

Novel 2-(5-Aryl-4,5-Dihydropyrazol-1-yl)thiazol-4-One as EGFR Inhibitors: Synthesis, Biological Assessment and Molecular Docking Insights

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Introduction: Epidermal growth factor receptor (EGFR) regulates several cell functions which include cell growth, survival, multiplication, differentiation, and apoptosis. Currently, EGFR kinase inhibitors are of increasing interest as promising targeted antitumor therapeutic agents.

Methods: Different thiazolyl-pyrazoline derivatives (**7a-o**) were synthesized and were first tested for anti-proliferative effect towards the A549 lung cancer cell line and the T-47D breast cancer cell line in MTT assay. Thereafter, thiazolyl-pyrazolines (**7b**, **7g**, **7l**, and **7m**) were subsequently evaluated for their PK inhibition for EGFR. Moreover, representative promising derivatives (**7g** and **7m**) in cytotoxic and PK inhibition assays were tested to investigate their impact on the apoptosis and cell cycle phases in T-47D cells in order to explore more insights into the antitumor actions of the target thiazolyl-pyrazolines. Furthermore, docking studies were accomplished to evaluate the patterns of binding of thiazolyl-pyrazolines **7b**, **7g**, **7l**, and **7m** in the EGFR active pocket (PDB ID: 1M17).

Results: Testing the thiazolyl pyrazoline compounds **7a-o** on A549 and T-47D cell lines showed IC_{50} arrays between 3.92 and 89.03 μ M, and between 0.75 and 77.10 μ M, respectively. Also, the tested thiazolyl-pyrazolines (**7b**, **7g**, **7l**, and **7m**) demonstrated significant sub-micromolar EGFR inhibitory actions with IC_{50} values 83, 262, 171 and 305 nM, respectively, in comparison to erlotinib (IC_{50} = 57 nM).

Discussion: Generally, it was observed that the tested thiazolyl pyrazolines showed more potent antiproliferative activity toward breast cancer cells T-47D than toward lung cancer cell lines A549. In particular, thiazolyl pyrazolines **7g** and **7m** showed the best activity against A549 cells (IC_{50} = 3.92 and 6.53 μ M) and T-47D cells (IC_{50} = 0.88 and 0.75 μ M). Compounds **7g** and **7m** provoked a sub-G1 phase arrest and cell apoptosis which are in agreement with the expected outcome of EGFR inhibition. Finally, the molecular docking of **7g** and **7m** in the active site of EGFR revealed a common binding pattern similar to that of erlotinib which involves the accommodation of the 1,3 thiazol-4-one ring and pyrazoline ring of target compounds in the binding region of erlotinib's quinazoline ring and anilino moiety.

Keywords: antitumor, EGFR inhibitors, pyrazolo, molecular docking, breast cancer, lung cancer

Introduction

Cancer stands as a baleful fatal challenge, in the current medical era, with regard to morbidity and mortality ranks second only to cardiovascular diseases and is expected to overtake the leading death cause globally in the near future.¹ Accordingly, World Health Organization (WHO) reported that the number of cancer deaths could reach 13 million by the end of 2030.^{1,2} It is estimated that one out of every five people under the age of 75 will develop cancer during their lifetime.¹⁻³ Most cancers are characterized by uncontrolled cell division, growth, and impaired proliferation because of the deregulation of the necessary proteins and enzymes that control cell division.⁴ Despite the numerous efforts to treat cancer diseases and the significant progress from cancer diagnosis to cancer treatment, drug developers worldwide face a slew of issues stemming from the several undesirable side effects of conventional non-selective chemotherapeutic agents such as systemic toxicity, decreased bioavailability, and drug resistance.⁵

The emergence of targeted therapies specifically the signaling-pathways-targeting chemotherapeutic agents construct a new era of selective chemotherapeutic hits that could attack cancer cells and/or the tumor micro-environment responsible for tumor proliferation, resulting in greater efficacy and minor side effects on normal cells.^{6,7} One of the most principal signaling pathways was the tyrosine kinase (TK) Epidermal Growth Factor Receptor (EGFR) that regulates several cell functions which include cell growth, survival, multiplication, differentiation, and apoptosis. The EGFR is activated as a result of conformational changes caused by binding interactions of the endogenous EGF ligand within EGFR extracellular binding domain.^{8,9} As a result, several tyrosine residues are autophosphorylated resulting in a downstream activation of Mitogen Activated Protein Kinase (MAPK) pathway through the phosphorylation of following proteins in the pathway. Remarkable mutations in multiple proteins of the MAPK pathway, including EGFR, have been linked to various cancer types which include the colorectal carcinoma, hepatocellular carcinoma, pancreatic cancer, breast cancer, as well as the non-small cell lung cancer.¹⁰ This explains why EGFR kinase inhibitors are of increasing interest as promising targeted antitumor therapeutic agents.

Both pyrazoline- and thiazole-bearing small molecules have variable forms of biological activities, including anti-inflammatory, anti-cholinesterase, antimalarial, anti-carbonic anhydrase, and antimicrobial.¹¹⁻¹⁷ In particular, pyrazolo- and thiazolo-based small molecules are significant organic compounds that have been extensively studied and reported for their antitumor activity.¹⁸⁻²¹ Surveying literature showed that there has been a remarkable rise in the amount of published studies investigating the antitumor and kinase inhibitory activities of both pyrazoles and thiazoles.^{9,21-27} For example, Akhtar et al²⁸ reported new pyrazoline-bearing EGFR inhibitors (Compound I, Figure 1) with potent cytotoxic effect against several human cancer cell lines which include breast cells MDA-MB231, and lung cells A549. Moreover, in 2021 Kamonpan et al²⁹ investigated the antitumor effect of a series of thiazole-based chalcone derivatives against lung carcinoma cells A549 overexpressing EGFR. Interestingly, compound II (Figure 1) showed promising inhibitory activities against both lung carcinoma cells (A549) growth and EGFR TK (IC₅₀ of 16.30 μM and 33 nM, respectively) upon comparison to the reference drug erlotinib.

In the last few years, many research groups were interested in designing and synthesizing diverse thiazolyl-pyrazoline hybrids in order to gain the most beneficial antitumor activity of both rings.³⁰⁻³³ In 2019, Mohamed et al³⁴ synthesized novel series of thiazolylpyrazolyl coumarin derivatives as new effective vascular endothelial growth factor receptor-2 (VEGFR-2) inhibitors. In particular, the polycyclic lead compound III (Figure 1) exhibited a remarkably potent VEGFR-2 inhibitory effect (IC₅₀= 34 nM), in addition to a significant growth inhibitory effect against different human cancer cell lines especially breast MCF-7 cells (IC₅₀= 5.41 μM). Moreover, another new series of dual EGFR/HER2 inhibitors based on a hybrid thiazolyl/pyrazoline scaffold has been reported recently which exhibited promising anti-proliferative activity towards lung A549 and breast MCF-7 cancer cell lines in MTT cytotoxicity assay.³⁵ Compound IV (Figure 1) inhibited HER2 and EGFR kinases possessing IC₅₀ of 2.28 μM and 4.34 μM, respectively. Moreover, it exerted moderate anti-proliferative effect against the two examined cell lines with IC₅₀ of 10.76 μM and 8.05 μM, respectively.³⁵ By the end of 2019, another study reported two series of dihydropyrazole thiazole hybrids, one of the promising derivatives was

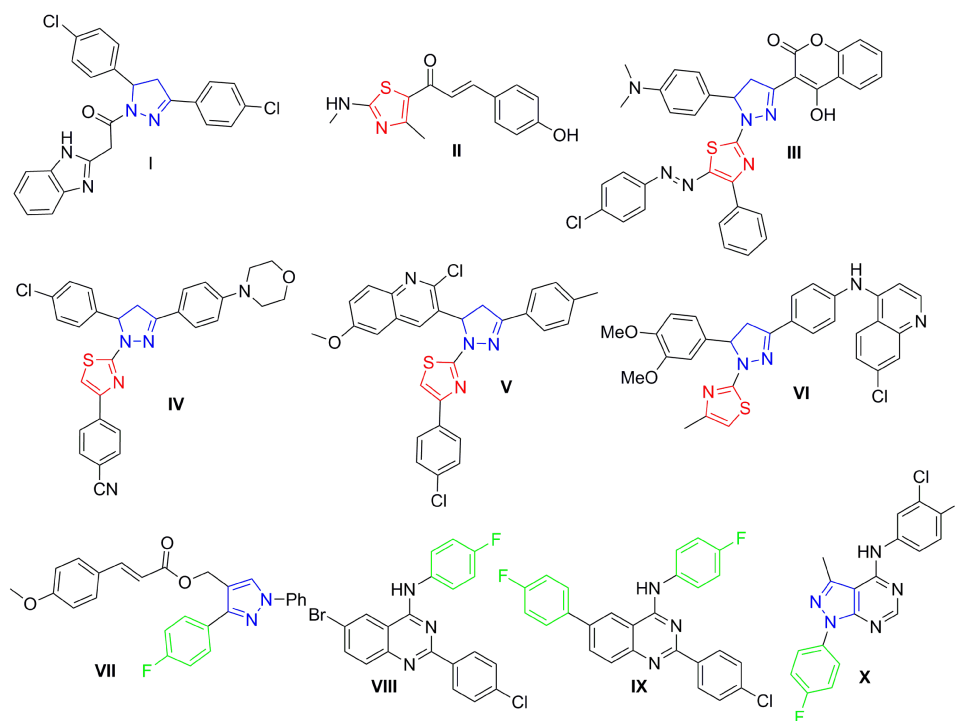


Figure 1 Structures of certain pyrazoline- and/or thiazole-bearing small molecules (I–X) endowed with anticancer and kinases inhibitory activities.

compound **V** (Figure 1) that exerted strong anti-proliferative impact against breast cancer cell line (MCF-7) (IC_{50} of 0.227 μ M) through inhibition of EGFR with an IC_{50} of 31.8 nM with a positive control (Gefitinib) IC_{50} of 29.16 nM.³⁶ Recently in 2021, novel thiazolopyrazoline-based small molecules were reported by Batran et al,³⁷ from which, compound **VI** (Figure 1) showed dual kinase inhibitory effect towards EGFR and HER2 with IC_{50} of 60 nM and 80 nM, respectively, versus 40 nM for erlotinib.

Several studies reported 4-fluorophenyl moiety as a key part of several potent EGFR inhibitors with pronounced cytotoxic activity on several cancer cell lines.^{38–40} The 4-fluorophenyl pyrazole derivative **VII** (Figure 1) showed a potent cytotoxic activity on MCF-7 breast cancer cell line with IC_{50} of 3.87 μ M and a promising EGFR and HER-2 inhibitory activity with IC_{50} of 4.87 and 6.23 μ M, respectively.³⁸ The 4-fluorophenyl quinazolines **VIII** and **IX** (Figure 1) exhibited promising cytotoxic activity on MCF-7 and HeLa cell lines with IC_{50} of 1.44 μ M and 2.70 μ M, respectively, on MCF-7 and IC_{50} of 4.97 μ M and 1.39 μ M, respectively, on HeLa cell lines. Moreover, they showed effective EGFR inhibitory activity with IC_{50} of 37.66 nM and 59.21 nM, respectively.³⁹ Finally, the 4-fluorophenyl pyrazolo[3,4-*d*]pyrimidine derivative **X** (Figure 1) proved prominent anticancer activity against OVCAR-4, NCI-H460, and ACHN cell lines with IC_{50} of 1.74, 4.44, and 5.53 μ M, respectively, more potent than the used reference standard (erlotinib); it inhibited EGFR and HER2 kinases at sub-micromolar level (IC_{50} of 0.18 and 0.25 μ M, respectively).⁴⁰

The pronounced cytotoxic activity of the pyrazoline/thiazole hybrid compounds **III–VI** and the 4-fluorophenyl derivatives **VIII–X** which attributed to their remarkable kinase inhibitory activity on EGFR, HER2, and/or VEGFR2 encouraged us to design and synthesize a novel series of 4-fluorophenyl substituted thiazolyl-pyrazoline derivatives (**7a–o**) (Figure 2). They were further evaluated for their cytotoxic activity against the lung cancer cell line A549 and the breast cancer cell line T-47D using MTT assay. Derivatives exhibited potent anti-proliferative action were subsequently evaluated for their PK inhibition for EGFR. Representative promising derivatives in cytotoxic and PK inhibition were further tested for their effect on cell apoptosis and cell cycle progression in breast cancer cell line (T-47D). In addition, molecular docking simulations were performed to predict the binding pattern of the target thiazolyl-pyrazoline hybrids in EGFR kinase domain (PDB ID: 1M17).

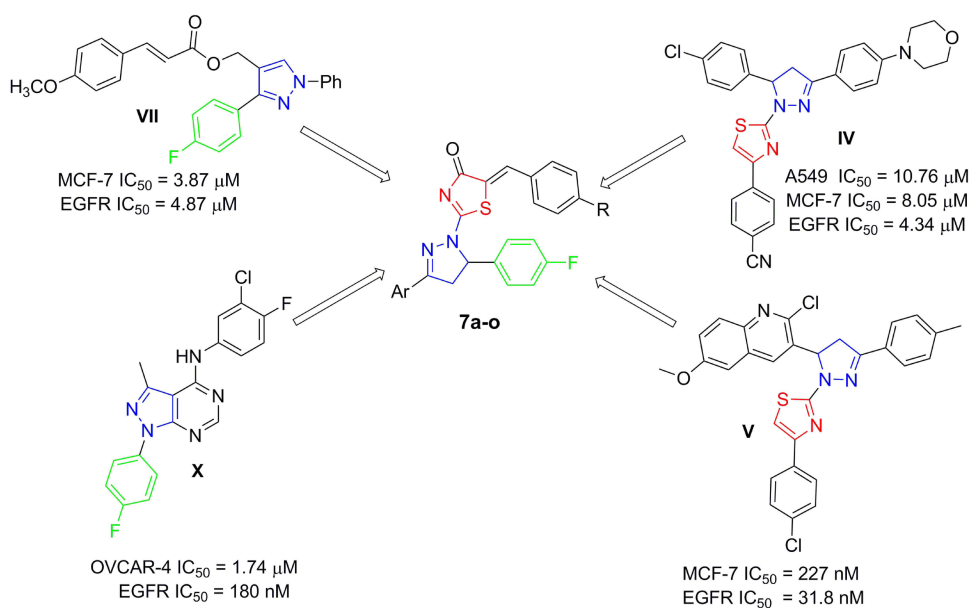


Figure 2 Design of the target thiazolyl pyrazolines **7a-o**, based on the structure of compounds **IV**, **V**, **VII** and **X**.

Materials and Methods

Chemical Synthesis

General

The proton NMR spectra were collected on a Bruker 400 MHz spectrometer for all herein prepared novel intermediates and final target thiazolyl pyrazolines. ^{13}C NMR spectra were done in deuterated dimethylsulfoxide (DMSO- d_6) using 100 MHz frequency. Chemical shifts (δ_H) have been reported relative to the solvent (DMSO- d_6) peaks. IR spectra were collected on Shimadzu FT-IR 8400S spectrophotometer. Elemental analyses (EI) were measured at the Regional Center for Microbiology and Biotechnology, Al-Azhar University, Egypt. Compounds **3a-c**, **4a-c** and **5a-b** were reported previously.^{41,42}

Synthesis of Thiazolidenone Derivatives **5a-c**

The appropriate pyrazoline thioamide derivative **4a-c** (6 mmol) was heated under reflux with equimolar amount of ethyl bromoacetate (1 g, 6 mmol) in absolute EtOH (30 mL), with TLC monitoring. After 4 hours, the starting materials were consumed, and the whole mixture was cooled to lab temperature. The resulting precipitate was collected with filtration, washed with petroleum ether and recrystallized from dioxane to produce the key thiazolidenone intermediates **5a-c** in a good yield (87–91%).

Synthesis of the Target Thiazolyl-Pyrazolines **7a-o**

To a hot stirring solution of thiazolidenone intermediates **5a-c** (1 mmol) in glacial acetic acid (10 mL), the appropriate aldehydes **6a-e** (1.1 mmol) and sodium acetate (0.1 g, 1.2 mmol) were mixed. The whole solution was heated for 5 hours then the resulted precipitate was collected by filtration on hot, washed using hexane and crystallized from DMF/ethanol mixture to provide the thiazolyl-pyrazolines **7a-o** in a good yield (78–90%).

Full characterisation (physical and spectral data) for the key intermediate (**5c**) and the target thiazolyl pyrazolines (**7a-o**) were presented in the [Supporting Information](#).

Biological Evaluation

All experimental procedures utilized in the biological cytotoxicity,^{43,44} Annexin V-FITC Apoptosis,⁴⁵ cell cycle,⁴⁶ and EGFR TK⁴⁷ assays were carried out as reported earlier and were shown in the [Supporting Information](#).

Molecular Docking Studies

Molecular docking was carried out using Molecular Operating Environment software (MOE, 2020.0901), using X-ray crystallographic structure of EGFR co-crystallized with erlotinib (PDB code: 1M17). The details of the utilized protocol as well as its validation were provided in the [Supporting Information](#).

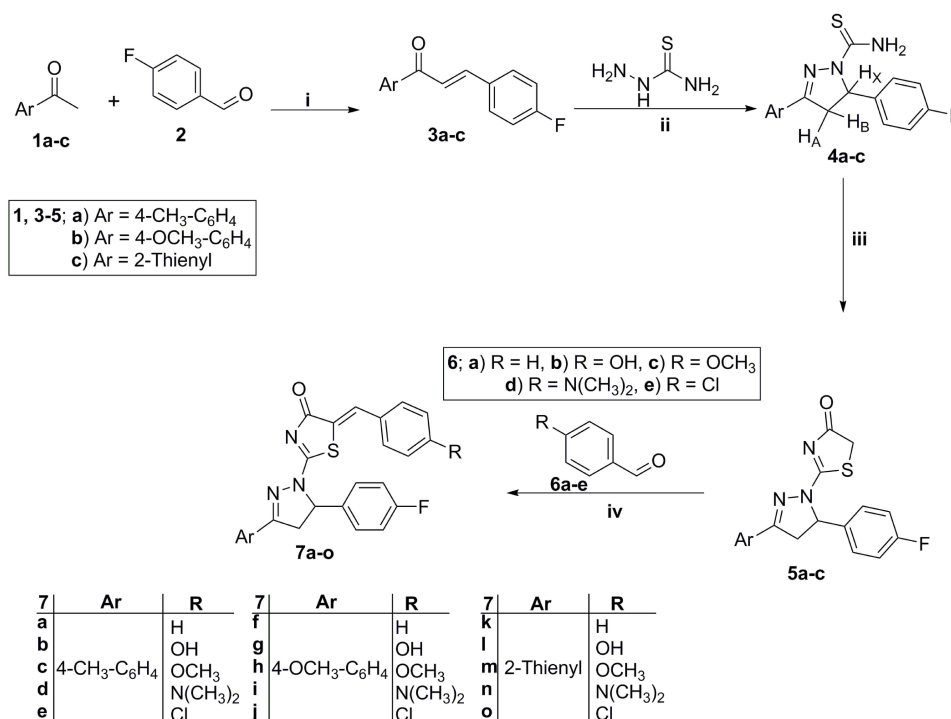
Results and Discussion

Synthesis of Compounds

The preparation of pyrazoline derivatives **7a-o** was done through the synthetic scheme depicted in [Scheme 1](#). The chalcones intermediate **3a-c** were prepared through a condensation reaction of different acetophenones **1a-c** with 4-fluorobenzaldehyde **2** via the reported procedures.⁴⁸ The previously prepared chalcones **3a-c** subjected to heterocyclization with thiosemicarbazide in basic condition (using NaOH) to furnish the pyrazoline derivatives **4a-c**. The pyrazoline derivatives **4a-c** were further cyclized with ethyl bromoacetate in absolute ethyl alcohol to produce the key thiazolidenone intermediates **5a-c**. Then, a Knoevenagel condensation reaction between thiazolidenones **5a-c** and different aldehydes **6a-e**, in refluxing acetic acid and sodium acetate, led to the production of thiazolyl-pyrazoline derivatives **7a-o**. [Scheme 1](#) illustrates these steps.

All of the prepared thiazolyl-pyrazolines **7a-o** showed satisfactory spectroscopic and analytical data that have complete agreement with respective structures. The proton NMR spectra of the targeted thiazolyl-pyrazolines clearly show a couple of doublets of doublet signals attributable to the diastereotopic protons (H_A and H_B) of $-CH_2-$ group at C-4 of the pyrazoline ring around δ 3.50 and 4.0 ppm. Moreover, a third doublets of doublet signal corresponding to the C-5 proton of the pyrazoline motif around δ 5.50 ppm because of the vicinal coupling between the non-equivalent methylene hydrogens at C-4 of the pyrazoline moiety.

In addition, the ¹H NMR spectra of thiazolyl-pyrazolines **7c**, **7f-j** and **7m** confirmed the presence of extra aliphatic signals attributable to the protons of the methoxy group around δ 3.85 ppm, while the spectra of thiazolyl-pyrazolines **7a-e** revealed the presence of another aliphatic signals for CH_3 group protons around δ 2.30 ppm. Moreover, the structure of thiazolyl-pyrazolines **7d**, **7i** and **7n** was confirmed with the existence of a singlet signal of $(N(CH_3)_2)$ protons at δ 3.0 ppm, whereas, the



Scheme 1 Preparation of key thiazolyl-pyrazolines **7a-o** products; Reagents and conditions: (i) 40% NaOH, 95% ethanol, stirring R.T. 8 hr; (ii) NaOH, ethanol, reflux 2 hr; (iii) Ethyl bromoacetate, absolute ethyl alcohol, reflux 4 hr; (iv) Glacial acetic acid, sodium acetate, reflux 5 hr.

structure of compounds **7b**, **7g** and **7l** was proven with the presence of an exchangeable proton of OH functionality at δ 10.10–10.21 ppm. Moreover, the ^{13}C NMR spectra of the targeted thiazolyl pyrazoline derivatives **7a–o** confirmed the presence of the two characteristic aliphatic signals of pyrazoline ring (C-4 and C-5) around δ 44.50 and 67.50 ppm, respectively.

Biological Evaluation

Using MTT assay, the novel new prepared thiazolyl-pyrazolines **7a–o** were first evaluated for their anti-proliferative effect on the lung cancer cell line A549 and the breast cancer cell line T-47D. Compounds exhibited potent anti-proliferative action, were subsequently evaluated for their PK inhibition for EGFR. Representative promising derivatives in cytotoxic and PK inhibition were tested on T-47D cell line apoptosis and cell cycle progression.

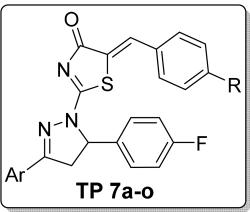
In vitro Cytotoxic Activity

It is well-reported that EGFR is frequently overexpressed in diverse human malignancies such as non-small cell lung cancer and breast cancer, as well as, it stands out as a key molecule in the initiation and progression for these tumors.^{49–52} In this study, A549 and T-47D cell lines were selected as representative ones for the NSCLC and breast cancer respectively. Using the MTT assay, all the prepared thiazolyl-pyrazolines **7a–o** were tested for their inhibitory effect on two different cell lines including A549 lung cancer and the T-47D breast cancer cell lines, and they were compared to Staurosporine and erlotinib as a standard reference anticancer drugs.⁵³ IC₅₀ values of target compounds against both cell lines are shown in Table 1.

Testing the thiazolyl pyrazoline compounds **7a–o** on A549 cell line showed an IC₅₀ array between 3.92 and 89.03 μM , with compounds **7b** and **7g** showing the most potent in vitro anti-proliferative impact with (IC₅₀ of 4.41 and 3.92 μM , respectively) comparable to staurosporine (IC₅₀ = 4.29 μM). Moreover, compounds **7l** and **7m** showed a moderate antiproliferative activity (IC₅₀ = 8.10 and 6.53 μM , respectively), whereas the remainder of compounds showed modest antiproliferative activities (IC₅₀ are between 10 and 100 μM) (Table 1).

Testing the thiazolyl pyrazoline compounds on T-47D cell line exhibited an IC₅₀ array between 0.75 and 77.10 μM . Compounds **7b**, **7g**, **7l** and **7m** showed more potent antiproliferative activity (IC₅₀ of 0.75, 0.88, 1.15, and 1.66 μM , respectively) than the used reference standard (staurosporine) (IC₅₀ = 6.83 μM). Moreover, compounds **7e** and **7c** showed moderate cytotoxic activity with IC₅₀ of 7.06 and 9.30 μM , respectively, while the remainder compounds showed modest

Table 1 IC₅₀ Values for Thiazolyl Pyrazoline Derivatives (TP **7a–o**) Towards Two Cancer Cell Lines; Lung (A549) and Breast (T-47D)

	Compound	Ar	R	IC ₅₀ (μM) ^a		
				A549	T-47D	
 <p>TP 7a–o</p>	TP 7a	<i>p</i> -CH ₃ -C ₆ H ₄	H	66.50 ± 3.38	17.10 ± 1.03	
	TP 7b	<i>p</i> -CH ₃ -C ₆ H ₄	OH	4.41 ± 0.59	1.15 ± 0.56	
	TP 7c	<i>p</i> -CH ₃ -C ₆ H ₄	O-CH ₃	11.72 ± 1.06	9.30 ± 0.07	
	TP 7d	<i>p</i> -CH ₃ -C ₆ H ₄	N-(CH ₃) ₂	28.80 ± 1.21	43.10 ± 2.61	
	TP 7e	<i>p</i> -CH ₃ -C ₆ H ₄	Cl	43.11 ± 3.70	7.06 ± 0.43	
	TP 7f	<i>p</i> -OCH ₃ -C ₆ H ₄	H	NA ^b	77.10 ± 4.68	
	TP 7g	<i>p</i> -OCH ₃ -C ₆ H ₄	OH	3.92 ± 0.18	0.88 ± 0.05	
	TP 7h	<i>p</i> -OCH ₃ -C ₆ H ₄	O-CH ₃	21.73 ± 1.96	14.4 ± 0.87	
	TP 7i	<i>p</i> -OCH ₃ -C ₆ H ₄	N-(CH ₃) ₂	78.24 ± 3.28	NA ^b	
	TP 7j	<i>p</i> -OCH ₃ -C ₆ H ₄	Cl	89.03 ± 4.62	55.10 ± 3.34	
	TP 7k	2-thienyl	H	37.49 ± 2.26	12.90 ± 0.78	
	TP 7l	2-thienyl	OH	8.10 ± 0.37	1.66 ± 0.1	
	TP 7m	2-thienyl	O-CH ₃	6.53 ± 0.23	0.75 ± 0.05	
	TP 7n	2-thienyl	N-(CH ₃) ₂	24.07 ± 1.83	29.10 ± 1.76	
	TP 7o	2-thienyl	Cl	33.81 ± 2.05	16.90 ± 1.03	
	Staurosporine				4.29 ± 0.72	6.83 ± 1.03
	Erlotinib				5.73 ± 0.69	8.14 ± 0.97

Notes: ^aIC₅₀ values are the average ± standard deviation of independent triplicates. ^bNA: entries with IC₅₀ values more than 100 μM .

antiproliferative activities with IC_{50} ranged between 10 and 100 μM . Generally, the tested pyrazoline derivatives showed more potent antiproliferative activity on the breast cancer cell line (T-47D) than on the lung cancer cell line (A549) (Table 1).

As can be seen in Table 1, the hydrophilic *p*-hydroxy substituted phenyl ring improves the antiproliferative activity of the tested derivatives against both used cell lines as indicated by the significant potency of thiazolyl pyrazoline **7b**, **7g** and **7l** with IC_{50} of 4.41, 3.92 and 8.10 μM , respectively, towards A549 cell line and IC_{50} of 1.15, 0.88 and 1.66 μM , respectively, against T-47D cell line compared to staurosporine (IC_{50} = 4.29 and 6.83 μM , respectively). Moreover, the *p*-methoxyphenyl derivatives **7c**, **7h** and **7m** showed potent to moderate antiproliferative activity against both used cell lines with IC_{50} of 11.72, 21.73 and 6.53 μM , respectively, against A549 cell line and IC_{50} of 9.30, 14.40 and 0.75 μM , respectively, against T-47D cell line. Thiazolyl pyrazoline **7g** and **7m** showed the most potent antiproliferative activity toward the tested cell lines with IC_{50} equal 3.92 and 6.53 μM , respectively, against A549 cell line and IC_{50} of 0.88 and 0.75 μM , respectively, toward T-47D cell line.

From the presented IC_{50} values in Table 1, we can draw valuable structure activity relationships (SARs). First, the obtained cell growth inhibition data ascribed to the target thiazolyl pyrazoline derivatives **7a–o** more enhanced activity toward breast T-47D cell line than lung A549 cell line, with an exception for *N,N*-dimethylamino bearing compounds **7d**, **7i** and **7n** which displayed slight better activity against A549 cell line (IC_{50} = 28.80 \pm 1.21, 78.24 \pm 3.28 and 24.07 \pm 1.83 μM , respectively) than T-47D cell line (IC_{50} = 43.10 \pm 2.61, > 100 and 29.10 \pm 1.76 μM , respectively), Table 1.

Upon investigation of impact of the substitution of the benzylidene motif, appended on C-5 of the thiazol-4-one ring, we can see that grafting of different substituents within the para position such as hydroxyl, methoxy, *N,N*-dimethylamino and chloro substituents, resulted in an enhanced anti-proliferative activity against lung A549 cell line. This could be evidenced by the lower IC_{50} values of the substituted thiazolyl pyrazoline derivatives **7b–d** (IC_{50} range: 4.41 \pm 0.59–43.11 \pm 3.70 μM), **7g–j** (IC_{50} range: 3.92 \pm 0.18–89.03 \pm 4.62 μM), and **7l–o** (IC_{50} range: 6.53 \pm 0.23–33.81 \pm 2.05 μM), comparing to their unsubstituted analogues **7a**, **7f** and **7k** (IC_{50} = 66.50 \pm 3.38, > 100 and 37.49 \pm 2.26 μM), respectively. In like manner, the para substitution of the benzylidene motif in the target thiazolyl pyrazolines with hydroxyl, methoxy, and chloro substituents boosted the anti-proliferative activity against breast T-47D cell line. Compounds **7b**, **7c** and **7e** displayed better anti-proliferative activity (IC_{50} = 1.15 \pm 0.56, 9.30 \pm 0.07 and 7.06 \pm 0.43 μM , respectively) than compound **7a** (IC_{50} = 17.10 \pm 1.03 μM), as well as thiazolyl pyrazolines **7g**, **7h** and **7j** were more potent than their unsubstituted counterpart **7f** (IC_{50} = 77.10 \pm 4.68 μM). On the other hand, para substitution of the benzylidene moiety with *N,N*-dimethylamino functionality resulted in target thiazolyl pyrazolines **7d**, **7i** and **7n** with decreased anti-proliferative activity against T-47D cell line (IC_{50} = 43.10 \pm 2.61, > 100 and 29.10 \pm 1.76 μM , respectively) than their unsubstituted counterparts **7a**, **7f** and **7k** (IC_{50} = 17.10 \pm 1.03, 77.10 \pm 4.68 and 12.90 \pm 0.78 μM , respectively).

Concerning the SAR about the aryl moiety appended to C-3 of the pyrazoline ring, it is worth mentioning that the bioisosteric replacement of the 4-methyl/methoxy phenyl ring with the 2-thienyl heterocycle led to an enhancement of the anti-proliferative activity toward both examined cell lines (A549 and T-47D), except the 4-hydroxyl derivatives **7b** and **7g**.

Furthermore, the cytotoxic activity of thiazolyl-pyrazolines **7b**, **7g**, **7l** and **7m** was assessed against non-tumorigenic breast MCF-10A cell line to explore their selectivity toward the cancer cells. Interestingly, the examined thiazolyl-pyrazolines exerted non-significant toxicity towards the normal breast MCF-10A cells with IC_{50} s equal 48.19 \pm 3.71, 55.03 \pm 4.53, 72.84 \pm 4.02 and 39.70 \pm 2.68, respectively (Table 2).

Table 2 Cytotoxic Activity of Thiazolyl-Pyrazolines **7b**, **7g**, **7l** and **7m** Against Non-Tumorigenic Breast MCF-10A Cell Line

Compound	IC_{50} (μM)
7b	48.19 \pm 3.71
7g	55.03 \pm 4.53
7l	72.84 \pm 4.02
7m	39.70 \pm 2.68

Table 3 Inhibitory Activity of **7b**, **7g**, **7l** and **7m** Against EGFR

Compound	IC ₅₀ (nM)
	EGFR
7b	83±4
7g	262±13
7l	171±9
7m	305±16
Erlotinib	57±3

EGFR Inhibitory Activity

Compounds with significant antiproliferative impact against the investigated cell lines, viz, **7b**, **7g**, **7l**, and **7m**, were biochemically assessed for their EGFR inhibitory activity and erlotinib was used as a reference standard, [Table 3](#) summarizes these results. When compared to the employed reference standard, erlotinib (IC₅₀ = 57 nM), the tried compounds demonstrated significant nanomolar inhibitory activities with IC₅₀ values of 83, 262, 171 and 305 nM, respectively.

Regarding the correlation of cytotoxicity with EGFR inhibition, it is worth mentioning that the cell growth inhibition profile of thiazolyl-pyrazolines **7b**, **7g**, **7l** and **7m** was found to be rather flat, where the measured IC₅₀ toward lung (A549) and breast (T-47D) cell lines ranged from 3.92 ± 0.18–8.10 ± 0.37 and 0.75 ± 0.05–1.66 ± 0.1 μM, respectively. The same could be observed for the resulting inhibitory activity against EGFR since the obtained IC₅₀ values span from 83 ± 4 to 305 ± 16 nM, with about 3-fold difference in activity.

Cell Cycle Analysis

Compounds with significant antiproliferative and EGFR inhibitory activities **7g** and **7m** were next evaluated for the impact on cell cycle using flow cytometry technique utilizing propidium iodide (PI) staining. Cell cycle parameters for T-47D cells were compared after incubation of test compounds for 24 hours and after using DMSO as a negative control. [Table 4](#) and [Figure S3](#) show the results of this experiment.

According to [Table 4](#), a significant increase of cells in the sub-G1 phase is seen, from 2.14% in control to 31.41 and 26.78% in cells incubated with **7g** and **7m**, respectively, which suggest a sub-G1 phase arrest and cell apoptosis which is the expected outcome of EGFR inhibition.^{54,55}

The higher efficiency of compound **7g** in cell arrest in the sub-G1 phase and in cell apoptosis which is seen in [Table 4](#) compared to its **7m** analogue agrees with its more potent antiproliferative effect on T-47D cell line as indicated by its IC₅₀ of 3.92 μM in comparison to that of **7m** (IC₅₀ of 6.53 μM).

Apoptosis Assay

The potent thiazolyl-pyrazolines **7g** and **7m** were investigated for action on cells apoptosis using Annexin V-FITC/propidium iodide dual-staining.⁵⁶ The apoptotic markers of the tested cell line, T-47D, were analyzed in the presence and absence of compounds **7g** and **7m**. Programmed cell death is characterized by the phosphatidylserine (PS) phospholipid translocation to the apoptotic cell surface. PS is stained with annexin V fluorescent conjugate then detected with flow cytometry. Additionally, T-47D cancer cells have been stained by the propidium iodide (PI) which is able to penetrate only the cells with broken plasma

Table 4 Effect of Thiazolyl-Pyrazolines **7g** and **7m** on the Cell Cycle Phases of T-47D Cells

Comp.	%G0-G1	%S	%G2/M	%Sub-G1
7g	32.6	30.74	5.24	31.42
7m	40.81	28.98	3.43	26.78
Control	51.71	32.73	13.42	2.14

Table 5 Distribution of the Apoptotic Cells in the AnnexinV Assay in T-47D Cells Upon Treatment with Thiazolyl Pyrazolines **7g** and **7m**

Comp.	Early Apoptosis (Lower Right %)	Late Apoptosis (Upper Right %)	Total (L.R % + U.R %)
7g	3.22	27.16	30.38
7m	6.28	18.97	25.25
Control	0.52	0.18	0.70

membranes; facilitating the differentiation among the early apoptotic cells (positive for PS, but negative for PI), late apoptotic and necrotic cells (positive for both PS and PI).

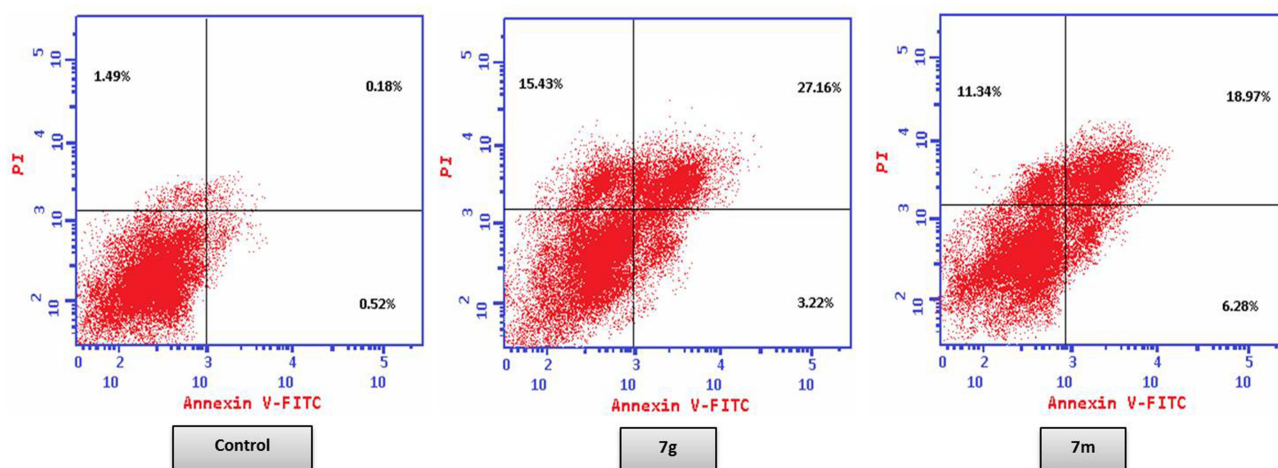
As can be seen in [Table 5](#) and [Figure 3](#), the total percentage of apoptotic cells in T-47D cell line increases because of compounds **7g** and **7m** treatment (30.38 and 25.25%, respectively) compared to non-treated cells (0.70%) which is a clear evidence of the apoptotic action of these two target compounds. Moreover, compounds **7g** and **7m** showed an increase in both early and late apoptotic phases where early phase was increased from 0.52 to 3.22 and 6.28 for **7g** and **7m**, respectively, while late phase was also increased from 0.18 to 27.16 and 18.97, respectively.

Likewise, The higher apoptotic effect of compound **7g** ([Table 5](#)) relative to its **7m** analogue agrees with its more potent antiproliferative effect on T-47D cell line, as indicated by its IC_{50} of 3.92 μ M in comparison to that of **7m** (IC_{50} of 6.53 μ M).

Docking Study

The molecular docking was performed to study the binding interactions between thiazolyl pyrazolines **7b**, **7g**, **7l**, and **7m** and the EGFR active pocket (PDB ID: 1M17)⁵⁷. The molecular docking procedure was first validated by self-docking of the known active co-crystallized ligand (erlotinib) in the proximity of the active site showing docking score (S) of -10.86 kcal/mol and RMSD of 1.46 Å. The ability of the docking protocol to predict the correct crystal structure pose was demonstrated by the presence of all key interactions between the residues in the active site and docked erlotinib indicating the suitability of it for the intended docking study. The key interactions are represented by the hydrogen bonding with Met 769, a water Hydrogen bond bridge with Thr 766 and cation-pi bond with Lys 721 (for further details, see [Supporting Information Figures S1](#) and [S2](#)).

The investigated compounds showed a common binding pattern which involves the accommodation of the 1,3-thiazol-4-one ring in the binding region of erlotinib's quinazoline ring involved in an essential hydrogen bond with the

**Figure 3** Pro-apoptotic effect of thiazolyl pyrazolines **7g** and **7m** toward breast cancer cells T-47D.

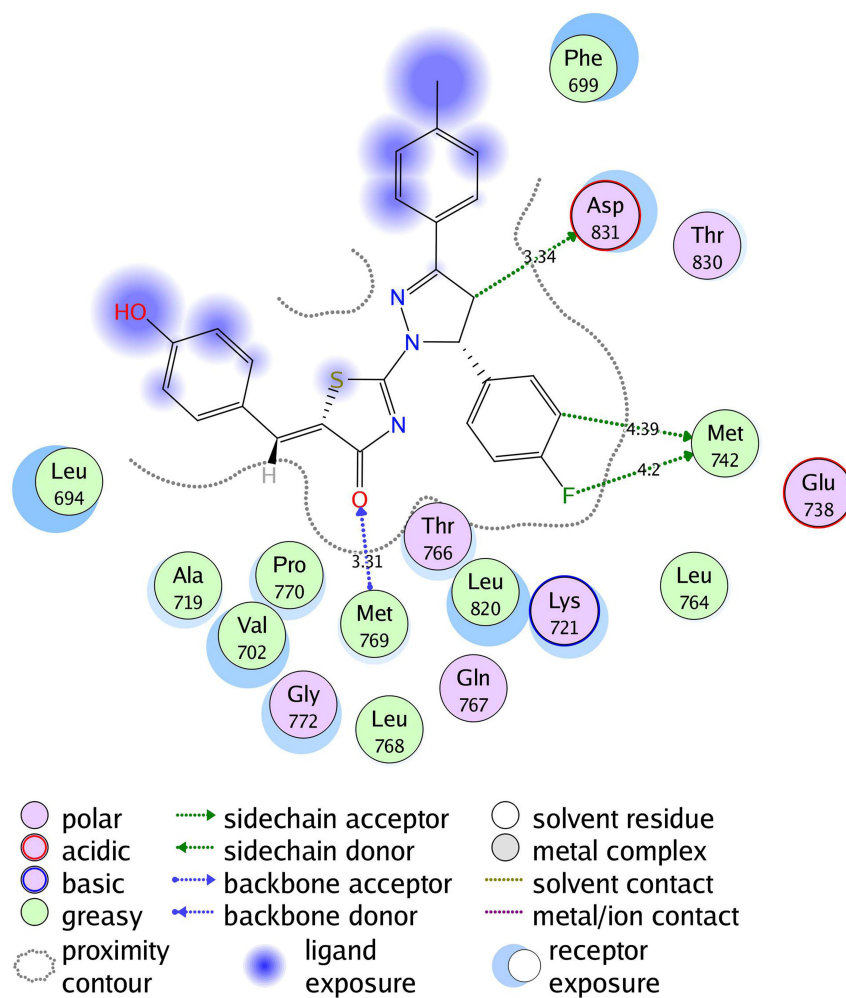


Figure 4 2D diagram of thiazolyl pyrazoline **7b** in the binding site of EGFR.

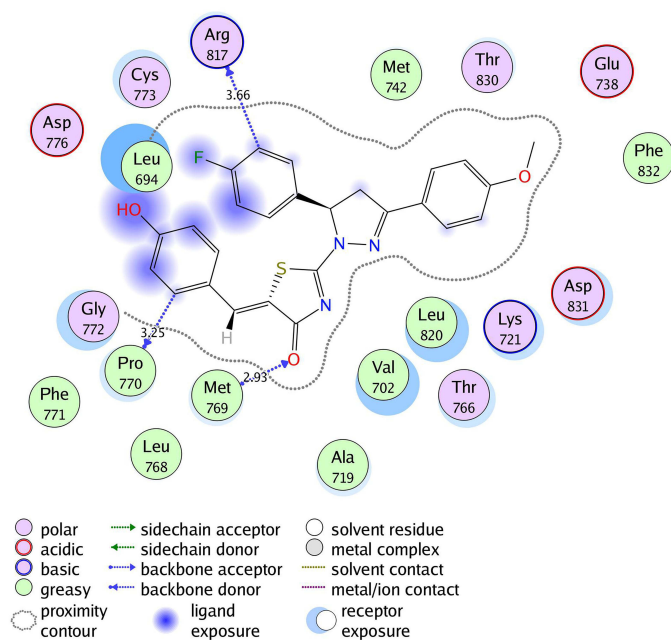


Figure 5 2D diagram for thiazolyl pyrazoline **7g** in the binding site of EGFR.

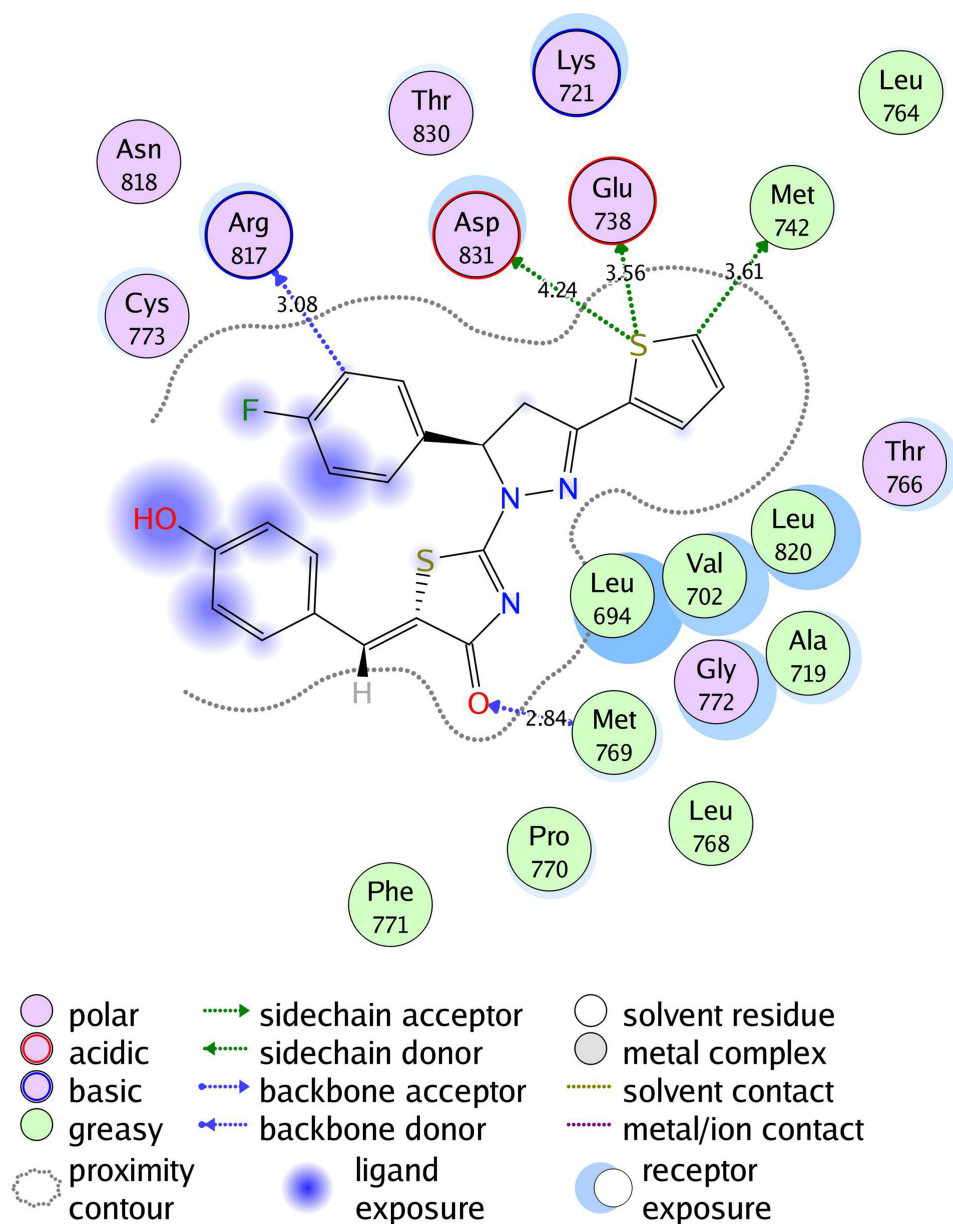


Figure 6 2D diagram of thiazolyl pyrazoline **7I** in the binding site of EGFR.

important residue Met 769 directing the 5-(*p*-hydroxy/*p*-methoxy phenyl) substitution towards the vicinity of the hydrophobic side chains of the amino acids Leu 694 and Leu 820.

The pyrazoline ring of the target compounds is accommodated in the binding region of erlotinib's anilino moiety directing the substitutions in position 3 and 5 of the pyrazoline ring towards both sides of the binding site interacting through several interactions with Glu738, Met742, and Asp831 on one side and Arg817 on the other side. The good activity of the tested thiazolyl pyrazoline derivatives was rationalized by their ability to interact with the key amino acids in the binding site as indicated by their docking binding pattern and docking score which ranged between -11.14 and -10.64 kcal/mol that was comparable to that of erlotinib ($S = -10.86$ kcal/mol) (Figures 4–7, and Table 6).

Conclusions

Novel synthesized thiazolyl-pyrazoline derivatives (**7a–o**) were investigated in vitro for their potential anticancer effect against the lung cancer cell line A549 and the breast cancer cell line T-47D in MTT assay. Generally, it was

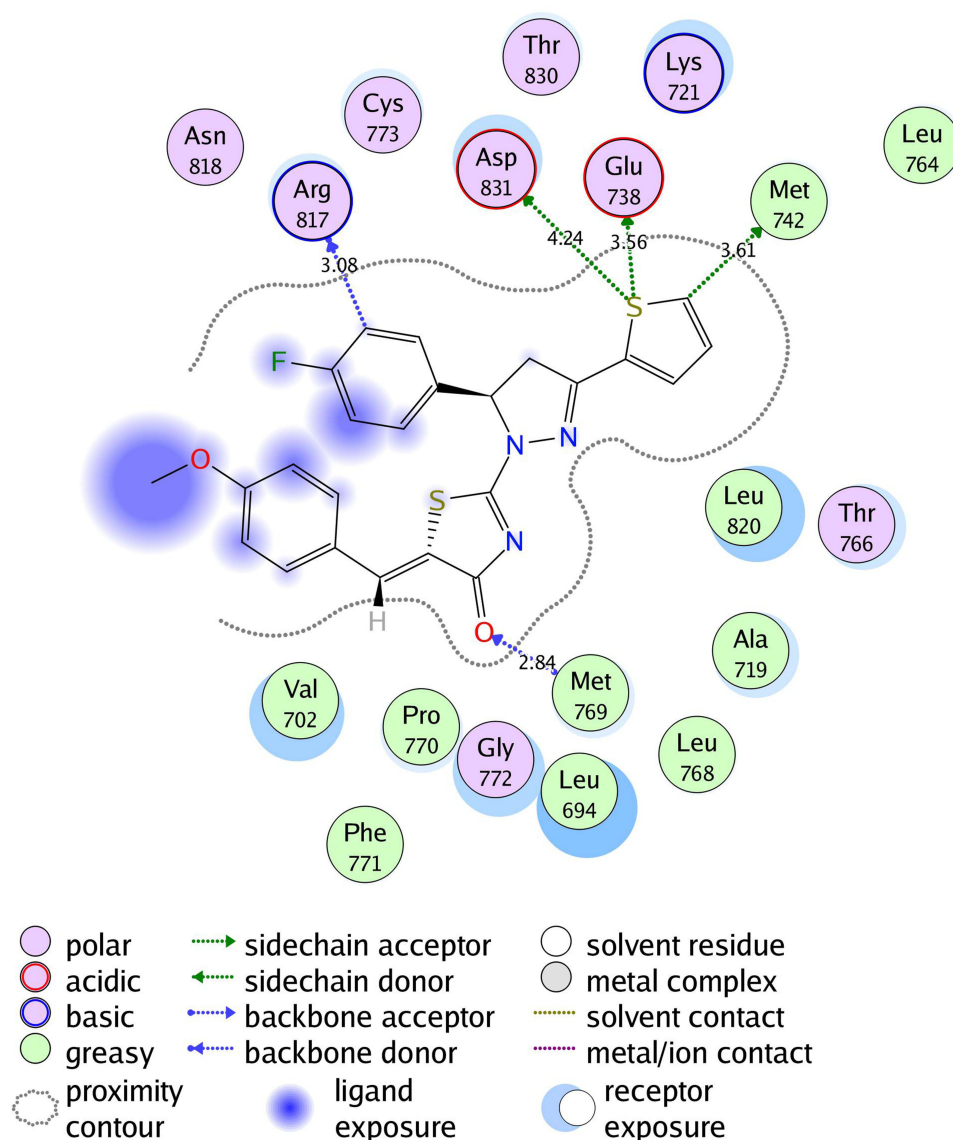


Figure 7 2D diagram of thiazolyl pyrazoline **7m** in the EGFR binding site.

observed that the tested thiazolyl pyrazolines showed more potent antiproliferative activity toward breast cancer cells T-47D than toward lung cancer cell lines A549. Four thiazolyl pyrazolines (**7b**, **7g**, **7l**, **7m**), out of fifteen, appeared to be potent cell growth inhibitors for both the examined cell lines. In particular, thiazolyl pyrazolines **7g** and **7m** showed the best activity against A549 cells ($IC_{50} = 3.92$ and $6.53 \mu\text{M}$, respectively) and T-47D cells ($IC_{50} = 0.88$ and $0.75 \mu\text{M}$, respectively). Further in vitro biochemical evaluation was performed for the potent hits (**7b**, **7g**, **7l**, **7m**) so as to assay their inhibitory effect for the EGFR kinase with respect to the approved EGFR inhibitor erlotinib. Target compounds demonstrated potent nanomolar inhibition activity with IC_{50} of 83, 262, 171 and 305 nM, respectively, in comparison to erlotinib ($IC_{50} = 57$ nM). Furthermore, a flow-cytometric cell cycle assay was carried out on the breast T-47D cells for the promising thiazolyl pyrazolines **7g** and **7m** so as to explore their cellular mechanism of action. Compounds **7g** and **7m** provoked a sub-G1 phase arrest and cell apoptosis which are in agreement with the expected outcome of EGFR inhibition. Finally, the molecular docking of **7g** and **7m** in the active site of EGFR revealed a common binding pattern similar to that of erlotinib which involves the accommodation of the 1,3 thiazol-4-one ring and pyrazoline ring of target compounds in the binding region of erlotinib's quinazoline ring and anilino moiety. Moreover, the ability of the tested thiazolyl pyrazolines to interact

Table 6 Docking Energy Scores (S) of Reference and Tested Thiazolyl Pyrazoline

Compound	Docking Score (S) (kcal/mol)
7b	-10.95
7g	-11.14
7l	-10.64
7m	-10.74
Erlotinib	-10.86

with the key amino acids in the binding site rationalizes their good activity as indicated by their docking binding pattern and docking score which ranged between -11.14 and -10.64 kcal/mol which is comparable to that of erlotinib (S = -10.86 kcal/mol).

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Disclosure

The authors declare no conflicts of interest in this work.

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