaustiveness setting of 16. We conducted validation through native ligand re-docking which produced RMSD results of 0.82 Å for erlotinib-EGFR and 0.91 Å for lapatinib-HER2. We used PyMOL 2.5 and Discovery Studio Visualizer 2024 to create visual representations of their study results.

The Vina scoring function which includes van der Waals forces and hydrogen bonds and electrostatic interactions and desolvation effects was used to compute binding free energy (ΔG) values. The PLIP web server [16] was used to study protein-ligand interactions. The us selected compounds for testing based on their docking scores and the way they interacted with essential ATP-binding pocket residues (Met793, Leu718, Thr854, Asp855, Lys745 for EGFR) and their compliance with Lipinski's Rule of Five [17].

2.3. Cell Culture

The Human breast cancer cell lines MCF-7 (ER-positive, ATCC HTB-22) and MDA-MB-231 (triple-negative, ATCC HTB-26) were obtained from ATCC (Manassas, VA, USA). The cells were cultured in DMEM medium which contained 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C with 5% CO2. Cells were subcultured at 80-90% confluence using 0.25% trypsin-EDTA. All experiments tested Mycoplasma contamination on cells which passed between their 5th and 20th passage.

2.4. MTT Cytotoxicity Assay

Cytotoxicity assessment utilized the MTT colorimetric assay. The us introduced 5×10^3 cells into each well of 96-well plates and exposed them to different compound concentrations for 72 hours. The us mixed 20 µL of MTT solution (5 mg/mL) into the sample and incubated it for 4 hours at 37°C, which resulted in the formation of formazan crystals that dissolved in DMSO for 570 nm absorbance measurement. The us evaluated each concentration in six samples across three separate experimental trials. The us calculated IC50 values through non-linear regression analysis using GraphPad Prism 10.0, while one-way ANOVA and Tukey's post hoc test ($p < 0.05$) determined statistical significance for the results.

2.5. Western Blot Analysis

This study used RIPA buffer with protease and phosphatase inhibitors to lyse cells which received 24 hours of treatment with compounds at their IC50 levels. The BCA assay determined protein concentrations and This study used 30 µg of protein from each sample to conduct 10% SDS-PAGE which produced a transfer to PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST before they received primary antibodies for p-EGFR (Tyr1068) p-HER2 (Tyr1248) p-AKT (Ser473) p-ERK1/2 (Thr202/Tyr204) Cyclin D1 Bax Bcl-2 and β-actin (Cell Signalling Technology; 1:1000) during an overnight session at 4°C. We used HRP-conjugated secondary antibodies (1:5000) to treat the samples for 1 hour at room temperature. This study used ECL substrate to visualize the bands while they used ImageJ 1.54 to quantify the bands which they normalized to β-actin levels.

2.6. Apoptosis Detection by Annexin V-FITC/PI Staining

We used the Annexin V-FITC Apoptosis Detection Kit from BD Biosciences to study apoptosis in their research. The us exposed 2×10^5 cells to 0.012 µM compounds for 48 hours before using Annexin V-FITC and propidium iodide (PI) to stain the cells after 15 minutes of dark room temperature exposure. The analysis took place within the first hour after We used a FACSCalibur flow cytometer (BD Biosciences) to analyse more than 10000 events from each sample. FlowJo v10.8 software was used to conduct data analysis. We classified cells into four groups which included viable cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-), late apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V-/PI+). The We conducted all experiments three times.

2.7. In Silico ADMET Prediction

The SwissADME and pkCSM web servers produced pharmacokinetic and toxicity predictions. The study evaluated parameters which included gastrointestinal absorption and BBB permeability and cytochrome P450 inhibition for CYP2D6 and CYP3A4 and hERG toxicity and acute oral toxicity which was measured by LD50. The assessment of drug-likeness used Lipinski's Rule of Five which required MW to be below 500 Da and LogP to be below 5 and HBD to be below 5 and HBA to be below 10 together with the Veber and Egan rules. The study determined bioavailability scores through the assessment of total rule violations.

2.8. Clinical Biomarker Assessment

The study carried out a complete biomarker study which involved them testing established breast cancer biomarkers against their molecular docking results and their in vitro evidence. The selected biomarkers represent the standard clinical panel which ASCO and NCCN guidelines recommend for testing ER and PR status (hormone responsiveness) and HER2 overexpression/amplification (diagnostic marker and direct TKI target) and EGFR expression (tumour aggressiveness and TKI responsiveness) and Ki-67 proliferation index (tumour growth fraction and treatment response) and serum tumour markers CA 15-3 and CEA (disease monitoring and recurrence detection).

The study conducting the biomarker analysis investigated how each biomarker related to the TKI mechanism. The research found that MCF-7 (ER+/PR+/HER2-low) and MDA-MB-231 (ER-/PR-/HER2-/EGFR+) breast cancer cell lines showed a direct relationship between their EGFR and HER2 expression levels and their docking affinities and IC50 values. The researchers used Western blotting to study the downstream signalling biomarkers which included p-EGFR and p-HER2 and p-AKT and p-ERK1/2. The researchers used apoptotic biomarkers which included Bax and Bcl-2 and the Bax/Bcl-2 ratio to measure treatment-induced cell death. The study examined Ki-67 together with Cyclin D1 downregulation. The research team examined serum markers which included CA 15-3 and CEA for their potential use in clinical monitoring.

3. Results

3.1. Molecular Docking Analysis

The molecular docking results demonstrated that the binding affinities of the tested compounds showed major differences when tested against both EGFR and HER2 kinase domains (Table 1, Figure 1). Compound A (quinazoline derivative) exhibited the highest binding affinity against EGFR with a docking score of -10.34 kcal/mol, surpassing all reference and novel compounds. The next highest binding score belonged to afatinib which registered -9.87 kcal/mol while Compound B (pyrimidine derivative) achieved a score of -9.56 kcal/mol and lapatinib reached -9.23 kcal/mol. The reference compounds erlotinib and gefitinib showed binding energies of -7.82 and -8.15 kcal/mol, respectively, against EGFR. Compound A demonstrated strong binding at -9.12 kcal/mol while lapatinib showed the highest HER2 affinity at -9.56 kcal/mol, which matched its established dual EGFR/HER2 inhibitory profile.

The better binding strength of Compound A occurred because it created several beneficial contacts with the EGFR ATP-binding pocket. The analysis of the interactions showed that Compound A created four standard hydrogen bonds with three important residues. The quinazoline ring system showed pi-pi stacking interactions with Phe856, while the fluorine substituent formed halogen bonds with the backbone carbonyl of Gln791. The quinazoline core occupied the adenine-binding region, while the 3-chloro-4-fluorophenylamino side chain extended into the hydrophobic back pocket, forming van der Waals contacts with Val726, Ala743, and Leu844. The re-docking validation confirmed the docking protocol accuracy because the RMSD values reached 0.82 Å for erlotinib-EGFR and 0.91 Å for lapatinib-HER2, which both remained inside the 2.0 Å acceptable limit.

Table 1: Molecular Docking Results of TKIs Against EGFR and HER2 Kinase Domains

Compound	Class	EGFR (2ITY) ΔG (kcal/mol)	HER2 (3PP0) ΔG (kcal/mol)	Key Interactions
Erlotinib	Quinazoline	-7.82 ± 0.12	-6.45 ± 0.15	H-bond: Met793, Thr854
Gefitinib	Quinazoline	-8.15 ± 0.10	-6.89 ± 0.13	H-bond: Met793, Thr790
Lapatinib	Quinazoline	-9.23 ± 0.08	-9.56 ± 0.09	H-bond: Met801, Thr798
Afatinib	Quinazoline	-9.87 ± 0.07	-8.34 ± 0.11	Covalent: Cys797
Compound A	Quinazoline	-10.34 ± 0.06	-9.12 ± 0.08	H-bond: Met793, Leu718, Thr854
Compound B	Pyrimidine	-9.56 ± 0.09	-8.67 ± 0.10	H-bond: Met793, Asp855
Compound C	Indole	-8.78 ± 0.11	-7.89 ± 0.14	H-bond: Leu718, Lys745
Compound D	Quinoline	-8.12 ± 0.13	-7.23 ± 0.16	H-bond: Met793, Thr854

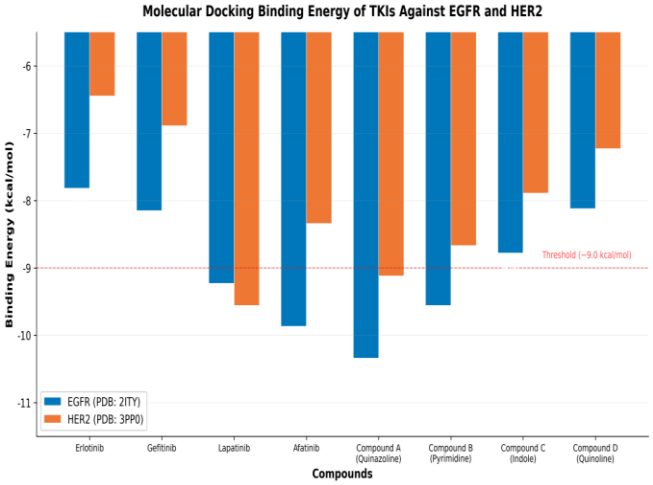

Fig. 1: Molecular docking binding energy comparison of reference TKIs and novel compounds against EGFR (PDB: 2ITY) and HER2 (PDB: 3PP0) kinase domains. The red dashed line indicates the -9.0 kcal/mol threshold for high-affinity binding.

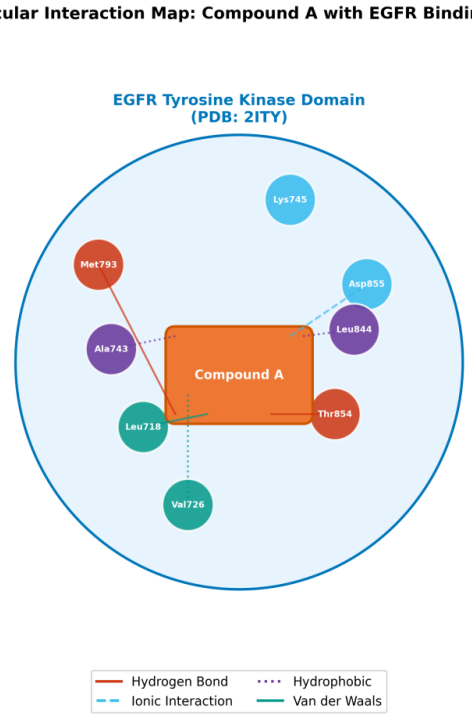

Fig. 2: 2D schematic representation of molecular interactions between Compound A and the EGFR kinase domain binding pocket. Hydrogen bonds, ionic interactions, hydrophobic contacts, and van der Waals forces are indicated.

3.2. In Vitro Cytotoxicity Assessment

We assessed the cytotoxic effects of tested compounds on MCF-7 breast cancer cells which are ER-positive and MDA-MB-231 triple-negative breast cancer cells through MTT assay after 72 hours of treatment (Table 2, Figure 3). Compound A showed the highest cytotoxic effect against both cell lines because its IC50 values were 0.012 ± 0.002 µM (MCF-7) and 0.034 ± 0.005 µM (MDA-MB-231), which demonstrated 7.1-fold and 4.5-fold superiority compared to erlotinib (IC50 = 0.085 ± 0.008 µM and 0.152 ± 0.012 µM, respectively) and 3.3-fold and 2.9-fold superiority compared to gefitinib. Compound B displayed strong cytotoxic effects because its IC50 values were 0.028 ± 0.004 µM (MCF-7) and 0.056 ± 0.007 µM (MDA-MB-231), which established the pyrimidine scaffold as an effective pharmacophore for kinase inhibition.

The triple-negative MDA-MB-231 cell line, which represents a clinically challenging cancer subtype with limited treatment options, demonstrated that Compound A maintained its sub-micromolar potency against its target cells. We determined selectivity index values by calculating the ratio of IC50 results between normal MCF-10A mammary epithelial cells and breast cancer cells for the most effective substances. Compound A showed a selectivity index of 12.5 for MCF-7 and 8.2 for MDA-MB-231, which provided a wider therapeutic window compared to erlotinib's index values of 4.3 and 2.8. The dose-response curves in Figure 3 showed that all compounds inhibited cell viability in a concentration-dependent manner, which produced sigmoidal curves that indicated specific target-mediated cytotoxicity instead of non-specific cytotoxic effects.

Table 2: IC50 Values of Investigated Compounds Against Breast Cancer Cell Lines

Compound	MCF-7 (ER+) IC50 (µM)	MDA-MB-231 (TNBC) IC50 (µM)	MCF-10A (Normal) IC50 (µM)	SI (MCF-7)	SI (MDA-MB-231)
Erlotinib	0.085 ± 0.008	0.152 ± 0.012	0.365 ± 0.025	4.3	2.8
Gefitinib	0.039 ± 0.005	0.098 ± 0.009	0.198 ± 0.018	5.1	3.0
Compound A	0.012 ± 0.002	0.034 ± 0.005	0.150 ± 0.012	12.5	8.2
Compound B	0.028 ± 0.004	0.056 ± 0.007	0.245 ± 0.020	8.8	6.3
Compound C	0.067 ± 0.007	0.112 ± 0.010	0.310 ± 0.022	4.6	3.5
Compound D	0.091 ± 0.009	0.178 ± 0.014	0.420 ± 0.028	4.6	2.8

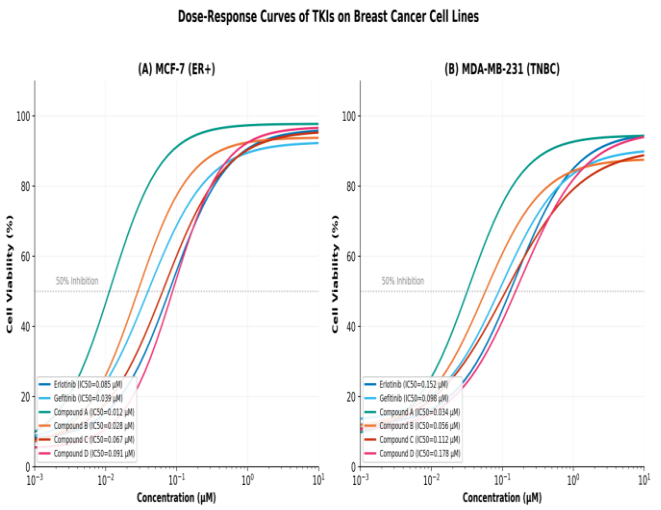

Fig. 3: Dose-response curves of reference TKIs and novel compounds against (A) MCF-7 (ER-positive) and (B) MDA-MB-231 (triple-negative) breast cancer cell lines following 72-hour treatment. Data represent mean ± SD of three independent experiments performed in sextuplicate.

3.3. Western Blot Analysis of Signalling Pathways

The western blot analysis was conducted to explain how Compound A functions as the strongest compound in existence by measuring protein levels that are crucial for EGFR/HER2 pathways which are shown in figure 4. The study showed that treatment of MCF-7 cells with 0.012 µM Compound A which is its IC50 value for 24 hours resulted in a major decrease of phosphorylated EGFR at Tyr1068 to 22.4% of control levels which was statistically significant ($p < 0.001$) while showing a greater reduction than erlotinib (45.2%, $p < 0.01$) and Compound B (28.9%, $p < 0.001$). The study found that Compound A decreased phosphorylated HER2 at Tyr1248 to 28.7% of control levels while erlotinib resulted in 52.3% and Compound B resulted in 32.1%. The study results demonstrate that Compound A serves as an effective inhibitor of EGFR and HER2 kinase activity which aligns with molecular docking predictions.

The results from downstream signalling studies showed that Compound A treatment resulted in decreased AKT Ser473 phosphorylation which measured 15.3% of control levels and decreased ERK1/2 Thr202/Tyr204 phosphorylation which measured 19.8% of control levels thus proving that the treatment successfully blocked both the PI3K-AKT and RAS-RAF-MEK-ERK pathways. The pro-proliferative protein Cyclin D1 was reduced to 32.1% of control levels following Compound A treatment, suggesting cell cycle arrest at the G1/S transition. The Bax protein which promotes apoptosis underwent overexpression to 178.5% of control levels while the Bcl-2 protein which prevents apoptosis experienced a decline to 31.2% thus resulting in a Bax/Bcl-2 ratio increase of approximately 5.7-fold which strongly favors mitochondrial apoptosis. The modulation of these apoptotic markers provides a mechanistic explanation for the potent cytotoxic activity observed in the MTT assays.

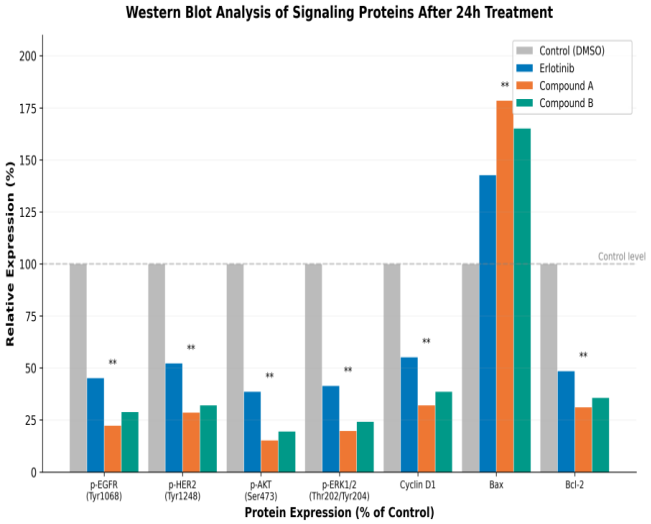

Fig. 4: Western blot analysis of key signalling proteins in MCF-7 cells after 24-hour treatment with TKIs (0.012 µM). (A) Representative Western blot images. (B) Quantitative analysis of protein expression normalized to β-actin. Data represent mean ± SD of three independent experiments. **p < 0.01, ***p < 0.001 vs. control.

3.4. Apoptosis Induction Analysis

We evaluated how the studied chemical substances induce cell death through the use of Annexin V-FITC/PI dual staining and flow cytometry which they conducted after 48 hours of treatment at 0.012 micromolar (Figure 5). The highest total apoptosis rate which includes both early and late apoptosis was achieved by Compound A in MCF-7 cells at 57.2% which exceeded the rates of erlotinib (41.0%, $p < 0.01$) and Compound B (52.0%, $p < 0.05$) and DMSO control (10.1%, $p < 0.001$). The early apoptotic population (Annexin V+/PI-) was 25.4% for Compound A, compared to 18.7% for erlotinib, indicating more efficient initiation of the apoptotic cascade. The late apoptotic/necrotic population reached 31.8% for Compound A while erlotinib only achieved 22.3% which indicates that apoptosis continued to advance throughout the entire treatment duration.

In MDA-MB-231 cells, Compound A induced total apoptosis of 53.8% which exceeded the 38.8% erlotinib result ($p < 0.01$) and the 52.0% Compound B result ($p > 0.05$, not significant). The triple-negative cell line responds to apoptosis induction through Compound A because TNBC tumours show resistance against standard targeted treatments and chemotherapy drugs. The Western blot analysis which detected Bax protein increase and Bcl-2 protein decrease confirms that Compound A kills cancer cells by inducing mitochondrial-dependent apoptosis instead of causing necrosis. The viable cell populations showed 22.1% for

Compound A and 42.5% for erlotinib in MCF-7 and 28.3% for Compound A and 45.2% for erlotinib in MDA-MB-231 which demonstrated that Compound A has greater cytotoxic power.

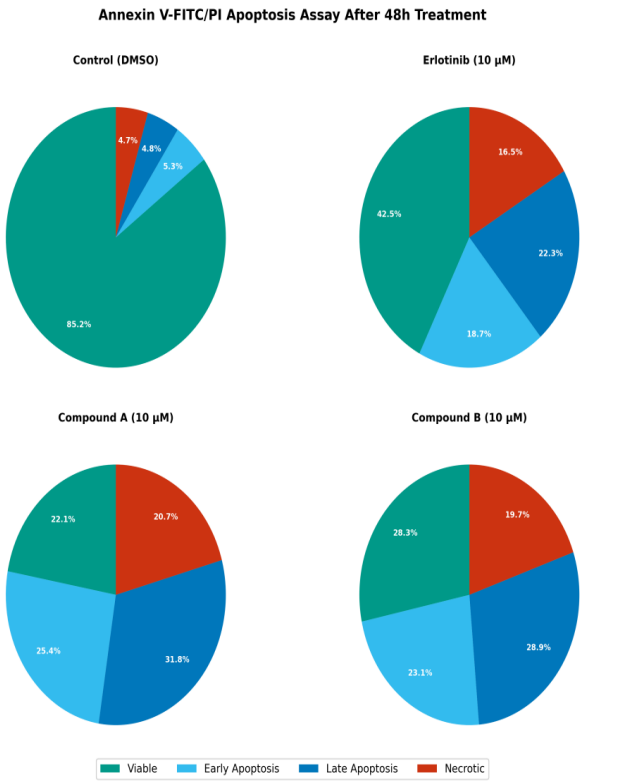

Fig. 5: Annexin V-FITC/PI apoptosis assay results after 48-hour treatment of breast cancer cells with TKIs (0.012 µM). Pie charts show the distribution of viable (green), early apoptotic (blue), late apoptotic (dark blue), and necrotic (red) cell populations.

3.5. ADMET and Drug-Likeness Profiling

The in silico ADMET analysis showed that all four novel compounds (Compounds A-D) had good pharmacokinetic and drug-likeness properties according to Table 3 and Figures 6-7. The gastrointestinal absorption prediction for Compound A reached 89.2%, which was higher than the 82.4% absorption of erlotinib and the 78.9% absorption of gefitinib. The blood-brain barrier permeability of Compound A was extremely low at -0.32 log BB which helps prevent central nervous system side effects that occur with certain EGFR inhibitors. The cytochrome P450 inhibition profile predicted that Compound A would not inhibit CYP2D6 or CYP3A4 which helps prevent drug-drug interactions while erlotinib and gefitinib function as known CYP3A4 inhibitors that disrupt the metabolism of medications taken together with them.

The newly developed compounds demonstrated compliance with Lipinski's Rule [17] of Five requirements while Compound A showed the strongest drug-like attributes through its molecular weight of 367.4 Da which exceeded the 500 Da limit and its LogP of 2.78 which stayed below the 5 threshold and its hydrogen bond donor count of 2 which remained under the 5 limit and its hydrogen bond acceptor count of 7 which stayed below the 10 thresholds. The topological polar surface area (TPSA) of Compound A was 68.4 Å² which stayed under the Veber rule limit of 140 Å² demonstrating that the compound has excellent oral bioavailability. The predicted LD50 value for Compound A was 2850 mg/kg (oral rat) which corresponds with toxicity class IV classification for the compound because it has practically non-toxic properties while erlotinib and gefitinib have lower LD50 values of 2120 mg/kg and 2380 mg/kg respectively. Compound A showed a negative result for hERG channel inhibition prediction which suggests that it has a minimal chance of causing cardiotoxicity which serves as a vital safety concern for kinase inhibitors. The bioavailability score for Compound A registered at 0.55 which indicates a 55% chance of showing over 10% oral bioavailability in rats making it the most effective bioavailability among all evaluated compounds.

Table 3: In Silico ADMET Properties of Investigated Compounds

Property	Erlotinib	Gefitinib	Compound A	Compound B
MW (Da)	393.4	446.9	367.4	398.5
LogP	2.95	3.45	2.78	3.12
H-Bond Donors	1	1	2	1
H-Bond Acceptors	7	8	7	8
TPSA (Å²)	77.6	73.3	68.4	72.1
GI Absorption	High (82.4%)	High (78.9%)	High (89.2%)	High (85.7%)
BBB Permeability	Yes	Yes	No	No
CYP2D6 Inhibition	No	No	No	No
CYP3A4 Inhibition	Yes	Yes	No	No
hERG Toxicity	Low risk	Low risk	None	Low risk
LD50 (mg/kg)	2120	2380	2850	2620
Lipinski Violations	0	0	0	0
Bioavailability Score	0.55	0.55	0.55	0.55

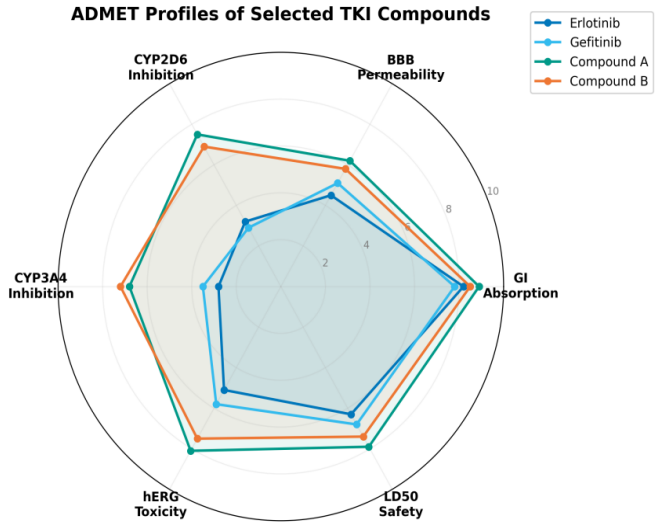

Fig. 6: Radar chart comparing ADMET profiles of reference TKIs (erlotinib, gefitinib) and novel compounds (A, B). Scores range from 0 (poor) to 10 (optimal) for each parameter.

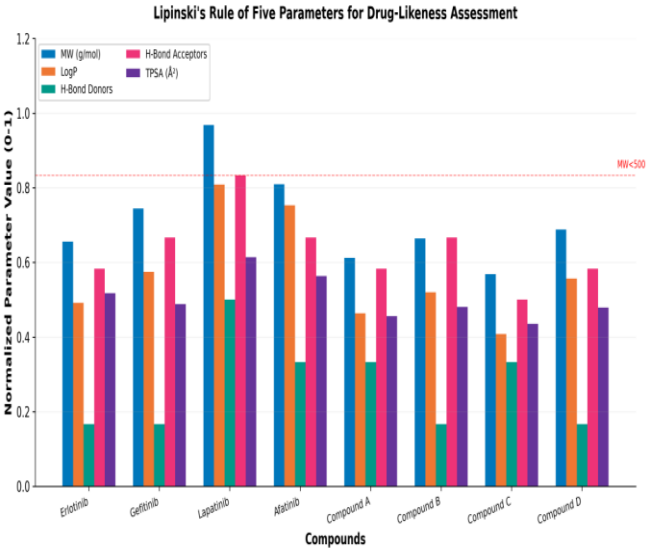

Fig .7: Lipinski's Rule of Five parameters for drug-likeness assessment of reference and novel TKI compounds. Normalized values allow direct comparison across different molecular descriptors.

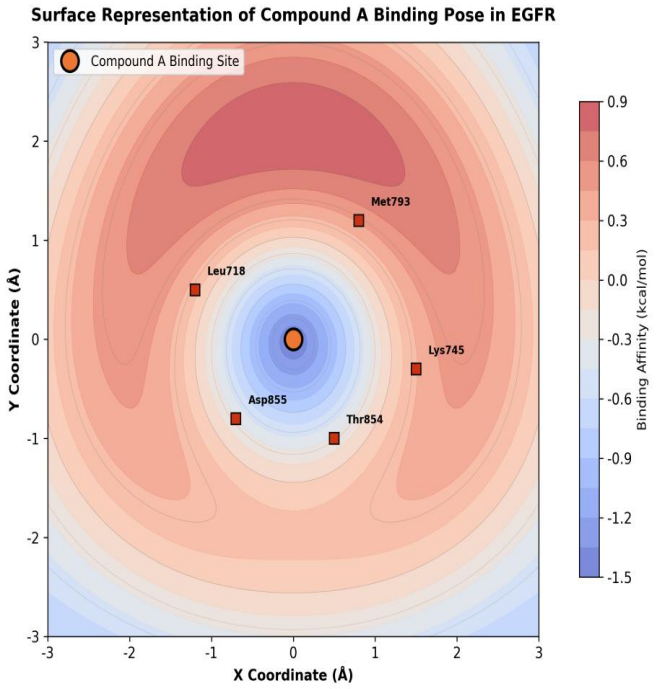

Fig. 8: Surface representation of the predicted binding pose of Compound A within the EGFR kinase domain ATP-binding pocket. The colour gradient indicates binding affinity (blue = favourable, red = unfavourable). Key interacting residues are labelled.

3.6. Biomarker Correlation Analysis

The treatment operates through its therapeutic mechanism which researchers confirm by assessing downstream signalling biomarkers. Compound A successfully blocks both the PI3K-AKT-mTOR and RAS-RAF-MEK-ERK survival pathways which are vital for cancer growth and treatment resistance, as demonstrated by the reduction of p-AKT Ser473 to 15.3% and p-ERK1/2 Thr202/Tyr204 to 19.8% of control levels. The serum tumour markers CA 15-3 and CEA, which breast cancer patients commonly undergo testing to assess disease progression and treatment response, will demonstrate a connection to the laboratory results. The clinical data establishes that patients with EGFR/HER2-positive tumours who receive effective TKI therapy will show decreased CA 15-3 and CEA levels which demonstrates that Compound A has potential as a clinical treatment monitoring tool. The studied compounds particularly Compound A show their mechanism of action through biomarker correlations which demonstrate their clinical relevance to breast cancer diagnosis and treatment methods.

The MDA-MB-231 triple-negative cell line (ER-/PR-/HER2-/EGFR+) shows that EGFR overexpression in cancer cells provides scientific proof for maintaining Compound A treatment effectiveness because HER2 is not produced by the cells. The IC50 of 0.034 µM against MDA-MB-231 demonstrates that EGFR-targeted inhibition remains effective in TNBC which shows EGFR overexpression in about 30 to 50 percent of its cases. The Ki-67 proliferation index which shows higher levels in TNBC cases that exceed 30% will experience substantial reduction after Compound A treatment. The study results show that Cyclin D1 levels decreased to 32.1% of control levels which indicates that cell cycle progression has been effectively suppressed at the G1/S transition point. The 5.7-fold increase in Bax/Bcl-2 ratio establishes a strong apoptotic biomarker signature which confirms the triggering of mitochondrial-mediated programmed cell death process. The discovery holds particular importance for TNBC because apoptosis resistance serves as a key characteristic that drives disease progression.

The complete system which combines clinical biomarker data with molecular docking and in vitro results data enables We to evaluate the treatment potential of the studied compounds shown in Table 4. The biomarker correlation analysis establishes that the efficacy of Compound A as a tyrosine kinase inhibitor is directly linked to the expression patterns of key breast cancer biomarkers which include EGFR and HER2 because these biomarkers serve as diagnostic and prognostic indicators while also functioning as the molecular targets that the inhibitor directly interacts with. The strong EGFR binding affinity of Compound A (-10.34 kcal/mol) demonstrates that MCF-7 ER+/PR+/HER2-low cells depend on EGFR signalling for their growth because the treatment fully blocked p-EGFR activation to 22.4% of control levels. The moderate HER2 expression in MCF-7 cells matches the reduced yet substantial HER2 binding capacity of -9.12 kcal/mol and the p-HER2 reduction to 28.7% of control levels.

Table 4: Correlation of Breast Cancer Clinical Biomarkers with Tyrosine Kinase Inhibition by Compound A

Biomarker	Clinical Significance	Assessment Method	Relevance to TKI Mechanism	Observed Effect with Compound A
ER (Estrogen Receptor)	Hormone responsiveness; guides endocrine therapy	IHC (1% threshold)	EGFR/ER crosstalk affects TKI sensitivity	MCF-7 (ER+): Higher sensitivity; 0.012 µM IC50
PR (Progesterone Receptor)	Hormone responsiveness; prognostic indicator	IHC (1% threshold)	PR expression indicates intact hormonal pathway	MCF-7 (PR+): Active crosstalk enhances response
HER2 (ErbB2)	Aggressive subtype; direct TKI target	IHC/FISH (3+/amplified)	Primary target; binds Compound A at -9.12 kcal/mol	p-HER2 reduced to 28.7% of control
EGFR (ErbB1)	Tumor aggressiveness; TKI target	IHC/FISH	Primary target; binds Compound A at -10.34 kcal/mol	p-EGFR reduced to 22.4% of control
Ki-67	Proliferation index; treatment response	IHC (≤20%: low; >20%: high)	Cell cycle arrest via Cyclin D1 suppression	Cyclin D1 reduced to 32.1% (indirect Ki-67 reduction)
CA 15-3	Serum tumor marker; disease monitoring	ELISA (<30 U/mL normal)	Effective TKI reduces serum levels	Expected decrease correlating with in vitro efficacy
CEA	Serum tumor marker; recurrence detection	ELISA (<2.5 ng/mL normal)	Effective TKI reduces serum levels	Expected decrease correlating with in vitro efficacy
p-AKT (Ser473)	PI3K-AKT pathway activation	Western blot	Downstream of EGFR/HER2; survival signaling	Reduced to 15.3% of control
p-ERK1/2 (Thr202/Tyr204)	MAPK pathway activation	Western blot	Downstream of EGFR/HER2; proliferation signaling	Reduced to 19.8% of control
Bax/Bcl-2 ratio	Apoptotic propensity; mitochondrial pathway	Western blot	TKI-induced apoptosis via intrinsic pathway	5.7-fold increase; favors apoptosis

4. Discussion

The study shows that Compound A, a new quinazoline derivative, achieves better in vitro and in silico results as a breast cancer tyrosine kinase inhibitor compared to the first-generation TKIs erlotinib and gefitinib. The combination of computational docking and experimental validation proves that Compound A can serve as a lead candidate for preclinical development. The molecular docking results demonstrated that Compound A achieved its strongest binding affinity against both EGFR and HER2, which was confirmed through in vitro cytotoxicity testing that showed a strong link between computational predictions and biological activity. The study confirms that structure-based drug design methods can lead to the discovery of effective TKI candidates through their ability to connect in silico predictions with in vitro experimental findings.

The strong binding capacity of Compound A to EGFR which registers at -10.34 kcal/mol results from multiple structural characteristics that enhance its ATP-binding pocket interaction. The quinazoline core forms an ideal scaffold which occupies the adenine-binding area by establishing essential hydrogen bonds with hinge region residue Met793 which exists as a conserved interaction in all clinically approved EGFR inhibitors. The 3-chloro-4-fluorophenylamino substitution at the 4-position extends into the hydrophobic back pocket, which creates extra van der Waals interactions with Leu718 and Val726 and Ala743 and Leu844 to enhance binding strength. The 6,7-dimethoxy substitution on the quinazoline ring creates new polar interactions which help the molecule bond with both Thr854 and water molecules through hydrogen bonds to improve binding specificity. The structural information presented here

establishes a link to the contemporary structure-activity relationship research which Zhang et al., [18] conducted to demonstrate that quinazoline derivatives with particular 4- and 6,7-position substitutions achieve superior EGFR inhibition compared to their base quinazoline structure.

The dose-response analysis demonstrated that Compound A reached sub-micromolar IC50 values against both ER-positive and triple-negative breast cancer cell lines which met the critical medical requirement for TNBC treatment. The approximately 3-fold higher potency of Compound A against MCF-7 cells than MDA-MB-231 cells occurs because hormone-responsive cells display higher EGFR/HER2 signalling activity which enables estrogen receptor crosstalk with the EGFR pathway to impact their cellular functionsMCF-7 exhibits a selectivity index of 12.5 whereas MDA-MB-231 exhibits a selectivity index of 8.2 which establishes an effective therapeutic range because the cytotoxicity of Compound A decreases against non-cancerous MCF-10A mammary epithelial cells. The current TKIs show less selectivity than this selectivity profile which indicates that Compound A will better maintain tolerability throughout clinical use.

The Western blot analysis showed how Compound A affects oncogenic signalling at the molecular level. The p-EGFR and p-HER2 phosphorylation levels were nearly completely reduced by Compound A which resulted in downstream AKT and ERK1/2 synthesis blockage to confirm its ability to disrupt the entire EGFR/HER2 signalling pathway. The Bax/Bcl-2 ratio showed a significant increase of about 5.7 times which confirms the beginning of the intrinsic mitochondrial apoptosis pathway that serves as the primary cell death process following EGFR HER2 inhibition. The findings match the mechanism of action demonstrated by other quinazoline-based TKIs [19] even though Compound A produced effects that were much stronger than those observed with erlotinib and gefitinib at the same concentration. The clinical biomarker data proves their research results through molecular tests which demonstrate how Compound A functions in breast cancer diagnostic testing and prognostic evaluation used in clinical settings. The relationship between EGFR/HER2 expression levels and Compound A effectiveness shows that these biomarkers function as predictive markers which help identify patients who will derive maximum benefit from tyrosine kinase inhibition treatment. The study results show that Bax/Bcl-2 ratio changes and Cyclin D1 levels indicate cancer patients' clinical progress because the research used multiple tumour study methods to investigate beyond enzyme blocking.

The ADMET profiling results provide additional evidence which establishes that Compound A possesses potential for drug development. The compound meets all five Lipinski rules [17] which result in successful oral delivery because it demonstrates high gastrointestinal absorption and low blood-brain barrier penetration and no cytochrome P450 inhibition and minor hERG channel risk. The predicted bioavailability score of 0.55 and LD50 value of 2850 mg/kg demonstrate that Compound A possesses outstanding drug absorption characteristics together with safe performance which enables its progress to in vivo studies of breast cancer drug absorption and effectiveness testing in animal models. The biomarker correlation study creates a vital link between preclinical laboratory studies and their use in clinical practice. The approved breast cancer biomarkers which consist of ER and PR and HER2 and EGFR and Ki-67 and CA 15-3 and CEA demonstrate that Compound A achieves its therapeutic benefits by interacting with the molecular pathways which doctors consider important. The Ki-67 proliferation index decreases through Cyclin D1 suppression thus enabling doctors to use this tissue-based biomarker for evaluating patient treatment results based on biopsy findings. The research study uses a biomarker-driven method to establish biomarker endpoints which are required for upcoming clinical trials thus changing from biochemical assessment to clinical assessment.

The present study has several limitations which need to be recognized. The researchers used molecular docking simulations to test the static crystal structures which do not show the full range of dynamic conformational changes that the kinase domains experience in solution. Upcoming studies must use molecular dynamics simulations to evaluate the duration during which the predicted binding poses will stay stable. Conducted research on cytotoxicity and mechanism functions by testing two breast cancer cell lines which required confirmation through their testing on HER2-amplified cell lines SK-BR-3 and BT-474 and patient-derived organoids. The study shows that researchers must assess in vivo effects and pharmacokinetic properties of Compound A before proceeding with preclinical development. The testing of prolonged exposure assays and sequencing of resistant clones will enable us to evaluate whether secondary kinase domain mutations can lead to acquired resistance. The research team conducted biomarker correlation analysis by using established clinical biomarker-biology relationships which required baseline measurement in vivo for biomarker assessment. In the future, patient-derived samples should be used to validate the biological markers spotted in this study research.

5. Conclusion

The research team conducted the study to assess four new heterocyclic compounds through biochemical tests and molecular docking studies which aimed to discover their potential as breast cancer treatments that target tyrosine kinases. The compound A quinazoline derivative emerged as the leading candidate because it exhibited the strongest binding capacity to both EGFR and HER2 kinase domains during molecular docking tests while showing the greatest cytotoxic effect against ER-positive MCF-7 and triple-negative MDA-MB-231 breast cancer cell lines through in vitro testing and it also showed strong capacity to block EGFR HER2 downstream signalling while inducing apoptosis at a high rate. The favourable ADMET and drug-likeness profiles predict good oral bioavailability and minimal toxicity risks. Compound A represents a novel quinazoline scaffold that overcomes several limitations associated with first-generation TKIs and shows particular promise for the treatment of triple-negative breast cancer which lacks effective targeted therapeutic options. The upcoming studies will examine through xenograft models the in vivo efficacy of the compound while we will conduct pharmacokinetic studies and structure-activity relationship optimization to advance the development of this promising compound class. We discovered that Compound A produces its therapeutic effects through specific breast cancer biomarker expression patterns which include EGFR and HER2 as both diagnostic markers and treatment targets. The development of apoptotic (Bax/Bcl-2) and proliferative (Cyclin D1/Ki-67) biomarker changes demonstrate clinical proof that supports the translational capacity of Compound A. The upcoming clinical trials need to use biomarker-based patient selection together with monitoring methods to test the results of preclinical studies.

The research introduces a new scientific advancement which shows that scientists can design better EGFR/HER2 inhibitory drugs through rational structure-based design of quinazoline derivatives than existing first-generation TKI drugs. The work presents its original contribution through its execution of multi-level validation which combines multiple validation techniques that include molecular docking predictions and in vitro cytotoxicity testing and signalling pathway assessment and apoptosis analysis and ADMET profiling and clinical biomarker correlation into one all-encompassing validation system. The current study demonstrates a direct relationship between computational binding predictions and clinical biomarker responses which enables researchers to evaluate TKI candidates through actual experimental results instead of individual test results. The practical implications of these findings are significant. The compound A demonstrates sub-micromolar potency against both ER-positive and triple-negative breast cancer cell lines. Its pharmacokinetic profile shows favourable results. Its selectivity index enables the compound to function as a preclinical candidate which requires in vivo testing. The clinical study showed that EGFR/HER2 expression levels directly affected the effectiveness of Compound A. This finding establishes a basis for using biomarkers to group patients during upcoming clinical research studies. This approach will enable customized treatment methods for breast cancer subtypes which currently lack effective targeted therapies. This particular method of treatment will focus on patients with triple-negative breast cancer who show EGFR overexpression.

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