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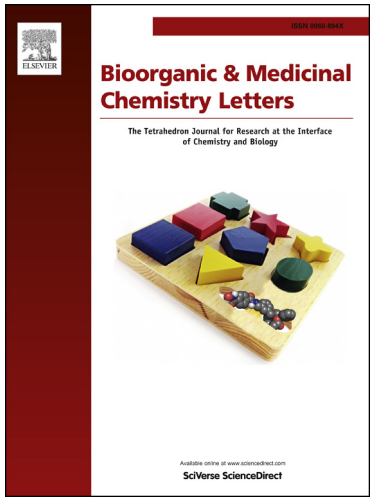

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Targeting EGFR/HER2 tyrosine kinases with a new potent series of 6-substituted 4-anilinoquinazoline hybrids: Design, synthesis, kinase assay, cell-based assay, and molecular docking

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Abstract

Coexpression of EGFR and HER2 has been found in many tumors such as breast, ovarian, colon and prostate cancers, with poor prognosis of the patients. Herein, our team has designed and synthesized new eighteen compounds with 6-substituted 4-anilinoquinazoline core to selectively inhibit EGFR/HER2 tyrosine kinases. Twelve compounds (**8a**−**8d**, **9a**, **9c**, **9d**, **10a**, **10c**, **11b**, **14**, **15**) showed nanomolar range of IC_{50} values on EGFR and/or HER2 kinases. Accordingly, a detailed structure activity relationship (SAR) was established. A molecular docking study demonstrated the favorable binding modes of **8d**, **9b**, **9d** and **10d** at the ATP active site of both kinases. A kinase selectivity profile performed for compound **8d** showed great selectivity for EGFR and HER2. In addition, **8d**, **9c**, and **9d** exerted selective promising cytotoxic activity over BT-474 cell line with IC_{50} values of 2.70, 1.82 and 1.95 µM, respectively. From these results, we report analogues **8d**, **9c**, and **9d** as promising candidates for the discovery of well-balanced compounds in terms of the kinase inhibitory potency and antiproliferative activity.

Keywords: Synthesis, EGFR/HER2 dual inhibitors, Antiproliferative activity, Bt-474 cell line, Molecular docking, kinase panel.

Protein tyrosine kinases play a crucial role in signal transduction pathways that control several cellular functions including proliferation, differentiation, migration, and angiogenesis [1]. Therefore, protein kinase inhibitors which target the elevated pathways are attractive candidates for cancer therapy [2]. Overexpression of the epidermal growth factor receptor (EGFR or ErbB1) and human epidermal growth factor receptor 2 (HER2 or ErbB2) which are members of the ErbB family of receptor tyrosine kinases (RTKs) is frequently observed in various solid tumors. Both kinases have been validated as rational targets for cancer therapy [3]. Stimulation of the epidermal growth factor (EGF) causes the ErbB receptors to form either homo- or heterodimers with the other ErbB family receptors, resulting in activation of the downstream signaling and consequently tumor cell growth promotion [4]. Coexpression of EGFR and HER2 has been reported in different tumors such as breast, ovarian, colon and prostate cancers [5-8]. Therefore, it is more effective to dual target EGFR/HER2 rather than just EGFR inhibition [9]. EGFR/HER2 inhibitors can block tyrosine kinase phosphorylation and inhibit the upregulated intracellular signals in cancer cells leading to loss of the tumor regulatory function. A variety of ATP-competitive EGFR/HER2 RTK dual inhibitors related to different scaffolds have been reported and many of them are currently in market or clinical trials for the treatment of cancer.

An important leading nucleus of EGFR/HER2 dual inhibitors is 4-anilinoquinazoline, exemplified by the lead compound lapatinib which was approved by the FDA for treatment of patients with HER2 overexpression metastatic breast cancer [9]. The binding mode of lapatinib with the catalytic domain of EGFR/HER2 kinases has been well studied in literature [10, 11]. Generally, the quinazoline ring is hydrogen bonded to the hinge region of the ATP binding cleft. The aniline group at C-4 of the quinazoline nucleus is oriented deep to fit in an adjacent back

pocket forming hydrophobic interactions. The SAR study of quinazoline was directed toward the modification of the C-4 aniline group which could achieve fine tuning of the kinase selectivity. Previous studies had established that the size and functionality of this pocket are main factors of the kinase inhibitor selectivity while the solubilizing moiety at C-6 of the quinazoline core act to improve the physical properties aiming for a favorable pharmacokinetic profile. In addition, many dual inhibitory candidates were designed to bind *via* hydrogen bonding or covalent bonding with Cys-773 in EGFR and Cys-805 in HER2 [12-16]. Although lapatinib therapy has proven effective, many patients fail to respond to treatment or become resistant due to different reasons which are still largely unknown [17-20]. Accordingly, new strategies and compounds are still in need for EGFR/HER2 inhibition. In this report, we focus our efforts on design and synthesis of new hybrid molecules with 6-substituted 4-anilinoquinazoline core to selectively inhibit EGFR and HER2 tyrosine kinases as depicted in Figure 1.

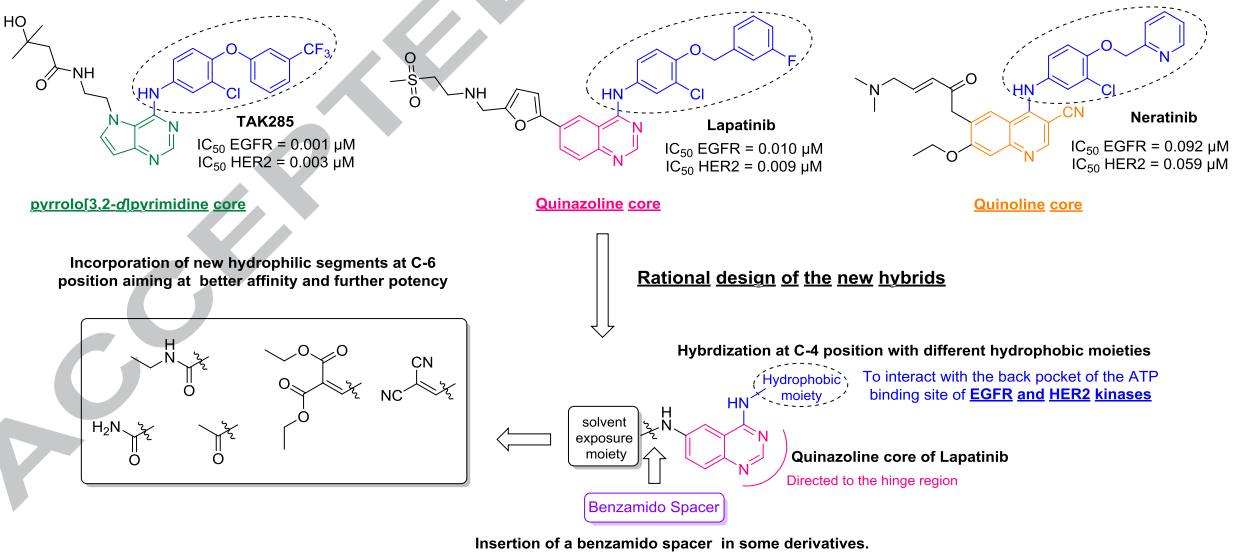
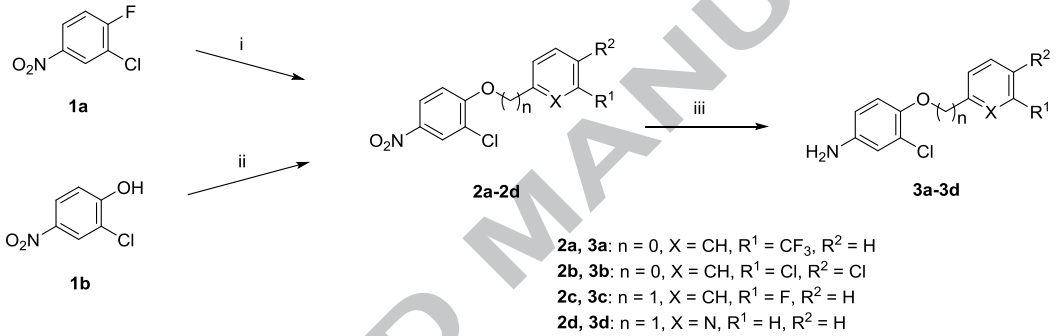


Figure 1. Rational design of the newly synthesized compounds.

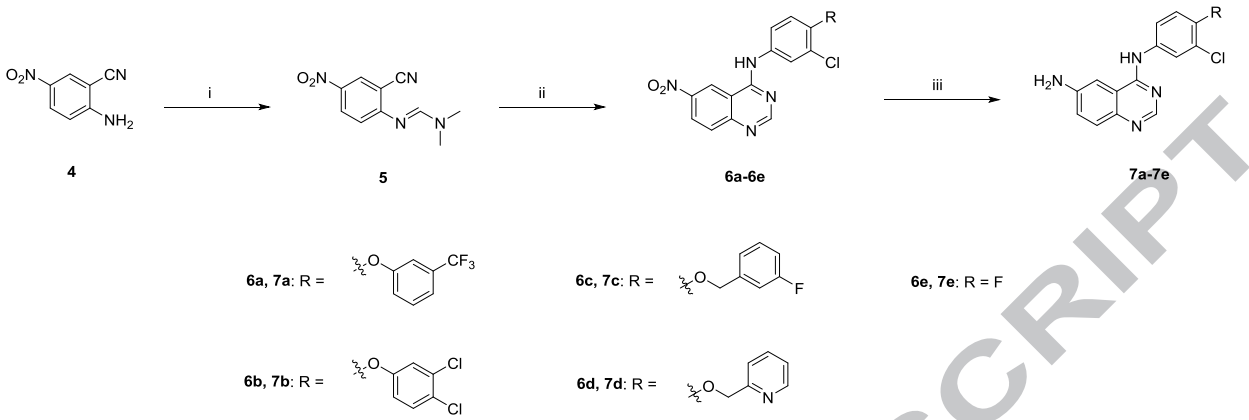
The new candidates possess different aniline patterns at C-4 in fashion similar to previously reported different potent dual inhibitors. Well-known various moieties were used including 3-chloro-4-((3-fluorobenzyl)oxy)aniline of lapatinib, 3-chloro-4-(pyridin-2-ylmethoxy)aniline of neratinib, 3-chloro-4-(3-(trifluoromethyl)phenoxy)aniline of TAK-285, and 3-chloro-4-fluoroaniline of gefitinib. In addition, a new aniline moiety (3-chloro-4-(3,4-dichlorophenoxy)aniline) was used to investigate the activity of compounds bearing 3,4-dihalo-substituted phenoxy segment. The aniline portion was designed to be directed into the hydrophobic deep pocket into the binding site. To achieve better affinity and further potency of the new candidates, the C-6 position was subjected to a variety of new different polar/solubilizing functional groups [21-24] including moieties of ureido (**8a**–**8c**), acetamido (**8d**), ethyl urea (**9a**–**9d**), diethyl aminomethylene malonate (**10a**–**10d**), and aminomethylene malononitrile (**11a**–**11c**). Moreover, to explore the effect of these solubilizing moieties at a distance from C-6 position of the quinazoline core, incorporation of amido spacer was applied in **14**, **15**, and **16**. The substitution patterns of the new agents aimed to improve potency and selectivity by incorporation of new moieties at C-6 of substituted 4-anilinoquinazolines and/or by varying the C-4 aniline moieties. All the target compounds were then tested for their inhibition profile over both EGFR and HER2 kinases in 10-dose IC_{50} mode. In addition, a detailed molecular docking study was carried out using GLIDE v 6.2 of Schrodinger suite 2014 [25, 26] as flexible docking program to predict the binding affinities and orientation of the target compounds **8d**, **9b**, **9d** and **10d** at the ATP binding site of EGFR/HER2-RTK. Moreover, a cell-based assay was performed to evaluate the antiproliferative activity of the new agents using the human breast carcinoma cell line (BT-474) which characterized by the overexpression of HER2.

The synthetic route adopted for preparation of the new 6-substituted amino-4-anilinoquinazoline derivatives **8a**−**8d**, **9a**−**9d**, **10a**−**10d**, **11a**−**11c**, **14**, **15**, and **16** is depicted in Schemes 1−4. Reaction of commercially available 2-chloro-1-fluoro-4-nitrobenzene (**1a**) or 2-chloro-4-nitrophenol (**1b**) with the appropriate phenol or arylmethyl bromide was carried out in the presence of potassium carbonate in dimethylformamide (DMF) or acetonitrile, followed by reduction of the nitro groups in **2a**−**2d** with platinum on carbon under hydrogen atmosphere to yield substituted anilines **3a**−**3d** in excellent yields as illustrated in Scheme 1.



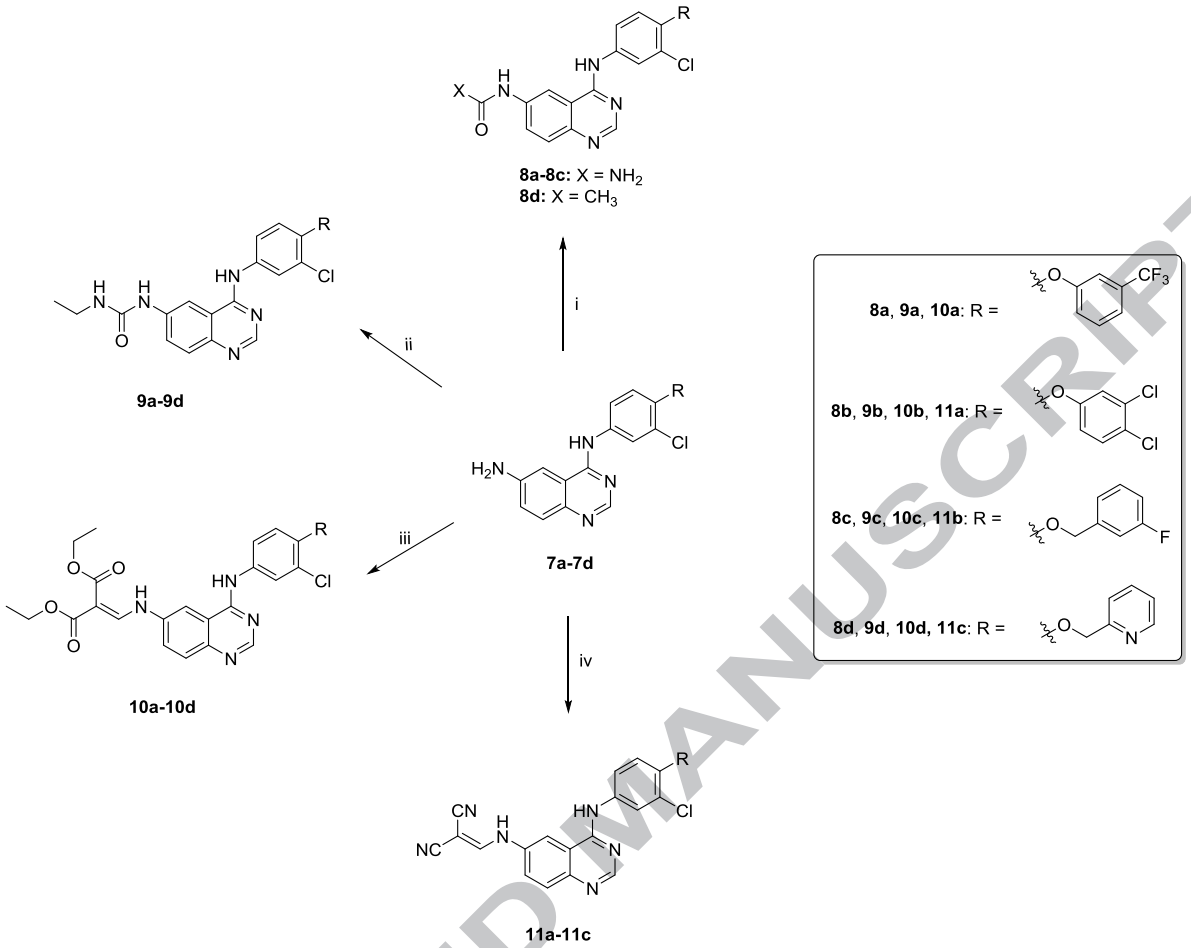
Scheme 1. Reagents and conditions: (i) appropriate phenol (**2a**, **2b**), CH_3CN, 85 ^oC, 5 h; (ii) appropriate arylmethyl bromide (**2c**, **2d**), K_2CO_3, DMF, 85 ^oC, 5 h; (iii) H_2, 10% Pt/C, CH_3OH, rt, 6 h.

The key intermediate 6-amino-4-anilinoquinazolines **7a**−**7e** were prepared following the literature methods illustrated in Scheme 2. 6-Nitro-4-arylaminoquinazoline derivatives **6a**−**6e** were synthesized in good yields through the reaction of 5-nitroanthranilonitrile (**4**) with dimethylformamide dimethyl acetal (DMF−DMA) affording the corresponding formamidine (**5**) followed by its reaction with appropriate anilines in acetic acid to give the 6-nitro-4-anilinoquinazolines **6a**−**6e**. The nitro groups in **6a**−**6e** were reduced by treating with Fe/acetic acid to afford the 6-aminoquinazolines **7a**−**7e**.



Scheme 2. Reagents and conditions: (i) dimethylformamide dimethyl acetal, 100 ^{o}C, 1.5 h; (ii) substituted aniline, glacial acetic acid, reflux, 1 h; (iii) Fe, AcOH, EtOH/H_2O (70%), reflux, 2 h.

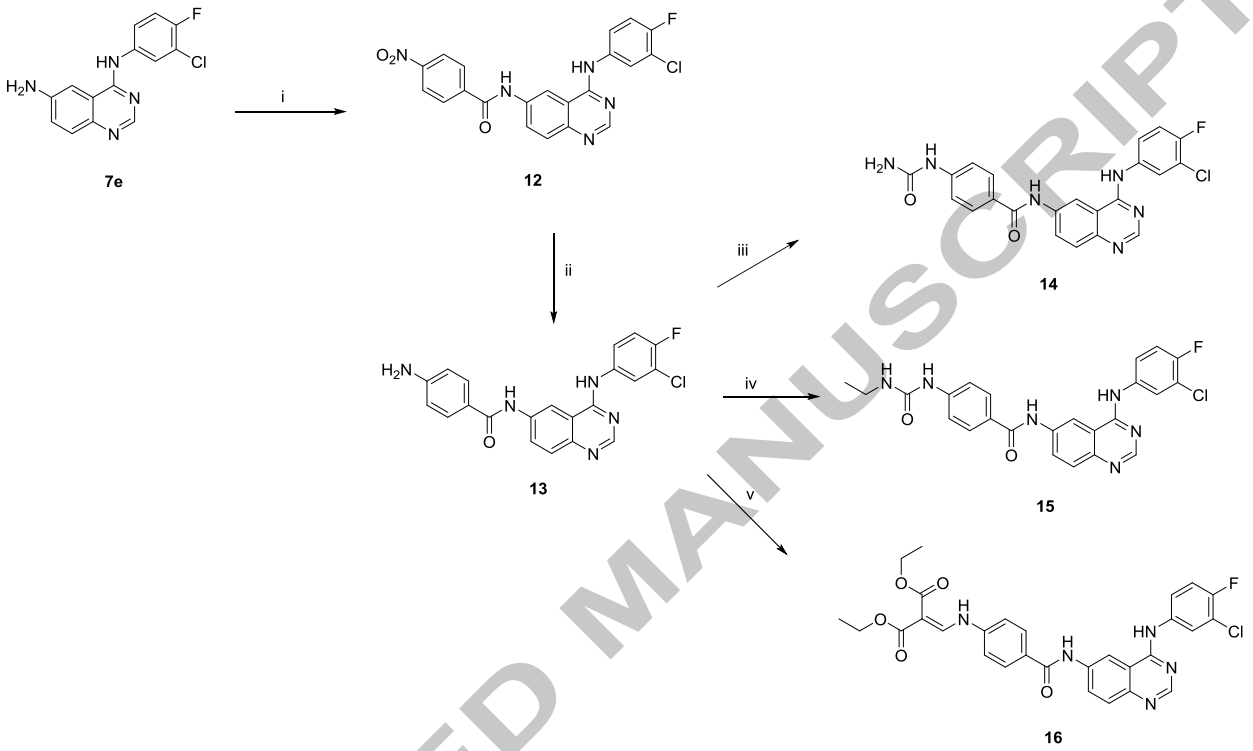
As shown in Scheme 3, the reaction of the amino derivatives **7a**−**7d** with potassium cyanate in acetic acid gave the corresponding 4-(substituted amino)-6 ureidoquinazolines **8a**−**8c**. The acetamido analogue **8d** was obtained by refluxing **7d** in acetic acid. Reaction with ethyl isocyanate provided the target ethyl urea derivatives **9a**−**9d**. Reaction with diethyl 2-(ethoxymethylene)malonate yielded diethyl 2-[(4-arylamino)quinazolin-6-ylaminomethylene]malonate analogues (**10a**−**10d**). Finally, compounds **11a**−**11c** were obtained through reaction with (ethoxymethylene)malononitrile.



Scheme 3. Reagents and conditions: (i) (**8a**−**8c**) KOCN, glacial acetic acid, reflux, 3 h, (**8d**) glacial acetic acid, reflux, 3 h; (ii) ethyl isocyanate, THF, 50 ^{o}C, 8 h; (iii) diethyl 2-(ethoxymethylene)-malonate, abs. EtOH, reflux, 3 h; (iv) (ethoxymethylene)malononitrile, abs. EtOH, reflux, 3 h.

With the aim of examining the effects of the polar/solubilizing functionality at a distance from C-6 position of the quinazoline core, a benzamido spacer was intentionally inserted directly attached to the quinazoline ring. Amidation of **7e** with 4-nitobenzoylchloride in the presence of TEA in THF provided *N*-(4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)-4-nitrobenzamide (**12**) in a good yield, followed by catalytic hydrogenation using platinum on carbon to reduce the

nitro group yielding the corresponding amine **13** (Scheme 4). Similar reactions to that of Scheme 3 were carried out for the synthesis of derivatives **14**−**16**.



Scheme 4. Reagents and conditions: (i) 4-nitrobenzoyl chloride, TEA, THF, 50 ^{o}C, overnight; (ii) H_2, 10% Pt/C, CH_3OH, rt, 6 h; (iii) KOCN, glacial acetic acid, reflux, 3 h; (iv) ethyl isocyanate, THF, 50 ^{o}C, 8 h; (v) diethyl 2-(ethoxymethylene)-malonate, abs. EtOH, reflux, 3 h.

To examine the final analogues potential to inhibit EGFR and HER2 kinases, all the newly synthesized compounds were tested over both targets at Reaction Biology Corporation using the 'HotSpot' assay platform in 10-dose IC_{50} mode with 3 fold serial dilutions starting at 20 μM. Kinase activity data were expressed as the percent remaining kinase activity in test samples compared to vehicle (dimethyl sulfoxide) reactions (for detailed information, see supplementary file). IC_{50} values were calculated and reported in Table 1. The pharmacological results offered twelve compounds with excellent inhibitory activities (nanomolar range of IC_{50} values on EGFR

and/or HER2 kinases). The structure-activity relationship (SAR) of the new analogues is indicated as follow: it was noted that all analogues at C-4 position with 3-chloro-4-(3,4-dichlorophenoxy)aniline (**9b**, **10b**, and **11a**) didn't show significant activity against both kinases, while other moieties exhibited good to excellent inhibitory activities. The results clearly demonstrated that derivatives containing ureido groups (**8a**−**8c**) exhibited promising activities with IC_{50} values of 0.004, 0.543, and 0.099 µM on EGFR and 0.077, 0.706, and 0.287 µM on HER2, respectively. Compound **8d** exerted 0.003 µM on EGFR and 0.016 µM on HER2, which are the best inhibitory activities in the series. It is worth mentioning that compound **14** with ureido moiety linked to the benzamido spacer exerted similar interesting results with IC_{50} values of 0.009 and 0.275 µM on EGFR and HER2, respectively. Incorporation of a small alkyl group (CH_3CH_2−) to the ureido part slightly decreased the potency against both kinases; compounds **9a**, **9d**, and **15** possessing ethyl ureas moieties showed inhibitory activities with IC_{50} values of 0.029, 0.227, and 0.031 µM on EGFR and 0.625, 1.390, and 0.855 µM on HER2, respectively. Compounds **10a**−**10d** with diethyl methylene malonate moieties exerted good to moderate activities on both kinases in low micromolar range. Conversely, replacement of the ureido moiety with aminomethylene malononitrile in **11a**−**11c** led to a marked reduction of the activity.

Table 1. IC_{50} values (μM) exhibited by the newly synthesized compounds on EGFR and HER2 tyrosine kinases.

Cpd	Kinase assay	
	EGFR IC_{50} (μM)[b]	HER2 IC_{50} (μM)[b]
8a	0.004	0.077
8b	0.543	0.706
8c	0.099	0.287
8d	0.003	0.016
9a	0.029	0.625
9b	> 10	> 10
9c	0.035	0.126
9d	0.227	1.390
10a	0.023	1.000
10b	> 10	> 10
10c	0.812	5.050
10d	1.020	3.290
11a	> 10	> 10
11b	0.445	2.720
11c	3.050	8.000
14	0.009	0.275
15	0.031	0.855
16	1.460	> 10
Lapatinib[a]	0.010	0.009

[a] Lapatinib was used as a positive control agent.

[b] Compounds were tested in a 10-dose mode with 3-fold serial dilution starting at 20 μM.

In order to provide deeper insights into the structure-activity relationship of the new series, compounds **8d**, **9b**, **9d** and **10d** were selected based on the potency and scaffold types and docked individually into the ATP-binding pockets of EGFR (PDB code: 1XKK) and HER2 (PDB code: 3RCD). The re-docked conformations of crystal ligands were kept as reference

structures for assessing the binding modes of the compounds in respective kinases (for detailed information about docking validation, see supplementary file).

Compound **8d**, with pyridine and acetamido groups at C-4 and C-6 respectively, produced outstandingly good result by mimicking lapatinib in geometry-wise and interaction-wise. As illustrated in Figure 2A, the pyridine moiety occupied a smaller hydrophobic pocket comprised of Met766, Cys775, Leu777 and Phe856. The quinazoline nucleus derived substantial binding energy via direct interaction with Met793 (hinge region) and water-mediated interactions with Thr854 (precedes DFG motif) and Thr790 (gatekeeper residue). The acetamido carbonyl formed one hydrogen bond with Cys797 which provided further hydrophobic stabilization to the terminal methyl group. **8d** pursued the similar binding pattern in HER2 (Figure 2B). The pyridine with an outward-facing conformation in the binding pocket was strengthened by Lys753 and Leu755. The interactions of quinazoline (Met801) and C-6 substituent (Cys805) were recreated.

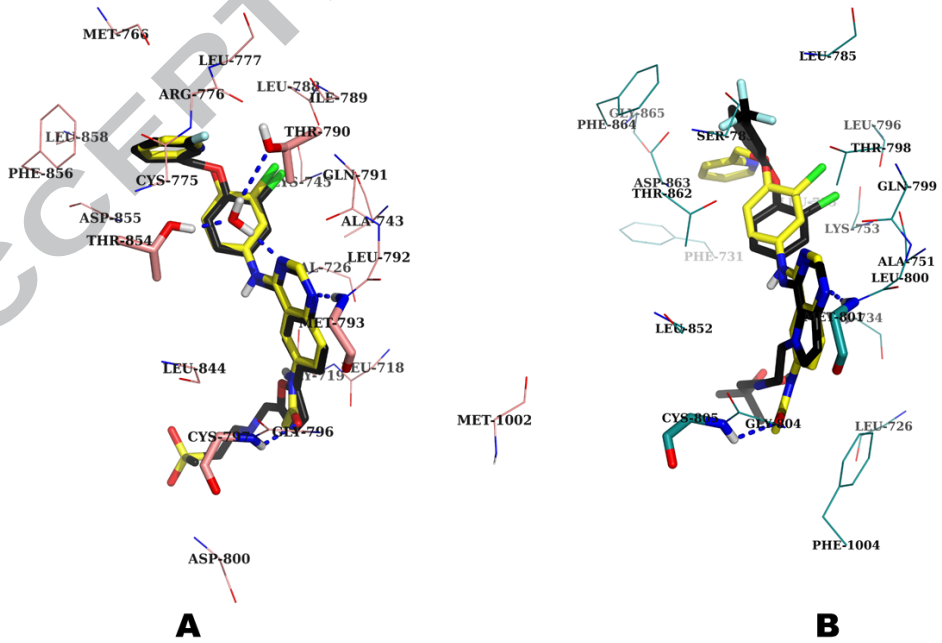


Figure 2. Top-scored poses of **8d** (yellow) aligned to the reference compounds in (A) EGFR (peach) and (B) HER2 (aqua). Hydrogen bonds are in blue dashed lines; the compounds, hydrogen bonding residues (Thr790, Met793, Cys797 and Thr854 in EGFR; Met801 and Cys805 in HER2) and conserved water molecule shown as sticks.

Compounds designed with bulky hydrophobic substituents at C-4 and C-6 proved to be non-productive in the docking experiments. In **9b** (Figure 3A and 3B), the dichlorophenyl and ethylurea at the suggested positions slightly dislocate the orientation of ligand from the proximity of functional residues (**8b**, with a similar scaffold composition at C-4 showed reasonable inhibition, this could be due to the C-6 ureido group which makes adequate polar contacts). **9d**, with pyridine substituent (C-4) and ethylurea (C-6) improved the performance both qualitatively and quantitatively in EGFR (Figure 4A) and HER2 (Figure 4B).

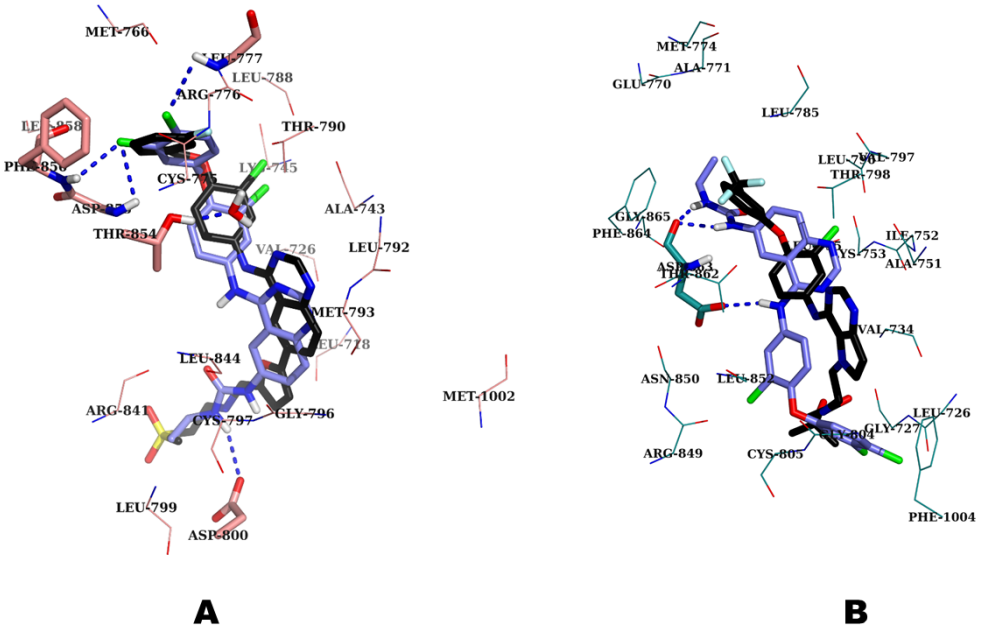


Figure 3. The docked conformations of **9b** (light violet) on superimposition with re-docked structures in (A) EGFR (peach) and (B) HER2 (aqua). Compounds, hydrogen bonding residues (Leu777, Asp800, Asp855 and Phe856 in EGFR; Asp863 in HER2) and conserved water

molecule shown as sticks; hydrogen bonds depicted as blue dashed lines. The ligand missed water-mediated interaction with Thr854 in EGFR.

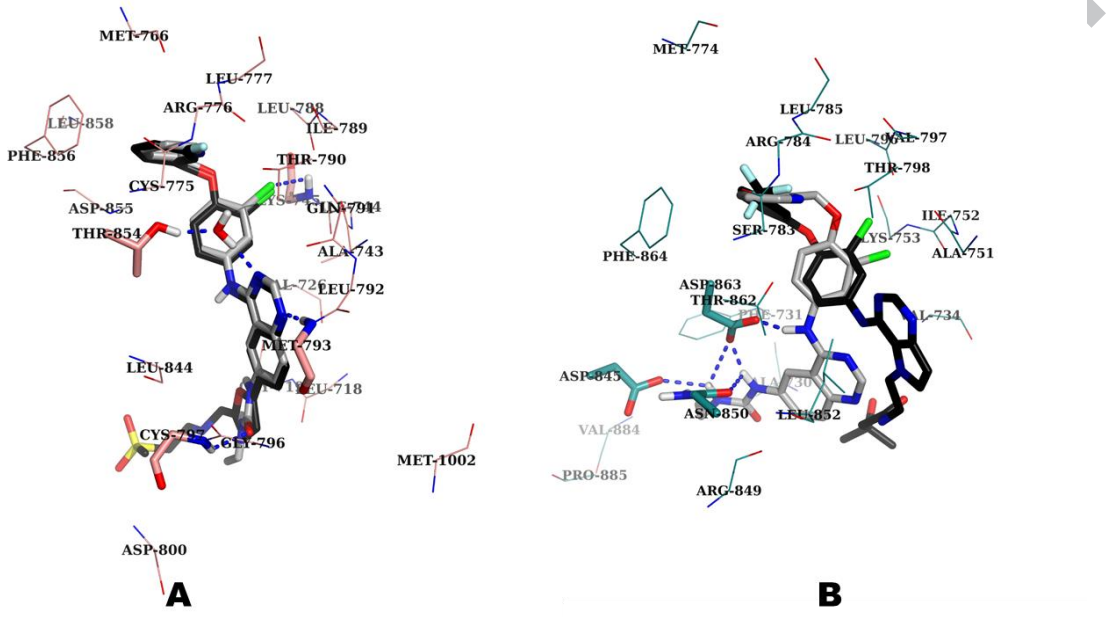


Figure 4. Superimposed **9d** poses (grey) and re-docked reference compounds (black) in (A) EGFR (peach) and (B) HER2 (aqua). Hydrogen bonds shown as blue dashed lines; ligands, hydrogen bonding residues (Lys745, Met793, Cys797 and Thr854 in EGFR; Asp845, Asn850 and Asp863 in HER2) and conserved water molecule displayed as sticks.

Covalent adduct formation between the inhibitor's reactive Michael Acceptor (MA) group and the receptor's cysteine nucleophile (Cys797 in EGFR and Cys805 in HER2) implied to enhance the potency of ligands [27-31]. In the present in silico investigation, covalent docking was attempted on **10d** because the ester MA at C-6 is approachable to the nearest conserved cysteine in the hinge region of the ATP-binding pocket. The docked models and interactions showed good alignment with re-docked ligands; expected covalent bonding with the targeted cysteines was also established (Figure 5).

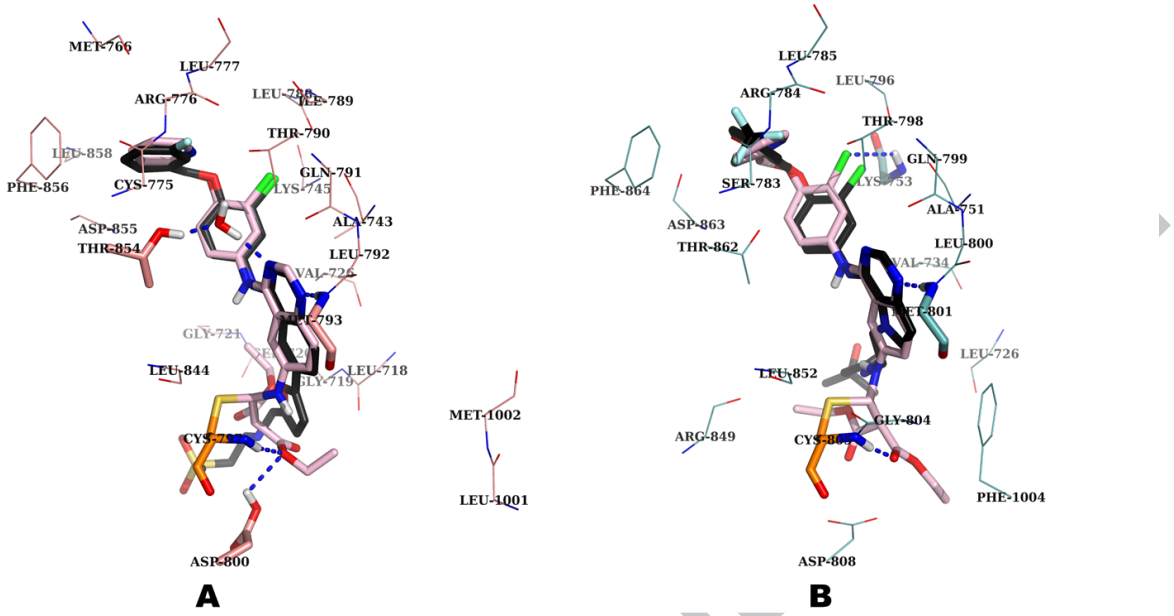


Figure 5. Top **10d** poses (light pink) aligned to re-docked crystal ligands (black) in (A) EGFR (peach) and (B) HER2 (aqua). Hydrogen bonds shown as blue dashed lines; conserved water molecule, hydrogen bonding residues (Met793, Cys797, Asp800 and Thr854 in EGFR; Lys753, Met801 and Cys805 in HER2) and covalent bonding cysteine (orange; Cys797 in EGFR and Cys805 in HER2) are in stick form.

The present docking computation created the virtual models of compounds bound with EGFR-HER2 kinase domains and their putative binding modes were deciphered with a special focus on the nature of C-4/C-6 substituents. Compounds bearing pyridine at C-4 and acetamido or ureido functional groups at C-6 found to be well-tolerated. The dual inhibitory activity of the investigated compounds was thus rationalized by the modeling studies and the later provides a foundation for further optimization.

The preliminary data for **8d** over the molecular level of EGFR and HER2 tyrosine receptors as well as its well-docked binding models showed promise and therefore it underwent further evaluation. **8d** was screened in a small panel of kinases including CDK2, c-MET, FAK, FGFR1,

IGF1R, VEGFR2, LCK, PKA, P38a, and MAPKAP kinase 2. As shown in Table 2, it exhibited no inhibition for all other tested targets except LCK kinase with IC_{50} of 1.22 µM. These results strongly indicated a great selectivity of the compound under test with its nanomolar potency over both kinases (EGFR and HER2) in comparison to the other tested targets.

Table 2. Kinase selectivity profile of compound **8d**.

Kinase	IC_{50} (µM)[a]
EGFR	0.003
HER2	0.016
CDK2	–
c-MET	–
FAK	–
FGFR1	–
IGF1R	–
VEGFR2	–
LCK	1.22
PKA	–
P38a	–
MAPKAPK2	–

[a] IC_{50} values were calculated using 10-dose mode with 3-fold serial dilution starting at 20 µM.
– No inhibition

In an effort to test the antiproliferative activity of the most potent derivatives, **8a**–**8d** and **9a**–**9d** were tested over BT-474 (HER2-overexpressing human breast cancer cell line) in a duplicate assay using 10-dose mode with 3-fold serial dilution starting at 20 µM. GI_{50} values were calculated and reported in Table 3. However the molecular activity of **8b** among the three ureido derivatives **8a**–**8c** was the modest, it interestingly showed higher cytotoxic activity over BT-474 cell line with GI_{50} value of 2.38 µM compared to that of **8a** and **8c** (>10 and 4.72 µM,

respectively). This may be attributed to the two chloro substituents on the phenoxy moiety of **8b** which could increase the lipophilic property and consequently the antiproliferative activity. Compound **8d** also exhibited low micromolar GI_{50} value of 2.7 µM. The hydrophobic methyl group in the acetamido moiety may play a role on such activity over the cell line. In respect to the ethylurea analogues **9a**–**9d**, compound **9a** exhibited better cytotoxic activity (GI_{50} = 3.55 µM) compared to its ureido derivative **8a** (GI_{50} >10 µM). On the other hand, **9b** didn't show any significant activity neither over the enzymatic nor the cellular level. **9c** and **9d** were the most active hybrids in this study with GI_{50} values of 1.82 and 1.95 µM, respectively. Finally, it was noted that compounds possessing moieties of 3-fluorobenzyloxy (**8c** and **9c**) and pyridylmethoxy (**8d** and **9d**) showed better antiproliferative activities than most analogues with phenoxy groups (**8a**, **9a**, and **9b**).

Table 3. GI_{50} values (µM) exhibited by **8a**–**8d** and **9a**–**9d** over BT-474 breast cancer cell line.

Cpd	GI_{50} (µM)[b]
8a	> 10
8b	2.38
8c	4.72
8d	2.70
9a	3.55
9b	> 10
9c	1.82
9d	1.95
Lapatinib[a]	0.93

[a] Lapatinib was used as a positive control agent.

[b] Compounds were tested in a duplicate assay using 10-dose mode with 3-fold serial dilution starting at 20 µM.

As further evaluation for the selective cytotoxicity of the most promising compounds (**8d**, **9c**, and **9d**), they have been tested over L132 normal human embryonic lung epithelium cell line using MTT assay. As illustrated in Table 4 and Figure 6, no significant inhibitory effects have been exhibited on the tested cells. (For more details about experimental procedure, see supplementary file).

Table 4. Cytotoxicity (IC_{50}, MTT assay) of **8d**, **9c**, and **9d** over L132 normal cell line.

Cpd	IC_{50} (µM)
8d	> 100
9c	> 100
9d	> 100

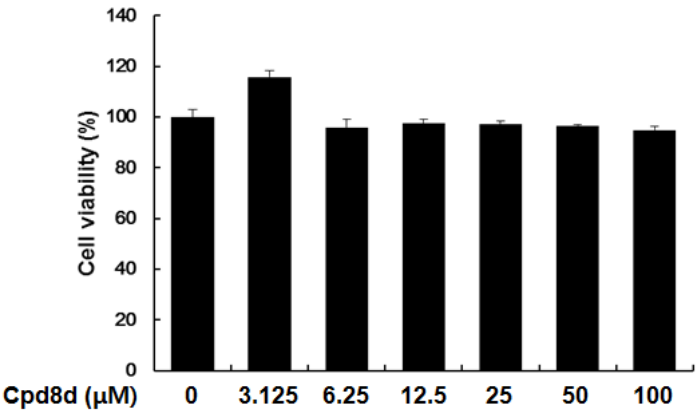


Figure 6. Cell viability of L132 normal cell line after treatment with compound **8d**.

As a conclusion, we have successfully prepared a new series of 4-anilinoquinazoline hybrids which exerted potent activity on both EGFR and HER2 kinases. Using the molecular docking approach, the binding modes of some selected compounds were modeled in the kinase domains. A cell-based assay was carried out to evaluate the antiproliferative activity of the most potent compounds. We believe that identification of analogues **8d**, **9c**, and **9d** with outstanding *in vitro*

profiles over the molecular and the cellular levels may offer an excellent framework for discovery of new potent and selective EGFR/HER2 dual inhibitors.

Acknowledgments

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The authors have declared no conflict of interest.

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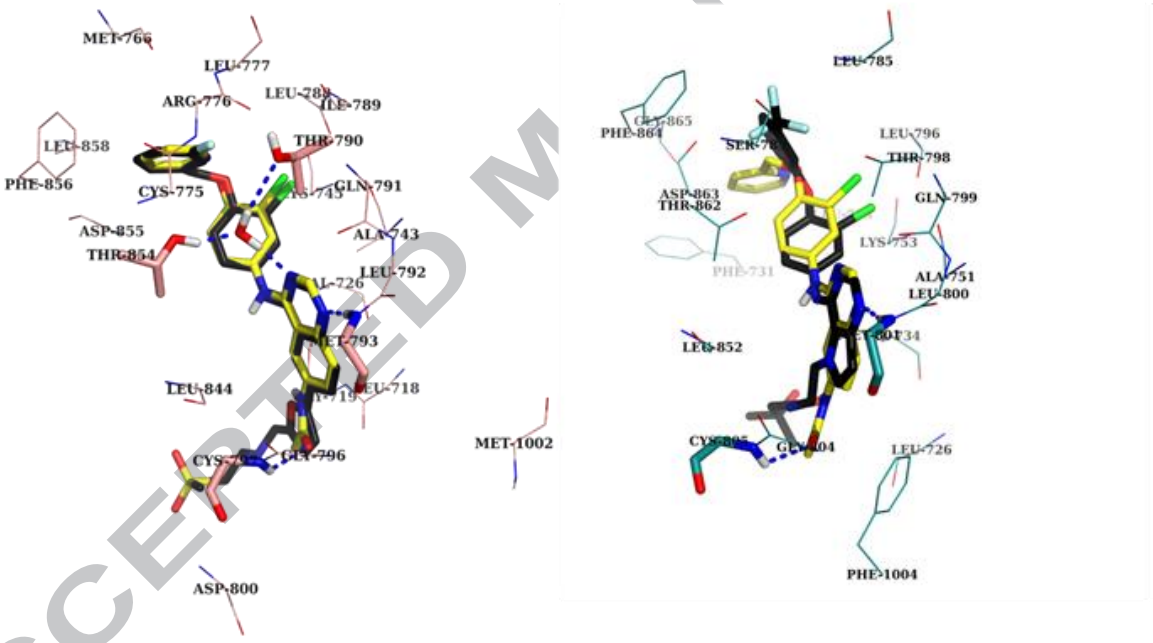
Graphical abstract



IC_{50} = 3 nM

IC_{50} = 16 nM

Compound **8d** alligned to the reference compounds
in the binding pockets of EGFR (A) and HER2 (B)