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# Synthesis and antitumor evaluation of novel 5-substituted-4-hydroxy-8-nitroquinazolines as EGFR signaling-targeted inhibitors

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**Abstract**—The synthesis and biological activity of a series of novel 5-substituted-4-hydroxy-8-nitroquinazolines that may function as inhibitors of EGFR- and/or ErbB-2-related oncogenic signaling are described. These compounds were prepared by $S_NAr$ reaction of 5-chloro-4-hydroxy-8-nitroquinazoline with alkyl or aryl amines, or alkyl alcohol as nucleophiles. Although the enzyme assay showed a weak inhibition effect against both EGFR and ErbB-2 tyrosine kinases, the cell-based antitumor activity turned out promising. Compounds having 5-anilino substituent exhibit high potency with 5-(4-methoxy)anilino-4-hydroxy-8-nitroquinazoline (**1h**) being the best dual EGFR/ErbB-2 inhibitors, which effectively inhibited the growth of both EGFR (MDA-MB-468, $IC_{50} < 0.01$ μM) and ErbB-2 (SK-BR-3, $IC_{50} = 13$ μM) overexpressing human tumor cell lines in vitro. More interestingly, the variation of the substituent(s) at the 3- and/or 4-position of the 5-anilino portion was found to modulate the selectivity and potency dramatically. However, compounds having an alkylamino or alkyloxy group at the 5-position of 4-hydroxy-8-nitroquinazolines are essentially inactive. These results are consistent with molecular modeling observations. This study was the first attempt to identify new structural types of dual EGFR/ErbB-2-related signaling inhibitors by incorporation of the anilino group at the 5-position of 4-hydroxy-8-nitroquinazolines' core structure, providing promising new templates for further development of potent inhibitors targeting both EGFR and ErbB-2 tyrosine kinases.


## 1. Introduction

Growth factor signaling pathways play a fundamental role in regulating key cellular functions, including cell proliferation, differentiation, metastasis, and survival.[1] An important mediator of growth-factor signaling pathways is the epidermal growth factor receptor (EGFR).[1] EGFR is a member of a family of four closely related receptors: EGFR (or ErbB-1), HER-2/*neu* (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4). The receptors exist as inactive monomers, which dimerize after ligand activation. This causes homodimerization or heterodimerization between EGFR and another member of the ErbB receptor family, with HER-2 being a preferred heterodimeric partner.[2] After ligand binding, the tyrosine kinase intracellular domain of the receptor is activated, with autophosphorylation of the intracellular domain, which initiates a cascade of intracellular events including activation of ras and mitogen-activated protein kinase followed by the activation of several nuclear proteins including cyclin D1, a protein required for cell cycle progression from G1 to S phase. The EGFR signaling is important for tumor cell proliferation, inhibition of apoptosis, angiogenesis, metastasis, and sensitivity to chemotherapy and radiotherapy. Overexpression of EGFR and ErbB-2 is common in a variety

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of major human solid tumors and has been correlated with poor prognosis in patients.[3] Consequently, inhibitors of the EGFR and/or HER-2 have emerged as promising anticancer agents, which was validated by the recent success in the clinical evaluation of EGFR/HER-2 TK inhibitors.[4]

Two therapeutic approaches have been used in clinical studies to inhibit the EGFR family members: humanized monoclonal antibodies (MAbs), and small molecule inhibitors of the EGFR tyrosine kinase (TKIs). MAbs are generally directed at the ectodomain of the EGFR to block ligand binding and receptor activation; TKIs prevent the autophosphorylation of the intracellular tyrosine kinase domain of the EGFR. The most promising small molecule selective EGFR-TKIs are currently three series of compounds, which include 4-anilinoquinazolines, 4-[ar(alk)ylamino]pyridopyrimidines, and 4-phenylaminopyrrolo-pyrimidines (Fig. 1).[5] Of these, the anilinoquinazolines are the most developed class of inhibitors. Figure 1 includes some examples in the quinazoline series that are currently approved drugs or in clinical trials.

The quinazoline scaffold provides the necessary binding properties for inhibition of the ErbB family of tyrosine kinases.[6] However, most of the structure–activity relationship studies were focused on the substitutions in the 6- and 7-positions of the quinazoline core structure as well as the substitutions at the 3′- and 4′-positions of the 4-anilino portion.[7–11] Few data have been published describing the antitumor activity of 5-substituted-4-hydroxyquinazolines. Since small substitution changes in kinase inhibitors greatly affect the kinase inhibition and drug properties, we were intrigued to determine the effect on the EGFR/ErbB-2 kinase activity resulting from the substitution at the 5-position of 4-hydroxyquinazoline core structure. Enlightened by the recently reported small molecule ErbB-2 TK inhibitor, namely B-17 (Fig. 1),[12] we put nitro group at the para-position of the 5-substituent of the quinazoline ring to see if it enhanced the interaction with the ErbB-2 tyrosine kinase. The aim of the study was to find new structural types of tyrosine kinase inhibitors which specifically target both EGFR and ErbB-2 TK. Dual inhibition of EGFR and ErbB-2 may offer increased activity over agents which target only one of these receptor kinases.

In this present work, we would like to report the synthesis and biological activity of a series of novel 5-substituted quinazoline derivatives represented by the general formula of **1** in Figure 1. The enzyme inhibitory activity as well as cellular activity in relevant tumor cell lines will be discussed to develop the structure–activity relationship of this new series. This work was the first attempt to explore the effect of 5-substitution on the EGFR/ErbB-2 kinase activity of the 4-hydroxyquinazoline series. Significantly, several of these compounds showed promising anti-proliferative effect against the EGFR and ErbB-2-overexpressing tumor cell lines.

## 2. Chemistry

We developed an efficient and facile synthesis approach to prepare a variety of quinazoline derivatives with various C-5 substituents. As depicted in Scheme 1, the straightforward six-step synthetic route allowed us to diversify position 5 of the quinazoline moiety via the key intermediate **6** at a later stage. Beginning with the commercially available 3-chloro-2-methyl aniline, the acetylation with acetyl chloride gave the protected aniline **2** in good yield (90%) using simple filtration isolation. Oxidation of the methyl group of **2** with $KMnO_4$ and $MgSO_4$ in refluxing water yielded **3**. Deacetylation of **3** in concentrated HCl solution at the temperature below 90 °C afforded **4** in excellent yields. The 5-chloroquinazoline **5** was prepared via Neimentowski synthesis by refluxing **4** in formamide,

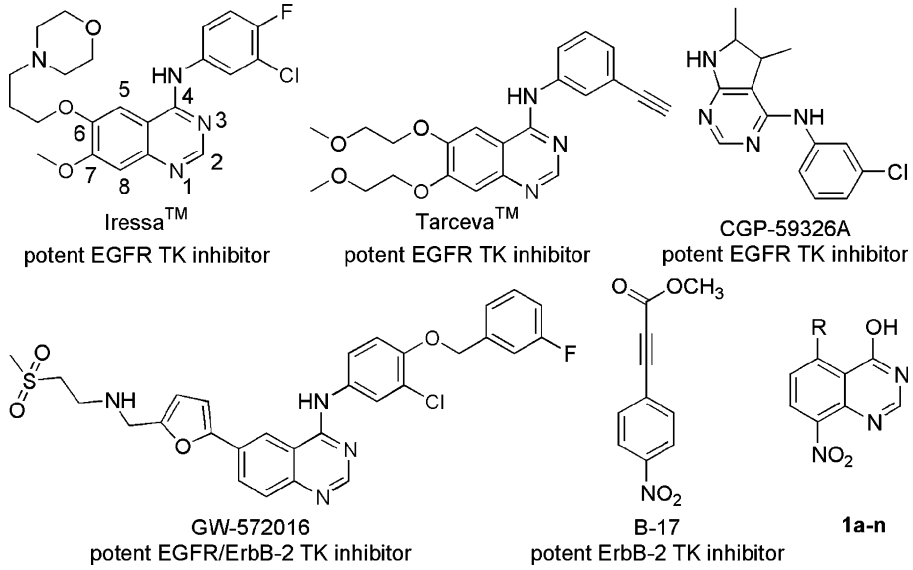
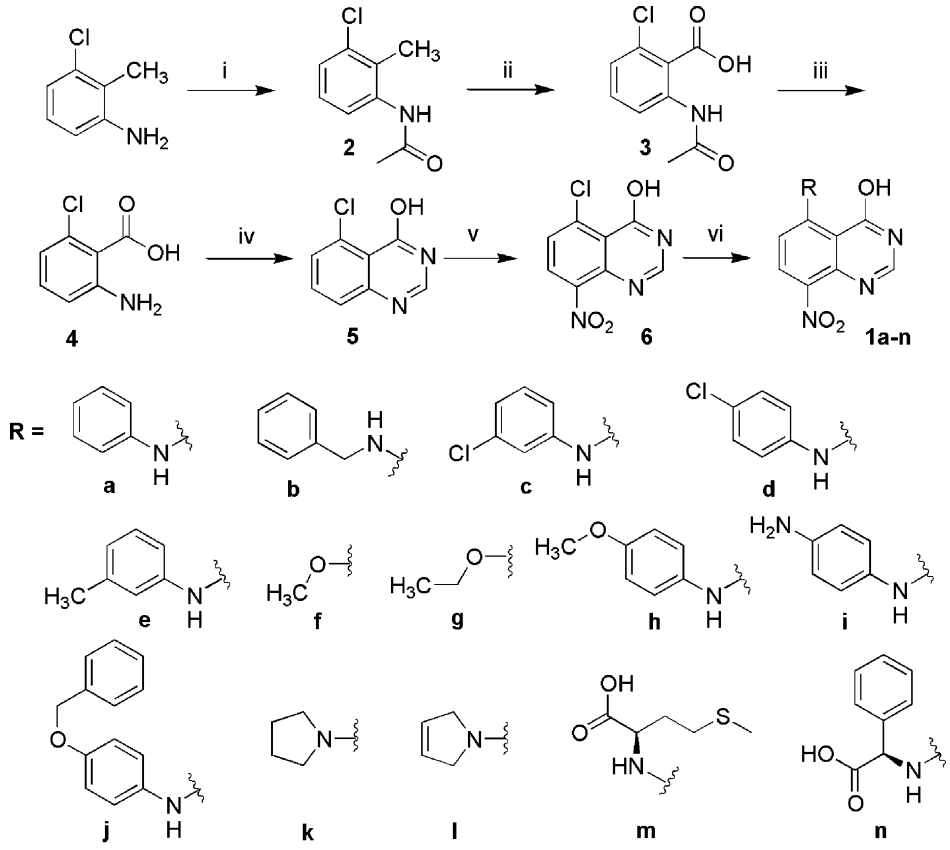



**Figure 1.** Representative small-molecule inhibitors of the tyrosine kinase domain of the EGFR. The quinazoline numbering convention is indicated.




**Scheme 1.** Reagents and conditions: (i) $CH_3COCl/CH_2Cl_2$, rt, 6 h, 90.2%; (ii) $KMnO_4$, $MgSO_4/H_2O$, reflux, 5 h, 65.7%; (iii) conc. HCl, 85 °C, 1.5 h, 98%; (iv) formamide, 130 °C for 45 min, 175 °C for 75 min, 67.6%; (v) conc. $H_2SO_4$, conc. $HNO_3$, 0 °C, 10 min, 51.6%; (vi) $RNH_2$ or ROH/RONa, THF, reflux, 12–24 h, 50–80%.

followed by an extractive basic workup. Treatment of **5** with the mixture solution of conc. $H_2SO_4$ acid and fuming $HNO_3$ acid furnished the selective nitration product **6**. Nucleophilic displacement of the key intermediate **6** with the appropriate secondary amine or alcohol in basic refluxing THF solution generated the desired final products, 5-substituted-4-hydroxy-8-nitroquinazolines (**1a–n**) in good yields (50–80%). The electron-withdrawing group nitro at the 8-position of the quinazoline ring is beneficial for the $S_NAr$ displacement reaction, and endows a large tolerance for the substitution in the 5-position of the quinazoline.

## 3. Results and discussion

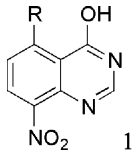
Kinase inhibitory activity of the compounds was evaluated using EGFR and ErbB-2 kinase activity assays by ELISA. The effect of the compounds on cell proliferation was measured using human tumor cell lines highly related with the aberrant EGFR signaling by sulforhodamine B assay.[13] Three human carcinoma cell lines were chosen for the cell proliferation assay: MDA-MB-468 (breast), which overexpresses EGFR, was used to determine the effectiveness of the EGFR TK inhibitory properties; SK-BR-3 (breast) overexpresses ErbB-2 and to a lesser extent EGFR and should be potently inhibited by a dual ErbB-2/EGFR TK inhibitor; MDA-MB-435 (breast) which is believed not to express either ErbB-2 or EGFR to a significant degree. Compounds known to inhibit ErbB-2 or EGFR tyrosine kinases were used as a positive control for the assays. The biological results for the 5-substituted-4-hydroxyquinazoline inhibitors are shown in Tables 1 and 2.

**Table 1.** Enzyme inhibitory activity of 4-hydroxy-8-nitroquinazolines with various C-5 substituents

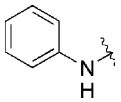
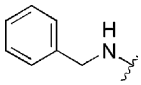
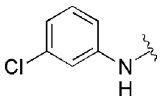
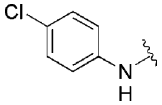
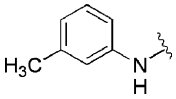
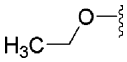
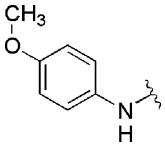
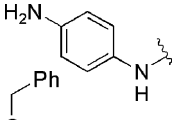
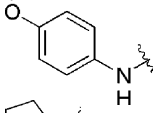
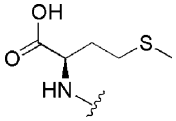
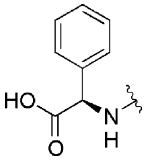
| Compound | Enzyme assays, inhibition % at 10 μM[a] | |
|---|---|---|
| | EGFR | ErbB-2 |
| **1a** | 17.7 | 2.4 |
| **1b** | 5.2 | 10.8 |
| **1c** | 17.1 | 0.3 |
| **1d** | 18.3 | 0 |
| **1e** | 0 | 4.8 |
| **1f** | 0 | 10.5 |
| **1g** | 3.9 | 0 |
| **1h** | 12.2 | 1.4 |
| **1i** | 0 | 0 |
| **1j** | 21.1 | 7.2 |
| **1k** | 0 | 4.1 |
| **1l** | 7.9 | 6.6 |
| **1m** | 5.6 | 3.7 |
| **1n** | 6.5 | 6.8 |
| **B17** | 28.0 | 2.3 |

[a] The percent inhibition of the kinase activity was generated by measuring the inhibition of phosphorylation of a peptide substrate added to enzyme reaction in the presence of 10 μm inhibitor.

**Table 2.** Inhibitory effect of compounds **1a–n** on the growth of tumor cell lines




1a-n

| Compound | R | Tumor cell $IC_{50}$ (μM)[a] | | |
|---|---|---|---|---|
| | | MDA-MB-468[b] | SK-BR-3[b] | MDA-MB-435[b] |
| **1a** |  | 0.48 | 33.4% inhibition at 10 μM | 10.0% inhibition at 10 μM |
| **1b** |  | 12.1% inhibition at 10 μM | No inhibition at 10 μM | 18.5% inhibition at 10 μM |
| **1c** |  | 32.5% inhibition at 10 μM | 8.0 | 42.8% inhibition at 10 μM |
| **1d** |  | 28.6% inhibition at 10 μM | 32.7% inhibition at 10 μM | 38.8% inhibition at 10 μM |
| **1e** |  | 15.2% inhibition at 10 μM | No inhibition at 10 μM | 15.8% inhibition at 10 μM |
| **1f** |  | 35.8% inhibition at 100 μM | 13.4% inhibition at 10 μM | 17.4% inhibition at 10 μM |
| **1g** |  | 32.3% inhibition at 10 μM | No inhibition at 10 μM | No inhibition at 10 μM |
| **1h** |  | <0.01 (75.5% inhibition at 0.01 μM) | 13.0 | <0.01 (76.0% inhibition at 0.01 μM) |
| **1i** |  | 4.0 | No inhibition at 10 μM | No inhibition at 10 μM |
| **1j** |  | 4.2 | 7.0 | 29.0% inhibition at 10 μM |
| **1k** |  | 23.3% inhibition at 100 μM | 19.5% inhibition at 100 μM | No inhibition at 10 μM |
| **1l** |  | 34.6% inhibition at 10 μM | No inhibition at 10 μM | No inhibition at 10 μM |
| **1m** |  | 19.9% inhibition at 100 μM | No inhibition at 10 μM | No inhibition at 10 μM |
| **1 n** |  | 29.2% inhibition at 100 μM | No inhibition at 10 μM | No inhibition at 10 μM |

[a] Cell line SK-BR-3 (breast carcinoma) overexpresses ErbB-2; cell line MDA-MB-468 (breast carcinoma) overexpresses EGFR; cell line MDA-MB-435 (breast carcinoma) is believed not to express EGFR or ErbB-2 to a significant extent.

[b] Dose–response curves were determined at five concentrations. The $IC_{50}$ values are the concentrations in micromolar needed to inhibit cell growth by 50% as determined from these curves.

The enzyme assay data are summarized in Table 1. Disappointingly, most compounds just display low to moderate inhibition against the enzyme activity of EGFR and ErbB-2 kinases at the concentration of 10 μM. To some extent, compounds **1a**, **1c**, **1d**, and **1j** exhibited a better ability to inhibit EGFR kinase than ErbB-2 kinase, whereas **1j** is a dual inhibitor of EGFR and ErbB-2 kinases. However, the cellular assay turned out more encouraging (Table 2). The 5-substituted quinazoline compounds show much more effectiveness in inhibiting the growth of the tumor cell lines known to express high levels of EGFR or ErbB-2. The inconsistency between the enzyme activity and the cellular efficiency could suggest that the new type of quinazoline compounds might function by inhibiting multiple key proteins involved in the EGFR signaling pathways, not only targeting the tyrosine kinase, thus leading to the significant antiproliferation effect against EGFR dependent tumor cell lines, which is highly relevant to the overexpression of EGFR or ErbB-2. The definite mechanism is still under study.

So, we used the cellular efficiency to investigate the SAR to determine the preferred substitutions of this novel quinazoline series as inhibitors of the EGFR-related tumors. Conventional quinazoline-based EGFR TK inhibitor requires an anilino substituent at the 4-position of the quinazoline scaffold for retaining high potency, thus 4-anilinoquinazolines are the most developed class of drugs that inhibit EGFR kinase intracellularly.[4] In our study with the 5-substituted-4-hydroxy-8-nitroquinazoline series, a similar observation was made; namely, the compounds not containing an anilino moiety at the 5-position such as **1b**, **1f**, **1g**, and **1k–n**, are significantly less potent than the compounds having a 5-anilino substitution, such as **1a**, **1c**, **1h**, **1i**, and **1j**, with respect to the inhibition of the proliferation of MDA-MB-468 and SK-BR-3 cell lines. Either 5-alkoxy (**1f**, **1g**) or 5-alkylamino (including the benzylamine) compounds (**1b**, **1k–n**) are essentially inactive. The results suggest that the anilino portion may play a strong role in determining the potency and selectivity of the quinazoline series as kinase inhibitors, regardless of the substitution position (4- or 5-) on the quinazoline ring. It is noteworthy that the substitution in the anilino portion is another important factor for the activity.

It is of interest to compare the inhibitory activities of the 5-anilinoquinazoline series with various substitutions at the 3′- and 4′-positions of the anilino moiety. The unsubstituted 5-aniline compound **1a** displayed excellent activity for the EGFR overexpressing cell line MDA-MB-468 ($IC_{50}$ = 0.48 μM) but was much less effective to inhibit the ErbB-2 overexpressing cell line SK-BR-3 ($IC_{50}$ > 10 μM). Interestingly, the selectivity was completely inversed by the introduction of 3-chloro substituent at the aniline ring, resulting in a selective ErbB-2 inhibitor (**1c**) with improved activity for the SKBR3 cell line ($IC_{50}$ = 8.0 μM) but poor potency in inhibiting the growth of MDA-MB-468 cell line ($IC_{50}$ > 10 μM). The utility of small lipophilic groups in the 3-position of the aniline ring was reported for EGFR inhibition.[14] In our case, 3-substitution seems more important for ErbB-2 inhibition. But the methyl substitution at the 3-position (**1e**) completely abolished the activity. With respect to the substituent effect at the 4-position of the anilino portion, the small lipophilic substituent was found to be detrimental to the activity of our 5-anilinoquinazoline series, e.g., the 4-chloro compound (**1d**) demonstrated much less activity in the two cell proliferation assays. Encouragingly, further elaboration of the 4-substitution in the anilino ring with electron-donating groups showed a remarkable positive response. The lipophilic electron-donating groups at the 4-position were beneficial, with the 4-methoxy and 4-benzyloxy derivatives (**1h**, **1j**) being the most potent dual inhibitors of EGFR and ErbB-2 signaling. The 4-methoxy-anilino substitution afforded the best dual inhibitor of the growth of both target cell lines (**1h**) ($IC_{50} \leqslant 0.01$ and 13.0 μM, for EGFR and ErbB-2, respectively). The large benzyloxy substitution at the 4-position (**1j**) conferred a better inhibitor for ErbB-2 ($IC_{50}$ = 7.0 μM) but an inferior inhibitor for EGFR overexpression cell line ($IC_{50}$ = 4.2 μM) compared to **1h**, suggesting that ErbB-2 inhibition prefers large substitution at the 4′-position of the 5-anilino substituent. In contrast, the small hydrophilic group at the 4′-position of the 5-aniline ring was disfavored for the ErbB-2 binding, whereby the 4′-amine group greatly reduced the activity for the ErbB-2 while retaining moderate potency against EGFR ($IC_{50}$ = 4.0 μM). So far, it is very apparent that for the novel 5-anilino-4-hydroxyquinazoline inhibitors of EGFR/ErbB-2-related tumors, the target affinity and selectivity could be modulated to a great extent via the substitutions on 5-anilino moiety, with the position and the nature of the substituent being the determining factors.

It was also evident that the 5-substituted quinazoline compounds are a much better inhibitor of the MDA-MB-468 and SK-BR-3 cell lines than the MDA-MB-435 line, except for compound **1h**. This is consistent with our proposed mechanism of cell growth inhibition being reliant, to some degree, on EGFR signaling. The observation that the most potent inhibitor **1h** does inhibit the growth of the MDA-MB-435 could suggest that this line has some dependence on EGFR or ErbB-2, even though these receptors are not expressed to a significant extent.

## 4. Molecular modeling

The earlier observations can be nicely rationalized from the results of molecular modeling experiments. Recently the X-ray crystal structure of EGFR kinase having a bound quinazoline-based ligand (GW572106) was reported.[15] GW572016 was a potent dual EGFR/ErbB-2 inhibitor, so the published coordinates from this X-ray structure was used as the starting point for the modeling studies in the present study. The automated molecular docking can produce several optional conformation of the inhibitor. The conformation of the inhibitor **1h** corresponding to the lowest binding energy was selected as the possible binding conformation, shown in Figure 2.

In the final model (Fig. 2), the N1 and N3 atoms of **1h** are hydrogen-bonded to the backbone carbonyl oxygen of Phe832 and the sidechain carboxyl oxygen of Asp831,

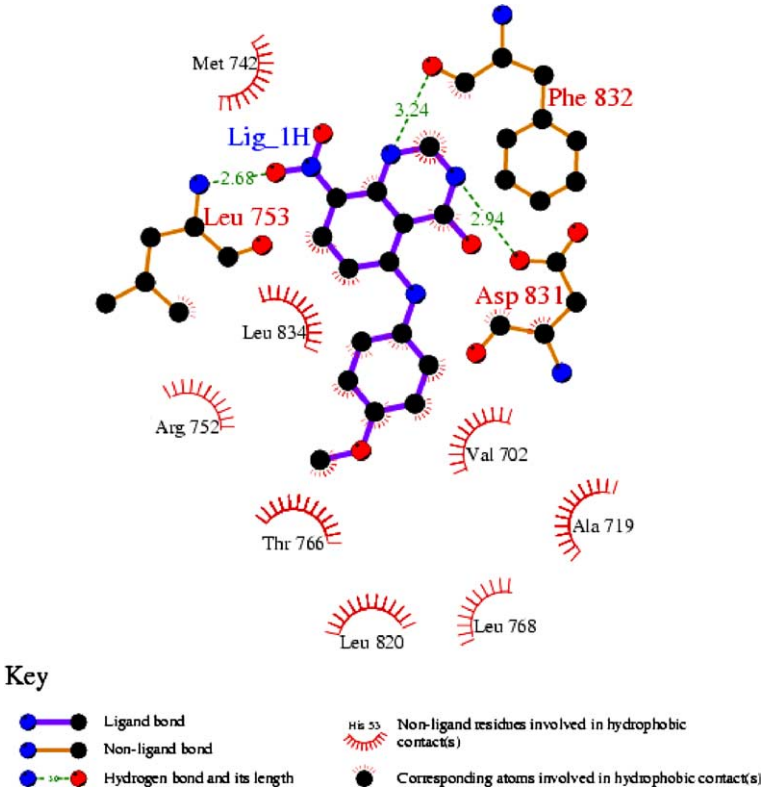



**Figure 2.** Two-dimensional representative of the interaction model of compound **1h** with the kinase domain of EGFR. This image was generated with LIGPLOT program.[16]

respectively. And the quinazoline ring forms hydrophobic interactions with residues Met742, Leu753, and Leu834. Of particular significance to the case of 5-substituted-8-nitroquinazoline series, the 8-nitro group of **1h** is involved in the interaction with the active site of EGFR via its oxygen atom forming hydrogen bond with the backbone NH of Leu753, and the 5-(4-methoxyaniline) moiety lies in a hydrophobic pocket containing Val702, Thr766, Ala719, Arg752, Leu820, and Leu768. We believe that this might account for the large difference in activity we see between the 5-alkoxy(or alkyamino-) and 5-anilinoquinazolines.

This binding mode is not similar to the original ligand GW-572016, which is much larger than the inhibitor **1h**. GW572016 has a bulky 6-anilino substituent reaching deep into an opened back pocket, which may result in the slow-off rate in dissociation. From the binding mode of **1h** with EGFR, we found that it just occupied the sub-pocket, suggesting why the 5-substituted-8-nitroquinazolines are not potent with respect to EGFR/ErbB-2 tyrosine kinase assay. This model will be helpful for our further structural elaboration of the novel quinazoline series to improve the kinase activity.

## 5. Conclusions

We have synthesized and assessed a series of novel 5-substituted-4-hydroxy-8-nitroquinazolines that may function as inhibitors of EGFR/ErbB-2-mediated cellular signaling pathways. The SAR was discussed in terms of the inhibitory activity against the proliferation of two human carcinoma cell lines known to express high levels of EGFR and ErbB-2. For the new 5,8-disubstituted quinazoline derivatives, the 5-anilino substitution is essential for its activity. Furthermore, the substitution on the 5-anilino portion has a substantial effect on the potency and selectivity of the resulting compound with respect to the inhibition of EGFR and ErbB-2 receptors. The 5-anilinoquinazoline (**1a**) was a selective inhibitor of EGFR, whereas the 5-(3-chloroanilino)-quinazoline (**1c**) displayed selective inhibition of ErbB-2. More significantly, two potent dual inhibitors (**1h** and **1j**) of EGFR and ErbB-2 were discovered by the alkoxy substitution at the 4′-position of 5-anilino substituent. A binding mode of these compounds autodocked into the active site of an EGFR kinase domain is helpful for explaining the SAR of 5-aniline. This study was the first attempt to identify new structural types of EGFR/ErbB-2 signaling inhibitors, by the incorporation of the anilino group at the 5-position of 4-hydroxy-8-nitroquinazolines' core structure, providing promising new templates for further development of potent inhibitors targeting both EGFR and ErbB-2 tyrosine kinases.

## 6. Experimental

### 6.1. Molecular modeling

The crystal structure of kinase domain of EGFR in complex with its inhibitor GW572016 (PDB entry code 1XKK)[15] was recovered from Brookhaven Protein

Database (PDB). The missed atoms and residues were modeled by Sybyl 6.8.[17] The kinds of atomic charges were taken as Kollman-united-atom[18] for the macromolecule and Gasteiger–Marsili[19] for the inhibitor.

To find the binding mode of the compound **1h** to the kinase domain of EGFR, the advanced docking program Autodock 3.0.3[20,21] was used to automatically dock the inhibitor to the enzyme. The Lamarckian genetic algorithm (LGA)[20] was applied to deal with the inhibitor–enzyme interaction. A Solis and Wets local search performed the energy minimization on a user-specified proportion of the population. The docked conformations of the inhibitor were generated after a reasonable number of evaluations.

The whole docking operation in this study could be stated as follows. First, the kinase domain of EGFR was checked for polar hydrogen and assigned for partial atomic charges, then a PDBQ file was created, and then the atomic solvation parameters were also assigned for the macromolecule. Meanwhile, some of the torsion angles of the inhibitor that would be explored during molecular docking stage were defined, allowing the conformation search for the ligand during the docking process. Second, the grid map with $60 \times 60 \times 60$ points and a spacing of 0.375 Å was calculated using the AutoGrid program,[20] to evaluate the binding energies between the inhibitor and the macromolecule. Third, some important parameters for LGA calculations were reasonably set on. Not only the atom types, but also the generations and the number of runs for the LGA algorithm were edited and properly assigned according to the requirement of the Amber force field. The maximum number of generations, energy evaluations, and docking runs were set to $3.0 \times 10^5$, $1.5 \times 10^7$, and 30, respectively. Finally, the docked complex of the inhibitor–enzyme for the inhibitor was selected according to the criterion of interaction energy combined with geometrical matching quality.

## 6.2. Biology

**6.2.1. EGFR and ErbB-2 kinase activity assays by ELISA.** The assay was performed in 96-well plates pre-coated with 20 μg/mL Poly(Glu, Tyr)$_{4:1}$ (Sigma) as a substrate. In each well, 85 μL of 8 μM ATP solution and 10 μL of the title compound were added at varying concentrations. AG825 and PD153035 were used as a positive control for ErbB-2 and EGFR kinase, respectively, and 0.1% (v/v) DMSO was the negative control. Experiments at each concentration were performed in triplicate. The reaction was initiated by adding 5 μL of ErbB-2-CD or EGFR kinase. After incubation for 1 h at 37 °C, the plate was washed three times with PBS containing 0.1% Tween 20 (T-PBS). Next, 100 μL antiphosphotyrosine (PY99) (1:500 dilution) antibody was added. After 1 h of incubation at room temperature, the plate was washed three times. Goat anti-mouse IgG horseradish peroxidase (100 μL) (1:2000 dilution) diluted in T-PBS containing 5 mg/mL BSA was added. The plate was reincubated at room temperature for 1 h, and washed as before. Finally, a 100 μL solution (0.03% $H_2O_2$, 2 mg/mL *o*-phenylenediamine in citrate buffer 0.1 M, pH 5.5) was added and incubated at room temperature until color emerged. The reaction was terminated by the addition of 100 μL of 2 M $H_2SO_4$, and A492 was measured using a multiwell spectrophotometer (VERSAmax$^{TM}$). The inhibition rate (%) was calculated using the equation:

$$\text{The inhibition rate}(\%) = [1 - (A_{492}/A_{492}\text{control})] \times 100\%.$$

**6.2.2. Cell growth inhibition assay.** Two human carcinoma cell lines, SKBR3 (breast carcinoma) and MDA-MB-468 (breast carcinoma), that were obtained from American Type Culture Collection (Rockville, MD), were used for the cell proliferation assay. Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA) in a highly humidified atmosphere of 95% air with 5% $CO_2$ at 37 °C. The growth inhibition was analyzed by the sulforhodamine B (SRB, Sigma) assay.[12] Briefly, the cells were seeded at 6000 cells/well in 96-well plates (Falcon, CA, USA), and allowed to attach overnight. The cells were treated in triplicate with grade concentrations of compounds at 37 °C for 72 h. Then, they were fixed with 10% trichloroacetic acid and incubated for 60 min at 4 °C. Then, the plates were washed and dried. SRB solution (0.4% w/v in 1% acetic acid) was added and the culture was incubated for an additional 15 min. After the plates were washed and dried, bound stain was solubilized with Tris buffer, and the optical densities were read on the plate reader (model VERSA Max, Molecular Devices) at 515 nm. The growth inhibitory rate of treated cells was calculated by the following formula: $[1 - (A_{515}\text{ treated}/A_{515}\text{ control})] \times 100\%$. The results were also expressed as $IC_{50}$ (the compound concentration required for 50% growth inhibition of tumor cells), which was calculated by the Logit method. The mean $IC_{50}$ was determined from the results of three independent tests.

## 6.3. Chemistry

Unless otherwise stated, the $^1$H NMR spectra were recorded on a Varian 400-MHz spectrometer. The data are reported in parts per million relative to TMS and referenced to the solvent in which they were run. Elemental analyses were obtained using a Vario EL spectrometer. Melting points (uncorrected) were determined on a Buchi-510 capillary apparatus. Low resolution mass spectra (EI) was determined on Finnigan MAT-95 mass spectrometer. The solvent was removed by rotary evaporation under reduced pressure, and flash column chromatography was performed on silica gel H (10–40 μm). Anhydrous solvents were obtained by redistillation over sodium wire.

**6.3.1. *N*-(3-Chloro-2-methylphenyl)-acetamide (2).** To the solution of 3-chloro-2-methyl aniline (2.34 g, 16.50 mmol) in $CH_2Cl_2$ (6 mL) was added acetic anhydride (2.53 g, 24.75 mmol) with stirring. The resultant

large gray precipitate of crude acetylated product was filtered with saction and washed with water. Recrystallization from EtOAc/MeOH = 10:1 afforded a pure white crystal (2.73 g, 90.2%). $^1$H NMR (DMSO-$d_6$, 400 MHz), $\delta$ 9.70 (s, 1H), 7.40 (d, $J$ = 8.40 Hz, 1H), 7.37 (t, $J$ = 8.01 Hz, 1H), 1.99 (s, 3H); MS (EI) $m/z$ 183 ($M^+$); Anal. Calcd. for ($C_9H_{10}ClNO$) C, H, N: 58.86, 5.49, 7.63.

**6.3.2. 2-(Acetylamino)-6-chlorobenzoic acid (3).** A mixture of compound **2** (3.3 g, 18.0 mmol), magnesium sulfate heptahydrate (4.3 g, 38.8 mmol), and water (80 mL) was heated to reflux (135 °C). A solution of potassium permanganate (11.4 g, 72.2 mmol) and water (100 mL) was added portionwise over 3 h. After the final addition, the mixture was refluxed for 2 h (160 °C), and the cooled mixture was filtered. The filtrate was acidified with hydrochloric acid (6 M) to pH 1.0. The crude product was precipitated as a white crystal that was collected by filtration and dried under high vacuum. Recrystalization from MeOH afforded a pure white solid product (2.5 g, 65.7%). $^1$H NMR (DMSO-$d_6$, 400 MHz), $\delta$ 9.70 (s, 1 H), 7.40 (d, $J$ = 8.04 Hz, 1H), 7.37 (t, $J$ = 8.01 Hz, 1H), 7.31 (d, $J$ = 8.06 Hz, 1H), 1.99 (s, 3H); MS (EI) $m/z$ 213 ($M^+$); Anal. Calcd. for ($C_9H_8ClNO_3$) C, H, N: 50.60, 3.77, 6.56. Found: 50.42, 3.54, 6.45.

**6.3.3. 5-Chloroquinazolin-4-ol (5).** Compound **3** (250 mg, 1.17 mmol) with 4 mL concentrated hydrochloric acid was heated for 1.5 h in an oil-bath at 80–90 °C, not higher than 90 °C, providing a white crystal on cooling. The solid was filtrated and recrystalized from MeOH to provide the 2-amino-6-chlorobenzoic acid (**4**), which was used directly for the next step. The mixture of **4** (1.2 g, 5.8 mmol) and 1.5 mL formamide was heated in an oil bath at 130 °C for 45 min and at 175 °C for 75 min. The cooled semi-solid mass was slurried with 1.5 mL of methyl-cellosove and dumped in 5 mL of cold water to yield a crude product. Purification from silica gel chromatography using EtOAc/PE = 1:1 afforded a white solid (0.75 g, 67.6%). $^1$H NMR (DMSO-$d_6$, 400 MHz), $\delta$ 12.09 (br, 1H), 8.07 (s, 1H), 7.71 (t, $J$ = 8.25 Hz, 7.7, 1H), 7.59 (d, $J$ = 8.0 Hz, 1H), 7.51 (d, $J$ = 7.43 Hz, 1H); MS (EI) $m/z$ 180 ($M^+$); Anal. Calcd. for ($C_8H_5ClN_2O$) C, H, N: 53.21, 2.79, 15.51. Found: 53.14, 2.72, 15,36.

**6.3.4. 5-Chloro-8-nitroquinazoline-4-ol (6).** The solution of compound **5** (155 mg, 0.86 mmol) in concentrated $H_2SO_4$ (3 mL) cooled to 0 °C was treated dropwisely (10 min) with fuming nitric acid ($HNO_3$, 3 mL). The reaction mixture was stirred for 10 min (0 °C) before being poured into crushed ice. The mixture was neutralized with 20% KOH with ice cooling. The product was collected by filtration and washed with $H_2O$ (2 × 6 mL). Recrystalization from EtOH–$H_2O$ gave 100 mg of pure compound **6** as a light yellow solid (193.5 mg, 51.6%). $^1$H NMR (DMSO-$d_6$, 400 MHz), $\delta$ 12.68 (br, 1 H), 8.27 (d, $J$ = 8.86 Hz, 1H), 8.22 (s, 1H), 7.75 (d, $J$ = 8.85 Hz, 1H); MS (EI) $m/z$ 225 ($M^+$); Anal. Calcd. for ($C_8H_4ClN_3O_3$) C, H, N: 42.59, 1.79, 18.63. Found: 42.47, 1.68, 18.60.

**6.3.5. 5-Anilino-8-nitroquinazolin-4-ol (1a).** To the solution of compound **6** (100 mg, 0.44 mmol) and 18 mL THF was added aniline (82 mg, 0.88 mmol). The mixture was refluxed for 24 h at 80–90 °C. The solution was concentrated and diluted with water (15 mL). After adding the HCl solution (20%) until pH 7, the resultant solution was extracted with EtOAc (2 × 20 mL), dried over $MgSO_4$, and then evaporated. The residue was purified by column chromatography (EtOAc/PE = 1:1) affording brown solid (90 mg, 72.5%). mp: 233–235 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 9.88 (br, 1H), 8.81 (s, 1H), 8.80 (d, $J$ = 9.08 Hz, 1H), 7.34 (d, $J$ = 8.9 Hz, 1H), 7.0–7.2 (m, 5H); MS (EI) $m/z$ 282 ($M^+$); Anal. Calcd. for ($C_{14}H_{10}N_4O_3$) C, H, N: 59.57, 3.57, 19.85. Found: 59.45, 3.44, 19.98.

**6.3.6. 5-(Benzylamino)-8-nitroquinazolin-4-ol (1b).** The compound **1b** was prepared in a similar procedure for **1a**, except for using benzylamine (94 mg, 0.88 mmol), affording orange solid (100 mg, 76.9%). mp: 182–184 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 10.00 (br, 1H), 8.68 (d, $J$ = 9.1 Hz, 1H), 7.2–7.3 (m, 5H), 6.77 (d, $J$ = 8.99 Hz, 1H), 4.25 (s, 2H); MS (EI) $m/z$ 296 ($M^+$); Anal. Calcd. for ($C_{15}H_{12}N_4O_3$) C, H, N: 60.81, 4.08, 18.91. Found: 60.72, 4.11, 18.87.

**6.3.7. 5-[(3-Chlorophenyl)amino]-8-nitroquinazolin-4-ol (1c).** The compound **1c** was prepared as described for **1a**, except for using 3-chlorobenzenamine (112 mg, 0.88 mmol), providing an yellow solid (110 mg, 79.5%). mp: 235–237 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 12.90 (br, 1H), 11.45 (s, 1H), 8.27 (s, 1H), 8.23 (d, $J$ = 9.07 Hz, 1H), 7.32 (t, $J$ = 7.93 Hz, 1H), 7.16 (d, $J$ = 9.07 Hz, 1 H), 7.16–7.07 (m, 2 H), 6.95 (d, $J$ = 7.96 Hz, 1 H); MS (EI) $m/e$ 316 ($M^+$); Anal. Calcd. for ($C_{14}H_9ClN_4O_3$) C, H, N: 53.09, 2.86, 17.69. Found: 53.24, 2.97, 17.57.

**6.3.8. 5-[(4-Chlorophenyl)amino]-8-nitroquinazolin-4-ol (1d).** The compound **1d** was prepared as described for **1a**, except for using 4-chloroaniline (112 mg, 0.88 mmol), yielding an yellow solid (106 mg, 76.8%). mp: 241–243 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 11.48 (br, 1H), 8.28 (s, 1H), 8.20 (d, $J$ = 9.08 Hz, 1H), 7.32 (d, $J$ = 8.51 Hz, 2H), 7.10 (d, $J$ = 9.06 Hz, 1H), 6.99 (d, $J$ = 8.52 Hz, 2H); MS (EI): 317 ($M^+$ + 1); Anal. Calcd. for ($C_{14}H_9ClN_4O_3$) C, H, N: 53.09, 2.86, 17.69. Found: 52.86, 3.21, 17.35.

**6.3.9. 5-[(3-Methylphenyl)amino]-8-nitroquinazolin-4-ol (1e).** The compound **1e** was prepared analogously to **1a**, except for using *m*-toluidine (95 mg, 0.88 mmol), affording a black brown solid (185 mg, 71.2%). mp: 215–217 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 12.85 (br, 1H), 11.42 (s, 1H), 8.26 (s, 1H), 8.18 (d, $J$ = 9.89 Hz, 1H), 7.15 (t, $J$ = 7.69 Hz, 1H), 7.08 (d, $J$ = 8.79 Hz, 1H), 6.93 (d, $J$ = 9.89 Hz, 1H), 6.78 (s, 1H), 6.76 (d, $J$ = 8.79 Hz, 1H), 2.23 (s, 3H); MS (EI) $m/e$ = 296 ($M^+$); Anal. Calcd. for ($C_{15}H_{12}N_4O_3$) C, H, N: 60.81, 4.08, 18.91. Found: 60.92, 4.11, 18.76.

**6.3.10. 5-Methoxy-8-nitroquinazolin-4-ol (1f).** To the solution of compound **6** (100 mg, 0.44 mmol) in

10 mL of methanol, sodium methanol was added (100 mg, 1.85 mmol). The mixture was refluxed for 24 h at 70 °C. The solution was concentrated and diluted with water (15 mL). HCl solution was added (20%) until pH 7, the resultant solution was extracted with EtOAc (2 × 20 mL). Afer usual work-up, the residue was purified by column chromatography (EtOAc/PE = 1:1) to afford a white solid (65 mg, 66.8%). mp: 209–210 °C, $^1$H MNR (DMSO-$d_6$, 300 MHz), $\delta$ 11.28 (br, 1 H), 9.13 (s, 1H), 8.67 (d, $J$ = 8.79 Hz, 1 H), 6.83 (d, $J$ = 8.66 Hz, 1 H), 3.90 (s, 3 H); MS (EI) $m/z$ 221 ($M^+$); Anal. Calcd. for ($C_9H_7N_3O_4$) C, H, N: 48.87, 3.19, 19.00. Found: 48.77, 3.23, 18.89.

**6.3.11. 5-Ethoxy-8-nitroquinazolin-4-ol (1g).** Compound **1 g** was prepared similar to **1f**, except using sodium ethanol (125 mg, 1.85 mmol) and 10 mL ethanol as a solvent. Purification by column chromatography (EtOAc/PE = 1:1) provided a white solid (80 mg, 77.6%). mp: 195–198 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 11.28 (br, 1H), 9.14 (s, 1H), 8.63 (d, $J$ = 8.20 Hz, 1H), 6.81 (d, $J$ = 8.40 Hz, 1H), 4.12 (m, $J$ = 6.9 Hz, 2H), 1.48 (t, $J$ = 6.9 Hz, 3H); MS (EI) $m/z$ 235 ($M^+$); Anal. Calcd. for ($C_{10}H_9N_3O_4$) C, H, N: 51.07, 3.86, 17.87. Found: 51.13, 3.68, 17.74.

**6.3.12. 5-[(4-Methoxyphenyl)amino]-8-nitroquinazolin-4-ol (1h).** The compound **1h** was prepared as described for **1a**, except for using 4-methoxybenzenamine (108 mg, 0.88 mmol), affording a black green solid (81 mg, 58.9%). mp: 200–203 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 12.65 (br, 1H), $\delta$ 11.40 (s, 1H), 8.24 (s, 1H), 8.14 (d, $J$ = 9.07 Hz, 1H), 7.02 (d, $J$ = 9.07 Hz, 1H), 6.95 (d, $J$ = 9.06 Hz, 2H), 6.85 (d, $J$ = 9.06 Hz, 2H), 3.73 (s, 3H); MS (EI) $m/e$ 312 ($M^+$); Anal. Calcd. for ($C_{15}H_{12}N_4O_4$) C, H, N: 57.69, 3.87, 17.94. Found: 57.79, 3.98, 17.67.

**6.3.13. 5-[(4-Aminophenyl)amino]-8-nitroquinazolin-4-ol (1i).** The compound **1i** was prepared as described for **1a**, except using 4-amino-phenylamine (48 mg, 0.44 mmol). Purification from column chromatography ($CHCl_3$/MeOH = 5:1) afforded a black-brown solid (68 mg, 51.4%). mp: 223–226 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 11.28 (br, 1H), 8.21 (s, 1H), 8.08 (d, $J$ = 9.06 Hz, 1H), 6.90 (d, $J$ = 9.07 Hz, 1H), 6.68 (d, $J$ = 8.52 Hz, 2H), 6.47 (d, $J$ = 8.79 Hz, 2H); MS (EI) $m/z$ $M^+$ = 297; Anal. Calcd. for ($C_{14}H_{11}N_5O_3$) C, H, N: 56.56, 3.73, 23.56. Found: 56.70, 3.46, 23.44.

**6.3.14. 5-[4-(Benzyloxy)phenyl]amino8-nitroquinazolin-4-ol (1j).** The compound **1j** was prepared in the same procedure as described for **1a**, except using 4-(benzyloxy)benzenamine (175 mg, 0.88 mmol), affording a yellow brown solid (110 mg, 64.7%). mp: 220–222 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 11.40 (br, 1H), 8.25 (s, 1H), 8.15 (d, $J$ = 9.07 Hz, 1H), 7.46–7.35 (m, 5H), 7.02 (d, $J$ = 9.07 Hz, 1H), 6.94 (s, 4H), 5.05 (s, 1H); MS (EI) $m/e$ 388 ($M^+$); Anal. Calcd. for ($C_{21}H_{16}N_4O_4$) C, H, N: 64.94, 4.15, 14.43. Found: 64.82, 4.45, 14.56.

**6.3.15. 8-Nitro-5-pyrrolidin-1-ylquinazolin-4-ol (1k).** The compound **1k** was prepared as described for **1a**, except using pyrrolidine (63 mg, 0.88 mmol), affording a brown solid (90 mg, 78.5%). mp: 208–210 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 12.30 (br, 1H), 8.12 (s, 1H), 8.09 (d, $J$ = 9.07 Hz, 1H), 6.72 (d, $J$ = 9.07 Hz, 1H), 3.29 (dt, 4H), 1.89 (dt, 4H); MS (EI) $m/e$ 260 ($M^+$); Anal. Calcd. for ($C_{12}H_{12}N_4O_3$) C, H, N: 55.38, 4.65, 21.53. Found: 55.41, 4.35, 21.78.

**6.3.16. 8-Nitro-5-(2H-pyrrol-1(5H)-yl)quinazolin-4-ol (1l).** The compound **1l** was prepared as described for **1a**, except using 2,5-dihydro-1H-pyrrole (61 mg, 0.88 mmol), providing a black brown solid (79 mg, 69.8%). mp: 154–160 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 12.41 (br, 1H), 8.11 (s, 1H), 7.99 (d, $J$ = 8.99 Hz, 1H), 6.79 (d, $J$ = 8.99 Hz, 1H), 6.79 (d, $J$ = 9.06 Hz, 1H), 5.91 (s, 2H), 3.98 (s, 4H); MS (EI) $m/e$ 257 ($M^+-1$); Anal. Calcd. for ($C_{12}H_{10}N_4O_3$) C, H, N: 55.81, 3.90, 21.70. Found: 55.79, 3.86, 21.92.

**6.3.17. 2-[(4-Hydroxy-8-nitroquinazolin-5-yl)amino]-4-(methylthio) butanoic acid (1m).** The compound **1m** was prepared as described for **1a**, except using 2-amino-4- (methylthio)butanoic acid (131 mg, 0.88 mmol). Purification by column chromatography ($CHCl_3$/MeOH = 2:1) afforded a brown solid (76 mg, 51.2%). mp: 214–217 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 12.78 (br, 1H), 10.34 (br, 1H), 8.23 (s, 1H), 8.11 (d, 1H, $J$ = 9.07 Hz), 6.91 (d, 1H, $J$ = 9.06 Hz), 4.01 (s, 1H), 2.37 (s, 2H), 2.2–1.8 (m, 5H); MS (EI) $m/e$ 338 ($M^+$); Anal. Calcd. for ($C_{13}H_{14}N_4O_5S$) C, H, N: 46.15, 4.17, 16.56. Found: 46.23, 4.25, 16.43.

**6.3.18. [(4-Hydroxy-8-nitroquinazolin-5-yl)amino](phenyl)acetic acid (1n).** The compound **1n** was prepared as described for **1a**, except for using 2-amino-2-phenylacetic acid (133 mg, 0.88 mmol). Purification by column chromatography ($CHCl_3$/MeOH = 2:1) provided a brown solid (74 mg, 49.7%). mp: >250 °C; $^1$H NMR (DMSO-$d_6$, 300 MHz), $\delta$ 12.80 (br, 1H), 11.25 (br, 1H), 8.22 (s, 1H), 7.91 (d, $J$ = 9.34 Hz, 1H), 7.25–7.12 (d, 5H), 5.12 (s, 1H); MS (EI) $m/e$ 340 ($M^+$); Anal. Calcd. for ($C_{16}H_{12}N_4O_5$) C, H, N: 56.47, 3.55, 16.46. Found: 56.22, 3.31, 16.28.

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