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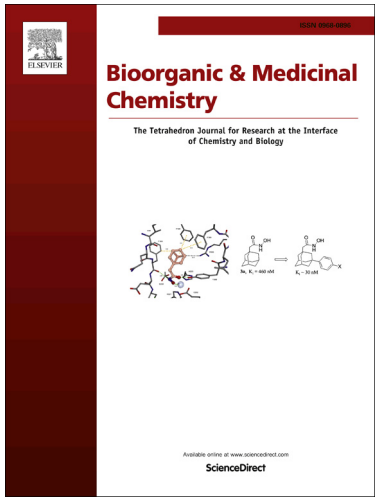

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Combination of 4-anilinoquinazoline and rhodanine as novel Epidermal Growth Factor Receptor tyrosine kinase inhibitors

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Abstract：A type of novel rhodanine-based 4-anilinoquinazoline，which designed the combination between quinazoline as the backbone and various substituted biological rhodanine groups as the side chain, have been synthesized, and their antiproliferative activities were also evaluated firstly. These compounds displayed good antiproliferative activity and EGFR-TK inhibitory activity. Among them, compound **8d** showed good inhibitory activity (IC_{50} = 2.7 μM for Hep G2, IC_{50} = 3.1 μM for A549) and molecular docking of **8d** into EGFR TK active site was also performed, this inhibitor well fitting the active site might well explain its excellent inhibitory activity.

Keywords: EGFR-TK inhibitors; 4-anilinoquinazoline; rhodanine ; structure-based design; molecular docking

1. Introduction

EGFR belongs to the ErbB family of receptor TKs that plays indispensable roles in cell proliferation, survival, adhesion, migration and differentiation. [1] The role of EGFR has been most thoroughly studied in breast cancer,[2] lung cancer (especially lung adenocarcinomas)[3–5] and in hormone-refractory prostate cancer.[6] Compounds that inhibit the kinase activity of EGFR after binding of its cognate ligand are of potential interest as new therapeutic antitumor agents. [7-8]

Among the EGFR TKIs, the 4-anilinoquinazolines play a key role and has been extensively studied in the past few years; the most representative compounds are gefitinib, erlotinib and lapatinib. [9-11] These TKIs generally designed to selectively inhibit EGF-stimulated signal transduction showed high potency in vitro, inhibiting EGFR at nanomolar concentration. It was concluded that the quinazoline core was the best scaffold for the development of EGFR inhibitors because any alteration of the nitrogen substitution pattern in the bicyclic ring resulted in less active compounds. From 2010 to present, many gefitinib, erlotinib and lapatinib analogs were reported in published patents and articles, and the focal points of the chemical space of these new irreversible and reversible inhibitors were centered on the 4-anilino headgroup moiety, 6-substituents and the bioisosteres of quinazoline ring, which have led to the development and the marketing of new series of antitumor agents. [12]

In our studies of the inhibition of EGFR by reversible and irreversible 4-anilinoquinazolines, many new 4,6-substituted-(diaphenylamino)quinazoline derivatives have been synthesized and displayed potent EGFR inhibitory activity,[13-15]modeling studies of the binding of reversible anilinoquinazoline with EGFR-TK has been developed. The results suggest that a binding mode whereby the C-6 position of the bicyclic chromophore point toward the solvent out of the ATP binding pocket. Also, in recent years, the structure-based approach has become very powerful for the discovery of novel lead compounds and for lead optimization, and new rhodanine-based derivatives as potent anticancer angents are reflected in our recent publication, the reported anti-apoptotic rhodanine-based acylsulfonamide

derivatives exhibited remarkable antitumor activity. [16] Based on our previous results, in the paper, by combining two distinct pharmacological properties 4-anilinoquinazoline and rhodanine in one molecule with a linker heterocyclic amino acid Pro, series of novel rhodanine-based 4-anilinoquinazoline derivatives were designed and synthesized as potent EGFR inhibitors, we postulated that these rhodanine-based 4-anilinoquinazoline derivatives may serve such a purpose while being topographically appropriate to maintain high affinity binding to EGFR proteins with potent antitumor activity and represent a novel approach to cancer therapy.

2. Results and Discussion

2.1 Chemsitry

Eighteen rhodanine-based 4-anilinoquinazoline derivatives **8a-8q**, were designed to perform comparative SAR studies. We designed a more efficient synthesis that allowed ring cyclization and incorporation of the 4-anilino group in a single step, as shown in Scheme 1. Commercially available 5-nitroanthranilonitrile **1** was converted into the corresponding formamidine **2** using DMF acetal. Heating a solution of formamidine **2** and 3-Cl-aniline in HOAc gave 6-nitro-4-(3-X-phenylamino)quinazolines **3** and. reduction of the nitro group of **3** with iron in HOAc yielded the intermediate 6-aminoquinazolines **4**. One the other hand, different substituted intermediates **5a–5q** and **6a–6q** were prepared by the Knoevenagel reaction of various substitute aldehydes with rhodanine, and methylated by CH3I. Then, the rhodanine derivatives **7a–7q** was synthesized from Pro with **6a–6q** and diisopropylethyl amine (DIEA). Finally, rhodanine derivatives **7a–7q**, 4-aminoquinazolines **4** and 1-(3-dimethylaminopropyl)-3- thylcarbodiimide hydrochloride (EDC) were dissolved in CH_2Cl_2 at room temperature to give the desired rhodanine-based 4-anilinoquinazoline derivatives **8a-8q**.

2.2. Antiproliferative assay

In our former reports, the N1, N3 of the 4-(phenylamino)quinazoline moiety would interact with active centre probably by hydrogen bond, water bridge or else. [13] So we saved the quinazoline skeleton in the new designed compunds. Recently, we

found that the thioxo-thiazolidinedione (rhodanine) derivatives have potent antiproliferative activities against HepG2. Thus, we postulated that the 4-(phenylamino)quinazoline core structure with active thioxo-thiazolidinedione groups may serve such a purpose while being topographically appropriate to maintain high affinity binding to EGFR protein with potent antiproliferative activity. To confirm our postulation, the synthesized rhodanine-based 4-anilinoquinazoline derivatives were evaluated for their antiproliferative activities against Hep G2 and A549 cells by applying the MTT colorimetric assay. The results were summarized in Table 1. Compounds were tested over a range of concentrations from 0.01 to 100 μg /ml, and the calculated IC_{50} values, that is, the concentration (μg /mL) of a compound that was able to cause 50% cell death with respect to the control culture, were reported differently according to different cancer cells. As expected, these compounds exhibited remarked effects on antiproliferative activities, and generally the results showed which those applied to Hep G2 cells would performed better than that in A549 cells. Especially, (S,E)-1-(5-((6-bromopyridin-2-yl)methylene)-4-oxo-4,5-dihydrothiazol-2-yl)-N-(4-((3-chlorophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8d**), with a bromopyridine group on the rhodanine moiety showed good inhibitory activity (IC_{50} = 2.7 μM for Hep G2 and IC_{50} = 3.1 μM for A549), comparable to the positive control gefitinib (IC_{50}=7.52 μM for Hep G2 and IC_{50}=5.51 μM for A549).

Structure-activity relationships in these new compounds demonstrated that compounds containing pyridine group on the rhodanine moiety (**8c**-**8f**) showed better activity than others, which is accord with the reported literature that pyridylrhodanine derivatives have potent anti-apoptotic activity. [17] Compound with substitution at the *meta* (**8i**) or *ortho* (**8h-8k, 8n, 8o**) on the phenyl ring at R position showed less potent activities against A549 cell line than those with substitution at the *para* position (**8m**, **8p**), and compound **8h**-**8p** displayed similar moderate activity against Hep G2 cell line, indicating relatively substituents on the phenyl ring at R position didn't input substantial effects on the antiproliferative capability against Hep G2 cell line of the compounds.

2.3. EGFR inhibitory activity and molecular docking

To evaluate the EGFR inhibitory potency of new compounds, their ability to block EGFR TK was tested in an EGFR TK assay. We chose theeight compounds behaved well for antiproliferation and their activities positively correlated with anti-proliferative activity, which they had the same trends (**Table 2**). This could indicate that rhodanine-based 4-anilinoquinazoline derivatives basically follow the EGFR inhibition path against cancer cell. As shown in Table 2, compound **8c** and **8d** displayed the most potent inhibitory activity (IC_{50} = 6.5 μM and 3.1 μM for EGFR).

To help understand the SARs observed at the EGFR and guide further EGFR inhibitors design, molecular docking of the most potent inhibitor **8d** into ATP binding site of EGFR kinase was performed on the binding model based on the EGFR complex structure (1M17.pdb). The binding model of compound **8d** and EGFR was depicted in Figure 1. In the binding model, compound **8d** is nicely bound to the region of EGFR, the quinazoline ring be inserted well inside the pocket. Outside the cleft, the N atom of 4-anilinoquinazoline core structure of **8d** forms hydrogen bond with the MET769 (OD···N: 2.4 Å), which made the combination ability of **8d** acting on EGFR. Meanwhile, the carbonyl group of rhodanine moiety forms hydrogen bond with the PRO770, the amide group which linked the 4-anilinoquinazoline core structure with rhodanine moiety forms hydrogen bond with the CYS773 of EGFR protein and play an important role in stabilizing the three dimensional structure of a protein, these results showed that the designed rhodanine-based 4-anilinoquinazoline derivatives were potential inhibitors of EGFR.

3. Conclusion

In summary, by combining two distinct pharmacological properties 4-anilinoquinazoline and rhodanine in one molecule with a linker Pro, a series of novel rhodanine-based 4-anilinoquinazoline derivatives were designed and synthesized potent EGFR Inhibitors. Four compounds containing pyridine group on the rhodanine moiety (**8c**-**8f**) showed better activity than others, which is accord with the reported literature that pyridyl-rhodanine derivatives have potent anti-apoptotic

activity The EGFR molecular docking experiment of compound **8d** indicated that **8d** was is well bound to the region of EGFR, the quinazoline ring be inserted nicely inside the pocket, the carbonyl group of rhodanine moiety forms hydrogen bond with the PRO770. In conclusion, these findings expand the chemical diverse of EGFR inhibitors, and similar strategies might be applied to the design of compounds able to form a covalent bond with a biological target.

4. Experimental

4.1 Chemistry General. All chemicals (reagent grade) used were purchased from Sigma–Aldrich (USA) and Sinopharm Chemical Reagent Co. Ltd.(China). NMR spectra were measured on a Varian Unity Inova 300MHz and 400MHz spectrometer at 25°C and referenced to Me4Si. Chemical shifts are reported in ppm (δ) using the residual solvent line as internal standard. Splitting patterns are designed as s, singlet; d, doublet; t, triplet; m, multiplet. HRMS spectra were acquiredon Bruker Esquire Liquid Chromatography-Ion Trap Mass Spectrometer. Melting points were determined on a XT4 MP apparatus (Taike Corp. , Beijing, China) and are as read. Analytical thin-layer chromatography (TLC) was performed on the glass-backed silica gel sheets (silica gel 60Å GF254). All compounds were detected using UV light (254 nm or 365 nm).

4.2. General procedure for the preparation of compounds 5a–5q

Rhodanine 1 (1.3 g, 10 mmol), substitute aldehyde (10 mmol) and sodium acetate (2.9 g, 35 mmol) were suspended in acetic acid (100 mL) and the mixture was refluxed for 24 h with N2. The resulting mixture was cooled to room temperature, and then 360 mL water was added. The precipitate that formed was filtered, washed with ethyl ether, and dried to give 5a–5q (Yield 43%-57%). **5k**: 1H NMR (300 MHz, DMSO-d6) d 7.38–7.58 (m, 3H), 7.64–7.80 (m, 5H), 7.82–7.92 (m, 2H).

4.3. General procedure for the preparation of compounds 6a–6q

Compound 5a-5q (1 mmol), DIEA (0.213 g, 1.5 mmol) were suspended in anhydrous ethanol (50 mL), then CH3I (0.215 g, 1.5 mmol) was slowly added within ten minutes, the mixture was stirred at room temperature for 6 h. Then 150 mL water was added,

the precipitate was filtered, and dried to give 6a-6q (Yield: 63%-79%). **6k**: 1H NMR (300 MHz, DMSO-d6) δ 2.85 (s, 3H), 7.38–7.56 (m, 3H), 7.72–7.81 (m, 4H), 7.84–7.92 (m, 3H).

4.4. General procedure for the preparation of compounds 7a–7q

Compound 6a-6q (0.5 mmol), DIEA (0.071 g, 0.5 mmol) and L- proline (0.058 g, 0.5 mmol) were suspended in ethanol (5 mL) and heated at reflux at 70–80 oC for 20 h. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The resulting solid was extracted with ethyl acetate for column chromatography. Column chromatography was performed using silica gel (200–300 mesh), eluted with ethyl acetate and petroleum ether (1:1, v/v) to give 7a–7q (Yield: 57%-68%). **7k**:1H NMR (400 MHz, DMSO-d6) 7.84–7.92 (m, 3H), 7.72–7.81 (m, 4H), 7.38–7.56 (m, 3H), 5.44 (d, J = 4.6 Hz, 1H), 4.04 (s, 1H), 3.86 (d, J = 10.1 Hz, 1H), 2.78 – 2.58 (m, 2H), 2.48 (s, 1H), 2.31 (s, 1H).

4.5. General procedure for the preparation of target compounds **8a**–**8q**

4-anilinoquinazoline 4 was synthesized as our previous report 12. Compound 7a-7q (0.1 mmol), EDCI (0.040 g, 0.2 mmol), compound 4 (0.014 g, 0.053 mmol) were suspended in pyridine (15 mL) and stirred at room temperature for 12 h. The mixture was diluted by EtOAc and washed with H2O and brine, dried over Na2SO4, and concentrated in vacuo. The residue was subjected to silica gel chromatography or crystallization if necessary to afford the compounds 8a–8q.

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(4-oxo-5-(thiophen-2-ylm ethylene)-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide (**8a**) yellow solid，yield：35%，Mp 214-215 oC. 1H NMR (400 MHz, CDCl3) δ 10.75 (s, 1H), 8.58 (s, 1H), 8.52 (s, 1H), 8.01 (d, *J* = 7.6 Hz, 1H), 7.94 (d, *J* = 4.6 Hz, 2H), 7.69 (d, *J* = 8.7 Hz, 1H), 7.60 (d, *J* = 4.9 Hz, 1H), 7.35 (s, 1H), 7.30 (d, *J* = 8.3 Hz, 1H), 7.22 (d, *J* = 9.0 Hz, 2H), 7.15 (s, 1H), 7.06 (d, *J* = 7.6 Hz, 1H), 5.44 (s, 1H), 4.07 (s, 1H), 3.90 (s, 1H), 2.64 (d, *J* = 23.3 Hz, 2H), 2.50 (s, 1H), 2.30 (s, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.4, 171.34, 168.95, 157.36, 153.16, 146.80, 141.08, 138.50, 136.58, 133.45, 132.67, 131.46, 129.97, 128.98, 128.63, 127.05, 126.78, 123.77, 122.93, 121.47,

120.44, 115.53, 111.74, 64.19, 49.81, 30.57, 23.69. HRMS: [M+Na$^+$]: Found m/z 583.0751, Calcd m/z 583.0856.

(S,E)-1-(5-((5-bromothiophen-2-yl)methylene)-4-oxo-4,5-dihydrothiazol-2-yl)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8b**) yellow solid，yield：52%，Mp 194-195 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.68 (s, 1H), 8.54 (d, *J* = 23.0 Hz, 2H), 8.03 – 7.88 (m, 2H), 7.79 (s, 1H), 7.67 (d, *J* = 9.0 Hz, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.22 (d, *J* = 8.7 Hz, 2H), 7.09 (dt, *J* = 12.5, 5.8 Hz, 3H), 5.39 (dd, *J* = 8.1, 4.1 Hz, 1H), 4.09 (dd, *J* = 19.6, 12.2 Hz, 1H), 3.87 (dd, *J* = 17.4, 7.2 Hz, 1H), 2.72 – 2.43 (m, 3H), 2.33 – 2.23 (m, 1H). ^{13}C NMR (101 MHz, DMSO) δ 178.25, 171.01, 168.87, 157.35, 153.15, 146.79, 140.73, 136.56, 133.80, 132.69, 132.30, 129.94 , 128.61, 127.64, 127.04, 124.47, 121.43, 120.50, 116.99, 115.53, 111.76, 64.27, 62.86, 49.90, 40.15, 30.60, 23.69. HRMS: [M+Na$^+$]: Found m/z 662.9825, Calcd m/z 662.9737.

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(5-((6-methylpyridin-2-yl)methylene)-4-oxo-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide (**8c**) yellow solid，yield：23%，Mp 197-198 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.84 (s, 1H), 8.64 (s, 1H), 8.52 (s, 1H), 8.02 (d, *J* = 7.5 Hz, 1H), 7.97 (s, 1H), 7.73 (d, *J* = 9.5 Hz, 1H), 7.67 (s, 1H), 7.61 (t, *J* = 7.7 Hz, 1H), 7.30 (d, *J* = 8.1 Hz, 1H), 7.23 (dd, *J* = 13.7, 8.2 Hz, 4H), 7.11 (d, *J* = 7.7 Hz, 1H), 7.05 (d, *J* = 7.6 Hz, 1H), 5.51 (s, 1H), 4.14 (s, 1H), 3.96 (d, *J* = 9.8 Hz, 1H), 2.66 (s, 3H), 2.51 (s, 3H), 2.29 (s, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.33, 172.01, 168.84, 157.35, 153.16, 150.93, 150.17, 146.81, 141.07, 136.56, 135.63, 132.65, 130.87, 129.97, 129.84, 128.65, 127.24, 124.17, 122.92, 121.45, 120.43, 115.53, 111.72, 64.27, 54.95, 49.85, 30.53 , 23.68. HRMS: [M+H$^+$]: Found m/z 570.1448, Calcd m/z 570.1401.

(S,E)-1-(5-((6-bromopyridin-2-yl)methylene)-4-oxo-4,5-dihydrothiazol-2-yl)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8d**) yellow solid，yield：47%，Mp 260-261 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.75 (s, 1H), 8.59 (s, 1H), 8.51 (s, 1H), 7.96 (d, *J* = 11.6 Hz, 2H), 7.71 (d, *J* = 9.1 Hz, 1H), 7.61 – 7.56

(m, 2H), 7.43 (d, *J* = 7.9 Hz, 1H), 7.37 (d, *J* = 7.5 Hz, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.22 (d, *J* = 8.5 Hz, 2H), 7.06 (d, *J* = 8.0 Hz, 1H), 5.48 (dd, *J* = 8.3, 4.1 Hz, 1H), 4.14 (d, *J* = 7.0 Hz, 1H), 3.98 (dd, *J* = 17.8, 7.6 Hz, 1H), 2.73 – 2.44 (m, 3H), 2.35 – 2.23 (m, 1H). ^{13}C NMR (101 MHz, DMSO) δ 183.93, 181.04, 174.33, 162.69, 158.48, 158.18, 152.13, 146.41, 145.90, 141.93, 139.28, 138.00, 135.29, 133.96, 133.13, 132.35, 131.56, 131.22, 128.25, 126.81, 125.78, 120.87, 117.31, 117.15, 69.56, 54.89, 35.74, 29.12. HRMS: [M+H$^+$]: Found m/z 636.0400, Calcd m/z 635.9340.

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(5-((2-chloropyridin-3-yl)methylene)-4-oxo-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide (**8e**) yellow solid，yield：46%，Mp 188-189 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.60 (s, 1H), 8.62 (s, 1H), 8.53 (s, 1H), 8.41 (d, *J* = 4.8 Hz, 1H), 8.08 – 8.01 (m, 2H), 7.93 (d, *J* = 8.9 Hz, 2H), 7.68 (d, *J* = 9.1 Hz, 1H), 7.40 – 7.34 (m, 1H), 7.30 (t, *J* = 7.8 Hz, 1H), 7.25 – 7.18 (m, 2H), 7.07 (d, *J* = 8.0 Hz, 1H), 5.43 (s, 1H), 4.05 (s, 1H), 3.88 (s, 1H), 2.82 – 2.38 (m, 3H), 2.31 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 177.76, 171.97, 168.74, 157.34, 153.18, 150.50, 150.07, 146.82, 141.07, 137.42 , 136.53, 134.34 , 132.67 , 129.97 , 128.83, 127.03, 124.27, 123.88 , 122.92, 121.43, 120.39, 115.53, 111.76, 64.40 , 62.84, 39.62, 30.52, 23.68. HRMS: [M+H$^+$]: Found m/z 590.0904, Calcd m/z .590.0854

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(4-oxo-5-(pyridin-3-ylmethylene)-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide (**8f**) yellow solid，yield：38%，Mp 230-231 ^{o}C. ^{1}H NMR (400 MHz, DMSO) δ 10.64 (s, 1H), 8.79 (s, 1H), 8.58 (dd, *J* = 29.4, 10.6 Hz, 3H), 8.01 (d, *J* = 7.6 Hz, 1H), 7.92 (s, 1H), 7.83 (d, *J* = 8.2 Hz, 1H), 7.76 (s, 1H), 7.68 (d, *J* = 8.9 Hz, 1H), 7.41 (dd, *J* = 8.0, 5.0 Hz, 1H), 7.30 (d, *J* = 8.2 Hz, 1H), 7.23 (d, *J* = 8.9 Hz, 2H), 7.06 (d, *J* = 8.1 Hz, 1H), 5.44 (dd, *J* = 8.5, 3.9 Hz, 1H), 4.09 (t, *J* = 14.1 Hz, 1H), 3.89 (dd, *J* = 17.6, 7.5 Hz, 1H), 2.69 (d, *J* = 7.5 Hz, 1H), 2.64 (d, *J* = 11.6 Hz, 1H), 2.51 (dd, *J* = 17.7, 10.1 Hz, 1H), 2.38 – 2.23 (m, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.93, 176.71, 169.16, 157.74, 157.36, 153.13, 150.64, 146.78, 141.10, 137.61, 136.66, 132.66, 132.25, 129.96, 128.61, 127.44, 127.02, 124.29, 123.22, 122.91, 121.47, 120.44, 115.54, 111.66, 64.00, 49.39, 30.40, 23.89. HRMS: [M+Na$^+$]: Found m/z 578.1135, Calcd m/z 578.1244.

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(4-oxo-5-(quinolin-2-yl methylene)-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide(**8g**)

yellow solid，yield：23%，Mp 217-218 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.84 (s, 1H), 8.63 (s, 1H), 8.50 (s, 1H), 8.23 (d, *J* = 8.4 Hz, 1H), 8.17 (d, *J* = 8.4 Hz, 1H), 7.98 (d, *J* = 6.2 Hz, 2H), 7.85 – 7.72 (m, 4H), 7.58 (t, *J* = 7.5 Hz, 1H), 7.51 (d, *J* = 8.4 Hz, 1H), 7.28 (s, 1H), 7.26 – 7.20 (m, 2H), 7.03 (d, *J* = 8.1 Hz, 1H), 5.54 (dd, *J* = 8.3, 4.0 Hz, 1H), 4.21 (dd, *J* = 14.1, 9.3 Hz, 1H), 4.07 – 3.99 (m, 1H), 2.76 – 2.49 (m, 3H), 2.38 – 2.27 (m, 1H). ^{13}C NMR (101 MHz, DMSO) δ 182.35, 174.69, 162.94, 158.73, 157.60, 152.54, 152.38, 146.66, 142.82, 142.22, 140.09, 138.24, 136.04, 135.53, 134.22, 134.04, 133.61, 132.52, 132.52, 129.80, 128.50, 127.19,127.05, 126.17, 126.03, 121.12, 117.41, 117.26, 69.69, 55.12, 36.01, 29.39. HRMS: [M+H$^+$]: Found m/z 606.1348, Calcd m/z 606.1401.

(S,E)-1-(5-(2-chlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)-*N*-(4-((3-chlor ophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8h**) yellow solid，yield：54%，Mp 181-182 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.70 (s, 1H), 8.58 (d, *J* = 42.3 Hz, 2H), 8.13 (s, 1H), 8.05 (d, *J* = 8.2 Hz, 1H), 7.95 (s, 1H), 7.66 (dd, *J* = 22.7, 8.3 Hz, 2H), 7.45 (d, *J* = 7.6 Hz, 1H), 7.34 (ddd, *J* = 22.3, 15.7, 7.8 Hz, 3H), 7.21 (d, *J* = 8.6 Hz, 2H), 7.06 (d, *J* = 7.9 Hz, 1H), 5.45 (dd, *J* = 8.5, 4.0 Hz, 1H), 4.06 (t, *J* = 12.2 Hz, 1H), 3.87 (dd, *J* = 17.4, 7.5 Hz, 1H), 2.81 – 2.43 (m, 3H), 2.30 (dd, *J* = 12.4, 5.8 Hz, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.13, 172.31, 168.84, 157.36, 153.18, 146.82, 141.09, 136.57, 134.29, 132.70, 132.34, 131.97, 131.67, 130.27, 130.12, 128.64, 128.06, 127.06, 125.81, 122.93, 121.46 , 120.42, 115.55, 111.78, 64.30, 49.88, 30.54, 23.69. HRMS: [M+H$^+$]: Found m/z 589.0955, Calcd m/z 589.0902.

(S,E)-1-(5-(3-chlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)-*N*-(4-((3-chlor ophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8i**) yellow solid，yield：23%，Mp 182-183 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.68 (s, 1H), 8.60 (s, 1H), 8.52 (s, 1H), 8.01 (d, *J* = 7.9 Hz, 1H), 7.94 (s, 1H), 7.68 (d, *J* = 11.9 Hz, 1H), 7.50 (s, 1H), 7.39 (s, 2H), 7.30 (d, *J* = 7.9 Hz, 1H), 7.21 (d, *J* = 10.2 Hz, 2H), 7.06 (d, *J* = 7.5 Hz, 1H), 5.37 (d, *J* = 57.4 Hz, 1H), 4.17 – 3.67 (m, 2H), 2.78 – 2.39 (m, 2H), 2.31 (s, 1H), 1.93 (s, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.42, 172.06, 168.86, 157.36, 153.17,

146.79, 141.07, 136.58, 136.29, 132.67, 132.68, 132.20, 131.32, 130.30, 129.99, 129.04, 128.66, 127.98, 127.03, 122.95, 122.52, 121.47, 120.45, 115.54, 111.70, 64.28, 49.89, 30.55, 23.70. HRMS: [M+Na^+]: Found m/z 610.9763, Calcd m/z 611.0902.

(S,E)-1-(5-(2-bromobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8j**) yellow solid，yield：37%，Mp 200-201 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.68 (s, 1H), 8.61 (s, 1H), 8.53 (s, 1H), 8.10 – 8.03 (m, 2H), 7.96 (s, 1H), 7.72 – 7.58 (m, 3H), 7.42 (t, *J* = 7.3 Hz, 1H), 7.30 (t, *J* = 8.2 Hz, 1H), 7.23 (d, *J* = 8.1 Hz, 3H), 7.07 (d, *J* = 8.1 Hz, 1H), 5.44 (d, *J* = 4.6 Hz, 1H), 4.04 (s, 1H), 3.86 (d, *J* = 10.1 Hz, 1H), 2.78 – 2.58 (m, 2H), 2.48 (s, 1H), 2.31 (s, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.20, 172.52, 168.83, 157.53, 153.36, 146.99, 141.27, 136.76, 133.95, 133.70, 132.85, 132.68, 131.75, 130.08, 128.97, 128.81, 127.25, 125.31, 123.12, 121.64, 120.61, 115.72, 111.93, 64.47, 50.17, 30.72, 23.88. HRMS: [M+H^+]: Found m/z 634.9476, Calcd m/z 634.9460.

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(5-(2-fluorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide (**8k**) yellow solid，yield：32%，Mp 182-183 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.73 (s, 1H), 8.62 (s, 1H), 8.53 (s, 1H), 8.01 (dd, *J* = 23.3, 17.2 Hz, 3H), 7.73 – 7.55 (m, 2H), 7.41 (dd, *J* = 12.7, 6.6 Hz, 1H), 7.32 – 7.27 (m, 1H), 7.25 – 7.19 (m, 2H), 7.16 – 7.10 (m, 1H), 7.06 (d, *J* = 7.3 Hz, 1H), 5.46 (dd, *J* = 8.4, 4.1 Hz, 1H), 4.08 (dd, *J* = 11.8, 6.5 Hz, 1H), 3.90 (t, *J* = 8.8 Hz, 1H), 2.78 – 2.44 (m, 3H), 2.36 – 2.25 (m, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.70, 172.59, 169.26, 159.25, 157.76, 153.58, 147.23, 141.49, 136.97, 133.08, 131.71, 130.37, 129.06, 128.88, 127.45, 125.72, 123.33 ,122.21, 122.05, 121.86, 120.83, 116.75, 116.46, 115.95, 112.16, 64.69, 60.20, 30.94, 21.20. HRMS: [M+H^+]: Found m/z 574.0360, Calcd m/z 574.0404.

(S,E)-1-(5-(3-bromobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8l**)

yellow solid，yield：43%，Mp 184-185 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.68 (s, 1H), 8.56 (d, *J* = 22.6 Hz, 2H), 8.07 – 7.90 (m, 2H), 7.68 (d, *J* = 13.6 Hz, 3H), 7.53 (d, *J* = 7.5 Hz, 1H), 7.45 (d, *J* = 7.4 Hz, 1H), 7.36 – 7.28 (m, 2H), 7.22 (d, *J* = 9.6 Hz,

2H), 7.07 (d, *J* = 7.8 Hz, 1H), 5.44 (s, 1H), 4.10 (s, 1H), 3.92 (s, 1H), 2.76 – 2.45 (m, 3H), 2.31 (s, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.00, 172.32, 168.83, 157.35, 153.17, 146.82, 141.08, 136.57, 133.80, 133.51, 132.66, 132.49, 131.55, 129.98, 128.80, 128.60, 127.06, 125.12, 122.93, 121.45, 120.42, 115.53, 111.74, 64.28, 49.90, 30.53, 23.69. HRMS: [M+H$^+$]: Found m/z 635.0439, Calcd m/z 634.9460.

(S,E)-1-(5-(4-chlorobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8m**) yellow solid，yield：49%，Mp 172-173 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.70 (s, 1H), 8.56 (d, *J* = 28.7 Hz, 2H), 8.03 (d, *J* = 8.2 Hz, 1H), 7.93 (s, 1H), 7.74 – 7.63 (m, 2H), 7.44 (q, *J* = 8.7 Hz, 4H), 7.29 (t, *J* = 8.1 Hz, 1H), 7.20 (d, *J* = 9.4 Hz, 2H), 7.06 (d, *J* = 7.8 Hz, 1H), 5.45 (dd, *J* = 8.5, 3.9 Hz, 1H), 4.13 – 4.02 (m, 1H), 3.89 (dd, *J* = 17.6, 7.3 Hz, 1H), 2.77 – 2.44 (m, 3H), 2.37 – 2.23 (m, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.62, 172.10, 168.90, 157.36, 153.17, 146.82, 141.09, 136.59, 134.49, 132.67, 131.21, 131.05, 129.97, 129.34, 128.66, 127.04, 122.93, 121.45, 120.42, 115.55, 111.72, 64.25, 49.80, 30.56, 23.70. HRMS: [M+H$^+$]: Found m/z 589.0959, Calcd m/z 589.0902.

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(5-(2-methoxybenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide (**8n**) yellow solid，yield：43%，Mp 252-253 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.83 (s, 1H), 8.62 (s, 1H), 8.53 (s, 1H), 8.18 (s, 1H), 8.02 (d, *J* = 7.4 Hz, 1H), 7.96 (s, 1H), 7.70 (s, 1H), 7.53 (d, *J* = 8.1 Hz, 1H), 7.38 (d, *J* = 7.2 Hz, 1H), 7.30 (d, *J* = 8.1 Hz, 3H), 7.24 – 7.21 (m, 2H), 7.04 (s, 2H), 6.92 (d, *J* = 8.3 Hz, 1H), 5.43 (s, 1H), 4.03 (s, 1H), 3.84 (s, 3H), 2.54 (d, *J* = 61.3 Hz, 4H), 2.26 (s, 1H). ^{13}C NMR (75 MHz, DMSO) δ 178.44, 172.07, 168.85, 157.35, 153.15, 146.79, 141.08, 136.58, 136.01, 133.95, 132.67, 131.04, 130.33, 129.95, 129.55, 129.28, 127.62, 127.02, 122.91, 121.46, 120.42, 115.54, 111.72, 64.27, 54.95, 49.85, 30.54, 23.68. HRMS: [M+H$^+$]: Found m/z 585.1452, Calcd m/z 585.1397.

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(5-(2-nitrobenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide (**8o**)

pink solid，yield：53%，Mp 187-188 °C. ^{1}H NMR (400 MHz, Acetone) δ 10.63 (s, 1H), 8.65 (s, 1H), 8.53 (s, 1H), 8.16 – 8.02 (m, 3H), 7.94 (s, 1H), 7.76 – 7.63 (m, 3H), 7.61 – 7.54 (m, 1H), 7.31 (t, *J* = 8.1 Hz, 1H), 7.22 (d, *J* = 8.7 Hz, 2H), 7.08 (d, *J* = 7.9 Hz, 1H), 5.41 (dd, *J* = 8.6, 4.1 Hz, 1H), 3.99 (d, *J* = 7.7 Hz, 1H), 3.79 (dt, *J* = 9.1, 6.0 Hz, 1H), 2.78 – 2.42 (m, 3H), 2.30 (dd, *J* = 12.5, 5.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 177.55, 172.34, 168.85, 157.37, 153.20, 147.91, 146.84, 141.10, 136.59, 134.48, 133.99, 132.71, 130.65, 130.13, 130.00, 129.32, 128.68, 127.05, 126.87, 125.30, 122.96, 121.47, 120.44, 115.57, 111.75, 64.30, 49.90, 30.55 , 23.71. HRMS: [M+H^{+}]: Found m/z 600.1198, Calcd m/z 600.1143.

(S,E)-*N*-(4-((3-chlorophenyl)amino)quinazolin-6-yl)-1-(5-(4-methoxybenzylidene)-4-oxo-4,5-dihydrothiazol-2-yl)pyrrolidine-2-carboxamide (**8p**)

yellow solid，yield：33%，Mp 176-177 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.82 (s, 1H), 8.58 (d, *J* = 46.2 Hz, 2H), 8.08 – 7.91 (m, 2H), 7.76 – 7.65 (m, 2H), 7.49 (d, *J* = 8.7 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.20 (d, *J* = 9.3 Hz, 2H), 7.05 (d, *J* = 7.7 Hz, 1H), 6.97 (d, *J* = 8.7 Hz, 2H), 5.45 (d, *J* = 4.4 Hz, 1H), 4.06 (s, 1H), 3.85 (s, 3H), 2.62 (dd, *J* = 51.5, 26.0 Hz, 4H), 2.29 (d, *J* = 5.5 Hz, 1H). ^{13}C NMR (75 MHz, DMSO) δ 179.06, 172.22, 169.05, 160.64, 157.36, 153.15, 146.79, 141.10, 136.64, 132.68, 131.51, 130.72, 129.97, 128.63, 127.04, 126.16, 125.71, 122.92, 121.47, 120.44, 115.55, 114.83, 111.69, 64.09, 55.47, 49.69 30.58, 23.71. HRMS: [M+H^{+}]: Found m/z 585.1450, Calcd m/z 585.1397.

(S,E)-1-(5-([1,1'-biphenyl]-4-ylmethylene)-4-oxo-4,5-dihydrothiazol-2-yl)-N-(4-((3-chlorophenyl)amino)quinazolin-6-yl)pyrrolidine-2-carboxamide (**8q**)

yellow solid，yield：42%，Mp 203-204 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.78 (s, 1H), 8.62 (s, 1H), 8.53 (s, 1H), 8.03 (d, *J* = 8.0 Hz, 1H), 7.95 (s, 1H), 7.83 (s, 1H), 7.74 – 7.66 (m, 3H), 7.62 (d, *J* = 8.6 Hz, 4H), 7.47 (t, *J* = 7.4 Hz, 2H), 7.40 (d, *J* = 7.5 Hz, 1H), 7.31 (d, *J* = 8.2 Hz, 1H), 7.22 (s, 1H), 7.06 (d, *J* = 7.9 Hz, 1H), 5.48 (s, 1H), 4.12 (d, *J* = 6.9 Hz, 1H), 3.92 (s, 1H), 2.79 – 2.46 (m, 3H), 2.33 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 178.80, 172.22, 168.96, 157.36, 153.16, 146.82, 141.38, 141.09, 138.99, 136.60, 132.78, 132.68, 130.26, 129.95, 129.08, 128.65, 128.47, 128.12,

127.39, 127.03, 126.78, 126.16, 122.92, 121.46, 120.42, 115.55, 111.72, 64.20, 49.76, 30.55, 23.70. HRMS: [M+Na^+]: Found m/z 653.1495, Calcd m/z 653.1605.

4.6. General procedure for preparation, purification of EGFR inhibitory assay

A 1.6 kb cDNA encoded for the EGFR cytoplasmic domain (EGFR-CD, amino acids 645-1186) were cloned into baculoviral expression vectors pBlueBacHis2B and pFASTBacHTc (Huakang Company China), separately. A sequence that encodes $(His)_6$ was located at the 5' upstream to the EGFR sequences. Sf-9 cells were infected for 3 days for protein expression. Sf-9 cell pellets were solubilized at 0 °C in a buffer at pH 7.4 containing 50 mM HEPES, 10 mM NaCl, 1% Triton, 10 μM ammonium molybdate, 100 μM sodium vanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 16 μg/mL benzamidine HCl for 20 min followed by 20 min centrifugation. Crude extract supernatant was passed through an equilibrated Ni-NTA superflow packed column and washed with 10 mM and then 100 mM imidazole to remove nonspecifically bound material. Histidinetagged proteins were eluted with 250 and 500 mM imidazole and dialyzed against 50 mM NaCl, 20 mM HEPES, 10% glycerol, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin for 2 h. The entire purification procedure was performed at 4°C or on ice.[18]

The EGFR kinase assay was set up to assess the level of autophosphorylation based on DELFIA/Time-Resolved Fluorometry. Compounds **8a-8d** were dissolved in 100% DMSO and diluted to the appropriate concentrations with 25 mM HEPES at pH 7.4. In each well, 10 μL compound was incubated with 10μL (5 ng for EGFR) recombinant enzyme (1:80 dilution in 100 mM HEPES) for 10 min at room temperature. Then, 10 μL of 5 × buffer (containing 20 mM HEPES, 2 mM $MnCl_2$, 100 μM Na_3VO_4, and 1 mM DTT) and 20 μL of 0.1 mM ATP-50 mM $MgCl_2$ were added for 1 h. Positive and negative controls were included in each plate by incubation of enzyme with or without ATP-$MgCl_2$. At the end of incubation, liquid was aspirated, and plates were washed three times with wash buffer. A 75 μL (400 ng) sample of europiumlabeled anti-phosphotyrosine antibody was added to each well for another 1 h of incubation. After washing, enhancement solution was added and the signal was detected by Victor (Wallac Inc.) with excitation at 340 nm and emission at

615 nm. The percentage of autophosphorylation inhibition by the compounds was calculated using the following equation: 100% - [(negative control)/(positive control – negative control)]. [15] The IC_{50} was obtained from curves of percentage inhibition with eight concentrations of compound. As the contaminants in the enzyme preparation are fairly low, the majority of the signal detected by the anti-phosphotyrosine antibody is from EGFR.

4.7 Molecular Docking Modeling.

The three-dimensional structures of the aforementioned compounds were constructed using Chem. 3D ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2010)], then they were energetically minimized by using MMFF94 with 5000 iterations and minimum RMS gradient of 0.10. The crystal structures of EGFR kinase (PDB code: (1M17.pdb)) complex were retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All bound waters and ligands were eliminated from the protein and the polar hydrogen was added to the proteins. Molecular docking of all twenty compounds was then carried out using the Discovery Studio (version 3.1) as implemented through the graphical user interface CDocker protocol.

5. Acknowledgments

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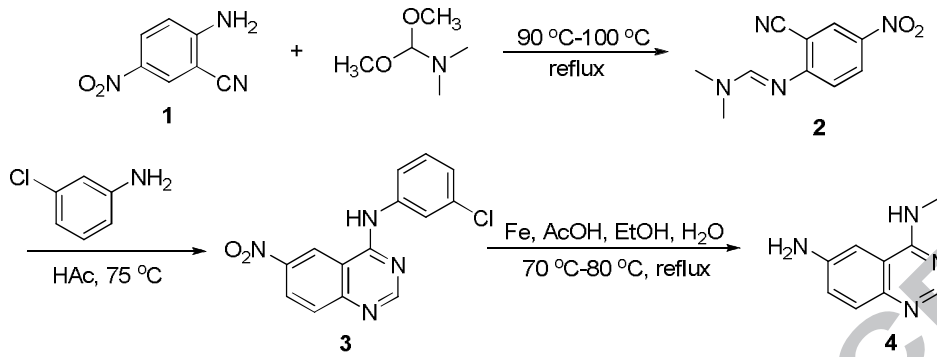
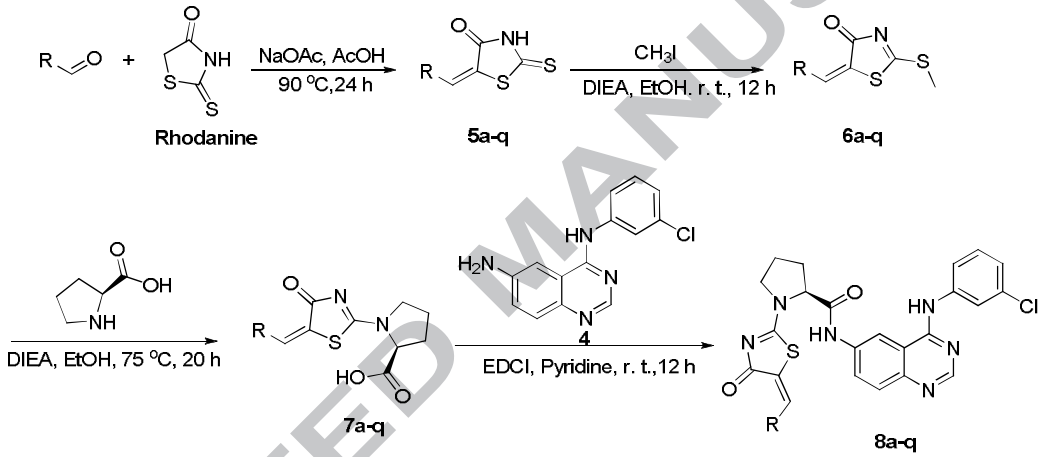


Scheme 1 Synthesis of 4-(phenylamino)quinazoline **4**



Scheme 2 Synthetic routes of target rhodanine-based 4-anilinoquinazoline derivatives **8a-8q**

Table 1. In vitro antiproliferative effects of synthesized compounds[a] **8a-8q**

Compound	IC_{50}(μM)		Compound	IC_{50}(μM)	
	A549 cells[a]	Hep G2 cells		A549 cells[a]	Hep G2 cells
8a	6.1±1.5	4.12±1.2	8j	＞50	24.3±0.2
8b	＞50	＞50	8k	34.5±4.2	18.2±1.6
8c	8.5±0.9	4.5±0.2	8l	＞50	21.3±3.3
8d	3.1±0.5	2.7±0.4	8m	18.7±0.6	10.5±1.4
8e	10.8±0.9	6.4±2.1	8n	＞50	22.1±0.8
8f	10.4±1.3	8.5±1.7	8o	＞50	32.5±2.6
8g	＞50	22.9±4.4	8p	31.7±2.7	19.0±1.5
8h	＞50	28.1±2.4	8q	24.2±1.8	15.4±3.3
8i	＞50	27.6±2.7	gefitinib	0.13±0.02	0.12±0.01

[a] Cell growth was measured using the MTT assay. Values are the average of two independent experiments run in triplicate. Variation was generally 1%.

Table 2 Inhibition (IC_{50}) of EGFR kinase

Compound	EGFR inhibition IC_{50}(μM)
8a	8.1±2.1
8b	31.4±5.2
8c	6.5±1.2
8d	3.1±0.8
8e	7.4±1.6
8f	9.8±2.2
8m	7.6±1.7
8q	13.2±3.8

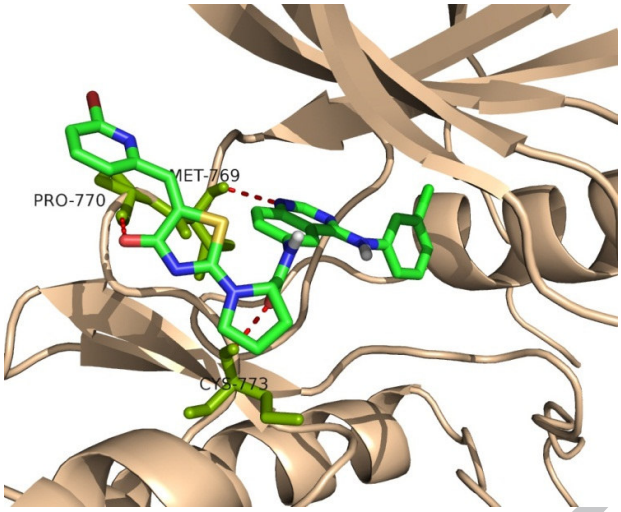


Figure 1. Molecular docking modeling of compounds **8d** with EGFR kinase(1M17.pdb): N atom of 4-anilinoquinazoline moiety of **8d** forms hydrogen bond with the MET769, the carbonyl group of rhodanine moiety forms hydrogen bond with the PRO770

Combination of 4-anilinoquinazoline and rhodanine as novel Epidermal Growth Factor Receptor tyrosine kinase inhibitors

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