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Toward discovery of mutant EGFR inhibitors; Design, synthesis and *in vitro* biological evaluation of potent 4-arylamino-6-ureido and thioureido-quinazoline derivatives

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Abstract:

A new series of 4-anilinoquinazolines with C-6 ureido and thioureido side chains and various substituents at the C-4 anilino moiety was designed, synthesized and evaluated as wild type (WT) and mutant EGFR inhibitors. Most of the compounds inhibited EGFR kinase wild type (EGFR WT) with IC_{50} values in the low nanomolar range (<0.495-9.05 nM) and displayed more potent cytotoxic effect in BaF/3 expressing EGFR WT than reference compound gefitinib. The anti-proliferative effect of all synthesized compounds against gefitinib insensitive double mutant cell lines Ba/F3 expressing Del19/T790M and Ba/F3 expressing L858R/T790M were assayed. Compounds **4d, 6f, 7e** showed significant inhibition (IC_{50}= 1.76-2.38 μM) in these mutant lines and significant Her2 enzyme inhibition (IC_{50}= 19.2-40.6 nM) compared to lapatinib (60.1 nM). The Binding mode of compounds **6d, 6f, 7a, 7b** and **8b** were demonstrated**.** Furthermore, growth inhibition against gefitinib insensitive cell lines PC9-GR4 (Del19/T790M) were tested, compounds **6f** and **7e** showed about eight and three folds respectively greater potency than gefitinib. Our structure-activity relationships (SAR) studies suggested that presence of ethyl piperidino urea/thiourea at 6-position and bulky group of (3-chloro-4-(3-fluorobenzyloxy)phenyl)amino at 4-position of quinazoline may serve as promising scaffold for developing inhibitors against wild type and mutant EGFR.

Keywords:

EGFR inhibitors; Quinazolines; Urea derivatives; Synthesis; EGFR-Her2 Dual inhibitors; EGFR L858R/T790 mutant; EGFR Del19/T790M mutant.

1. Introduction:

The epidermal growth factor receptor (EGFR, Her1, ErbB1) is a member of four homologous members of ErbB family, along with Her2/ ErbB2, Her3/ ErbB3 and Her4/ ErbB4. They play an important role in many of the signaling processes that regulate numerous cellular functions of cell growth, differentiation, and angiogenesis [1, 2]. This signaling cascade is initiated upon ligand binding, through formation of hetero- and homo-dimers where hetero-dimerization with ErbB2 is the preferred partner for maximum signaling [3]. Overexpression and mutations of EGFR and ErbB2 have been observed in various human cancers including breast, lung, colon, and prostate cancers and are associated with poor prognosis [4]. Therefore, inhibition of EGFR and erbB2 kinase activity has emerged as a promising approach to cancer therapy [3]

Several EGFR inhibitors have been approved by FDA for treatment of cancer [5, 6]. Gefitinib and erlotinib are first generation EGFR inhibitors that are used for treatment of non-small lung cancer. Lapatinib is dual EGFR/ Her2 dual inhibitor approved for the treatment of Her2 positive breast cancer and also is investigated in phase Π clinical trials for lung cancer treatment [5, 7]. T790M mutation has caused resistance to the first generation and hence second generation irreversible inhibitors were generated to overcome resistance, these inhibitors, as Afatinib and Dacomitinib, have a Michael acceptor group at the 6-position of quinazoline that bind to a unique cysteine residue (cys 797) to overcome drug resistance and increase selectivity towards ErbB family [8], (**figure 1**). A drawback of these irreversible covalent inhibitors is that they might react with related off-target proteins, giving rise to unexpected and increased toxicity [9]. Therefore, it is important to develop inhibitors with reduced toxicity while retaining activity towards resistant cell lines.

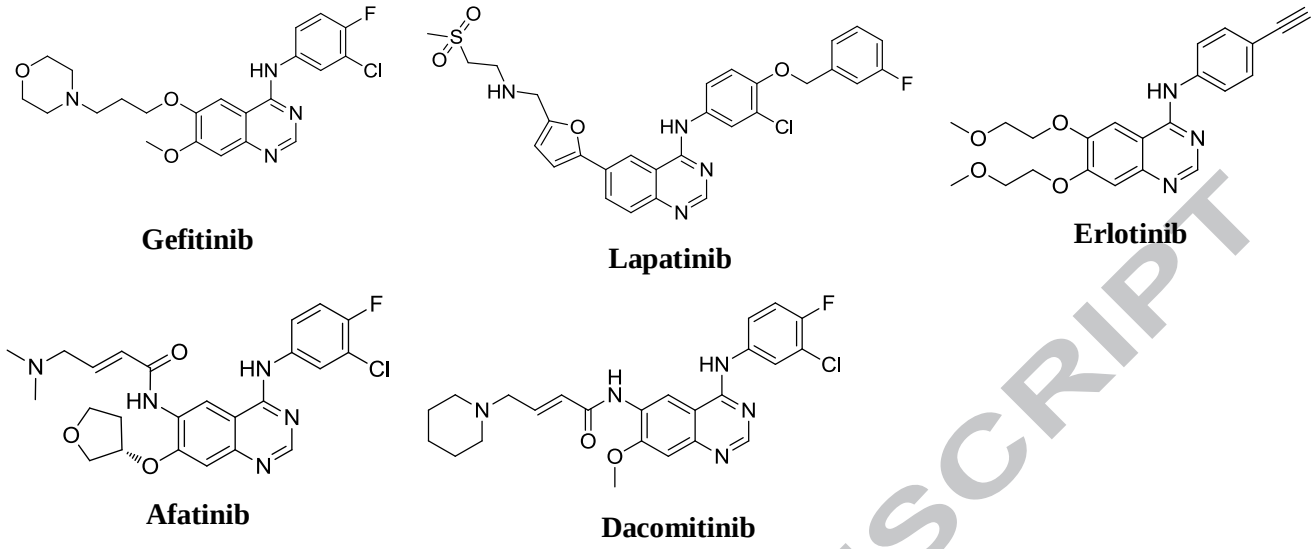

Figure 1: EGFR TK inhibitors

EGFR L858R and EGFR Del19 are activating mutations of EGFR that function by increasing the affinity with ATP. These mutations represent a common mechanism of tumor development [23, 29]. On the other hand, the T790M mutation is a secondary deactivating mutation of a key amino-acid. This mutation induces resistance to the available first generation TKIs (reversible inhibitors), causes loss of binding affinity to the active site and has been detected in 50% of NSCLC patients that acquired resistance [7, 10]. Therefore, it is a major challenge against EGFR driven cancers to develop inhibitors against the double mutants L858R/T790M and Del19/T790M that activates EGFR and prevents the inhibition [7].

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2. Rationale:

In our previous publication, Mowafy *et al* [2], a novel series of 6- urea anilinoquinazoline derivatives was synthesized and showed promising potent and selective inhibition towards EGFR-TK both biochemically as enzyme inhibitors and *in-vitro* cytotoxic effect against high EGFR expressing cell lines. Molecular modeling supported those findings by diplaying extra hydrogen bonding of 6-urea substitution to unique Cysteine (cys 797) of EGFR.

In addition, it has been reported that Her2 amplification is a potential mechanism of acquired resistance to EGFR inhibition in EGFR mutant lung cancers [11]. Thus, combining dual EGFR-Her2 inhibiton and designing lapatinib analogues could be an effective strategy for treatment of EGFR mutant tumors.

Based on these findings, this urea series was further investigated and optimized. Novel 6-ureido- and 6-thioureido-4-arylaminoquinazoline derivatives were designed and synthesized based on structure–activity relationship of the previously reported EGFR inhibitors[12]; the anilinoquinazoline core was retained with several modifications at the 6-position side chain by attaching different urea and thiourea derivatives as additional hydrogen bond acceptor functions. Also, different substitution pattern have been introduced at 4-anilino position with electron withdrawing group at 3'/and 4' position in a fashion similar to gefitinib, erlotinib and lapatinib, The effect of these changes on selectivity, potency against EGFR kinase as well as wild and mutant EGFR expressing cells lines was investigated, (**figure 2**).

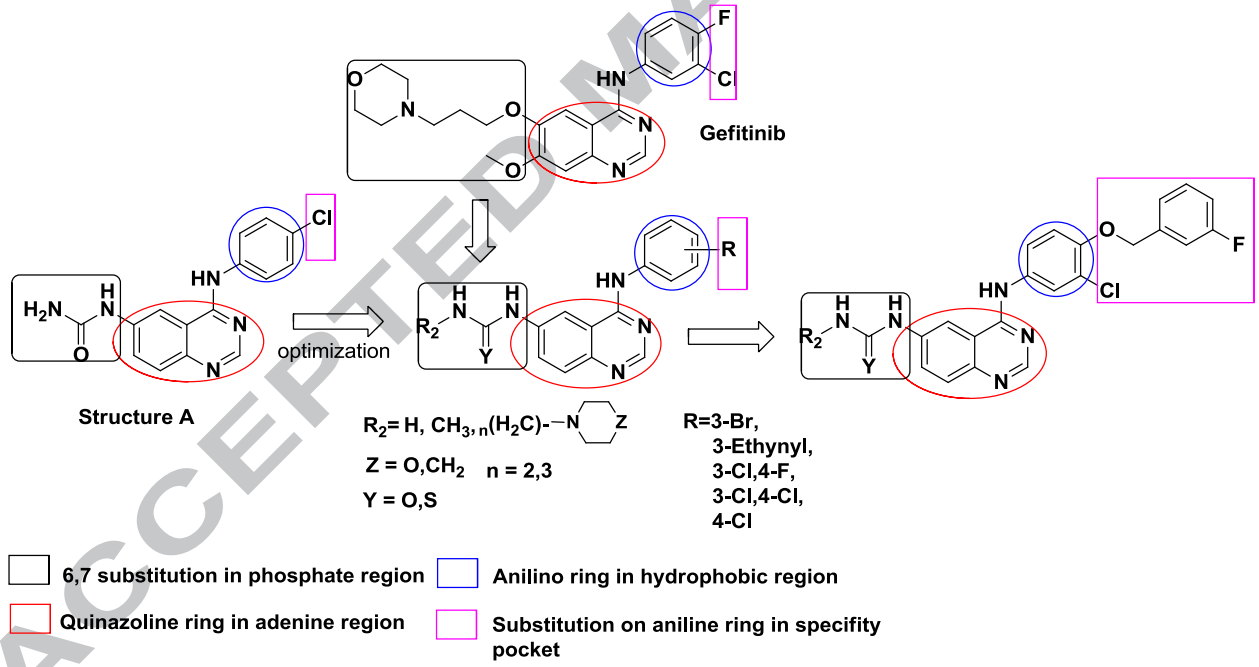


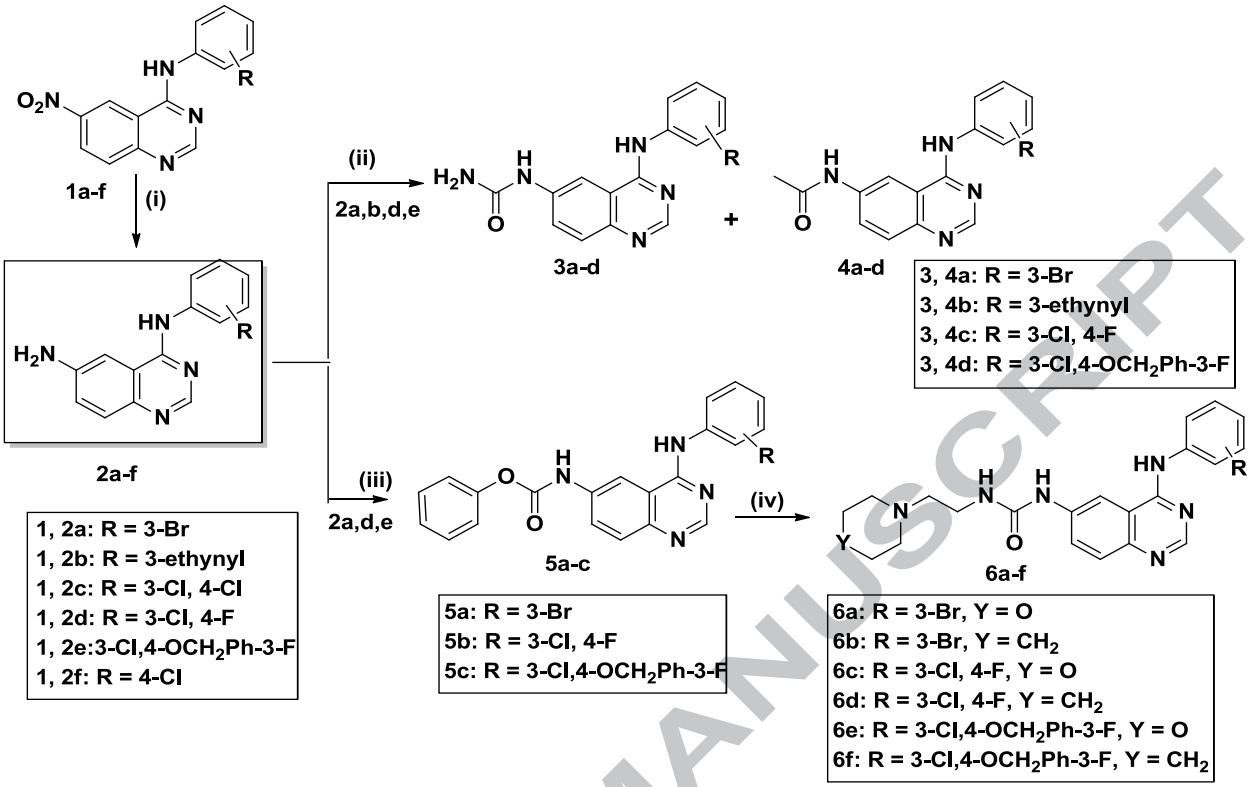
Figure 2: Rationale and Design

Gefitinib and structure **A** reported in our previous publication were both used as lead compounds [2].Several optimizations have been introduced to urea at the 6-position to give the substituted ureas and

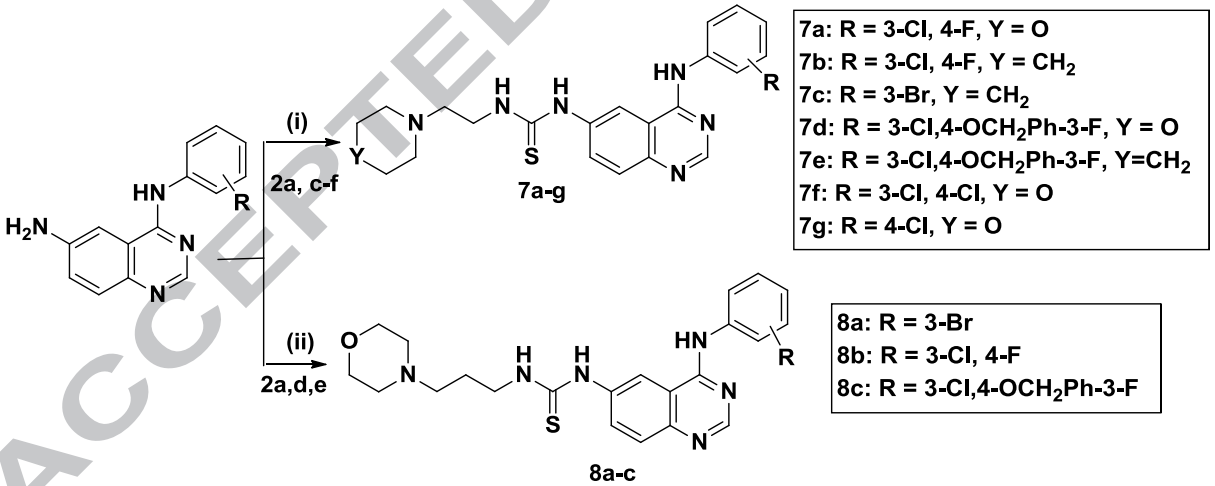
thioureas using the following strategies: (i) substitution of the N-terminal of the urea with ethyl piperidino and/ or morpholino moieties; (ii) replacement of ureas with thioureas and substitution of the N-terminal of the thioureas with ethyl or propyl side chain linked to piperidino and/ or morpholino moieties; (iii) different substitution pattern at the 4-anilino position to mimic gefitinib, erlotinib and lapatinib.

3. Chemistry

The synthesis of the new 6-ureido- and 6-thioureido-4-arylaminoquinazolines derivatives (target compounds) is shown in Schemes 1 and 2. The 6-nitroquinazolines **(1a–f)** were generated following published literature methods from 5-nitroanthranilo nitrile by refluxing with dimethylformamide dimethylacetal (DMF–DMA) to give the corresponding formamidine, which was then reacted with substituted anilines in acetic acid [2, 13]. The nitro derivatives **(1a-f)** were reduced to their corresponding the 6-aminoquinazolines **(2a-f)** by refluxing with $SnCl_2$/acetic acid under nitrogen. The corresponding ureas (**3a-d)** and acetamido derivatives (**4a-d)** were synthesized out of the same reaction by stirring of key intermediates (**2a-e)** with potassium cyanate in glacial acetic acid, the acetamido derivatives (**4a-d**) were generated as side products of this reaction, they were isolated, purified and characterized through NMR (^{1}HNMR, ^{13}CNMR, ^{1}H–^{15}N HSQC and ^{1}H–^{13}C HSQC) and MS. The phenyl carbamate derivatives (**5a-c)** were prepared by reacting the respective 6-aminoquinazolines with phenyl chloroformate, they further underwent nucleophilic reaction with various amines to afford the corresponding compounds (**6a-f).** In scheme 2, the thiourea derivatives **(7a-g, 8a-c)** were obtained via the reaction of key intermediates (**2a, c-f)** with the appropriate isothiocyanate.



Scheme 1. Reagents and conditions: (i) $SnCl_2$/MeOH, reflux; (ii) KCNO/ glacial Acetic acid, stir at rt; (iii) Phenyl chloroformate, DIPEA, THF, 5 °C, 3h; (iv) 2-Morpholinoethylamine/ 2-Piperidinylethylamine, THF, 70 °C, 4 h.



Scheme 2. Reagents and conditions: (i) 2-(4-Morpholinyl)ethyl-isothiocyanate/ 2-(1-Piperidinyl)ethyl isothiocyanate, THF, 0 °C /rt; (ii) 3-(4-Morpholinyl)propyl isothiocyanate, THF, 0 °C /rt

4. Results and discussion:

The synthesized compounds were evaluated in enzyme-based and cell-based assays for their ability to inhibit EGFR wild type (EGFR WT), and the proliferation against Ba/F3 cell lines expressing wild type EGFR, transformed Ba/F3 cells harboring L858R (**Table 1, 2**) and Ba/F3 cells harboring double mutants either L858R/T790M or Del19/T790M and (**figure 3, 4**).

a. Enzyme and cellular WT/L858R BaF/3 inhibition of EGFR

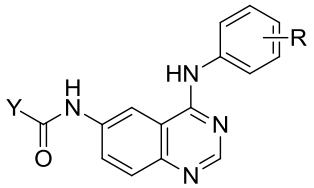
As shown in **table 1, 2**, compounds (**3a-d, 4a-d, 6a–d, 7a–d, f, g** and **8b, c**) revealed inhibition of WT EGFR activity with an IC_{50} = <0.495–9.05 nM

First, the influence of the substitution of the N-terminal of the urea on EGFR inhibition was investigated, **table 1**. The acetyl derivatives (**4a-c**); which are classical isosteres to the urea derivatives (**3a-c**), showed almost similar enzyme affinity and EGFR inhibition to their corresponding urea derivatives (**3a-c**), however, they showed better cytotoxicity in WT and L858R Ba/F3 cell lines; where the 3-bromo acetamido derivative (**4a)**, showed an IC_{50} of 0.041 µM in Ba/F3 cells expressing WT EGFR and an IC_{50} of 0.032 µM, in Ba/F3 L858R EGFR cells compared to compound (**3a)**, which showed an IC_{50} of 0.273 µM in Ba/F3 WT EGFR cells and IC_{50} of 0.315 µM in Ba/F3 cells expressing L858R EGFR. This may be attributed to the higher lipophilicity of acetamido group compared to urea moiety. However, for the benzyloxy analogues, the urea derivative (**3d)** showed better affinity and cytotoxicity profile than the corresponding acetamido derivative (**4d).** As for substituting the N-terminal with ethyl piperidino and/ or morpholino moieties **(6a-f),** the structure activity relationship (SAR) for enzyme inhibition seems to be rather flat while interestingly substitution of urea with ethyl piperidine showed dramatic increase in growth inhibition in Ba/F3 cells expressing WT EGFR, where the 3-bromo derivative, (**6b)** and the 3-chloro, 4-fluoro derivative, (**6d)** showed IC_{50}s of 0.021 µM and 0.062 µM respectively compared to the 3-bromo derivative **(3a)** and the 3-chloro, 4-fluoro derivative, (**3c)** which showed IC_{50} of 0.273 µM and 0.315 µM, respectively. In contrast, for benzyloxy derivatives; the unsubstituted urea derivative **3d** showed slightly better inhibition in the cell based assay than substituted ones **6e** and **6f.**

Second, we investigated the influence of the replacement of the linker at the six position of quinazoline from urea linked compounds (**6b, 6c, 6d**) to thiourea analogues (**7c, 7a, 7b**), respectively, **table 2**; In general, On comparing EGFR inhibition and cell based assay for both cases, the urea linker showed better EGFR inhibition and cytotoxic effect than the corresponding thiourea analogues; where compound (**6d)** inhibited EGFR enzyme with IC_{50} of 1.06 nM and showed antiproliferative IC_{50} of Ba/F3 cells expressing WT EGFR of 0.062 μM versus (3.70 nM and 0.080 μM) for compound **(7b),** respectively.

Third, the effect of increasing carbon chain length from two to three carbons was also studied, three carbon derivatives demonstrated better inhibition for EGFR and Her2 on comparing (**7a, 8b**) and (**7d, 8c**)

Fourth, as for 4-anilino substitution, all substitution showed nanomolar EGFR inhibition, where as the 3-Cl, 4-F anilino in compound (**8b)** showed highest EGFR inhibition of (<0.495 nM) which is more potent than gefitinib (IC_{50} = 0.509 nM). As for 3,4 dichloroanilino substitution in compound (**7f)**, showed the least inhibition among the tested compound series (IC_{50} = 9 nM). The bulky substitution of (3-chloro-4-(3-fluorobenzyloxy)phenyl)amino similar to that of Lapatinib allowed dual inhibition of Her2 and EGFR; where Her2 inhibition was verified for the following compounds (**3d**, IC_{50} = 26.8 nM), (**4d**, IC_{50} 32.3= nM), (**6f**, IC_{50} = 19.2 nM), (**7d**, IC_{50} 33.1= nM), (**7e**, IC_{50} =40.6 nM) and (**8c**, IC_{50} = 12.1 nM)



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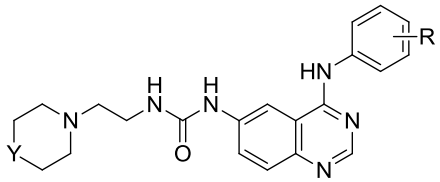

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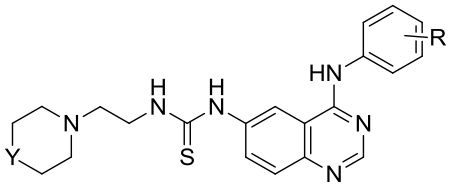
Table 1: The effect of newly synthesized compounds (**3a-d**, **4a-d**, **6a-f**) on EGFR/Her2 enzymatic activity and antiproliferation against Ba/F3expressing EGFR WT and L858R

	R	**Y**	**EGFR/Her2 IC_{50} (nM)**	**Ba/F3 EGFR (IC_{50}, μM)**	
				WT	**L858R**
3a	3-Br	NH_2	0.803	0.273	0.315
4a	3-Br	CH_3	1.01	0.041	0.032
3b	3-Ethynyl	NH_2	2.22	0.194	0.364
4b	3-Ethynyl	CH_3	2.04	0.039	0.068
3c	3-Cl,4-F	NH_2	0.854	0.315	0.401
4c	3-Cl,4-F	CH_3	0.662	0.264	0.371
3d	3-Cl,4-OCH_2-Ph-3F	NH_2	2.03 / Her2: 26.8	0.186	0.407
4d	3-Cl,4-OCH_2-Ph-3F	CH_3	3.96/Her2: 32.3	0.41	NT
6a	3-Br	O	1.43	0.269	NT
6b	3-Br	CH_2	0.772	0.021	NT
6c	3-Cl,4-F	O	2.92	0.087	0.053
6d	3-Cl,4-F	CH_2	1.06	0.062	0.077
6e	3-Cl,4-OCH_2-Ph-3F	O	NT	0.259	>0.5
6f	3-Cl,4-OCH_2-Ph-3F	CH_2	Her2: 19.2	>0.5	>0.5
gefitinib			0.509	0.127	0.022
lapatinib			Her2: 60.1		



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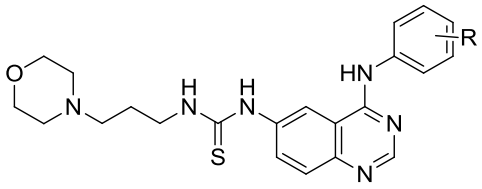

(8)

Table 2: The effect of compounds (**7a-g**, **8a-c**) on EGFR/Her2 enzymatic activity and antiproliferation against Ba/F3expressing EGFR WT and L858R

	R	**Y**	**EGFR /Her2 IC_{50} (nM)**	**Ba/F3 EGFR (IC_{50}, µM)**	
				WT	**L858R**
7a	3-Cl,4-F	O	2.25	0.150	0.316
7b	3-Cl,4-F	CH_2	3.70	0.080	0.154
7c	3-Br	CH_2	1.81	0.076	0.069
7d	3-Cl,4-OCH_2-Ph-3F	O	2.26 / Her2: 33.1	0.332	0.267
7e	3-Cl,4-OCH_2-Ph-3F	CH_2	Her2: 40.6	0.296	>0.5
7f	3-Cl,4-Cl	O	9.05	0.772	0.653
7g	4-Cl	O	5.06	0.066	0.065
8a	3-Br	-	NT	0.247	0.347
8b	3-Cl,4-F	-	<0.495	0.390	0.513
8c	3-Cl,4-OCH_2-Ph-3F	-	1.50 / Her2: 12.1	0.271	>0.5
gefitinib			0.509	0.127	0.022
lapatinib			Her2: 60.1		

b. Testing cytotoxic effect of synthesized compounds on double mutants Ba/F3 L858R/T790M and Ba/F3 Del19/T790M:

All the synthesized compounds were screened against the double mutant Ba/F3 L858R/T790M and Del19/T790M cell lines at single high concentration of 10 μM to identify the most interesting compounds, as shown in **figure 3** and **4.**

Among all tested compounds, compounds (**4d, 6f** and **7e),** having the bulky substitution of (3-chloro-4-(3-fluorobenzyloxy)phenyl)amino, showed the highest inhibition against both double mutants. To determine potency, the IC_{50} of those compounds were determined, (**table 3)**. The compounds showed significant inhibition of both double mutants of Ba/F3 harboring L858R/T790M or Del T790M compared to gefitinib whose IC_{50} could not be attained at the maximum tested concentration of 10 uM.

Table 3: Antiproliferative activity of **4d**, **6f**, **7e** on transformed Ba/F3 cells

Compound	**Ba/F3 EGFR (IC_{50}, μM)**	
	L858R/T790M	**Del19/T790M**
4d	2.10	2.28
6f	2.03	2.38
7e	1.76	2.31
gefitinib	>10	>10

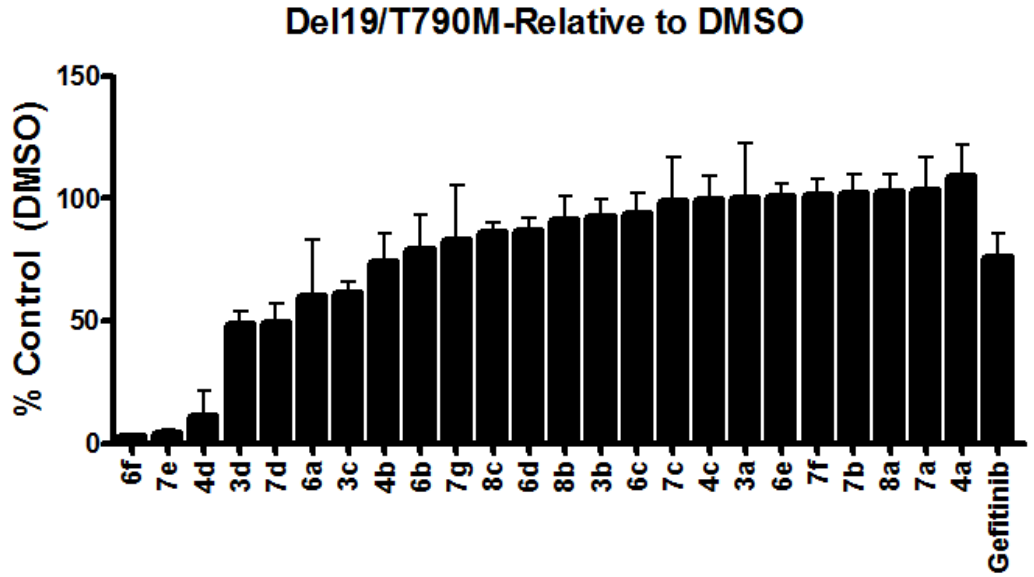

Figure 3: Assay of growth percent of double mutant del19/T790M Ba/F3 cells after adding 10 uM of compounds, and plotted relative to DMSO

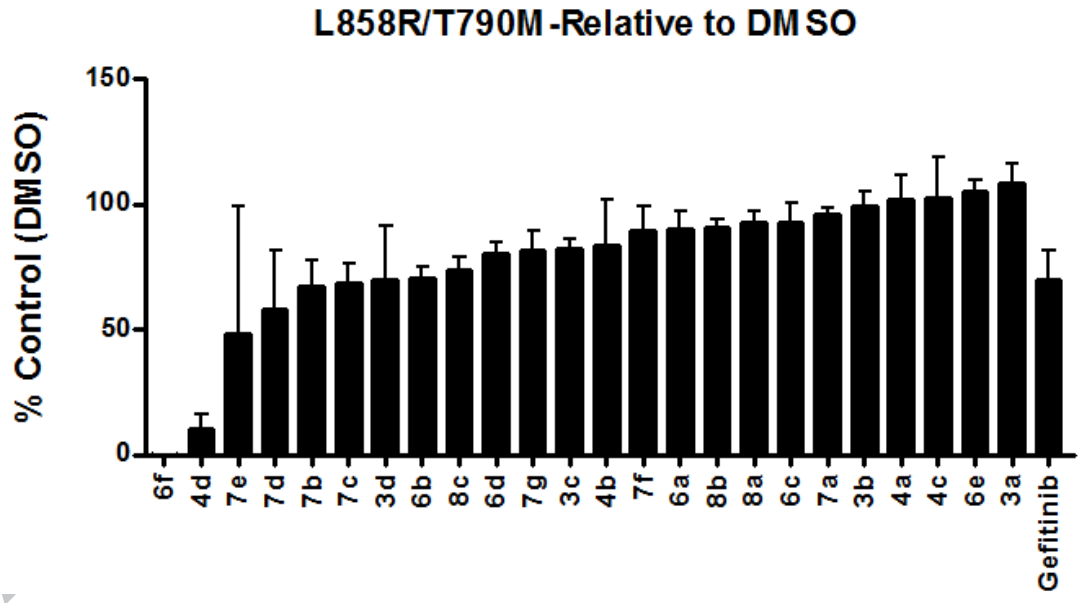

Figure 4: Assay percent growth after treating Ba/F3 double mutant L858R/T790M with 10 uM of compounds and plotted relative to DMSO

c. Testing cytotoxic effect on PC9 and mutant PC9-GR4 cells

To further investigate the cytotoxic effect of the new series, the most potent compounds**, (4d, 6f, 7e**) in addition to **(3c, 4c)** (gefitinib analogues) for comparison purposes were tested against one NSCLC cell line, PC9 cells; that was reported to be of high dependency on the EGF receptor/ERK1/2 and Akt pathway for its survival and proliferation compared to other NSCLC and it is sensitive towards gefitinib [14] and also compounds were tested against mutant PC9-GR4 that has T790M mutation (Del19/T790M) cells that is gefitinib insensitive [10], (**table 4, figure 5a** and **5b)**.

Most compounds tested retained activity against mutant cell lines except **3c** and showed better cytotoxic profiles especially for compounds **6f** and **7e** which showed about eight and three times greater potency respectively when compared to gefitinib.

Table 4: Antiproliferative activity of compounds on transformed PC9 and PC9-GR4

Compound	**PC9 (IC_{50}, µM)**	**PC9-GR4 (IC_{50}, µM)**
3c	0.9611	21.7
4c	0.2247	12.62
7e	2.156	5.701
4d	0.6616	8.313
6f	1.583	2.157
gefitinib	0.063	16.33

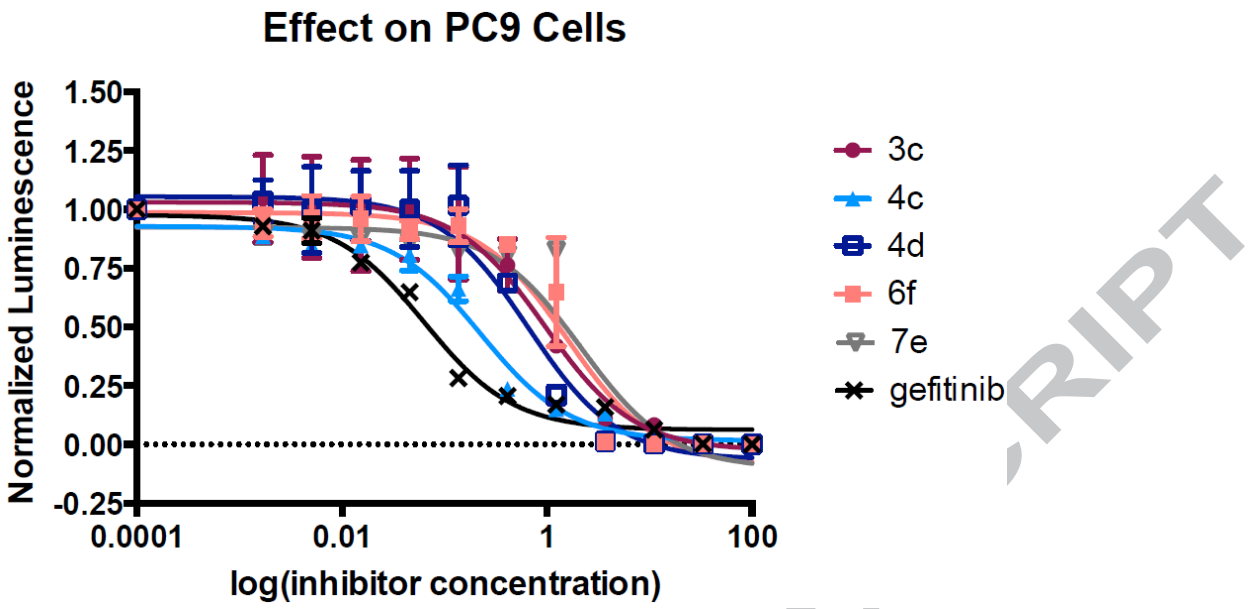

Figure 5a: PC9 cells were treated with compounds (**3c, 4c, 7e, 4d, 6f** and gefitinib) at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls

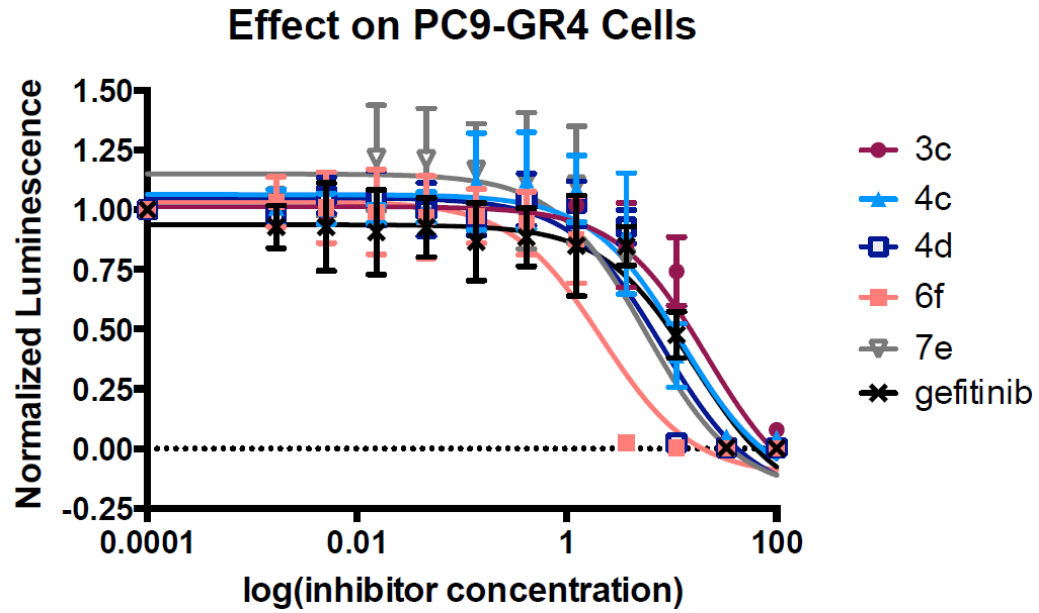

Figure 5b: PC9-GR4 cells were treated with compounds (**3c, 4c, 7e, 4d, 6f** and gefitinib) at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls

Molecular docking and Binding mode of compounds 6d, 6f, 7a, 7b and **8b**

The results of *in vitro* activity which showed that urea derivative (**6d**), displayed lower IC_{50} than corresponding thiourea derivatives (**7b**). Also three carbon linker (**8b**) showed lower IC_{50} than corresponding two carbon linker derivative (**7a**). Moreover, compound (**6f**) was found to be the most active compound against double mutant cell lines Ba/F3 expressing Del19/T790M and Ba/F3 expressing L858R/T790M. Therefore, a docking study was performed to explain differences in activity and predict the binding modes and orientation of the synthesized compounds (**6d, 7a, 7b, 8b and 6f)** at the active site of the ATP binding site of EGFR-TK.

The coordinates of the EGFR-TK structure were obtained from the crystal structure of EGFR (PDB code 1XKK) with its inhibitor, the 3D proposed binding mode of compounds **6d, 7a, 7b** and **8b** were generated by docking simulation, (**figure 6, 7**). Compounds showed a docking pattern model showed similar to lapatinib [15]; (i) The N-1 nitrogen of the quinazoline scaffold was hydrogen-bonded to the main chain NH of the hinge region Met793, (ii) The 4-anilino moiety occupied a deep back hydrophobic pocket (iii) The C-6 urea-linked side chains were positioned at the solvent interface of EGFR where the (O/S) atom of the urea/thiourea derivatives form a hydrogen bond with Cys797, which was absent in reference compound gefitinib which could enhance the binding affinity of these compounds.

For urea derivatives **6d** with IC_{50} = 1.06 nM forms four hydrogen bond; N-1 of quinazoline with Met793, oxygen of urea with Cys797, NH of the piperridine forms a hydrogen bond with Asp800 and fluorine atom makes hydrogen bonding to Lys745 with a docking score of (cDocker energy = **-29.32)**, **figure 6A**. On the other hand, compound **7b** with IC_{50} = 3.70 nM, forms only two hydrogen bonds with N-1 of quinazoline with Met793 and the sulphur of thiourea forms hydrogen bond with Cys797 with lower docking score (Cdocker energy= **-24.08)**, see **Figure 6B**. This may be attributed to the lower electronegativity of Sulphur atom compared to oxygen, thus making weaker hydrogen bonding.

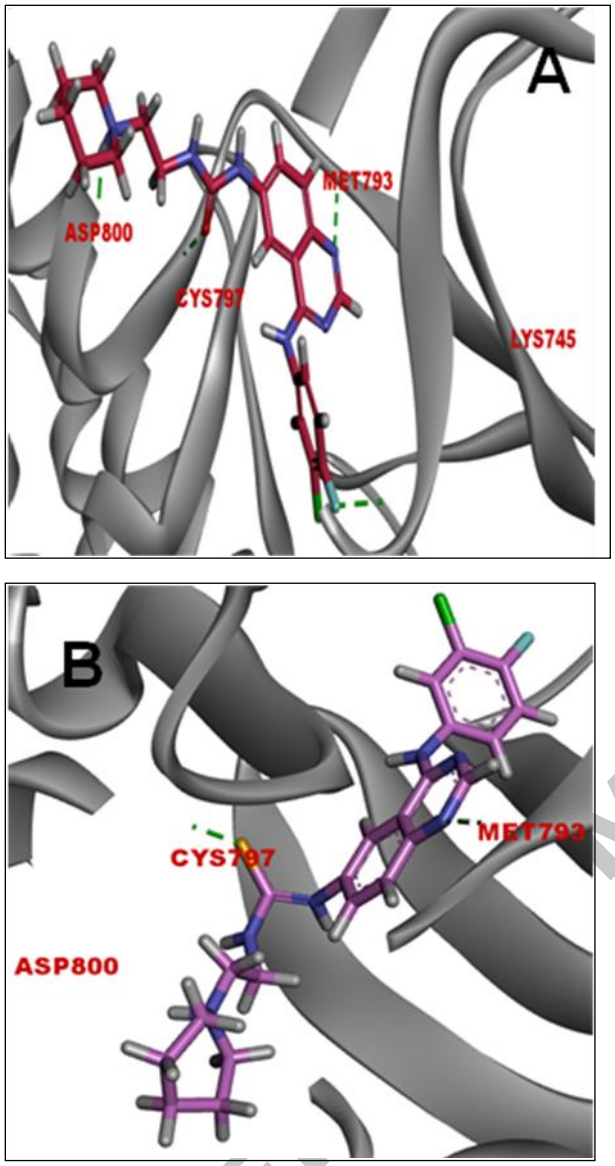

Figure 6: Proposed binding modes of compound **6d** (A) and compound **7b** (B); compound **6d** (Cdocker energy= -29.32) form four hydrogen bonding with Met793, Cys797, Asp800 and Lys745, while compound **7b** (Cdocker energy= -24.08)forms only two hydrogen bonding Met793, Cys797

Moreover, on comparing three carbon linker derivative (**8b,** IC_{50}=<0.495) with two carbon linker derivative (**7a,** IC_{50}= 2.25nM), the higher inhibition of three carbon linker compound (**8b)** may be attributed to forming of two extra interactions (carbon- hydrogen bond) between morpholine ring and Arg841 and Ser720 where both bonds are absent in two carbon linker (**7a)**, (**figure 7A, 7B)**

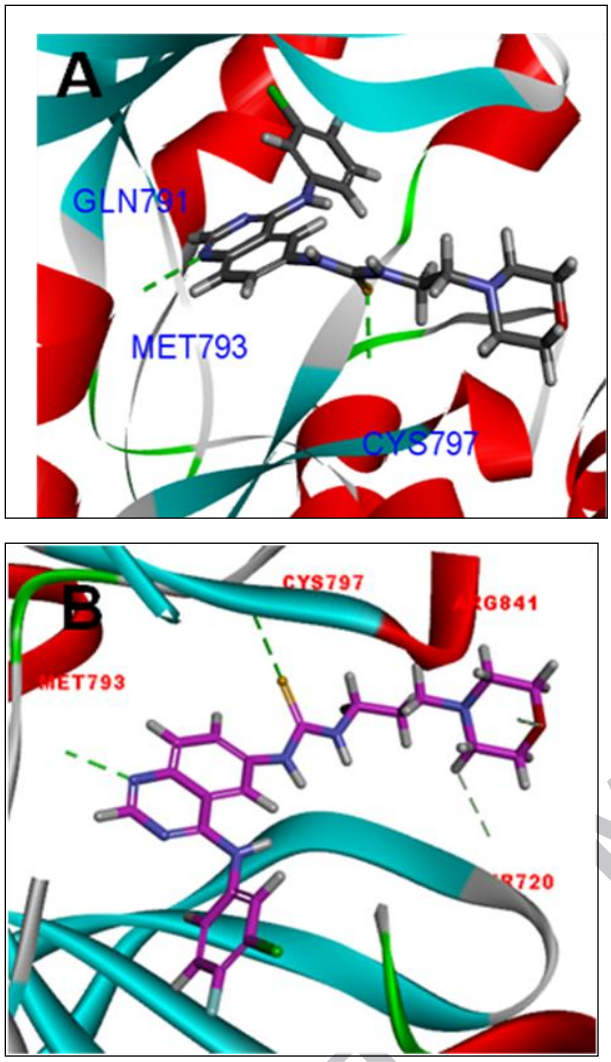

Figure 7: Proposed binding mode of compounds **7a** (A) and **8b** (B); both compounds makes two hydrogen bonds with Met793 and Cys797, while compound 8b forms other two weak interactions (carbon hydrogen bond) with Arg841 and Ser720

Next, we explored the binding mode of (**6f**); the most promising compound against double mutant cell lines Ba/F3 expressing Del19/T790M and Ba/F3 expressing L858R/T790M, The crystal structure of double mutant (PDB 3W2O) was used, **figure 8**. Compound (**6f**) forms hydrogen bond with Met793, Cys797 and Phe858 in addition to another interaction (salt bridge) with Asp800, **figure 8**.

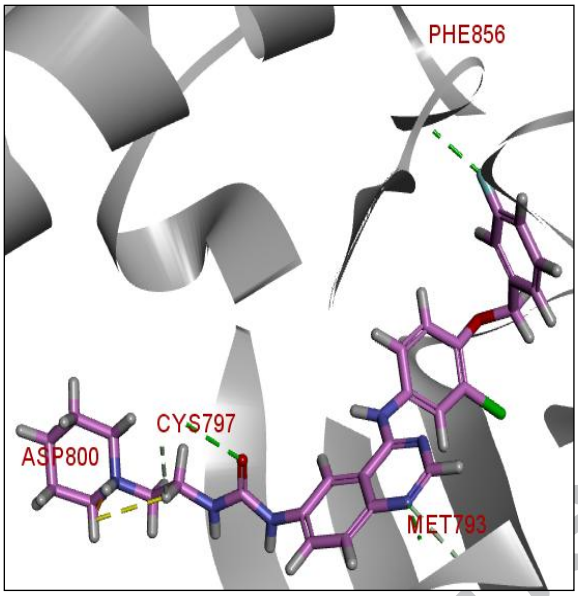

Figure 8: Proposed binding mode of compounds 6f on crystal structure (PDB 3W2O); it makes three hydrogen bonds with Met793, Cys797 and Phe856 and salt bridge with Asp800

Western Blot Analysis:

In addition to the kinase and cellular inhibitory activities, we further investigated if the compounds inhibit the phosphorylation of EGFR and the activation of downstream signaling Pathways in PC9 and PC9-GR4 cell lines. Compounds (**3c, 4c, 7e, 4d, 6f** and gefitinib) were selected for further experiments. PC9 and PC9-GR4 cells were incubated with 10 uM of selected compounds for 6 h, see **Figure 9a, 9b**. Potent inhibition of EGFR phosphorylation was observed with almost complete inhibition observed at PC9 cells and much less inhibition was observed at resistant PC9-GR4. Inhibition of EGFR phosphorylation was accompanied by a less concomitant inhibition of Akt phosphorylation.

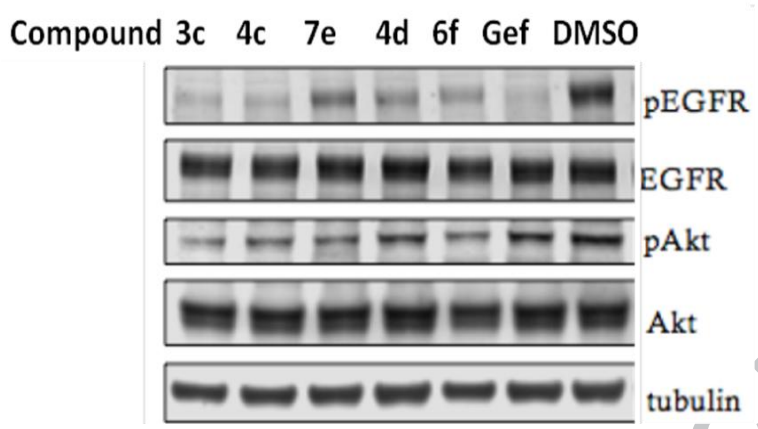

Figure 9a: Effect of inhibition of phosphorylation of EGFR and AKT after incubation for 6h with 10uM of corresponding compounds **(3c, 4c, 7e, 4d, 6f and gefitinib)** against PC9 cell lines

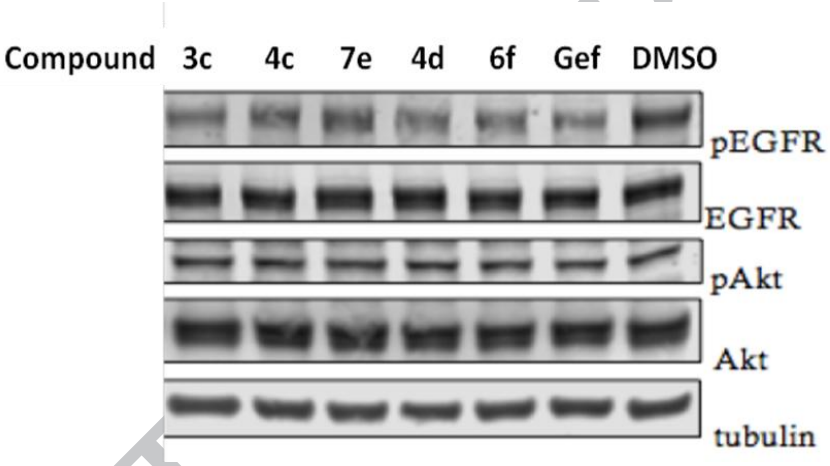

Figure 9b: Effect of inhibition of phosphorylation of EGFR and AKT after incubation for 6h with 10uM of corresponding compounds **(3c, 4c, 4d, 6f, 7e and gefitinib)** against PC9-GR4 cell lines

d. Testing cytotoxic effect on BT-474 cells

In addition, to investigating the effect of on Her2 enzyme inhibition **table 1**, **2,** we investigated cytotoxic effect of most promising lapatinib analogues on Her2 overexpressing cell lines BT-474, **figure 10** and **table 5.**

All Compounds tested for Her2 enzyme inhibition were more active than reference compound lapatinib, **table 1, 2**. While on testing compounds **4d, 6f, 7e** for their cytotoxic effect on BT-474, lapatinib had the highest cell growth inhibition followed by **4d** and then **7e** and **6f,** respectively**.**

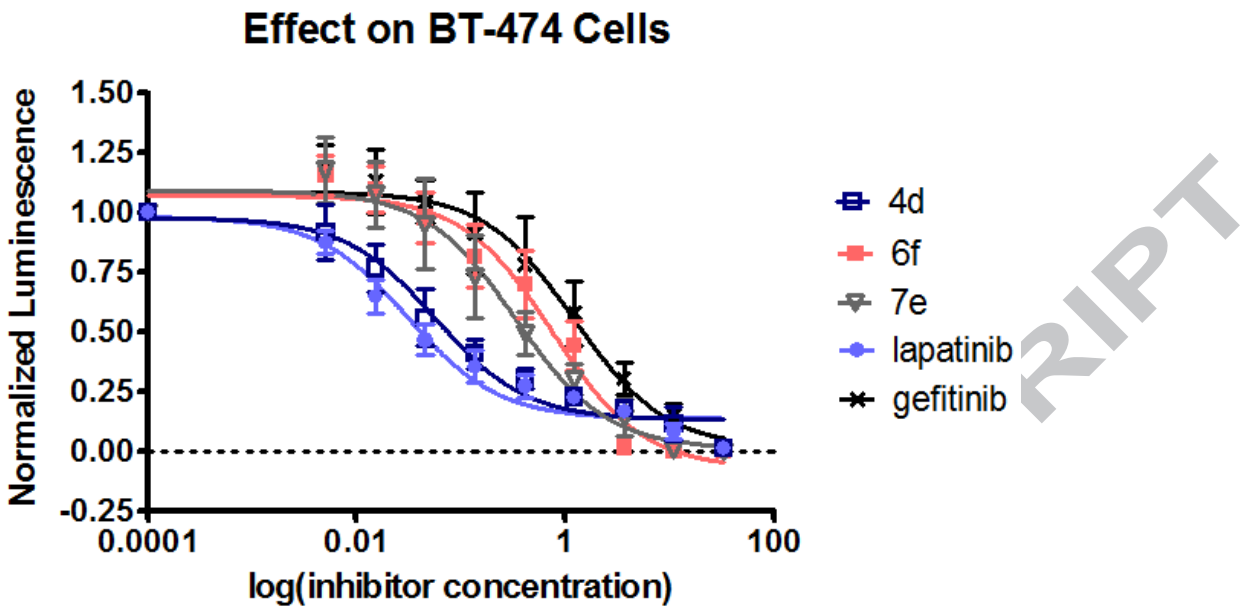

Figure 10: BT-474 cells were treated with compounds (**4d, 6f, 7e**, gefitinib and lapatinib) at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls

Table 5: Cytotoxic effect of compounds on BT-474

Compound	**BT-474 (IC_{50}, µM)**
4d	0.057
6f	0.76
7e	0.34
lapatinib	0.031
gefitinib	1.24

5. Final Conclusion

A new series of 4-anilinoquinazolines with C-6 ureido and thioureido linked side chains was designed, synthesized and evaluated as WT and mutant EGFR inhibitors. Most of the compounds showed IC_{50} values of WT EGFR kinase in the low nanomolar range (<0.495-9.05 nM) and displayed more potent cytotoxic effect in Ba/F3 expressing WT EGFR than reference compound gefitinib and inhibited EGFR activity and EGFR autophosphorylation in the PC9 NSCLC cell line. Among those, compounds **4d, 6f, 7e** showed significant inhibition in both double mutant Ba/F3 cell lines expressing Del19/T790M and Ba/F3 expressing L858R/T790M and showed significant inhibition of Her2 enzyme activity (IC_{50}= 19.2-40.6 nM) compared to lapatinib (60.1 nM). Furthermore, compounds **6f** and **7e** successfully retained activity in PC9-GR4 cells (Del19/T790M), these are the most promising compounds. Our SAR studies reveal that presence of ethyl piperidino urea/thiourea at 6-position and bulky group of (3-chloro-4-(3-fluorobenzyloxy)phenyl)amino at 4-position of quinazoline is the most potent against resistant double mutants cell lines which can be utilized in future studies.

In addition, compound **6f** showed higher sensitivity to resistant cell lines compared to gefitinib; **6f** was 8 times more potent in PC9-GR4 cells and more than 5 times potent against double mutant Ba/F3 expressing Del19/T790M and Ba/F3 expressing L858R/T790M than gefitinib, while **6f** was 25 times less potent in PC9 cells and had an IC_{50} greater than 500 nM in Ba/F3 WT EGFR cells.

Based on results of this study, we concluded that compounds targeting dual inhibition of EGFR and ErbB2 afforded superior inhibition and better targeting of mutant cell lines over agents that target only EGFR such as gefitinib. Dual inhibitors retained considerable activity towards double mutant del19/T790M and L858R/T790M without the cytotoxicity of covalent inhibitors that can bind off targets. In addition, they inhibit Her2 overexpression which may occur as a potential mechanism of acquired resistance of EGFR inhibition [11].

6. Experimental:

6.1 Chemistry

Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and were used without further purification. ^{1}H NMR spectra were recorded on a 400 MHz (Varian 7600 AS) or 500 MHz (Bruker Avance III) instrument, and chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Coupling constants (J) are reported in Hz. Spin multiplicities are described as s (singlet), brs (broad singlet), t (triplet), q (quartet), and m (multiplet). Mass spectra were obtained on a Waters Micromass ZQ instrument. Preparative HPLC was performed on a Waters Symmetry C18 column (19 mm × 50 mm, 5μm) using a gradient of 5−95% acetonitrile in water containing 0.05% trifluoroacetic acid (TFA) over 22 min (28 min run time) at a flow rate of 20 mL/min. Purification of compounds was performed with either a Teledyne ISCO CombiFlash Rf system or a Waters Micromass ZQ preparative system. Purities of assayed compounds were in all cases greater than 95%, as determined by reverse-phase HPLC analysis.

General method for preparation of 6-nitro-4-arylaminoquinazoline 1a-f

According to reported procedures: A mixture of N'-(2-cyano-4-nitrophenyl)-N,N-dimethylformamide (3.0 g, 13.7 mmol) and appropriate aniline (15.1 mmol) in glacial acetic acid was stirred and refluxed for about 2 h, reactions were monitored with TLC. After the reaction was completed the mixture was filtered while hot. The solid was washed with diethyl ether and dried to yield 3a-f [16, 17]

4-(3,4-Chlorofluorophenylamino)-6-nitroquinazoline 1a: Yield 93% as reported [13, 18]

4-(3,4-Dichlorophenylamino)-6-nitroquinazoline 1b: yield (86%); mp 297–298^{o}C , as reported **[13]**

4-(4-Chlorophenylamino)-6-nitroquinazoline 1c :Yield 87% as yellow crystals, mp 264^{o}C , (as reported) [17]

4-(3-Bromophenylamino)-6-nitroquinazoline 1d: Yield 76.5% as yellow crystals, mp 267-270 ^{0}C, (as reported) [17]

4-(3-Ethynylphenylamino)-6-nitroquinazoline 1e: Yield 95% as yellow crystals, mp 271-272 ^{0}C, (as reported) [13]

4-[3-Chloro-4-(3-fluorobenzyloxy)phenylamino]-6-nitroquinazoline 1f: as reported [19]

Amino-4-arylaminoquinazoline 2a-f:[16]

Following a published procedure [16], a mixture of the nitroquinazoline derivative (5 mmol) and stannous chloride (25 mmol) in MeOH (20 mL) was stirred for 1 h at reflux. Excess MeOH was removed under reduced pressure; the remaining residue was dissolved in EtOAc (200 mL) and made alkaline with an aqueous solution of NaHCO3 and stirred for about one h. The resulting mixture was filtrated under vacuum through celite. The organic phase was separated from the aqueous phase and the aqueous phase was extracted with EtOAc (2x 20 mL), the organic fractions were combined, washed with brine, and concentrated under reduced pressure. The crude product was purified with column chromatography (Dichloromethane/Methanol =9:1) to obtain the corresponding aminoquinazoline derivative **2a-f.**

N^4-(3,4-Chloroflorophenyl)-4,6-quinazolinediamine 2a: yield 82%; as reported *[18]*

N^4-(3-Bromophenyl)-4,6-quinazolinediamine 2b: Yield 78%, mp 267-270°C , as reported [17, 18]

N^4-(3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)quinazoline-4,6-diamine 2C: yield 70%, as reported [19]

N4-(4-Ethynylphenyl)quinazoline-4,6-diamine (2d): Yield 62%, mp 110–111°C , (as reported) [13]

N^4-(4-Chlorophenyl)quinazoline-4,6-diamine (2e): Yield 88% as yellow crystals, mp 237-239°C (as reported) [16]

3,4-Dichlorophenyl)quinazolin-4,6-diamine (2f): yield 5.5 g (86%); mp 243–244°C, as reported [13]

General Method for preparation of 4-(arylamino)-6-ureidoquinazoline: [2]

A mixture of appropriate amine (0.01 mol) and potassium cyanate (0.011 mol, 0.89 g) in glacial acetic acid was stirred at RT for 1 h. The product was poured over cold water and either filtered off or extracted with ethyl acetate, washed with brine, dried over anhydrous sodium sulphate, filtered, and concentrated. The crude product was concentrated and purified with reversed preparative HPLC to give desired product. On purification of the product, we found two products urea

and acetamido, both were separated, characterized and tested, Acetamido product comes out as result of side acetylating reaction

1-(4-((4-Bromophenyl)amino)quinazolin-6-yl)urea 3a : yield 30%, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 10.98 (br. s., 1 H), 9.21 (s, 1 H), 8.74 (s, 1 H), 8.58 (d, *J*=1.88 Hz, 1H), 7.96-7.93 (m, 1 H), 7.92 (dd, *J*=8.89, 1.88 Hz, 1 H), 7.75 (d, *J*=9.16 Hz, 1 H), 7.67 (d, *J*=8.93 Hz, 1 H), 7.46-7.32 (m, 2 H), 6.21 (br. s., 2 H), ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 160.89, 159.36, 156.22, 150.07, 141.12, 139.74, 131.07, 128.78, 128.64, 127.08, 123.52, 122.82, 121.64, 115.11, 110.18, MS (ESI) m/z 458.29 (M + H)+.

1-(4-((3-Ethynylphenyl)amino)quinazolin-6-yl)urea 3b : yield 35%, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.94 (br. s., 1 H), 9.07 (s, 1 H), 8.71 (s, 1 H), 8.55 (d, *J*=1.96 Hz, 1 H), 7.92 (dd, *J*=9.19, 2.15 Hz, 1 H), 7.85-7.78 (m, 1 H), 7.73 (d, *J*=9.00 Hz, 1 H) 7.70 - 7.63 (m, 1 H), 7.47-7.35 (m, 2 H), 6.15 (br. s., 2 H), 4.19 (s, 1 H), MS (ESI) m/z 304.34 (M + H)+.

1-(4-((3-Chloro-4-fluorophenyl)amino)quinazolin-6-yl)urea 3c : yield 49%, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.82 (br. s., 1 H, exchangeable by D_2O), 9.04 (s, 1 H, exchangeable by D_2O), 8.68 (s, 1 H), 8.52 (d, *J*=1.96 Hz, 1 H), 7.97 (dd, *J*=6.85, 2.54 Hz, 1 H), 7.88 (dd, *J*=9.00, 2.35 Hz, 1 H), 7.72 (d, *J*=9.00 Hz, 1 H), 7.68-7.59 (m, 1 H), 7.45 (t, *J*=9.00 Hz, 1 H), 6.13 (br. s., 2 H, exchangeable by D_2O), MS (ESI) m/z 332.39 (M + H)+

1-(4-((3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)urea 3d [20]: yield 32%, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.03 (br. s., 1 H, exchangeable by D_2O), 9.11 (s, 1 H, exchangeable by D_2O), 8.74 (s, 1 H), 8.58 (d, *J*=1.96 Hz, 1 H), 7.95 (dd, *J*=9.19, 2.15 Hz, 1 H), 7.85 (d, *J*=2.44 Hz, 1 H), 7.76 (d, *J*=9.00 Hz, 1 H), 7.58 (dd, *J*=9.20, 2.44 Hz, 1 H 1 H), 7.47 - 7.44 (m, 1 H), 7.38 - 7.24 (m, 3 H), 7.18 (td, *J*= 9.2, 2.4, Hz, 1 H), 6.20 (br. s., 2 H, exchangeable by D_2O), 5.28 (s, 2 H), ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 163.64, 161.71, 159.35, 156.18, 151.75, 149.99, 141.05, 139.91, 131.62 , 131.13, 131.06, 128.53, 126.57, 124.81, 123.84 , 121.59, 115.33, 115.17, 114.65, 114.44 , 110.22, 69.85, MS (ESI) m/z 438.44 (M + H)+.

N-(4-((4-Bromophenyl)amino)quinazolin-6-yl)acetamide 4a : yield 29%, ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 11.07 (br. s, 1 H, exchangeable by D_2O), 10.50 (s, 1 H, exchangeable by D_2O),

8.87 (d, *J*=1.88 Hz, 1 H), 8.78 (s, 1 H), 7.96 (t, *J*=1.88 Hz, 1 H), 7.93 (dd, *J*=9.16, 2.00 Hz, 1 H), 7.81 (d, *J*=8.88 Hz, 1 H), 7.67 (dd, *J*=8.83, 1.88 Hz, 1 H), 7.45-7.32 (m, 2 H), 2.09 (s, 3 H), ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 169.32, 159.57, 150.88, 139.65, 139.21, 131.08, 129.39, 128.89, 127.14, 123.55, 123.08, 121.64, 114.92, 112.64, 24.39, MS (ESI) m/z 457.30 (M + H)+.

N-(4-((3-Ethynylphenyl)amino)quinazolin-6-yl)acetamide 4b : yield 32%, ^{1}H NMR (400 MHz, Solvent) δ ppm 10.96 (br. s., 1 H), 10.42 (s, 1 H), 8.83 (d, *J*=2.00 Hz, 1 H), 8.73 (s, 1 H), 7.89 (dd, *J*=8.90, 2.05 Hz, 1 H), 7.84-7.79 (m, 1 H), 7.76 (d, *J*=9.00 Hz, 1 H), 7.65 - 7.71 (m, 1 H), 7.41 (t, *J*=8.0 Hz, 1 H), 7.31 (d, *J*=8.0 Hz, 1 H), 4.19 (s, 1 H), 2.09 (s, 3 H), MS (ESI) m/z 303.35 (M + H)+.

N-(4-((3-Chloro-4-fluorophenyl)amino)quinazolin-6-yl)acetamide 4c : yield 38%, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.80 (br. s., 1 H, exchangeable by D_2O), 10.39 (s, 1 H, exchangeable by D_2O), 8.79 (d, J=2.4Hz, 1 H), 8.69 (s, 1 H), 7.97 (dd, *J*=6.85, 2.54 Hz, 1 H), 7.86 (dd, *J*=9.00, 1.96 Hz, 1 H), 7.77 (d, *J*=9.00 Hz, 1 H), 7.66 - 7.63 (m, 1 H), 7.44 (t, *J*=9.00 Hz, 1 H), 2.08 (s, 3 H), MS (ESI) m/z 331.4 (M + H)+.

N-(4-((3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)acetamide 4d : yield 30%, ^{1}H NMR (400 MHz, Solvent) δ ppm 11.07 (br. s., 1 H), 10.49 (s, 1 H), 8.87 (d, J=2Hz, 1 H), 8.79 (s, 1 H), 7.94 (dd, *J*=9.00, 2.15 Hz, 1 H), 7.87 (d, *J*=2.54 Hz, 1 H), 7.83 (d, *J*=9.00 Hz, 1 H), 7.60 (dd, *J*=8.90, 2.54 Hz, 1 H), 7.48 - 7.45 (m, 1 H), 7.38 - 7.27 (m, 3 H), 7.17 (td, J=8.8, 2.4 Hz, 1 H), 5.30 (s, 2 H), 2.15 (s, 3 H), ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 169.25, 163.65, 161.71, 159.34, 156.49, 151.59, 151.12, 139.93, 138.94, 131.12, 131.06, 126.38, 124.60, 123.84, 123.82, 121.28, 115.33, 115.16, 114.65, 114.44, 112.62, 69.85, 24.37, MS (ESI) m/z 437.45 (M + H)+.

General Method for preparation of phenyl (4-(arylamino)quinazolin-6-yl)carbamate 5a-c [8]

To a solution of corresponding amine (0.21 mmol) in THF (2 mL) containing DIPEA (75 µL, 0.43 mmol), phenyl chloroformate (30 µL, 0.24 mmol) was added in dropwise at 5 ^{0}C for 1 h under nitrogen. The reaction was monitored by TLC, the reaction mixture was diluted with ethyl acetate and washed with saturated sodium carbonate solution, dried with anhydrous Na_2SO_4,

filtered and concentrated. The crude solid was purified by column chromatography (dichloromethane: ethyl acetate = 1:1)

Phenyl (4-((3-bromophenyl)amino)quinazolin-6-yl)carbamate 5a : yield 43% as light yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.58 (br. s., 1 H, exchangeable by D_2O), 9.90 (s, 1 H), 8.62 (s, 1 H), 8.59- 8.55 (m, 1 H), 8.14 (t, *J*=1.96 Hz, 1 H), 7.87- 7.71 (m, 3 H), 7.52- 7.41 (m, 2 H), 7.38- 7.20 (m, 5H), MS (ESI) m/z 435.35 (M + H)+.

Phenyl (4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)carbamate 5b: yield 41% as yellow solid^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 10.61 (br. s., 1 H, exchangeable by D_2O), 9.95 (s, 1 H, exchangeable by D_2O), 8.62 (d, *J*=2.20 Hz, 1 H) 8.57 (s, 1 H), 8.12 (dd, *J*=6.87, 2.50 Hz, 1 H), 7.86- 7.81 (m, 2 H), 7.81-7.78(m,1H), 7.54-7.38 (m, 3 H), 7.35- 7.16 (m, 3 H), MS (ESI) m/z 409.52 (M + H)+.

Phenyl (4-((3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)carbamate 5c : yield 43% as light yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.53 (br. s, 1 H, , exchangeable by D_2O), 9.77 (s, 1 H, , exchangeable by D_2O), 8.57 (d, *J*=1.17 Hz, 1 H), 8.51 (s, 1 H), 7.93 (d, *J*=2.74 Hz, 1 H), 7.82 -7.74 (m, 2 H), 7.66 (dd, *J*=9.00, 2.35 Hz, 1 H), 7.49 - 7.39 (m, 3 H), 7.34 -7.12 (m, 7 H), 5.23 (s, 2 H), MS (ESI) m/z 515.19 (M + H)+.

General Method for preparation of 1-(4-(Arylamino)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)urea 6b,d,f

A mixture of corresponding carbamate (0.5 mmol) and 2-Piperidinylethylamine **(**131.3 µl, 1 mmol) was stirred in THF (3 mL). The reaction mixture was heated to reflux for 3 h. The reaction was cooled to room temperature; the crude reaction mixture was extracted three times with ethyl acetate (10 mL x 3). The combined organic phase was wash with saturated sodium bicarbonate, dried with Na_2SO_4 and concentrated. The residue was purified by preparative HPLC to provide the desired product

1-(4-((4-Bromophenyl)amino)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)urea 6b : yield 87% as yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.45 (br. s, 1 H, exchangeable by D_2O), 9.41 (s, 1 H, exchangeable by D_2O), 9.22 (br. s., 1 H, exchangeable by D_2O), 8.67 (s, 1H), 8.52 (d, J= 2Hz, 1 H), 8.12- 8.08 (m, 1 H), 7.96 (dd, *J*=9.20, 2.15 Hz, 1 H), 7.77 (d, *J*= 9.00 Hz, 2 H),

7.48- 7.30 (m, 2 H), 3.71- 3.49 (m, 6 H), 3.17- 3.07 (m, 2 H), 2.95- 2.91 (m, 2 H), 1.87- 1.55 (m, 4 H), MS (ESI) m/z 469.35 (M + H)+.

1-(4-((3-Chloro-4-fluorophenyl)amino)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)urea 6d : yield 85% as yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.83 (br. s, 1 H, exchangeable by D_2O), 9.49 (s, 1 H, exchangeable by D_2O), 9.20 (br. s., 1 H, exchangeable by D_2O), 8.73 (s, 1 H), 8.57 (d, *J*=2.35 Hz, 1 H), 7.97 (dd, *J*=9.00, J=2 Hz, 1 H), 7.79 (d, *J*=9.00 Hz, 1 H), 7.49-7.47 (m, 1H), 7.45 (t, *J*=9.00 Hz, 1 H), 7.29 (dd, *J*=9 Hz, J=2 Hz, 1 H), 3.56- 3.44 (m, 4 H), 3.22- 3.09 (m, 2 H), 3.03- 2.79 (m, 2 H), 1.81 (m, 2 H), 1.56 - 1.74 (m, 4 H), MS (ESI) m/z 443.57 (M + H)+.

1-(4-((3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)urea 6f : yield 89% as yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.00 (br. s, 1 H, exchangeable by D_2O), 9.55 (s, 1 H, exchangeable by D_2O), 9.22 (br. s, 1 H, exchangeable by D_2O), 8.75 (s, 1 H), 8.60 (d, J=1.96 Hz, 1 H), 7.99 (dd, *J*=9.00, 2.00 Hz, 1 H), 7.85 (d, *J*=2.00 Hz, 1 H), 7.79 (d, *J*=9.00 Hz, 1 H), 7.59 (dd, *J*=8.61, 2.35 Hz, 1 H), 7.52-7.39 (m, 1 H), 7.24 - 7.36 (m, 2 H), 7.17 (td, *J*=8.8, 2.00 Hz, 1 H), 7.07 (t, *J*=5.60 Hz, 1 H), 5.28 (s, 2 H), 3.64 - 3.39 (m, 4 H), 3.19-3.15 (m, 2 H), 3.03-2.79 (m, 2 H), 1.79 - 1.83 (m, 2 H), 1.54 - 1.75 (m, 4 H), MS (ESI) m/z 452.97 (M + H)+.

General Method for preparation of 1-(4-(arylamino)quinazolin-6-yl)-3-(2-morpholinoethyl)urea 6a,c,e [8]

A mixture of appropriate carbamate (0.5 mmol) and 2-Morpholinoethylamine **(**131.3 µl, 1 mmol) was stirred in THF (3 mL). The reaction mixture was heated to reflux for 3 h. The reaction was cooled to room temperature; the crude reaction mixture was extracted three times with ethyl acetate (10 mL x 3). The combined organic phase was wash with saturated sodium bicarbonate, dried with Na_2SO_4 and concentrated. The residue was purified by preparative HPLC to provide the desired product

1-(4-((4-Bromophenyl)amino)quinazolin-6-yl)-3-(2-morpholinoethyl)urea **6a:** Yield 87% as yellowish solid**,** ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.89 (br. s, 1 H, exchangeable by D_2O), 9.87 (br. s, 1 H, exchangeable by D_2O), 9.52 (s, 1 H, exchangeable by D_2O), 8.77 (s, 1 H), 8.60

(d, *J*=1.96 Hz, 1 H), 8.03-7.99 (m, 1 H), 7.81-7.79 (d, *J*=9 Hz, 1H), 7.75-7.73 (d, *J*=8Hz, 1H), 7.44-7.39 (m, 2 H), 7.01 (t, *J*= 5.48 Hz, 1 H), 3.98 (s, 2 H), 3.56- 3.50 (m, 6 H), 3.25 (t, *J*= 6.06 Hz, 2 H), 3.16 (br. s, 2 H), MS (ESI) m/z 471.33 (M + H)+.

1-(4-((3-Chloro-4-fluorophenyl)amino)quinazolin-6-yl)-3-(2-morpholinoethyl)urea 6c : Yield 91 % as yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.53 (br. s., 1 H, exchangeable by D_2O), 10.18 (br. s., 1 H, exchangeable by D_2O), 9.42 (s, 1 H, exchangeable by D_2O), 8.74 (s, 1 H), 8.59 (d, *J*=2 Hz, 1 H), 8.08- 7.94 (m, 1 H), 7.81 (d, *J*=9.00 Hz, 1 H), 7.78-7.64 (m, 1 H), 7.51 (t, *J*=9.00 Hz, 1 H), 7.12- 6.96 (m, 1H), 3.82- 3.97 (m, 4 H), 3.56- 3.51 (m, 4 H), 3.26 (t, *J*=5.8 Hz, 2 H), 3.16 (s, 2 H), MS (ESI) m/z 445.33 (M + H)+.

1-(4-((3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)-3-(2-morpholinoethyl)urea 6e : Yield 85% as yellow solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.99 (br. s., 1 H, exchangeable by D_2O), 9.86 (br. s., 1 H, exchangeable by D_2O), 9.54 (s, 1 H, exchangeable by D_2O), 8.75 (s, 1 H), 8.59 (d, *J*=1.96 Hz, 1 H), 7.99 (dd, *J*=9.00, 1.96 Hz, 1 H), 7.85 (d, *J*=2.44 Hz, 1 H), 7.79 (d, *J*=9.00 Hz, 1 H), 7.59 (dd, *J*=9.00, 2.35 Hz, 1 H), 7.49-7.44 (m, 1 H), 7.35- 7.26 (m, 2 H), 7.18 (td, *J*=8.8, 1.88 Hz, 1 H), 7.04 (t, *J*=8.8 Hz, 1 H), 5.28 (s, 2 H), 4.07- 3.60 (m, 6 H), 3.58 - 3.40 (m, 2 H), 3.26 -3.22 (m, 2 H), 3.13 (br. s, 2 H), MS (ESI) m/z 551.28 (M + H)+.

General Method for preparation of 1-(4-(arylamino)quinazolin-6-yl)-3-(2-morpholinoethyl)thiourea 7a, d, f, g

A mixture of appropriate amine (0.11 mmol) and 2-(4-Morpholinyl)ethyl isothiocyanate (31.2 µL , 0.22 mmol) was stirred in 2 ml THF, the reaction mixture was heated at 60^0C. The reaction was monitored with TLC. The reaction mixture was then diluted with ethyl acetate and washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude product was concentrated and purified with reversed preparative HPLC to give desired product

1-(4-((3-Chloro-4-fluorophenyl)amino)quinazolin-6-yl)-3-(2-morpholinoethyl)thiourea 7a : yield 75%, ^{1}H NMR (500 MHz, Acetone) δ ppm 10.38 (br. s., 1 H, exchangeable by D_2O), 10.28 (br. s., 1 H, exchangeable by D_2O), 8.55 (s, 1 H, exchangeable by D_2O), 8.37 (s,1H), 8.14 (d, J=2 Hz, 1 H), 7.82 (dd, *J*=8.8 Hz, J= 2Hz,1 H), 7.78 (m, 2 H), 7.71 (d, J=8.8Hz, 1H), 7.32 (t,

J=9.2 Hz,1 H), 3.62-3.55 (m, 2 H), 3.44 (t, 4 H, J=4 Hz), 2.57 (s, 2 H), 2.42 (s, 4 H), MS (ESI) m/z 461.31 (M + H)+.

1-(4-((3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)-3-(2-morpholinoethyl)thiourea 7d: yield 77% , ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 10.85 (br. s., 1 H, exchangeable by D_2O), 10.52 (br. s., 1 H, exchangeable by D_2O), 10.03 (br. s., 1 H, exchangeable by D_2O), 8.83 (s, 1 H), 8.51(d, *J*=2 Hz,1 H), 8.43-8.41 (m, 1 H), 8.01 (dd, *J*=9.00, 2 Hz, 1 H), 7.93 (d, *J*=2.44 Hz, 1 H), 7.82 (d, *J*=8.85 Hz, 1 H), 7.66 (dd, *J*=9.00, 2.55 Hz, 1 H), 7.54 - 7.45 (m, 1 H), 7.38 - 7.29 (m, 2 H), 7.25 - 7.15 (m, 1 H), 5.35 (s, 2 H), 4.11-3.86 (m, 4 H), 3.80 (br. s., 2H), 3.52 (br. s., 2 H), 3.36 (t, *J*=6.26 Hz, 2 H), 3.17 (br. s, 2H), MS (ESI) m/z 567.58 (M + H)+.

1-(4-((3,4-Dichlorophenyl)amino)quinazolin-6-yl)-3-(2-morpholinoethyl)thiourea 7f : yield 79%, ^{1}H NMR (400 MHz, CD_3OD) δ ppm 10.34 (s, 1H, exchangeable by D_2O), 10.27 (s, 1H, exchangeable by D_2O), 9.78 (s, 1H, exchangeable by D_2O), 8.72 (s,1H), 8.45 (d, 1H, J = 2 Hz), 8.27 (dd, J = 8.8 Hz, J= 2Hz, 1 H), 8.16 (d, *J*=2 Hz, 1 H), 7.89 (d, J = 8.8 Hz, 1H), 7.82 (dd, *J*=8.8 Hz, J=2Hz, 1 H), 7.51 (d, J=8.8Hz, 1H), 4.08 (t, J=5.6 Hz, 2H), 3.92-3.89 (m, 4H), 3.53 (t, J=5.6 Hz, 4H), 3.27-3.24 (m, 2H), MS (ESI) m/z 477.3 (M + H)+.

1-(4-((4-Chlorophenyl)amino)quinazolin-6-yl)-3-(2-morpholinoethyl)thiourea 7g : yield 80%**,** ^{1}H NMR (400 MHz, acetone) δ ppm 10.80 (s, 1 H, exchangeable by D_2O), 10.2 (s, 1H, exchangeable by D_2O), 9.15 (s, 1 H, exchangeable by D_2O), 8.90 (s,1H), 8.80 (d, , J=2 Hz, 1H), 8.42 - 8.39 (dd, J = 9 Hz, J=2Hz, 1 H), 8.05-8.03 (d, *J*=8.8 Hz, 1 H), 7.93-7.91 (d, *J*=8.8 Hz, 2 H), 7.50-7.48 (d, *J*=8.8 Hz, 2 H), 4.22- 4.15 (s, 2 H), 4.03 (s, 4 H), 3.68 (t, J=5.6 Hz, 2 H), 3.63-3.57 (m, 2 H), 3.41- 3.28 (m, 2 H), MS (ESI) m/z 443.38 $(M + H)^+$.

General Method for preparation of 1-(4-(arylamino)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)thiourea 7b, c, e

To a solution of appropriate amine (0.11 mmol) in 2 ml THF, 2-(1-piperidinyl)ethyl isothiocyanate (36.1 µL , 0.22 mmol) was added, the reaction mixture was heated at 60^0C. The reaction was monitored with TLC. The reaction mixture was then diluted with ethyl acetate and

washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude product was concentrated and purified with reversed preparative HPLC to give desired product

1-(4-((3-Chloro-4-fluorophenyl)amino)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)thiourea 7b: yield 85%, ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ ppm 10.36 (s, 1 H, exchangeable by D_2O), 10.25 (s, 1 H, exchangeable by D_2O), 9.37 (s,1H, exchangeable by D_2O), 8.63 (s, 1 H), 8.45 (d, J=2Hz, 1 H), 8.28 (dd, J = 9 Hz, J= 2.4 Hz, 1 H), 7.89 (d, J=2.4, 1H), 7.84 (dd, J=2.4,J=9 Hz, 1H), 7.62-7.68 (m, 1 H), 7.09 (t, J=9 Hz, 1 H), 4.09-4.01 (m, 2H), 3.68 - 3.72 (m, 2 H), 3.35 (s, 4 H), 2.08 - 2.12 (m, 2 H), 1.80-1.85 (m, 4 H), MS (ESI) m/z 459.45 (M + H)+.

1-(4-((3-Bromophenyl)amino)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)thiourea 7c : yield 77%, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.38 (br. s., 1 H, exchangeable by D_2O), 10.28 (br. s., 1 H, exchangeable by D_2O), 9.19 (s, 1 H, exchangeable by D_2O), 8.73 (s, 1 H), 8.48 (d, J=2.4Hz, 1 H), 8.29 (dd, J = 9 Hz, J= 2.4 Hz, 1 H), 8.08 (m, 1 H), 7.86-7.84 (m, 1 H), 7.79 (d, J=9Hz,1H), 7.36-7.34 (m, 2H), 3.87 -3.83 (m, 2 H), 3.46 - 3.43 (m, 2 H), 3.24 - 3.21(m, 2 H), 2.93 -2.85 (m, 2 H), 1.76-1.73 (m, 2 H), 1.62 - 1.53 (m, 4 H), MS (ESI) m/z 485.34 (M + H)+.

1-(4-((3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)thiourea 7e: yield 76 %, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.61 (br. s, 1 H, exchangeable by D_2O), 10.39 (br. s, 1 H, exchangeable by D_2O), 9.26 (br. s, 1 H, exchangeable by D_2O), 8.77 (s, 1 H), 8.51 (d, *J*=1.88 Hz, 1 H), 8.42-8.44 (m, 1 H), 8.08-7.87 (m, 2 H), 7.81 (d, *J*=9.00 Hz, 1 H), 7.66 (dd, *J*=9.00, 2.35 Hz, 1 H), 7.40 - 7.52 (m, 1 H), 7.31-7.28 (m, 2 H), 7.17 (td, *J*=8.80, 2.35 Hz, 1 H), 5.27 (s, 2 H), 4.02- 3.81 (m, 2H), 3.51- 3.48 (m, 2 H), 3.24- 3.19 (m, 2 H), 2.84 - 3.05 (m, 2 H), 1.81- 1.78 (m, 2 H), 1.71- 1.51 (m, 2 H), 1.47 - 1.12 (m, 2 H), MS (ESI) m/z 565.60 (M + H)+.

General Method for preparation of 1-(4-(arylamino)quinazolin-6-yl)-3-(3-morpholinopropyl) thiourea 8a-c

To a solution of appropriate amine (0.11 mmol) in 2 ml THF, 3-(4-Morpholinyl)propyl isothiocyanate (37.45 µL , 0.22 mmol) was added, the reaction mixture was heated at 60^0C. The reaction was monitored with TLC. The crude product was concentrated and purified with reversed preparative HPLC to give desired product

1-(4-((3-Bromophenyl)amino)quinazolin-6-yl)-3-(3-morpholinopropyl)thiourea 8a: yield73%, ^{1}H NMR (500 MHz, Acetone) δ ppm 10.52 (br. s, 1 H, , exchangeable by D_2O), 10.17 (br. s., 1 H, exchangeable by D_2O), 9.78 (br. s, 1 H, exchangeable by D_2O), 8.80 (s, 1 H), 8.52 (d, *J*=2.00 Hz, 1 H), 8.39-8.34 (m, 1 H), 8.13- 8.09 (m, 1 H), 7.94 (d, *J*=9.00 Hz, 1 H), 7.81 (d, *J*=8.8 Hz, 2 H), 7.42- 7.39 (m, 2 H), 4.03-3.71 (m, 6 H), 3.67-3.59 (m, 2 H), 3.31-3.22 (m, 2 H), 3.21- 3.00 (m, 2 H), 2.18-2.03 (m, 2 H), MS (ESI) m/z 501.31 (M + H)+.

1-(4-((3-Chloro-4-fluorophenyl)amino)quinazolin-6-yl)-3-(3-morpholinopropyl)thiourea 8b: yield 75%, ^{1}H NMR (500 MHz, Acetone) δ ppm: 10.52 (br s, 1H, exchangeable by D_2O), 10.13 (br s, 1H, exchangeable by D_2O), 9.76 (br s, 1H, exchangeable by D_2O), 8.73 (s, 1H), 8.45 (d, J= 2Hz, 1 H), 8.24-8.31 (m, 1H), 8.03-8.06 (m, 1H), 7.87 (d, J=8.8 Hz, 1H), 7.77 (d, J=8.8 Hz , 1H), 7.45 (t, J=9.2Hz, 1H), 4.06-3.85 (m, 4H), 3.81-3.75 (m, 4H), 3.44-3.41 (m, 2 H), 3.25 (t, J=8 Hz, 2H), 2.20 - 2.29 (m, 2H), ^{13}C NMR (126 MHz, DMSO-d_6) δ ppm 205.58, 181.80, 159.52, 158.63, 155.62, 151.35, 134.92, 133.95, 126.58, 125.32, 125.28, 119.80, 119.65, 117.54, 117.37, 63.74, 54.31, 51.56, 42.78, 41.66, 23.35, MS (ESI) m/z 475.67 (M + H)+.

1-(4-((3-Chloro-4-((3-fluorobenzyl)oxy)phenyl)amino)quinazolin-6-yl)-3-(3-morpholinopropyl)thiourea 8c: yield 65%, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.75 (br. s., 1 H , exchangeable by D_2O), 10.23 (br. s., 1 H, exchangeable by D_2O), 9.83 (br. s., 1 H, exchangeable by D_2O), 8.79 (s, 1 H), 8.52 (d, *J*=1.96 Hz, 1 H), 8.45 - 8.43 (m, 1 H), 7.96 (d, *J*=8.71 Hz, 1 H), 7.91 (d, *J*=2.34 Hz, 1 H), 7.81 (d, *J*=9.00 Hz, 1 H), 7.64 (dd, *J*=9.00, 2.35 Hz, 1 H), 7.49 -7.46 (m, 1 H) 7.36 - 7.25 (m, 2 H), 7.17 (td, *J*=8.71, 1.96 Hz, 1 H), 5.28 (s, 2 H), 4.02-3.91 (m, 2 H), 3.51 - 3.70 (m, 4 H), 3.44 - 3.41 (m, 2 H), 3.24-3.10 (m, 2 H), 3.05 (br. s., 2 H), 2.08-1.87 (m, 2 H), MS (ESI) m/z 581.59 (M + H)+.

6.2 Biology Evaluation

6.2.1 Kinase Assay:

In vitro kinase assays were conducted at Invitrogen (Madison, WI) using the SelectScreen Kinase Profiling Service. The biochemical assay employs a fluorescence-based, coupled-enzyme

format and is based on the differential sensitivity of phosphorylated and non-phosphorylated peptides to proteolytic cleavage.

6.2.2 Cell proliferation and growth assays:

Ba/F3 cell proliferation assay: Growth and inhibition of growth was assessed by MTS assay according to previously established methods [21, 22]. All experiments were repeated at least three times. The data was graphically displayed using GraphPad Prism, (GraphPad Software; www.graphpad.com).

PC9 and PC9-GR4 cell proliferation assay: Compound efficacy against cell proliferation was conducted in 96-well plates. Compounds were added in 3-fold serial dilutions to form 12 point dose titration. After 72 hour incubation with compounds, cell viability was measured using CellTiter-Glo (Promega,Wisconsin), and IC_{50} values were determined by GraphPad Prism

BT-474 cell proliferation assay: Compound efficacy against cell proliferation was conducted in 96-well plates. Compounds were added in 3-fold serial dilutions to form 9 point dose titration. After 72 hour incubation with compounds, cell viability was measured using CellTiter-Glo (Promega,Wisconsin), and IC_{50} values were determined by GraphPad Prism

6.2.3 Western Blotting

Cells were grown to half confluence in six-well plates, and then treated with indicated concentrations of compounds in 3 ml total media for 6 hours. At desired time point, cells were harvested by trypsinization and washed once with cold PBS (350g, 5min). Cells were lysed by re-suspending in 90ul ice-cold RIPA buffer, together with protease and phosphatase inhibitor tablet. Samples were lysed on ice for 30 mins, with occasional agitation. Then clarified by centrifugation (14,000 rpm, 10min, 4°C)., After BCA analysis to quantify proteins, samples were prepared in 4x LDS buffer + 5% beta mercaptoethanol, then boiled for 5 minutes.

Western blot analyses were conducted after separation by SDS/PAGE electrophoresis and transfer to nitrocellulose membranes. Immunoblotting was performed according to the antibody manufacturers' recommendations. Anti-phospho-Akt (Ser-473), anti-total-Akt, and antiEGFR

antibodies were obtained from Cell Signaling Technology. The phospho-EGFR (pY1068) antibodies were purchased from Biosource International Inc

Molecular modeling: The proteins used for the docking were downloaded from the RCSB Protein Data Bank (PDB IDS: 2ITY and 3W2O). All molecular modeling calculation and docking studies were carried out using GOLD 4.1 [26]. The protein structure was prepared using protein preparation protocol of Accelry's discovery studio 2.5 and all the water molecules in the protein were deleted. The ligands were drawn using default sketching tools of Accelry's discovery studio 2.5 and then energy minimized prepared using ligand preparation protocol of Accelry's discovery studio 2.5. Docking was performed for compounds **6d, 7a, 7b** and **8b** with 2ITY and compounds **6f** with 3W2O. The kinase scoring function was used to score the resulted docking poses

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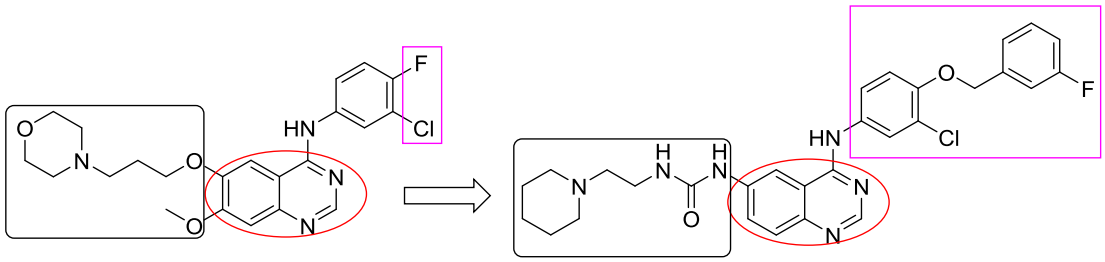

Gefitinib

Ba/F3 L858R/T790M, IC_{50} >10 μM

Ba/F3 Del19/T790M, IC_{50} >10 μM

PC9-GR4, IC_{50} = 16.33 μM

6f

Ba/F3 L858R/T790M, IC_{50} = 2.03 μM

Ba/F3 Del19/T790M, IC_{50} = 2.38 μM

PC9-GR4, IC_{50} = 2.157 μM

- 6-ureido and thioureido 4-anilinoquinazolines were designed and synthesized
- Final compounds displayed potent inhibitory activity on wild and mutant EGFR cell lines and ERB2
- Inhibition of wild and gefitinib resistant cell lines without off target cytotoxicity of covalent inhibitors