

Anti-breast cancer activity of selected 1,3,5-triazines via modulation of EGFR-TK

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Abstract. The present study investigated the effect of certain 1,3,5-triazine derivatives on epidermal growth factor receptor-tyrosine kinase (EGFR-TK). The results suggested that 1,3,5-triazine derivatives exhibited optimal drug likeness via molinspiration and proved to be effective drug candidates as potential anticancer agents. The molecules were demonstrated to interact with key catalytic residues of EGFR, including Asn818, Lys721, Leu694, Val702 and Met742 as demonstrated in molecular docking experiments. Compound 1d was demonstrated to be the most potent analogue with an inhibitory constant of 0.44 nM, against EGFR-TK in *in-vitro* enzyme inhibition assay. Compound 1d was further evaluated for its effect on the cellular viability of three breast cancer cell lines, including highly metastatic MDAMB231, human epidermal growth factor receptor 2-positive BT474 and estrogen receptorpositive MCF7 cells, using an MTT assay and apoptosis analysis. Western blot analysis was performed to examine the levels of β -catenin in the control and treated cells. Based on the findings of the current study, the chemical 1,3,5-triazine series are potential novel inhibitors of EGFR-TK and β -catenin signaling, and may be potent anti-breast cancer agents.

Introduction

Breast cancer is the leading cause of cancer-associated mortality in women (1,2). The mortality rate is >450,000 individuals per year (3). The World Health Organization's previous data suggested the number of breast cancer cases has been gradually increasing worldwide since 2008 (4). Despite the availability of advanced therapeutic modalities,

current options to manage breast cancer remain inadequate (5,6). Using drugs to treat or manage the progression of breast cancer is the recommended option, however, its efficacy has been seriously compromised due to the development of resistance (7,8). Therefore, it is vital to identify drugs that are able to target novel sites and act in a specific manner. Previous studies reported that epidermal growth factor receptor-tyrosine kinase (EGFR-TK) was aberrantly overexpressed in breast cancer (9,10). As a result, EGFR-TK has attracted interest as a potential target from a number of studies across the globe searching for novel therapy agents (11-13). Various studies have reported that EGFR and Wnt signaling cascades are associated with and conserved in neoplasia. EGFR and Wnt signaling pathways are considered to serve key roles in embryonic development and cell proliferation. It is well established that dysregulation of these two pathways frequently leads to tumorigenesis with a poor prognosis. EGFR mutations are associated with a better prognosis in non-small cell lung cancer patients with unmethylated Wnt antagonists compared with patients with methylations, which points to a functional cooperation. Thus, it is hypothesized that activation of β -catenin by inactivation of E-cadherin activates EGFR, and thus effectively creates a positive feedback loop and potential targets for anticancer agents.

Due to their excellent pharmacological activity, 1,3,5-triazine derivatives have received considerable attention from medicinal chemists (14). These derivatives have been demonstrated to exert strong antibacterial (15,16), antiviral (17), antifungal (18), antimalarial (19,20), antidiabetic (21) and cystic fibrosis transmembrane conductance regulator-modulatory effect (22). Previously, these derivatives have been demonstrated to exert potent anticancer activity and it has been reported that substitution of various chemical groups on 1,3,5-triazine exhibited pronounced effect on the tumor cell proliferation (13,23). Therefore, in the present study, the EGFR-TK inhibitory activity of certain previously reported 1,3,5-triazines as anti-breast cancer agents were investigated. Furthermore, the effect of the compounds were evaluated on multiple breast cancer cell lines, specifically, the highly metastatic MDA-MB-231, human epidermal growth factor receptor 2 (HER2)-positive BT-474 and estrogen receptor (ER)-positive MCF7 cell lines. The effect of the compounds on β -catenin was also assessed.

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Key words: 1,3,5-triazines, docking study, epidermal growth factor receptor-tyrosine kinase, breast cancer, Wnt/ β -catenin

Materials and methods

Synthesis of compounds. The synthesis of the compounds was described in a previous study and the purity of the compounds was assessed on the basis of standard melting point (21).

Molinspiration. Physicochemical parameters have a vital role in the modulation of the bioactivity of chemical entities. Molinspiration (www.molinspiration.com), web-based software was used to obtain various parameters, including octanol/water partition coefficient (MiLogP), topological polar surface area (TPSA) and drug likeness. The MiLogP is calculated using the method developed by Molinspiration by summing the fragment-based contributions and correction factors. The method is robust and is able to process practically all organic and most organometallic molecules. TPSA is calculated based on the methodology published by Ertl *et al.* (24) as a sum of the fragment-based contributions (25) in which O- and N-centered polar fragments are considered and the surface areas that are occupied by oxygen and nitrogen atoms and by hydrogen atoms attached to them are calculated. TPSA has been demonstrated to be a useful factor for characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability and blood brain barrier penetration. Therefore, TPSA is associated with the hydrogen bonding potential of a compound. The compounds were evaluated on the basis of these parameters.

Docking study. Docking calculations were performed using Docking Server (26). Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged and rotatable bonds were defined. Docking calculations were carried out on compounds (ligands) using the EGFR kinase domain model (Protein code: 1M17.pdb; rcsb.org/3d-view/1M17). Essential hydrogen atoms, Kollman united atom type charges and solvation parameters were added with the aid of AutoDock tools (27). Affinity (grid) maps of 60x60x60 Å grid points and 0.375 Å spacing were generated using the Autogrid program. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of van der Waals and the electrostatic forces, respectively. Docking simulations were performed using the Lamarckian genetic algorithm and the Solis-Wets local search method (28). Initial position, orientation and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 10 different runs that were set to terminate following a maximum of 250,000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å and quaternion, and torsion steps of 5 were applied.

Cell lines. Three breast cancer cell lines, specifically, the highly metastatic MDA-MB-231, HER2-positive BT-474 and ER-positive MCF7 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), RPMI (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and α -minimal essential medium (Sigma-Aldrich, Merck KGaA), for the MDA-MB-231, HER2-positive BT-474 and ER-positive MCF7 cells, respectively. Cells were cultured

at 37°C with 5% CO₂ and 100% humidity. The medium was supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin.

MTT assay. MTT (Sigma-Aldrich; Merck KGaA) assay was used to evaluate the effect of compounds (1a-1d) on cell proliferation capacity. Cells were cultured in a 96-well plate at a density of 7x10³ cells/well and in a volume of 200 µl. Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO). The cells were then treated with compound 1d (0, 10, 25 and 50 nM). The final concentration of the solvent in the medium was 0.5%. At appropriate time intervals, the medium was removed and replaced with 200 µl 0.5 mg/ml MTT in the growth medium and then the plates were transferred to a 37°C incubator for 3 h. Then the medium was removed and the purple formazan crystals were dissolved in DMSO (200 µl/well). Absorbance was determined on an ELISA plate reader (Biotek Instruments, Inc., Winooski, VT, USA) with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain the sample signal optical density (OD; 570-630 nm).

EGFR-TK inhibitory activity. Kinase activity was determined using Kinase-Glo Plus luminescence kinase assay kit (Promega Corporation, Madison, WI, USA) by calculating the amount of adenosine triphosphate (ATP) remaining in the kinase reaction solution. The luminescent signal is correlated with the residual amount present and it was inversely associated with kinase activity. The tested compounds were diluted to 100 mM in 10% DMSO, then 5 ml the dilution was added to a 50 ml reaction. All of the enzymatic reactions were performed at 30°C for 40 min using a 50 ml reaction volume containing 10 mM MgCl₂, 40 mM Tris, (pH 7.4), 0.1 mg/ml bovine serum albumin, 0.2 mg/ml poly (Glu, Tyr) substrate, 10 mM ATP and EGFR. The plate was incubated for 5 min at room temperature then 50 ml Kinase-Glo Plus Luminescence kinase assay reagent was added to each reaction. ADP-Glo assay kit (Promega Corporation) is a protein kinase assays used to determine IC₅₀ values in which adenosine diphosphate (ADP) generation was measured which leads to an increase in the luminescence signal. The reaction mixture was incubated in a 96-well plate at 30°C for 30 min, following the incubation period 25 ml ADP-Glo reagent was added to terminate the assay. The 96-well plate agitated for 30 min at ambient temperature and incubated, then 50 ml kinase detection reagent was added. The 96-well plate was read using the ADP-Glo Luminescence reader. All the assay components were added to the blank control except the substrate. The blank control value was used to correct the activity for each protein kinase target is determined.

Apoptosis assay. Cells at a density of 1x10⁵ cells/well were cultured in 96-well plates in medium supplemented with 10% FBS for 24 h and was followed by treatment with compound 1d (0, 10, 25 or 50 nM). After 48 h, cells were collected by 300-350 x g for 5 min at room temperature, rinsed with PBS, fixed in 4% paraformaldehyde for 30 min at room temperature and then rinsed again with PBS to remove the fixing agent. The fixed cells were resuspended in PBS that already containing 5 µg/ml Hoechst 33258 and incubated at room temperature for 15 min in the dark room. The cells were examined to record

Table I. Molecular property calculation of target compounds.

Compound	Mutagenic	Tumorigenic	Irritant	Reproductive effective	ClogP	Solubility	MW	TPSA	Drug likeness	Drug score
1A	+++	+	+++	+	3.51	-5.31	378.0	125.4	2.11	0.2
1B	+++	+	+++	+	2.16	-5.46	408.0	177.4	1.63	0.2
1C	+++	+	+++	+	1.67	-6.23	468.0	217.0	-3.25	0.09
1D	+++	+	+++	+	4.73	-6.78	446.0	125.4	3.78	0.13

+++ , highlytoxic; + , non toxic; MW, molecular weight; TPSA, total polar surface area; Calculated logPClogP.

the percentages of apoptotic cells by determining the nuclear condensation and chromatin fragmentation via fluorescence microscopy. A total of 250 nuclei from random microscopic fields were examined for the quantification of the apoptotic rate.

Western blot analysis. Total proteins were extracted using a RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China). A bicinchoninic protein assay kit was used to quantify protein concentrations. Then, the protein samples were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk powder overnight at 4°C. Primary antibodies against β -catenin (~90 kDa) and β -actin were purchased from Santa Cruz Biotechnology, Inc., (anti- β -catenin; cat. no. sc-7963; 1:200; anti- β -actin, cat. no. sc-130301; 1:10,000) and incubated overnight at 4°C. The secondary antibody, goat anti-mouse immunoglobulin (Ig) G-horseradish peroxidase, was purchased from Santa Cruz Biotechnology, Inc., (cat. no. sc-2005; 1:5,000) and incubated at 37°C for 1 h. Finally, the bands were visualized using an enhanced chemiluminescence kit (Solarbio, Beijing, China) and analyzed by Image pro plus (version 9.3; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. All experiments were done in triplicate or more. One-way analysis of variance was used to estimate overall significance followed by post-hoc Tukey's tests corrected for multiple comparisons. Data are presented as the mean \pm standard deviation of the mean. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Molecular properties of 1,3,5-triazines. In order to find a probable candidate for an anti-breast cancer agent as shown in Fig. 1, four previously reported 1,3,5-triazine compounds were selected for the present study. The molecular properties of the compounds are presented in Table I, where the molecules were assessed on the parameters of the Lipinski's rule of five (29). The molecules demonstrated no violations of the rules and proved to be effective drug candidate to be act as anticancer agent.

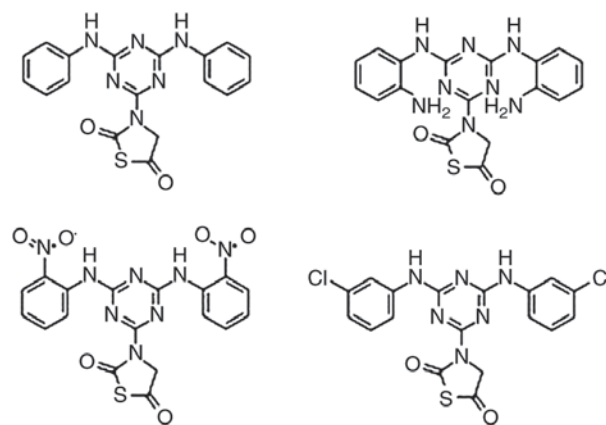


Figure 1. Structures of the selected compounds.

Docking analysis of 1,3,5-triazine at the EGFR-TK. As the drug likeness profile of the 1,3,5-triazine derivatives was good, docking analysis with EGFR-TK protein domain was performed. The results have been presented in Table II. It has been demonstrated that all compounds exhibited good inhibition constants against the target protein ranging from 0.44 nM to 3.10 μ M and engaging the key active site as shown in Fig. 2. The molecules also demonstrated considerable interaction with key catalytic residues, including Asn818, Lys721, Leu694, Val702, Met 742 and other residues as exhibited in Table II. Particularly, in the case of compound 1a, as presented in Fig. 3, it had an inhibitory constant (K_i) of 3.10 μ M with polar interaction with Lys721 and Asn818. It also had interaction with Leu694, Val702 and Leu678. Compound 1b exhibited marked improvement in the K_i value as a result of improved binding contacts with the target protein, including additional residues Arg817, Met442 and halogen binding with Glu738, Asp831, and Lys721, Fig. 4. Fig. 5 demonstrated that compound 1c exhibited 1.80 μ M via interacting with Thr766, Leu694 and Met742. Compound 1d had the most potent activity, where the compound exhibited K_i value of 0.44 nM with improved contacts in the 3D protein structure (Fig. 6). The docking interactions were further demonstrated to be in agreement with the 2D H-plot, as shown in Fig. 2.

In vitro EGFR-TK inhibitory activity. As a result of the excellent drug likeness profile and considerable docking affinity of the 1,3,5-triazine derivatives, these derivatives

Table II. Scoring and interacting residue of the compounds against the epidermal growth factor receptor tyrosine kinase domain.

Code	Est. free energy of binding (kcal/mol)	Est. inhibition constant (Ki)	vdW + Hbond + desolvenergy	Electrostatic energy	Total intermolecular energy	Polar	Hydrophobic	Halogen bond
1a	-7.51	3.10 μ M	-8.26	-0.06	-8.32	Lys721, Asn818	Leu694, Val702, Leu678	-
1b	-7.91	1.60 μ M	-8.51	-0.14	-8.64	Lys721, Arg817, Asn818	Leu694, Val702, Met442	Glu738, Asp831, Lys721
1c	-7.84	1.80 μ M	-8.93	-0.06	-8.99	Thr766	Leu694, Met742, Leu764	Thr766, Asp831
1d	-8.66	0.44 nM	-9.32	-0.06	-9.37	Asp831	Leu694, Met742, Leu820	Asp831

Est., estimated; vdW, van der Waals; Hbond, hydrogen bond; dsolv.

were synthesized and tested for inhibitory activity against EGFR-TK. The results are presented in Table III.

It has been demonstrated that synthesized derivatives exhibit excellent inhibitory activity compared with dacomitinib as a standard in the present study. For instance, compound 1d was demonstrated to exhibit the most potent activity among all the tested analogues. The lowest activity against EGFR was reported by compound 1a. The other compounds demonstrated mild to moderate activity. These results further confirmed the EGFR-TK inhibitory effect of 1,3,5-triazines as determined in docking experiments.

In vitro anticancer activity. Subsequent to determining the inhibitory activity, compound 1d was further analyzed for anticancer activity in breast cancer cell lines, specifically, the highly metastatic MDA-MB231, HER2-positive BT-474 and ER-positive MCF7 cell lines. The compound was synthesized as described elsewhere and characterized using the melting point as compared with the reported value (21). As presented in Fig. 7, it has been demonstrated that the cellular viability of all the cells were decreased in a significant manner ($P < 0.05$). Out of the tested cells, compound 1d causes inhibition of the BT-474 and least activity against MCF-7 at 50 nM. The inhibitory activity of compound 1d was revealed to be concentration dependent with maximum at 50 nM and minimum at 10 nM. Whereas 25 nM demonstrated considerable effects on the viability of cells.

The effect of compound 1d was further evaluated by measuring the apoptosis of the all three tested breast cancer cells. The cells were treated with the vehicle (0 nM) and different concentrations (10, 25 or 50 nM) of the compound 1d for 48 h. The apoptotic rates were determined via the staining of all three cells treated with compound 1d or the control cells with Hoechst 33258. As shown in Fig. 8, compound 1d increased in apoptosis in all three cells. At the highest concentration of compound 1d (50 nM), the level of apoptosis in all the cells were demonstrated to be increased significantly ($P < 0.05$; Fig. 8).

The results suggested that compound 1d causes significant increases in the apoptotic rates. The effect of compound 1d on the degradation of β -catenin was also evaluated. The total protein was isolated and the expression levels of β -catenin were determined by immunoblot analysis using β -actin as a loading control. As shown in Fig. 9, it has compound 1d decreased the protein expression of β -catenin in MDA-MB-231 compared with the control. These results indicated that compound 1d caused a significant decline in the protein expression of β -catenin expression in the MDA-MB-231 cells ($P < 0.05$). Thus, compound 1d may exert an anticancer effect via inhibition of the β -catenin signaling pathway.

Discussion

The burden of cancer and the multifactorial etiology has attracted thousands of studies across the world to investigate this disease (2). Cancer can be induced by the aberrant expression of receptors and growth factors (3). It can also be modulated via various oncogenic activators and inactivation of tumor suppression genes (5). Various intracellular pathways have also been demonstrated to be deregulated in cancer.

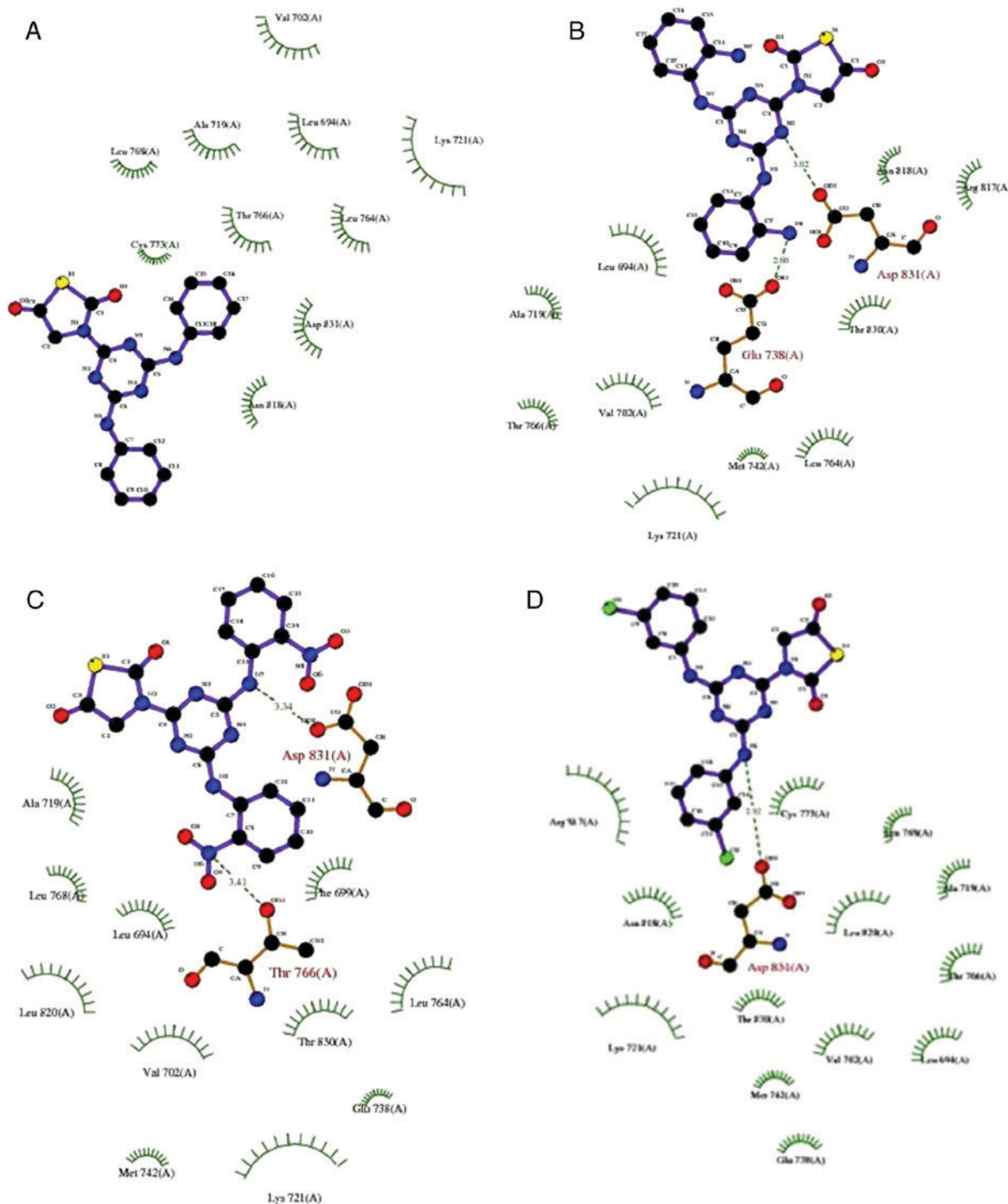


Figure 2. Docking mode of compounds in the active site of the target three dimensional structure of epidermal growth factor receptor-tyrosine kinase protein. (A) 1a, (B) 1b, (C) 1c and (D) 1d.

Consequently, the advancement of novel clinical techniques advances the understanding of the underlying mechanisms behind the generation and progression of cancer (6). This leads to the development of novel treatments targeted to specific molecules and pathways, which are considered to be critical for the development of cancer. Among the well-known targets, EGFR a transmembrane growth factor receptor TK was demonstrated to be frequently overexpressed in epithelial tumors. Particularly in breast cancer, EGFR has a major role in promoting cellular proliferation and tumor growth (9).

It is considered to be a proto-oncogene, which encodes a 170 kDa transmembrane protein that results in the dimerization and autophosphorylation of the receptor in presence of the EGFR ligand. EGFR activation leads to the recruitment of downstream signaling molecules, which have critical roles in cellular proliferation, survival and migration. EGFR has been reported to be overexpressed in the majority of the triple receptor-negative breast tumors (30). The canonical Wnt signaling pathway (β -catenin dependent) regulates numerous genes that are responsible for diverse cellular functions

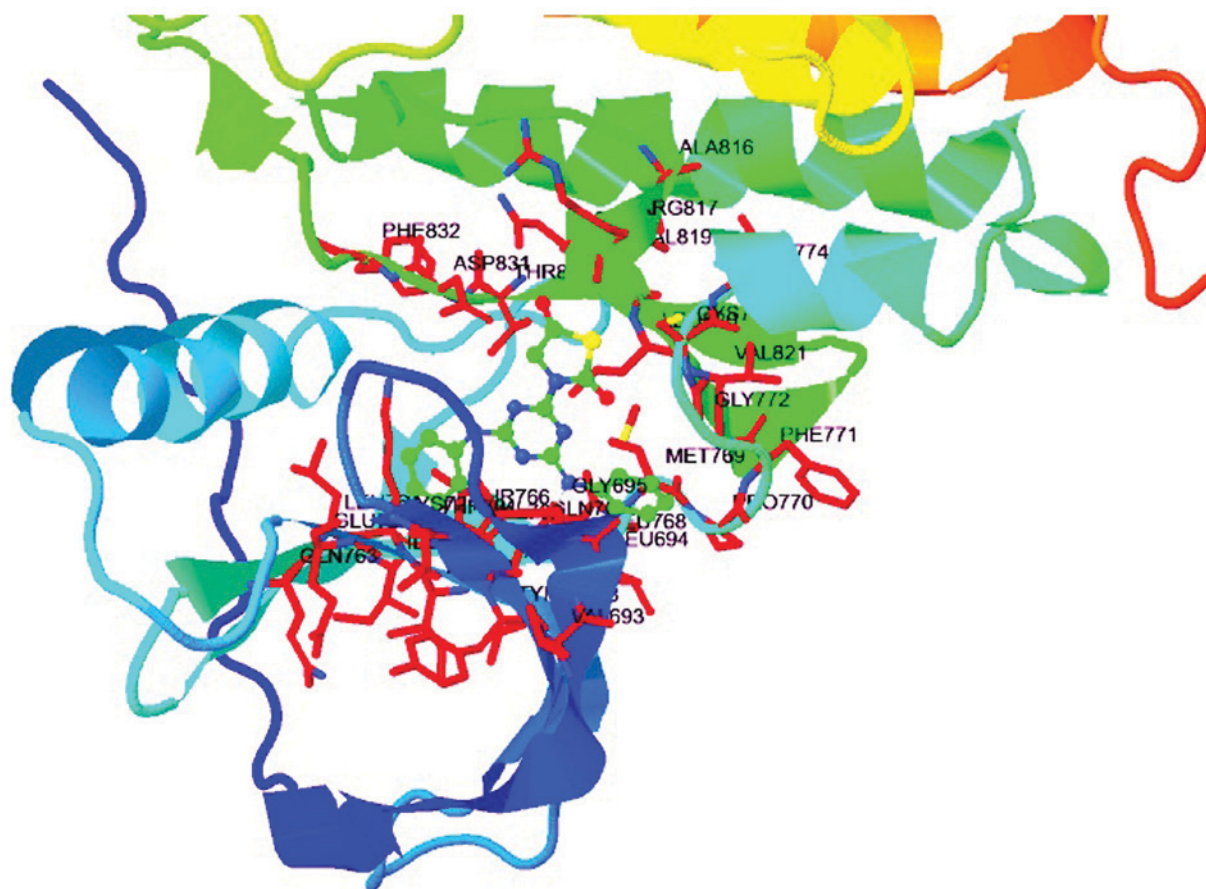


Figure 3. Docked interaction of compound 1a in the active site of crystal structure of epidermal growth factor receptor-tyrosine kinase.

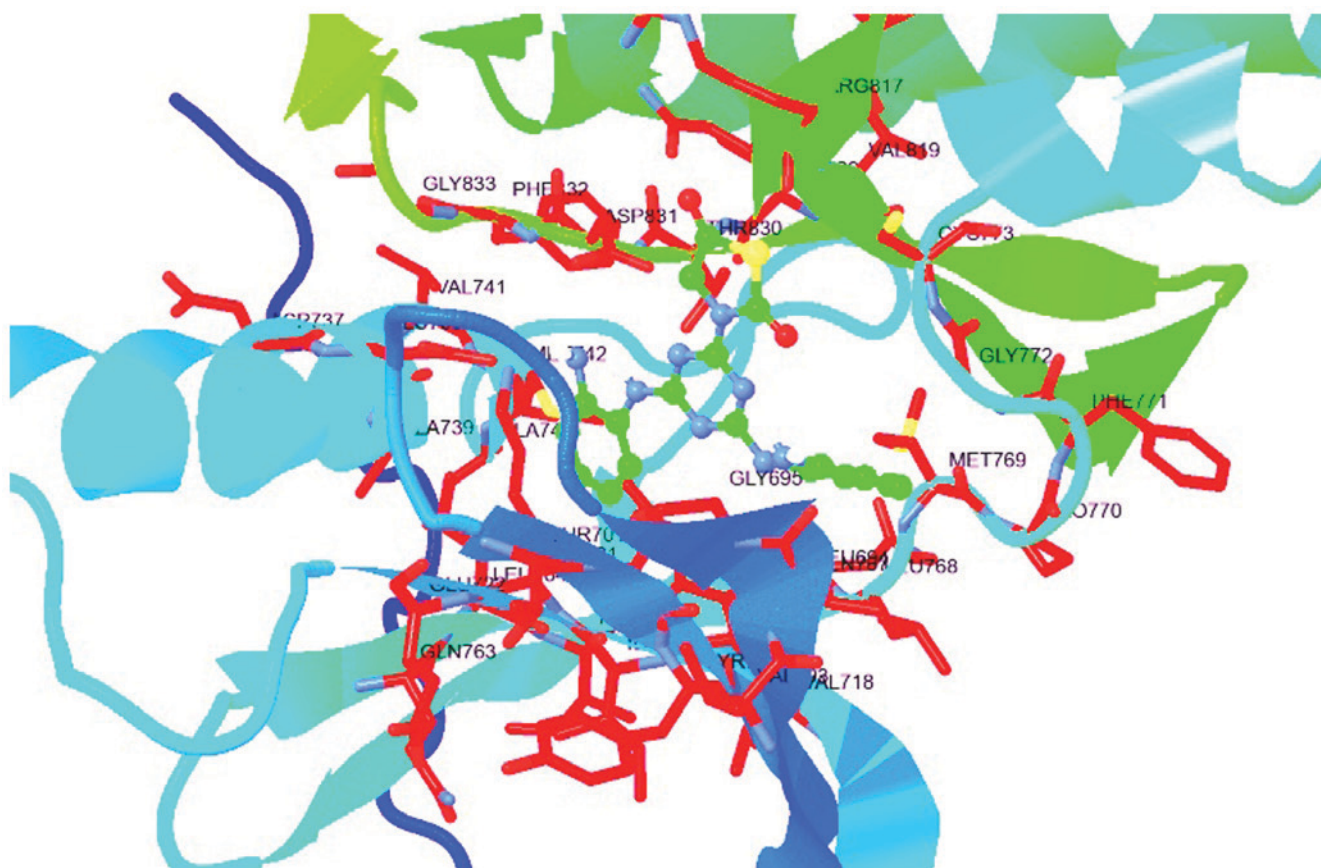


Figure 4. Docked interaction of compound 1b in the active site of crystal structure of epidermal growth factor receptor-tyrosine kinase.

Table III. Epidermal growth factor receptor-tyrosine kinase inhibitory activity of target molecules.

Compound	IC ₅₀ (in μ M)
1a	112.45±21.52
1b	34.05±4.11
1c	8.45±0.65
1d	2.54±0.22
Dacomitinib	0.06±0.01

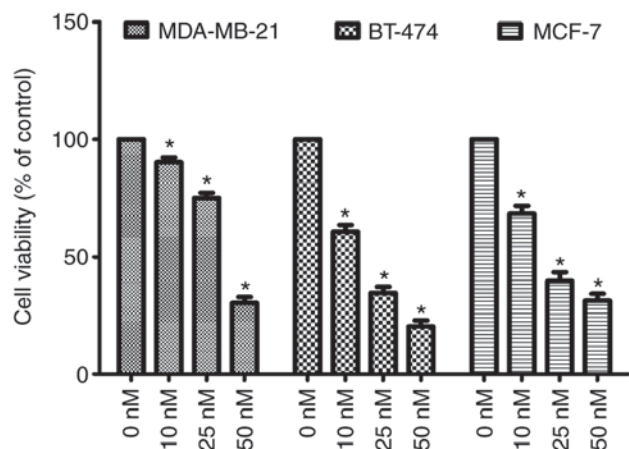


Figure 7. Effect of compound 1d on the viability of cells. (n=3). *P<0.05 vs. control.

negative breast cancer and is associated with a poor clinical outcome (12). Thus, the selective inhibition of EGFR-TK and β -catenin offers a number of advantages. 1,3,5-Triazines are well known for their anticancer activity and are an important group of agents among novel drugs with potential for cancer therapy. Recently certain 1,3,5-triazines have been demonstrated to exhibit anti-EGFR-TK inhibitory activity (31). As a result, in the present study the putative EGFR-TK inhibitory activity of specific 1,3,5-triazine derivatives was investigated (21). As presented in Table I, it has been found compounds demonstrated a considerable druglikeness score, as indicated by Lipinski's rule of five. Lipinski's rule of five, also known as the Pfizer's rule of five, or simply the rule of five is used to evaluate drug-likeness or to determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a suitable candidate as an orally active drug in humans. The rule was formulated by based on the observation that the majority of orally administered drugs are relatively small and moderately lipophilic molecules (29). The rule describes the molecular properties important for drug pharmacokinetics in the human body, including their absorption, distribution, metabolism and excretion. However, the rule does not predict if a compound is pharmacologically active. Thus, the compounds were investigated to determine if they have sufficient efficacy to act as drug molecules. The molecules were analyzed for docking with EGFR-TKs. In previous studies, it has been demonstrated that potent EGFR inhibitors were able to interact with the EGFR protein and the docking

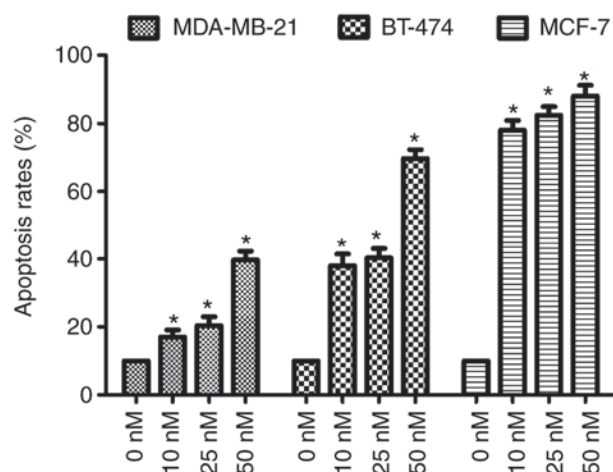


Figure 8. Effect of compound 1d on the apoptosis of cells. (n=3). *P<0.05 vs. control.

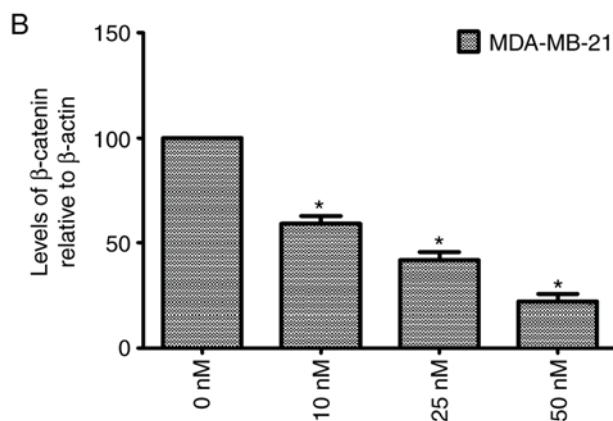
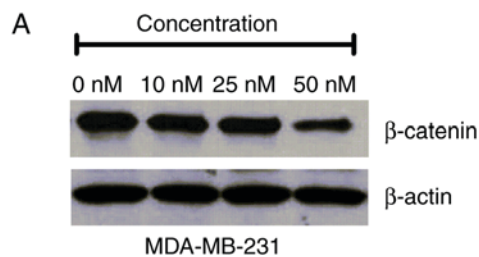


Figure 9. Effect of compound 1d on expression levels of β -catenin. (A) Levels of β -catenin proteins relative to β -actin levels in all cells. (B) Representative blot of MDA-MB-231 cell lysates. (n=3). *P<0.05 vs. the control.

results revealed that three amino acids Leu694, Lys721 and Asp831 located in the binding pocket of the protein had a vital role in binding with compounds (10-13). The compounds in the present study interacted with these residues and similarly exhibited EGFR-TK inhibitory activity. The compounds also significantly inhibited EGFR-TK in an enzymatic inhibitory assay (Table III). Thus, it was hypothesized that these derivatives may have potential to inhibit EGFR in a breast cancer cell system assay. As confirmed by the *in vitro* analysis, the most active compound exhibited potent anticancer activity in the all tested cell lines in a dose-dependent manner, with the highest in the activity at 50 nM. The results of the study were in accordance with a previous study where 1,3,5-triazine attenuated

EGFR-TK with anti-breast cancer activity (30). The activity against apoptosis on different cells was investigated. Apoptosis is evaded by cancer cells, resulting in malignant cells that will not die spontaneously. The mechanism of apoptosis is multifaceted and involves a number of signaling pathways. Defects can occur at any point along these pathways, leading to malignant transformation of the affected cells, tumor metastasis and resistance to anticancer drugs. The tested compound exhibited potent induction of apoptosis. The effect of compound 1d on β -catenin was also investigated via western blot analysis. Compound 1d reduced the expression of β -catenin in the MDA-MB-321 cells at various doses, with prominent activity at 50 nM dose. However the present study lacks ITC data for each compound and the effect on non-cancerous cells to further understand the spectrum of activity.

In conclusion, a series of 1,3,5-triazines were investigated as inhibitors of β -catenin and EGFR-TK inhibitors, and acted as potent anti-breast cancer agents. Currently, the more data is being acquired on the inhibitory mechanism of these compounds via ITC experiments and effect on non-cancerous cells, which will be reported in due course. Thus, derivatives may act as potent anti-breast cancer agents.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JH designed the study. WY and YZ performed the experiments. WY, YZ and JH analyzed the data and wrote the paper.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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