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ORIGINAL RESEARCH

Targeting the Inactive Conformation of the Epidermal Growth Factor Receptor Identifies EG31: A Novel Small Molecule Inhibitor Effective Against Normal and 5-Fluorouracil-Resistant Triple Negative Breast Cancer Cells

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Background: Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer characterized by the absence of estrogen, progesterone, and HER2 receptors, making it difficult to treat with targeted therapies. The Epidermal Growth Factor Receptor (EGFR) is overexpressed in TNBC and is crucial in promoting tumor growth and survival. Despite chemotherapeutics like 5-fluorouracil (5-FU), resistance remains a clinical challenge, underscoring the need for novel therapeutic strategies.

Methods: High-throughput virtual screening (HTVS) was employed to identify inhibitors targeting the inactive conformation of EGFR. The top-ranked compounds underwent molecular dynamics (MD) simulations and binding free energy calculations using Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA). MDA-MB-231, Hs578T, and 5-FU resistant- MDA-MB-231/5-FU^R cells were utilized to assess the anti-proliferative, EGFR, and apoptosis.

Results: HTVS identified EG31 showing strong binding affinities towards EGFR. MD simulations confirmed the stable binding of EG31 to EGFR, as indicated by consistent Root Mean Square Deviation and hydrogen bond patterns throughout the simulation. MMPBSA calculations revealed highly favorable binding free energies. EG31 inhibited MDA-MB-231 and Hs578T proliferations with GI_{50} values of 498.90 nM and 740.73 nM, respectively. The compound decreased EGFR-positive populations and favored early and late-phase apoptosis in these cells. Furthermore, EG31 retained the anti-proliferative efficacy in the MDA-MB-231/5-FU^R cells, while 5-FU lost its effectiveness by 6-fold.

Conclusion: This study identified EG31 targeting the inactive conformation of EGFR, offering a promising therapeutic approach to overcome 5-FU resistance in TNBC. Further research could enhance treatment efficacy and provide a new avenue for managing this challenging cancer subtype.

Keywords: triple-negative breast cancer, epidermal growth factor receptor, 5-fluorouracil resistance, molecular dynamics simulation, small molecule inhibitors

Introduction

Triple-negative breast cancer (TNBC) is a particularly aggressive and challenging subtype of breast cancer (BC) that accounts for 10–20% of all breast cancer diagnoses.[1,2] Unlike other forms of BC, TNBC is characterized by the absence of estrogen receptors (ER), progesterone receptors (PR), and HER2 amplification, which makes it unresponsive to hormonal therapies and HER2-targeted treatments.[3–5] This lack of targeted therapeutic options, coupled with its aggressive nature, contributes to the poor prognosis associated with TNBC. Patients with TNBC often experience higher rates of recurrence and metastasis, leading to lower overall survival rates compared to those with other BC subtypes.[4] As a result, chemotherapy remains the primary treatment option for TNBC, yet the rapid development of drug resistance frequently limits its efficacy.

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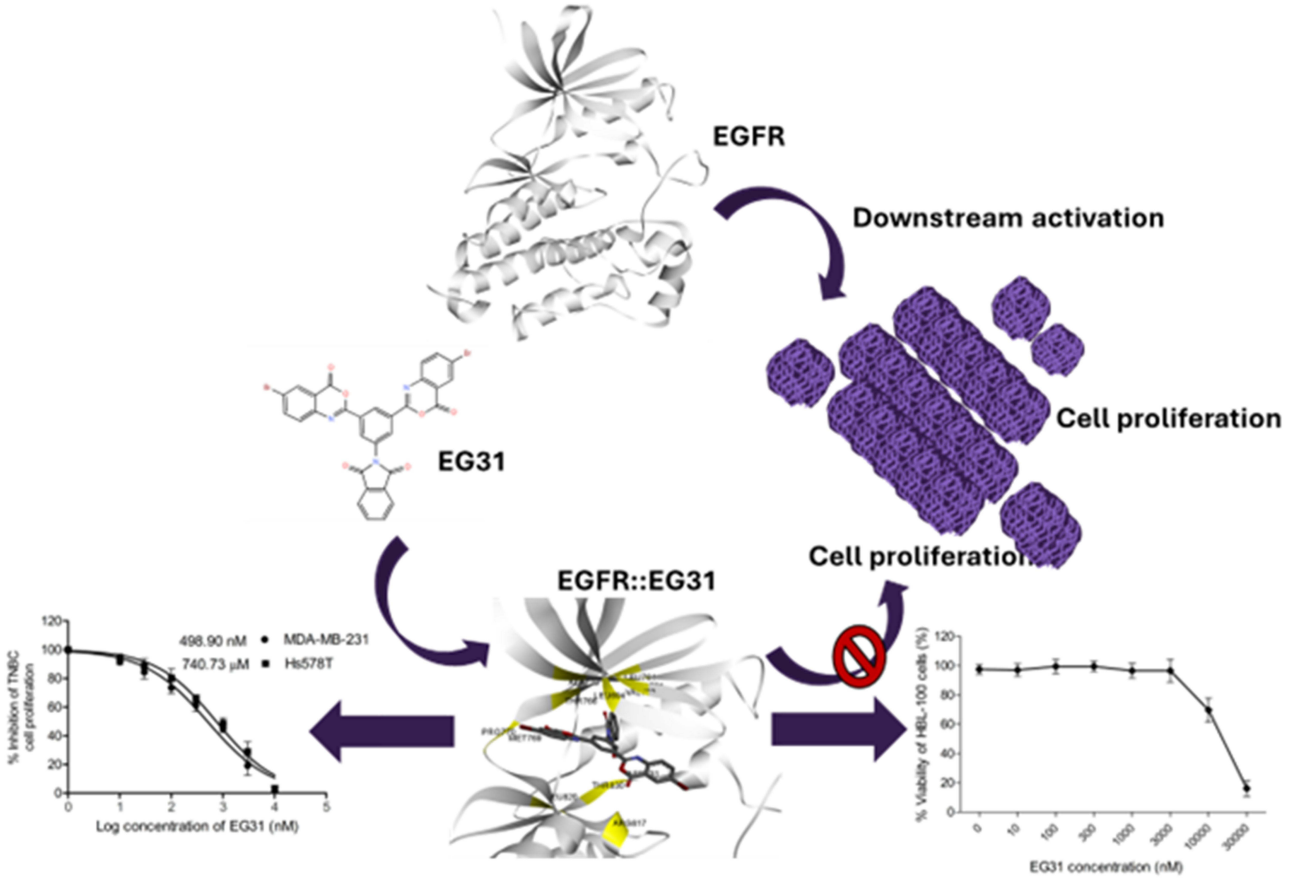
Graphical Abstract



The pathogenesis of TNBC is driven by several key molecular pathways that contribute to tumor initiation, growth, and metastasis.[6,7] Among these, the Epidermal Growth Factor Receptor (EGFR) signaling pathway plays a significant role. EGFR, a transmembrane receptor tyrosine kinase, is often overexpressed in TNBC and is associated with poor clinical outcomes. Upon activation, EGFR triggers a cascade of downstream signaling pathways, including the PI3K/AKT and RAS/RAF/MEK/ERK pathways, which promote cell proliferation, survival, angiogenesis, and metastasis.[8,9] The critical role of EGFR in TNBC underscores its importance as a therapeutic target, as its activation is directly associated with the aggressive behavior of TNBC tumors.

In clinical practice, chemotherapeutic agents such as 5-fluorouracil (5-FU) have been widely used to treat TNBC. 5-FU is a pyrimidine analog that inhibits thymidylate synthase, disrupting DNA synthesis and leading to cell death.[10,11] Despite its initial effectiveness, developing resistance to 5-FU is a significant hurdle in treating TNBC. Additionally, 5-FU resistance is proven to cause cross-resistance with other vital chemotherapeutic drugs like vinorelbine, paclitaxel, and gemcitabine in TNBC.[12] Therefore, 5-FU resistance is the first choice for evaluating the resistance effects in TNBC cells.

Resistance mechanisms are multifactorial, involving alterations in drug metabolism, enhanced DNA repair, and the activation of survival pathways like those mediated by EGFR.[13,14] As EGFR activation can drive resistance to 5-FU, patients with TNBC often experience limited long-term benefits from chemotherapy, necessitating the exploration of alternative therapeutic strategies. The resistance of TNBC to chemotherapy,[5] particularly 5-FU, highlights the urgent need for novel treatment approaches. Targeting EGFR has been identified as a potential strategy to overcome chemoresistance in TNBC.[15] However, traditional EGFR inhibitors, which typically target the receptor's active conformation,

have shown limited success due to the emergence of resistance mechanisms such as mutations in the EGFR gene that alter its binding affinity or the activation of compensatory signaling pathways.[16,17] These challenges underscore the need for innovative EGFR inhibitors to target the receptor and circumvent these resistance mechanisms effectively.

Developing novel EGFR inhibitors that can inhibit the receptor even in the presence of mutations or in its inactive conformation is crucial for improving outcomes in TNBC patients. Such inhibitors would block EGFR signaling more effectively and increase TNBC cells' sensitivity to chemotherapeutic agents like 5-FU. By preventing the activation of downstream survival pathways, these next-generation inhibitors can potentially restore the efficacy of chemotherapy and reduce the incidence of resistance, offering new hope for patients with TNBC. The search for such therapeutic agents is an area of active research that seeks to provide more effective and durable treatment options for this challenging cancer subtype.

Methods

Cell Culture and Reagents

Triple-negative breast cancer (TNBC) cell lines (eg, MDA-MB-231, Hs578T) and non-cancer normal breast cell line (HBL-100) were obtained from ATCC, Rockville, MD, USA, and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained in a humidified incubator at 37°C with 5% CO_2. EG31 was from ChemBridge Corporation, San Diego, CA, USA. 5-FU and other biochemical reagents used in this study were sourced from Sigma Aldrich (St. Louis, MO, USA). The Annexin V assay kit (Catalog number CBA059) was purchased from Merck Millipore (Burlington, MA, USA). EGFR- FITC tagged Monoclonal Antibody (ICR10) was from Thermo Fisher Scientific (USA).

High-Throughput Virtual Screening (HTVS)

To identify potential small molecule inhibitors targeting the inactive conformation of EGFR, a high-throughput virtual screening (HTVS) was conducted using the SiBioLEAD platform (ref) preloaded with the ChemBridge library, which contains approximately 850,000 compounds. The EGFR structure in its inactive conformation (PDB ID: 4HJO) was retrieved from the Protein Data Bank (PDB) and prepared for docking using standard procedures. Compounds preloaded in the SiBioLEAD HTVS platform were filtered based on molecular weight (350–750 Da) and Lipinski's Rule of Five. The docking calculations were performed using Autodock-Vina, which runs on the SiBioLEAD online platform (https://sibiolead.com/).

Molecular Dynamics (MD) Simulation

The top-ranked compounds identified from HTVS were subjected to GROMACS molecular dynamics (MD) simulations to assess their binding stability and interaction with EGFR. The EGFR-ligand complex was simulated using the SiBioLEAD MD simulation platform (https://sibiolead.com/),[18,19] and the systems were solvated in a triclinic box containing Simple Point Charge (SPC) water molecules. To mimic physiological conditions, 0.15M NaCl was added to neutralize the system. Energy minimization was performed using the steepest descent method, followed by a 300-picosecond (ps) equilibration period. The production MD simulation was run for 100 nanoseconds (ns) using GROMACS software, with the trajectory data collected for analysis. Root Mean Square Deviation (RMSD) and hydrogen bond (H-bond) analysis were performed to evaluate the stability of the ligand binding throughout the simulation.

Binding Free Energy Calculation (MMPBSA)

To quantify the binding affinity of the selected compounds to EGFR, Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) binding energy calculations were conducted. Fifty frames were extracted from the 100-ns MD simulation trajectory for each EGFR-ligand complex. The gmx_MMPBSA tool as part of the SiBioLEAD MD simulation platform was utilized to calculate the binding free energy,[19] which includes contributions from van der Waals interactions, electrostatic interactions, polar solvation energy, and non-polar solvation energy. The average binding free energy across all frames was reported, and compounds with highly negative binding energies were considered strong candidates for further experimental validation.

Proliferation Assay

The MTT test was used to measure cell growth, as described earlier.[20] Standard growth media was used to seed 5×10^3 cells/well of HBL-100, MDA-MB-231, MDA-MB-231/5-FU^R, or Hs578T into 96-well plates. For 48 hours, the cells were exposed to different doses of 5FU or EG31. After the treatment, the medium was aspirated, and the cells were treated for four hours with 100 µL of MTT solution (1 mg/mL). After dissolving the formazan crystals in 200 µL of DMSO, the absorbance at 560 nm was determined. GraphPad Prism 6.0 was utilized to compute the percent inhibition to ascertain the GI_{50} values.

Flow Cytometry EGFR Assay

EGFR of untreated and EG31-treated MDAMB-231 and Hs578T cells was analyzed using flow cytometry. Following cell harvesting, the concentration was increased to 1×10^6 cells/mL. The cells were then fixed with 2% paraformaldehyde and rinsed with PBS. After blocking the cells for 30 minutes at room temperature with a 2% BSA-PBS solution, the cells were treated for 60 minutes at room temperature with a 0.5 µg/test dilution of EGFR Monoclonal Antibody (ICR10), FITC (Catalog # MA5-28104). The cells were washed twice with PBS and resuspended in a PBS buffer. Flow cytometric analysis was performed by acquiring 10,000 events using the Guava easyCyte flow cytometer, and the data were analyzed with ExpressPro Software from Millipore (Burlington, MA, USA). The percentage of positive EGFR cells was examined.

Annexin V Assay

MDA-MB-231 and Hs578T cells were cultivated on 6-well plates at a density of 0.5×10^6 cells/well and subjected to 500 nM and 740 nM of EG31 concentrations (Near GI_{50} doses) treatments, respectively, with suitable untreated controls. For the next 48 hours, the cells were cultured at 37°C with 5% CO_2. Following incubation, the cells were removed, washed using a kit-provided buffer, and dark-stained for 15 minutes using 0.25 µg/mL Annexin V reagent. After two further washes, the cells were resuspended in a kit buffer containing 0.5 µg/mL of propidium iodide. Using a Guava easyCyte system, flow cytometry was used to collect data from 10,000 events. InCyte software was then used to analyze the data to distinguish between early and late apoptosis or healthy and apoptotic cells. The results were displayed using GraphPad Prism (6.0) software.

Establishment of MDA-MB-231/5-FU^R Cells and Cell Proliferation Assay

MDA-MB-231 was used to create 5-FU-resistant cells (MDA-MB-231/5-FU^R) by subjecting it to escalating 5-FU doses, as previously mentioned in protocol.[12] An initial 5-FU concentration of 3.84 µM in DMEM + 10% FBS was applied to MDA-MB-231 cells. Next, the drug concentration was raised 1.25 times for each resistance step, from 3.84 µM to 23.0 µM. At each stage, cells were cultivated for at least four weeks, with a medium change occurring every three days. Fifteen days before every experiment, the MDA-MB-231/5-FU^R cells were free of any drug treatments. A cell proliferation assay was performed on these cells, as described in section 2.5.

Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism software (version 6.0; La Jolla, CA, USA). Significant results were determined using a two-tailed Student's *t*-test or one-way ANOVA, followed by post-hoc tests where applicable. A p-value of <0.05 was considered statistically significant.

Results

High-Throughput Virtual Screening Identifies Targeted Molecules Against EGFR Inactive Conformation

To discover novel small molecules targeting the EGFR kinase, the inactive conformation of EGFR bound to erlotinib (PDB ID: 4hjo) was retrieved from the Protein Data Bank. Visualization of this conformation highlighted the potential binding region of erlotinib within the kinase domain of EGFR (Figure 1a and b). A detailed protein-ligand interaction analysis identified key EGFR residues involved in binding to erlotinib (Figure 1b). This analysis also revealed a ligand-

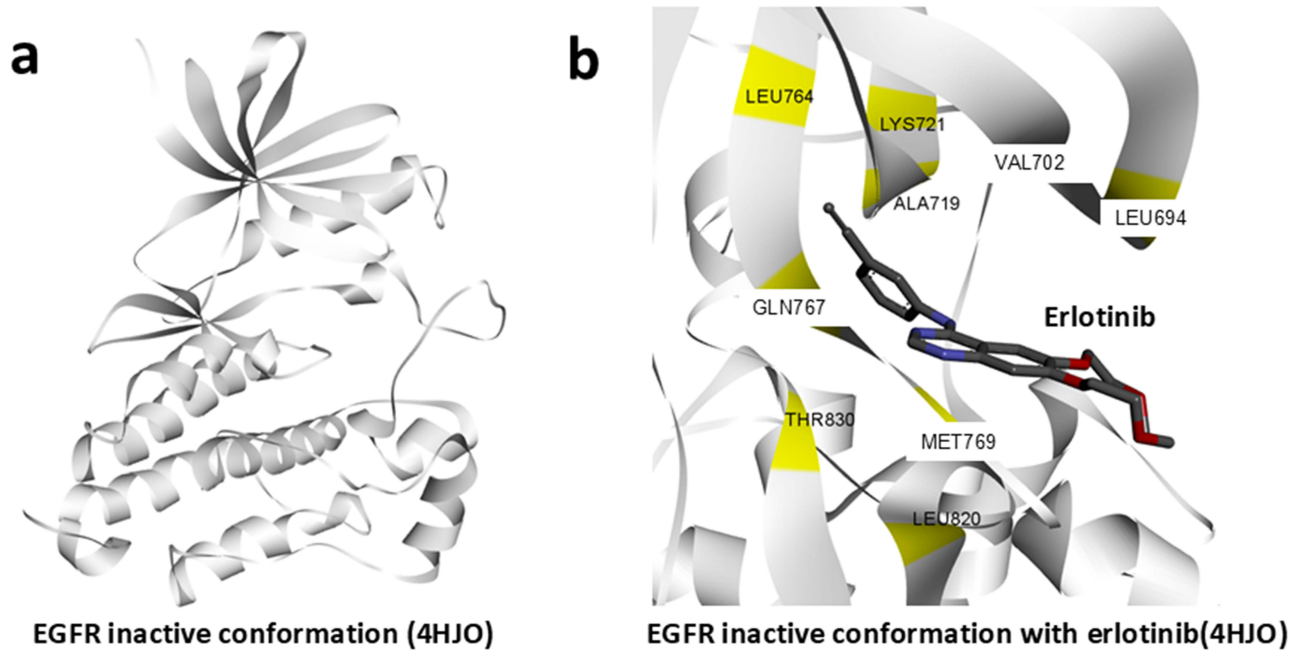


Figure 1 Structural Analysis and Visualization of EGFR in Inactive Conformation. (**a**) Crystal structure of EGFR in its inactive conformation (PDB ID: 4HJO), highlighting the kinase domain and key residues involved in ligand binding. (**b**) Close-up view of the erlotinib binding site within the EGFR kinase domain, showing critical interactions with key residues.

binding cavity in the kinase domain's inactive conformation. To identify the most effective molecules targeting EGFR, this inactive conformation was selected for further study (Figure 1b). A diversity-based high-throughput virtual screening (D-HTVS) approach was employed to screen small molecules with high affinity for the inactive kinase domain of EGFR from the ChemBridge library. From an initial pool of around 850,000 compounds, each with a molecular weight ranging from 350 to 750 Da, D-HTVS identified 1187 potential candidates based on their docking scores (Figure 2a). The top five compounds, ranked by their predicted docking scores, are listed in Figure 2b. Further refinement was conducted by filtering out compounds with docking scores below −13.2 kcal/mol, a threshold representing less than three standard deviations from the mean (Figure 2b). The highest-ranked compound, EG31, chemically identified as 2-[3,5-bis (6-bromo-4-oxo-4H-3,1-benzoxazin-2-yl)phenyl]-1H-isoindole-1,3(2H)-dione, achieved a promising docking score of −13.5 kcal/mol, warranting a more detailed analysis. The 2D structure of EG31 is shown in Figure 2c.

Protein-Ligand Interaction Analysis Shows EG31 Binds Effectively to EGFR Inactive Conformation

The number and strength of interactions between EG31 and EGFR were examined using the protein-ligand interaction profiler from Discovery Studio Visualizer. Amino acid residues contributing to the predicted interaction energy were analyzed. Results show that EG31 fits well within the kinase pocket of the EGFR (Figure 3a). Compound EG31 interacts with crucial amino acid residues of EGFR, forming a variety of interactions, including 4 hydrogen bonds with Asp831, Lys221, Thr830, and Met769, and 4 π-sigma bonds with Thr766, Key694, and Val702 (Figure 3b and c). Collectively, these analyses indicate that EG31 fits well within the kinase domain of EGFR, suggesting that EG31 may inhibit the activation of EGFR kinase.

Atomistic Molecular Dynamic Simulation Shows the Stability of EG31 to EGFR

To gain a thorough understanding of the binding stability and dynamics of EG31 in complex with EGFR, a detailed 100-nanosecond (ns) fully solvated atomistic molecular dynamics (MD) simulation was performed. The EGFR::EG31 complex was immersed in a triclinic simulation box, fully solvated with Simple Point Charge (SPC) water molecules. Counterions (NaCl) were introduced to simulate physiological conditions, resulting in a 0.15M NaCl concentration. Before the main simulation, a critical energy minimization step involving 5000 iterations of the steepest descent method

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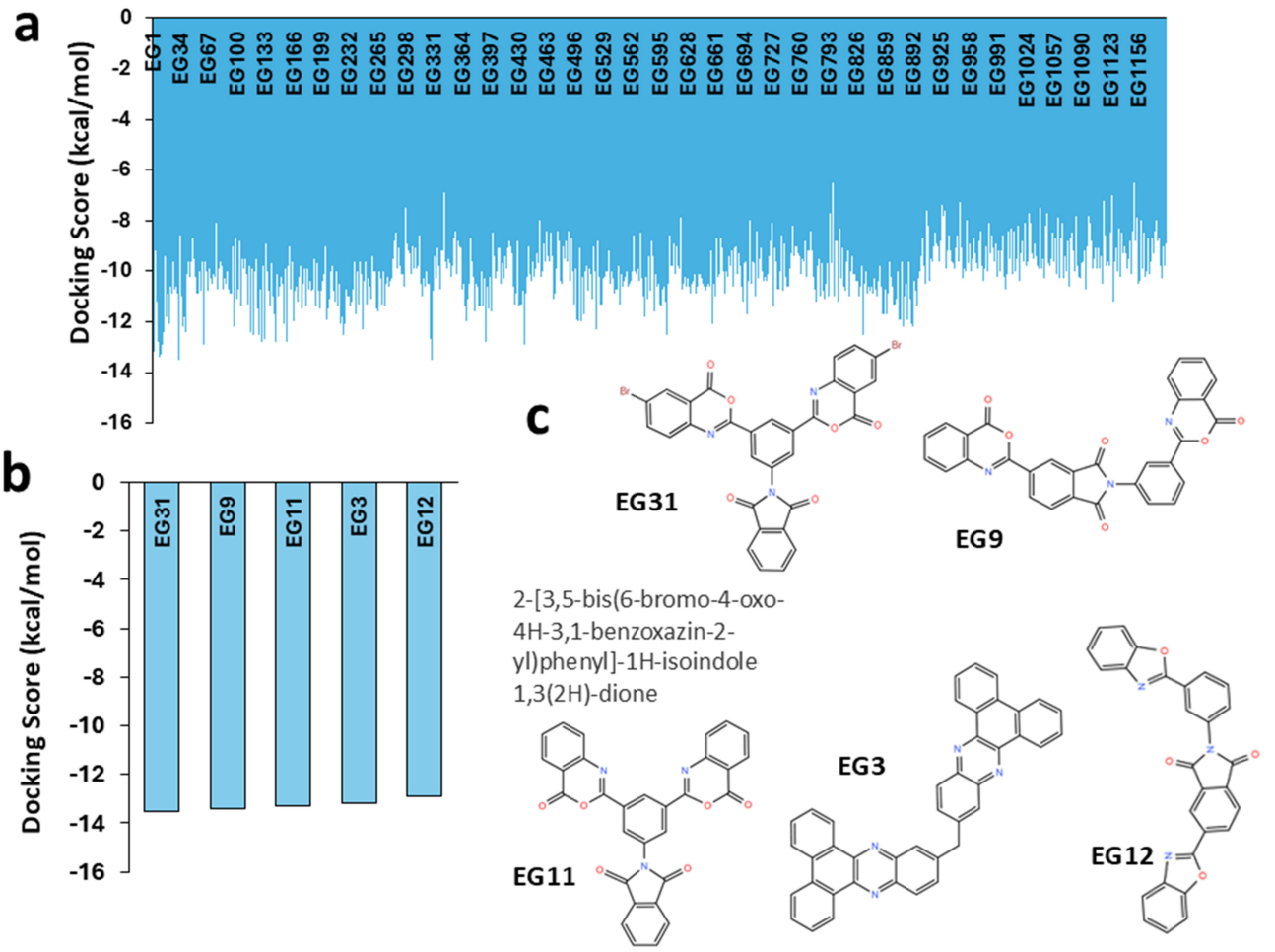

Figure 2 High-Throughput Virtual Screening (HTVS) Results. (**a**) Distribution of docking scores for the 1187 compounds selected from the ChemBridge library. (**b**) Docking scores of the top 5 compounds identified from HTVS targeting the inactive conformation of EGFR. (**c**) 2D chemical structures of the top-ranked compounds, illustrating the key functional groups contributing to its high affinity for EGFR.

was conducted to resolve steric clashes and prepare the system for equilibration. The system was then equilibrated for 300 picoseconds to stabilize before commencing the 100ns simulation. GROMACS simulation software, accessed via a web-based application (www.sibiolead.com), was used to track the dynamic behavior of EG31 bound to EGFR over the simulation period. The Root Mean Square Deviation (RMSD) of EG31 was monitored throughout, with values consistently around 0.04 nm, indicating stable binding interactions (Figure 4a).

Furthermore, hydrogen bond (H-bond) analysis revealed a stable and robust H-bond pattern for EG31 throughout the simulation, signifying persistent interactions between the ligand and EGFR (Figure 4b). Snapshots taken at various stages of the 100ns simulation confirmed the consistent and stable binding of EG31 to EGFR (Figure 4c and d). Furthermore, to better understand the efficacy of EG31, a control simulation was performed for a known EGFR drug, erlotinib. For this, the crystal of EGFR bound to EG31 (4HJO) was simulated for 100ns under the same conditions as that used for EG31. Results showed that EG31 has better ligand RMSD and H-Bond stability when compared to erlotinib (Supplementary Figure 1a and b). Snapshots of simulation trajectories taken before and after 100ns simulation show the binding fluctuations of erlotinib (Supplementary Figure 1c and d). These results collectively underscore the stability and sustained nature of the EG31-EGFR binding, offering key insights into their dynamic interaction.

MMPBSA-Based Gibbs Binding Free Energy Estimation of EG31 Binding to EGFR

To further investigate the binding stability of EG31 with EGFR, binding energy calculations using the Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) method were performed. For this purpose, 50 frames from the 100-nanosecond

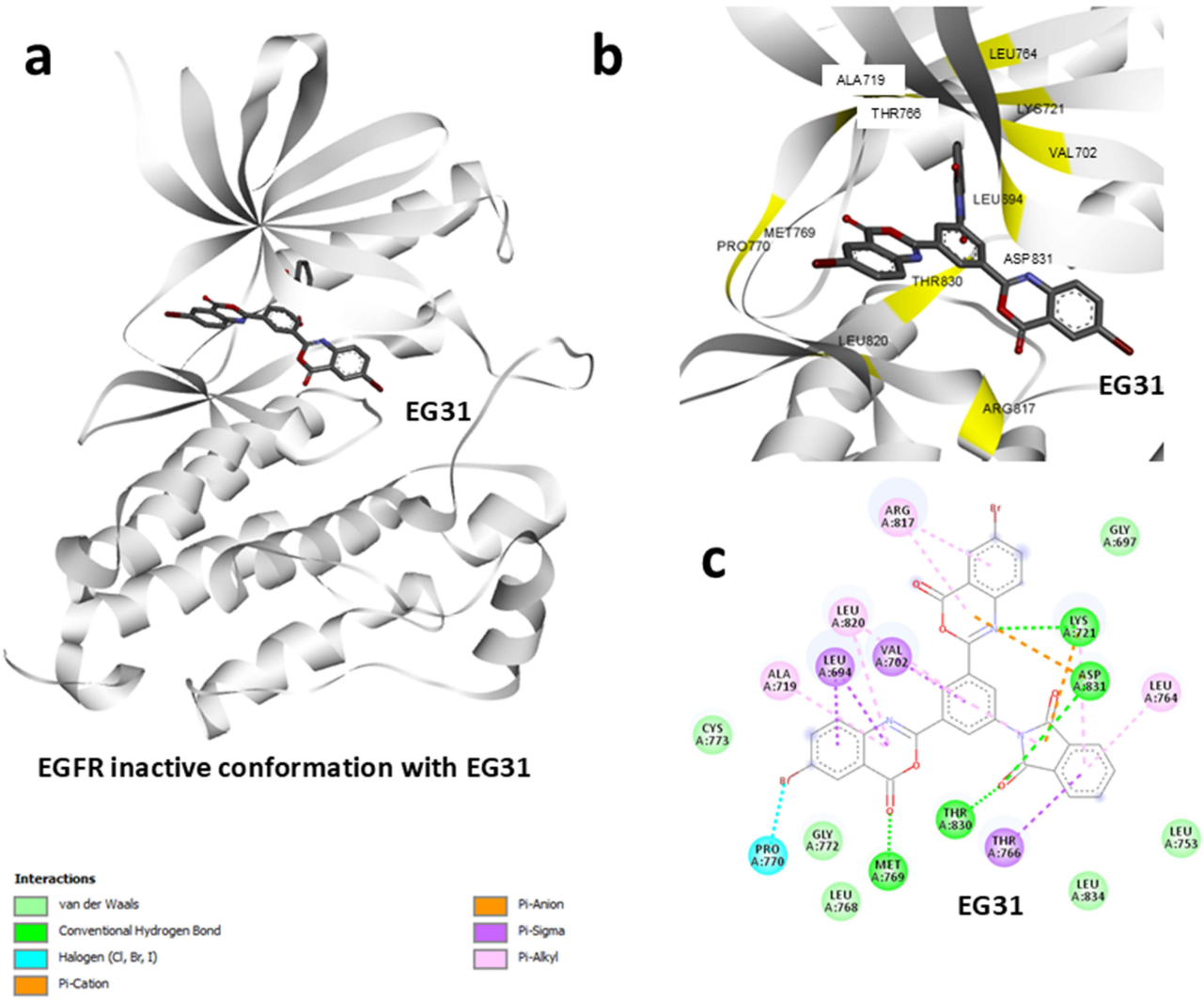

Figure 3 Protein-ligand interaction analysis. (**a**) Three-dimensional representation depicting EG31::EGFR complex. (**b**) Protein-ligand interaction analysis showing the binding mode and interactions of EG31 with EGFR. (**c**) 2D representation of the number and type of interactions between EG31 and EGFR.

(ns) simulation trajectory were analyzed. The gmx_MMPBSA tool was used to estimate the binding free energy. The analysis yielded a highly favorable binding energy of −52.57 kcal/mol for EG31, as shown in Figure 5. This value indicates a strong and stable interaction between EG31 and EGFR, with the negative binding energy reflecting an energetically favorable binding process, further confirming the high affinity of the ligand for the protein. To compare the Gibbs binding free energies with control (standard) drugs, MMPBSA-based binding free energies were calculated for erlotinib from 100ns simulation trajectories (Supplementary Figure 1e), and results showed that EG31 has better binding free energies (−52.57 kcal/mol), compared to erlotinib (−34.01 kcal/mol). Overall, the Molecular Dynamics (MD) simulation results and the MMPBSA-based binding energy calculations suggest that EG31 binds avidly and stably to EGFR.

EG31 Inhibited Proliferation, Reduced EGFR Positive Population, and Induced Apoptosis in TNBC Cells

The ability of EG31 to inhibit TNBC cell proliferation was assessed using the MTT assay. Figure 6a shows that the compound had dose-dependent effects on MDA-MB-231 and Hs578T cell growth, with GI_{50} values of 498.90 nM and 740.73 nM, respectively. Different dosages of EG31 were evaluated on HBL-100 cell proliferation to determine the tolerance of these biologically active levels in normal, noncancerous breast cells. Up to 3000 nM, the chemical did not

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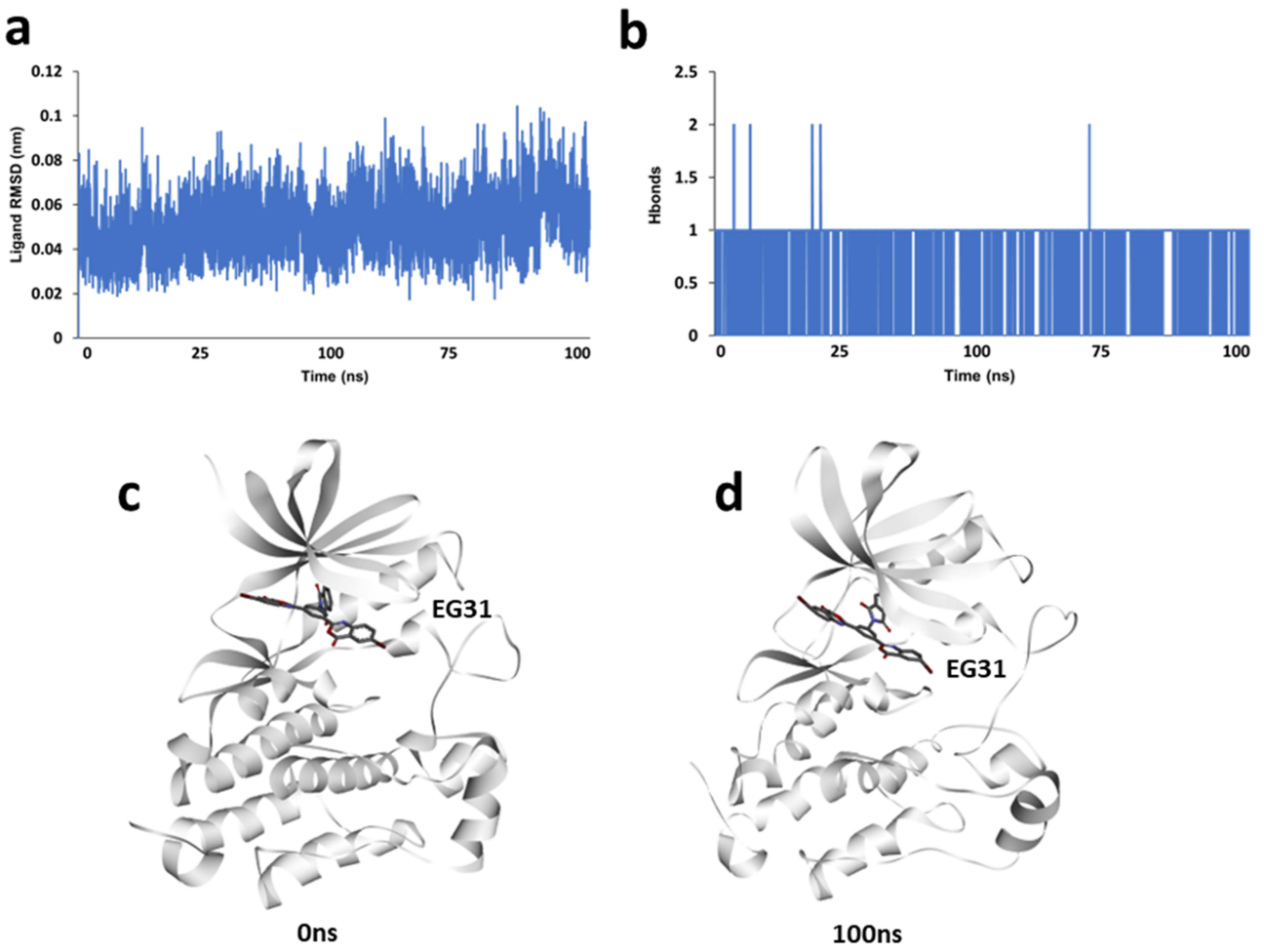

Figure 4 Molecular Dynamics (MD) Simulation of EGFR-Ligand Complexes. (**a**) Root Mean Square Deviation (RMSD) of the EGFR-ligand complex over a 100-nanosecond (ns) MD simulation, indicating the stability of the binding interaction. (**b**) Hydrogen bond (H-bond) analysis showing the number of H-bonds between EGFR and the ligand throughout the simulation. (**c** and **d**) Snapshots of the EGFR-ligand complex at different time points during the MD simulation, illustrating consistent and stable binding.

affect the growth of Vero cells (Figure 6b). To supplement the computational predictions, we examined the expression of EGFR in MDA-MB-231 and Hs578T cells with their near GI_{50} concentrations in these cells. After EG31 treatment, the percentage of EGFR-positive cells in MDA-MB-231 and Hs578T cells decreased from 39.47±3.54% to 07.27±1.02% and 23.52 ±2.57% to 8.71 ± 1.83%, respectively, compared to the corresponding controls (Figure 6c). Apoptosis analysis showed that EG31 treatment raised the percentage of early and late apoptotic cells in both TNBC cell types (Figure 6d). In MDA-MB-231 cells and Hc578T cells, EG31 treatment increased early apoptosis to 30.78% and 28.48%, respectively, compared to 2.90% and 2.18% in the corresponding controls (Figure 6d). MDA-MB-231 cells revealed a 17.45% positive population and Hc578Tcells had 9.62% for late-phase apoptosis (Figure 6d).

Effect of EG31 and 5 FU in MDA-MB-231/5-FU^R Cell Proliferation

The efficacy of 5-FU in the normal MDA-MB-231 and 5-FU resistant MDA-MB-231/5-FU^R cells was analyzed. 5-FU inhibited the proliferation of normal MDA-MB-231 with a GI_{50} value of 30.08 µM, while it lost the efficacy by 6-fold in the MDA-MB-231/5-FUR cells with a GI_{50} value of 181.35 µM (Figure 7a). However, EG31 retained efficacy in controlling the proliferation of MDA-MB-231/5-FU^R cells with a GI_{50} value of 519.5 nM (Figure 7b).

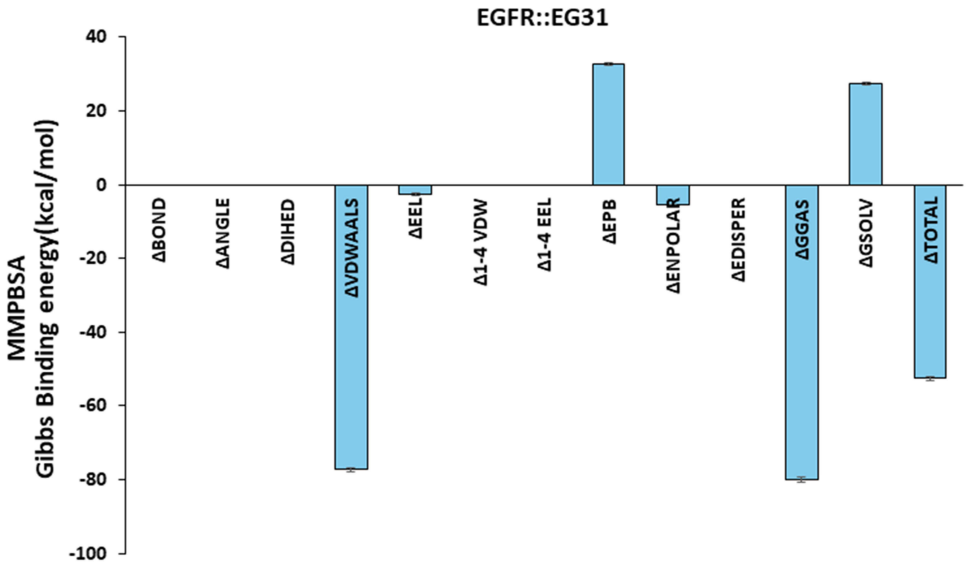

Figure 5 Binding Free Energy Calculations (MMPBSA). Binding free energy estimates for the top-ranked EGFR inhibitors, calculated using MMPBSA over 50 frames extracted from the 100-ns MD simulation trajectory.

Discussion

5-FU is a widely used chemotherapeutic agent in treating various cancers, including TNBC.[21] Despite its efficacy, the development of resistance to 5-FU remains a significant clinical challenge in BC therapy.[11] These resistance mechanisms not only limit the therapeutic potential of 5-FU but also necessitate the exploration of novel therapeutic strategies to overcome resistance and improve patient outcomes. EGFR is frequently overexpressed in TNBC and has been implicated in developing resistance to various chemotherapeutic agents, including 5-FU.[22–24] Thus, targeting EGFR represents a promising approach to overcoming 5-FU resistance in breast cancer, especially when conventional therapies fail to achieve the desired therapeutic effect. Recent research has highlighted the importance of targeting the inactive conformation of EGFR as a potential strategy for overcoming drug resistance.[25–27] The inactive conformation of EGFR presents a unique binding pocket that can be exploited to develop small molecule inhibitors. Unlike traditional EGFR inhibitors that target the active conformation, compounds targeting the inactive state can effectively inhibit receptor activation, even in cases where mutations confer resistance to active-site inhibitors.[28,29] This approach offers the potential to bypass common resistance mechanisms and achieve more sustained inhibition of EGFR signaling.

EG31 is a small molecule that has shown promise in targeting the inactive conformation of EGFR. High-throughput virtual screening (HTVS) is a powerful tool for identifying potential small molecule inhibitors that can bind to this inactive conformation of EGFR.[30,31] In the case of EG31, a diversity-based HTVS was conducted using the ChemBridge library, which contains approximately 850,000 compounds. The MD simulation provided valuable insights into the stability of the EG31-EGFR interaction over time. The Root Mean Square Deviation (RMSD) of EG31 was monitored throughout the simulation, with values consistently around 0.04 nm, signifying a stable binding interaction. This stability is crucial, suggesting that EG31 can bind to EGFR even under dynamic cellular conditions.[32] Furthermore, the stability of EG31 binding was predicted through Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) binding energy calculations. The results revealed a highly favorable binding energy estimate of −52.57 kcal/mol for EG31, which is indicative of a robust and energetically favorable interaction between the small molecule and the inactive EGFR conformation.[19] The negative binding energy supports the notion that EG31 has a high affinity for EGFR and is likely to be effective in inhibiting its activity.[19,33] Comparing the binding efficacies of EG31 with a known EGFR drug, erlotinib, showed that EG31 has a better binding affinity for EGFR. Comparing the molecular dynamic simulation and MMPBSA results demonstrated the superior binding affinity of EG31 compared to the known EGFR drug, suggesting that EG31 could be a better candidate for inhibiting EGFR activity.

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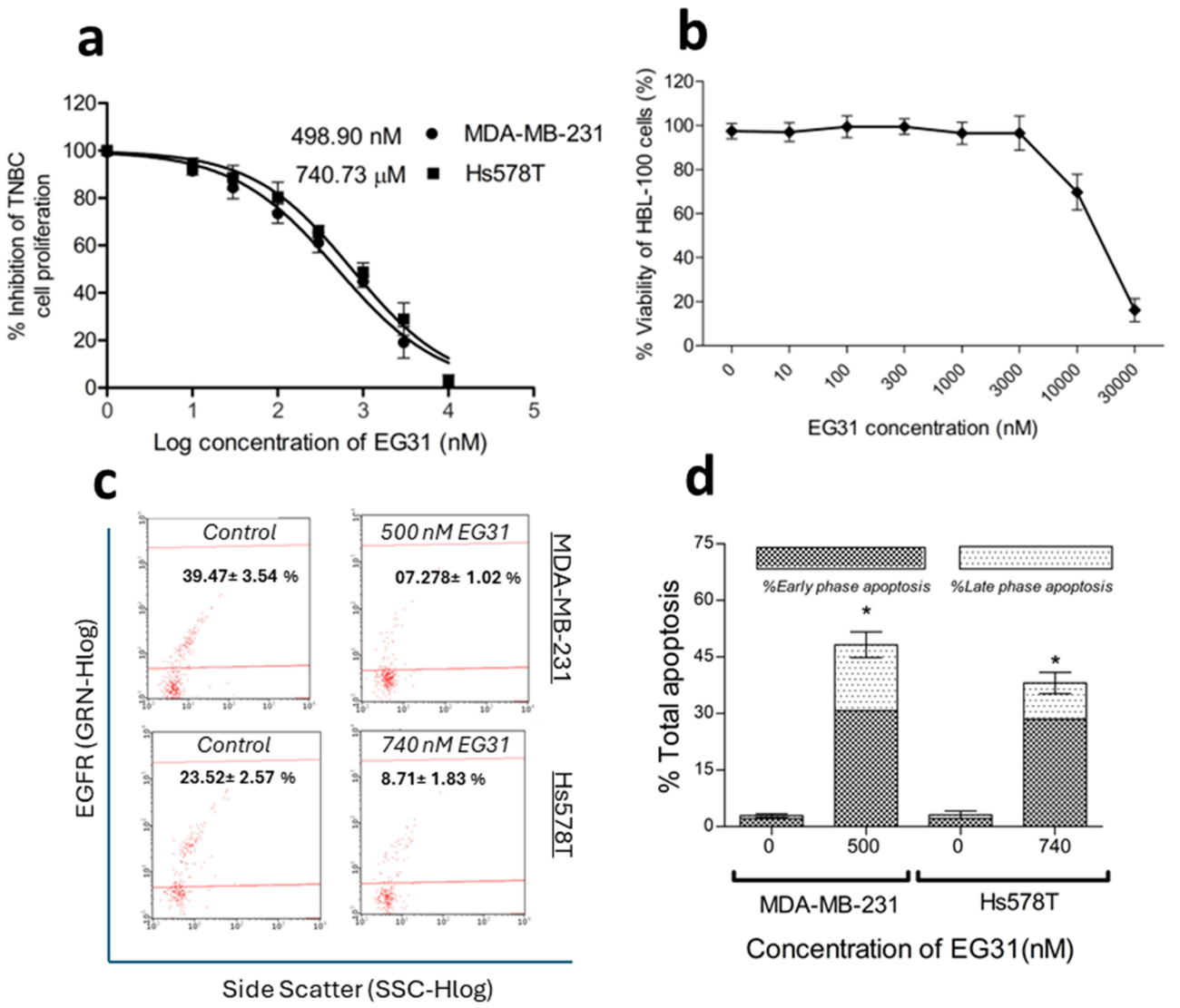

Figure 6 Effect of EG31 in TNBC cells. (**a**) GI_{50} values for cell proliferation in EG31-treated MDA-MB-231 and Hs578T cells. (**b**) Effect of EG31 at different doses on the survival of HBL-100 noncancerous breast cells. GraphPad Prism version 6.0 was utilized to examine the mean ± SD values obtained from the MTT assay, which evaluated cell viability and proliferation. (**c**) The proportion of EGFR-positive cells in MDA-MB-231 and Hc578T cells was assessed via flow cytometry. Treatment with EG31 led to a decrease in the EGFR-positive population in both MDA-MB-231 and Hc578T cells. The values presented represent the mean ± standard deviation (SD) from three independent experiments. (**d**) The results of the Annexin V assay demonstrate that EG31 administration induces both early- and late-phase apoptotic cells in MDA-MB-231 and Hs578T cells. Experiments were conducted in triplicate, and the mean ± SD findings are shown. * represents statistical significance; *P*-value <0.05 compared to respective controls.

EGFR overexpression is observed in at least 50% of TNBC patients.[22] Several EGFR-targeted antibody/drug conjugates have shown great potential for TNBC treatment and are currently undergoing clinical trials for TNBC patients.[34] Studies indicate excellent growth inhibition of breast cancer cells due to the inhibitor binding to the dimerization arm of EGFR and inhibiting the signaling pathway to reduce cell proliferation.[35] In par with these observations, In vitro tests showed that EG31 effectively inhibited the proliferation of TNBC cells. Additionally, EG31 was nontoxic to noncancerous breast cells at several folds of concentration in terms of its biological efficacy in the TNBC cells. This large window may be considered a benefit for a safer EG31 therapeutic dosage.[36] Alongside this, the EGFR inhibitory efficacy of EG31 was in line with the computational predictions. Previous studies have reported that inhibitors targeting EGFR can induce apoptosis in TNBC cells.[37,38] The induction of apoptosis is considered the major mechanism for anticancer effects. Cancer cells harboring mutant EGFRs depend on them for their survival and,

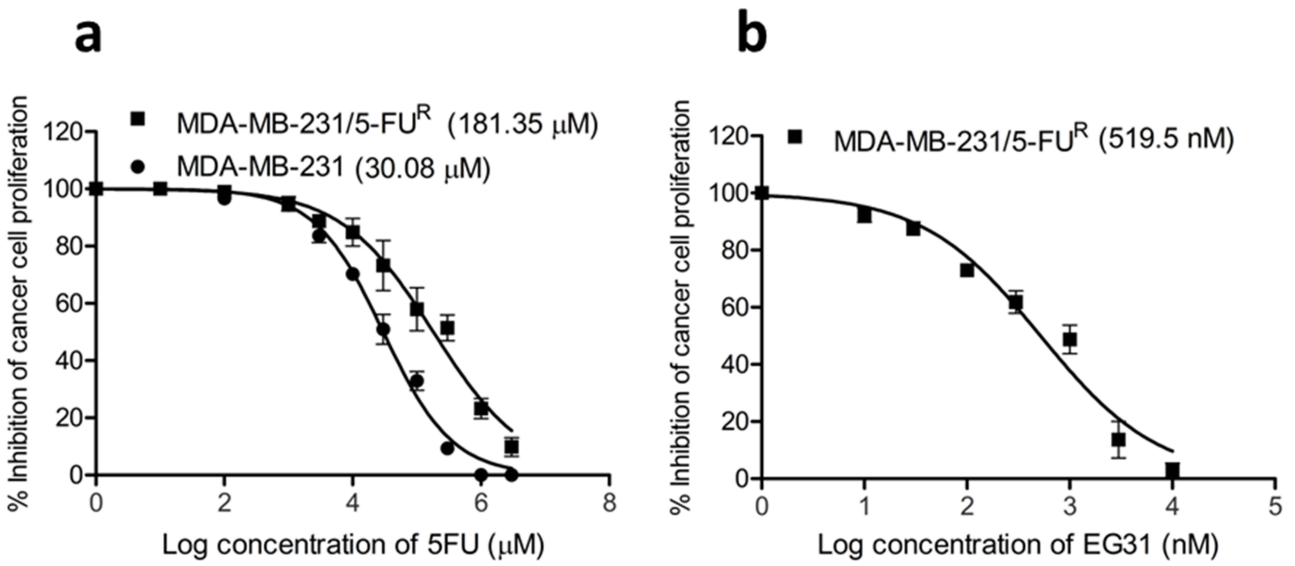

Figure 7 Antiproliferation in normal and 5-FU resistant TNBC cells. (**a**) Effect of 5-FU in normal MDA-MB-231 cells and 5-FU resistant MDA-MB-231/5-FU^R cells in controlling cell proliferation. (**b**) Efficacy of EG31 to control the cell proliferation in the 5-FU resistant MDA-MB-231/5-FU^R cells. Cell proliferation was assessed using the MTT assay, and results were analyzed using GraphPad Prism version 6.0 software to determine GI_{50} concentration.

consequently, undergo apoptosis following the inhibition of EGFR tyrosine kinase by small molecules.[39] In this study, the apoptosis-inducing property of EG31 highlights its potential as a promising therapeutic agent for TNBC.

Binding to the inactive state of EGFR could prevent the receptor from undergoing the conformational changes required for activation, thereby inhibiting downstream signaling pathways contributing to cell proliferation and survival.[40–42] This mechanism of action may prove particularly effective in TNBC cells that have developed resistance to 5-FU and other chemotherapeutic agents. In this study, EG31 was computationally assessed to bind the inactive state of EGFR and was proven effective in both normal and 5-FU resistance cells, where 5-FU lost activity several folds. As demonstrated by molecular simulations and binding energy studies, the strong and stable binding of EG31 to inactive EGFR highlights its potential as a valuable addition to TNBC therapy. Further research and clinical investigations are warranted to fully elucidate the therapeutic potential of EG31 in combating 5-FU-resistant TNBC.

Conclusion

The challenge of 5-FU resistance in triple-negative breast cancer (TNBC) remains a significant barrier to effective treatment, given the aggressive nature and high recurrence rate of TNBC. Overcoming resistance to 5-Fluorouracil (5-FU), a key chemotherapeutic agent, is essential for improving patient outcomes. One promising strategy involves targeting the inactive conformation of the epidermal growth factor receptor (EGFR), which plays a critical role in cancer progression and resistance. Small molecules like EG31, designed to bind specifically to the inactive form of EGFR, have shown the potential to disrupt signaling pathways that drive resistance, thereby restoring drug sensitivity. Unlike conventional inhibitors, EG31's unique mechanism could reduce resistance development while enhancing therapeutic efficacy, especially when combined with existing treatments like 5-FU. Future studies should focus on understanding the molecular mechanisms of EG31, optimizing its properties, and evaluating its effectiveness in clinical settings. Additionally, identifying predictive biomarkers could facilitate personalized treatment approaches. In conclusion, targeting the inactive conformation of EGFR with small molecules like EG31 offers a promising strategy to overcome 5-FU resistance in TNBC, paving the way for more effective therapeutic interventions.

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Disclosure

The authors report no conflicts of interest in this work.

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