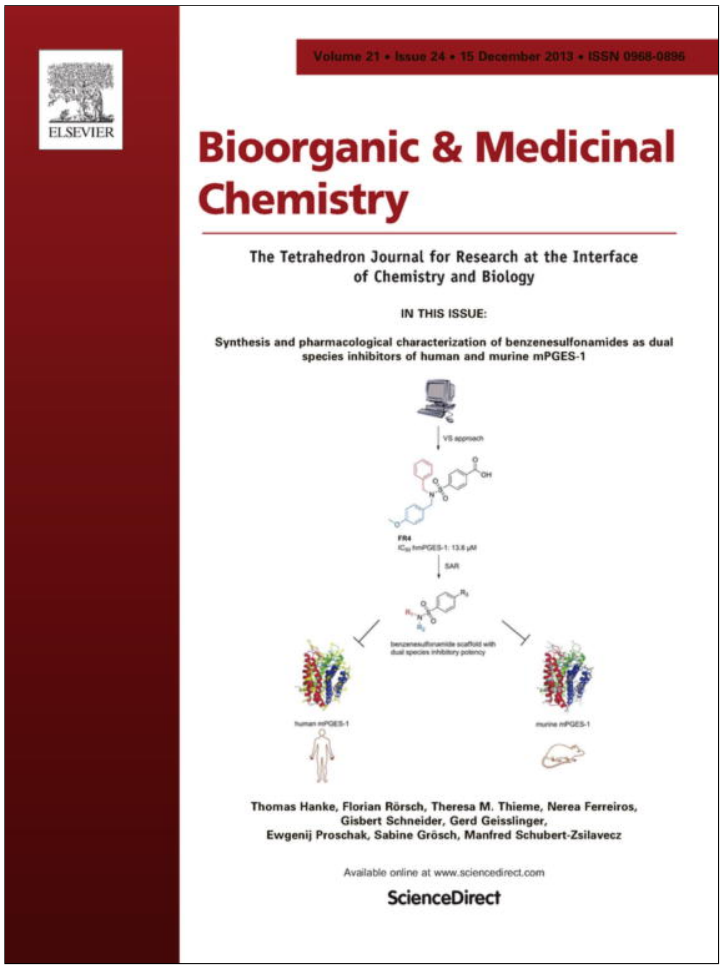

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Design, synthesis and biological evaluation of novel 4-anilinoquinazolines with C-6 urea-linked side chains as inhibitors of the epidermal growth factor receptor

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ABSTRACT

A novel series of anilinoquinazoline compounds with C-6 urea-linked side chains was designed and synthesized as reversible inhibitors of epidermal growth factor receptor (EGFR) based on the structure–activity relationships (SARs) of anilinoquinazoline inhibitors. All compounds demonstrated good inhibition of EGFR wild type (EGFR wt) (IC_{50} = 0.024–1.715 μM) and inhibited proliferation of A431cell line (IC_{50} = 0.116–22.008 μM). The binding mode of compounds **8a**, **8d**, **8k** and **8o** was consistent with the biological results. Moreover, compounds **8k** and **8l** almost completely blocked the phosphorylation of EGFR in A431 cell line at 0.01 μM. Interestingly, all of the compounds also demonstrated moderate inhibition of EGFR/T790M/L858R (IC_{50} = 0.049–5.578 μM). In addition, compounds **8f** and **8h** blocked the autophosphorylation of EGFR in NCI-H1975 cells at high concentration (10 μM), and compound **8f** was confirmed to be an irreversible inhibitor through the dilution method. Importantly, the compounds with C-6 urea-linked side chains which did not contain Michael acceptors demonstrated moderate to strong irreversible EGFR inhibition.

1. Introduction

The epidermal growth factor receptor (EGFR, erbB1, Her1) is a member of the erbB receptor protein tyrosine kinase (PTKs) family,[1,2] which includes erbB2/Her2/neu, erbB3/Her3, and erbB4/Her4 and plays a critical role in many of the signal transduction pathways that regulate numerous cellular functions, such as proliferation, differentiation, migration, angiogenesis and apoptosis.[3–5] The activation of EGFR through homo- or hetero-dimerization followed by autophosphorylation of each tyrosine residue within the intracellular kinase domain can activate downstream signaling, such as Ras/Raf/MAPK, and the PI3K/AKt/mTOR pathways.[1,6] EGFR overexpression has been observed in many types of tumor, including non-small cell lung cancer, breast, head and neck, colorectal, ovarian, and bladder cancers.[7]

EGFR has been clinically validated as a rational target for cancer therapy, and several small molecule inhibitors have been developed and released (Fig. 1).[8] The first generation of EGFR inhibitors include EGFR-specific inhibitors, **1** (Gefitinib),[9] **2** (Erlotinib),[10] and **4** (Icotinib),[11] for the treatment of non-small cell lung cancer, and EGFR/Her2 dual inhibitors 3 (Lapatinib)[12] for the treatment of Her2 positive breast cancer. Although **1** and **2** are effective in the treatment of non-small cell lung cancer in patients, resistance to EGFR-specific inhibitors has been clinically observed and has been associated with the T790M mutation of the EGFR-specific tyrosine kinase.[13] The second generation of EGFR inhibitors containing a Michael acceptor group at the 6-position, such as compounds **5** (Canertinib),[14] **6** (Afatinib/BIBW2992),[15] and **7** (Neratinib/HKI-272),[16] are irreversible inhibitors and have been clinically observed to overcome drug's resistance. Compared to conventional reversible ATP-competitive inhibitors, the advantages of the irreversible inhibitors are high selectivity for erbB family TKs, which have the conserved cysteine residue and the formation of covalent bonds with cysteine residues.[17] Despite the potential advantages of irreversible inhibitors, a drawback is that these covalently binding kinase inhibitors might indiscriminately react with related non-target proteins, giving rise to increased and unexpected toxicity.[18] Therefore, it has become a high priority to develop inhibitors with reduced toxicity while being able to overcome the problem of drug resistance.

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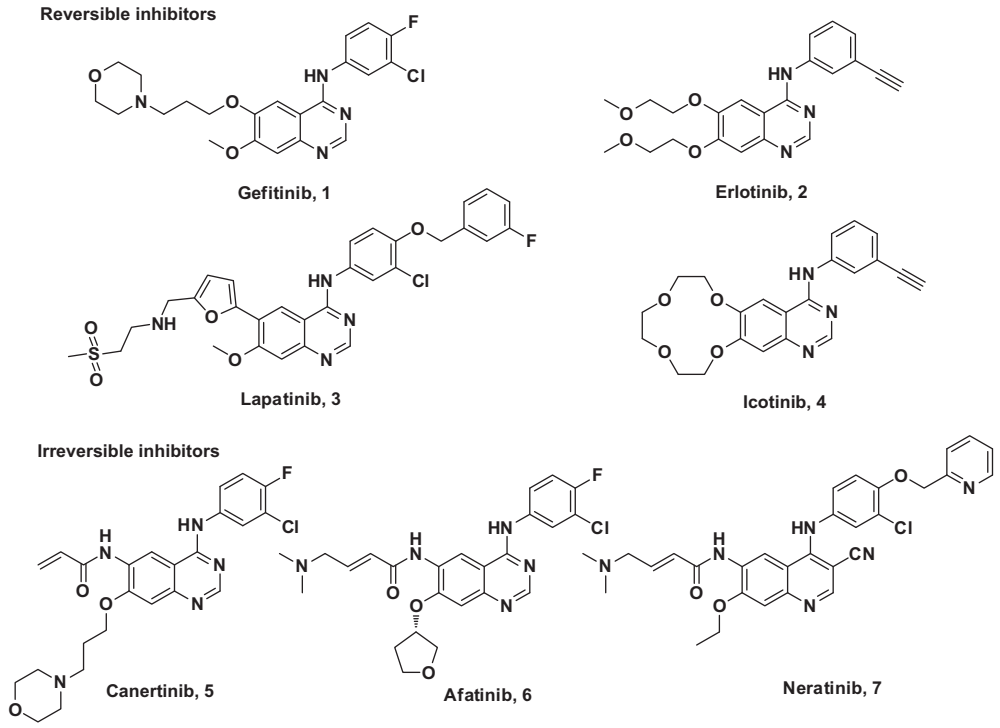


Figure 1. Chemical structures of reversible and irreversible EGFR inhibitors.

The anilinoquinazoline core has been extensively used in the design of TK inhibitors that mimic the adenine moiety of ATP. Retaining the anilinoquinazoline core, we present a new sub-family of 4-anilinoquinazolines with C-6 urea-linked side chains as potent irreversible EGFR inhibitors.

2. Design

Upon further investigation of the SARs (structure–activity relationships) of previously reported EGFR inhibitors, the 4-anilinoquinazoline moiety has been widely studied as an important fragment that binds competitively to the ATP binding pocket of intracellular kinase domains and blocks the induction of downstream signaling networks mediated by tyrosine kinase. The (*S*)-tetrahydrofuran-3-ol moiety of Afatinib can improve the solubility of the compound, and thus has also been included in the design. The urea moiety contains two hydrogen bond donors and one hydrogen bond acceptor, which may form specific interactions with certain enzyme residues, and many small molecular inhibitors containing the urea moiety have been in clinical trials,[19] and was therefore included in small molecule design (Fig. 2).

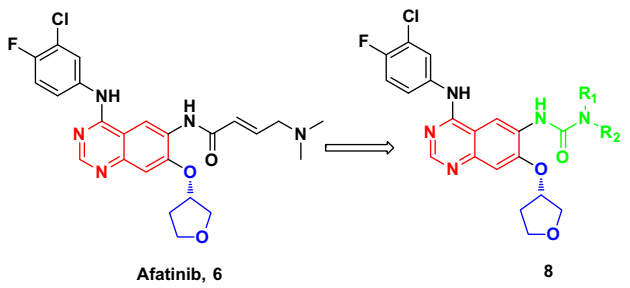


Figure 2. Design strategy of novel EGFR inhibitors.

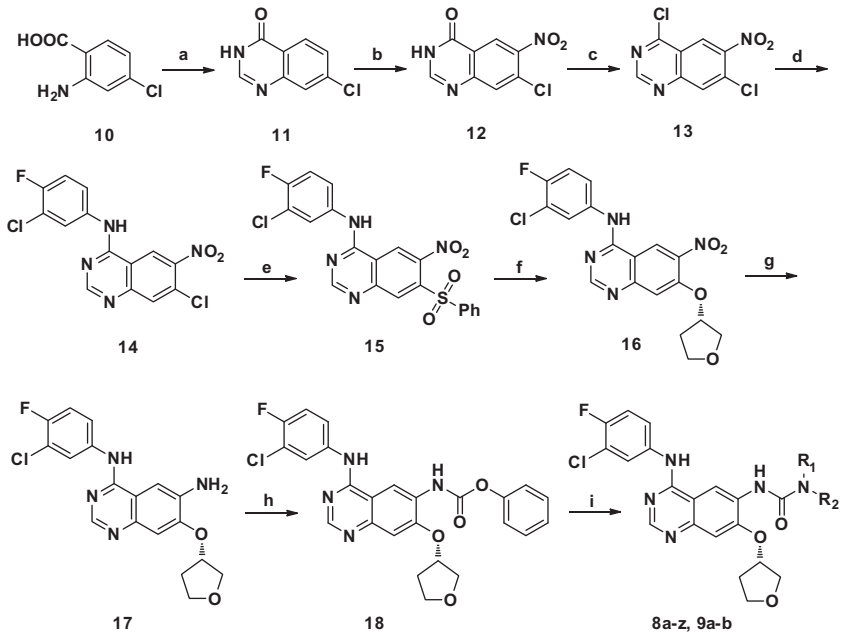
3. Chemistry

Compounds **8a**–**z** and **9a**–**b** were synthesized according to Scheme 1. The key intermediate **17** was generated from commercially available compound **10** via cyclization, nitrification, chlorination, amination, sulfonylation, nucleophilic substitution and reduction, similar to the synthesis of Afatinib.[20] The phenylcarbamate **18** was obtained from compound **17** with phenyl chloroformate, which underwent a nucleophilic reaction with various amines to yield the desired compounds **8a**–**z** and **9a**–**b**.

4. Results and discussion

4.1. Kinase and cellular inhibition of EGFR by the compounds

All the synthesized compounds (**8a**–**z** and **9a**–**b**) were evaluated in enzyme-based and cell-based assays for their capacity to inhibit EGFR wild type (EGFR wt) and the proliferation of human epithelial carcinoma cell line A431 (Table 1). We designed and optimized the urea-linked side chains through the following strategies: (i) the mono-, or di-substituted of the N-terminal of the urea; (ii) increasing the substituted C-chain of the N-terminal of the urea; (iii) chain or cycle di-substitution of the N-terminal of the urea. Firstly, we investigated the influence of mono-substitution of the N-terminal of the urea on EGFR inhibition. As shown in Table 1, all of the compounds (**8a**–**z**, and **9a**–**b**) demonstrated inhibition of EGFR (IC_{50} = 0.024–1.715 μM). For example, compound **8a**, which contained a hydroxyethyl group on the N-terminal of the urea, inhibited EGFR (IC_{50} = 0.027 μM). However, replacement of the hydroxyethyl group with a methoxyethyl group (compound **8b**, IC_{50} = 0.479 μM), notably reduced the inhibitory activity, likely because the urea-linked side chain of compound **8b** did not form interactions with the enzyme. Compound **8c**, which contained a methylsulfonylethyl group on the N-terminal of the urea, did not show a significant change in kinase inhibition (IC_{50} = 0.041 μM). When the N-terminal of the urea was replaced with a



Scheme 1. Reagents and conditions: (a) microwave, 60% power, 5 min; (b) H_2SO_4/HNO_3 (1:1), 90 °C, 1 h; (c) $POCl_3$, 90 °C, 2 h; (d) 3-chloro-4-fluoroaniline, dioxane, 90 °C, 3 h; (e) $PhSO_3Na$, 90 °C, 2 h; (f) (*S*)-tetrahydrofuran-3-ol, NaH, DMF, 0 °C to rt, 4 h; (g) Reney-Ni, H_2, DMF, 4 h; (h) phenyl carbonochloridate, DIPEA, THF, 5 °C, 3 h; (i) different substituted amine, THF, 70 °C, 4 h.

dimethylaminoethyl group (compound **8d**, IC_{50} = 0.635 μM) or a diethylaminoethyl group (compound **8e**, IC_{50} = 0.365 μM), EGFR inhibition was notably reduced. However, compared to compound **8e**, compounds **8f** (IC_{50} = 0.068 μM), **8g** (IC_{50} = 0.165 μM), and **8h** (IC_{50} = 0.082 μM), demonstrated improved inhibition by 9.3-, 3.8-, and 7.7-fold, respectively. We also investigated the SARs of the compounds through increasing the C-chain of the N-terminal of the urea (compounds **8i**–**l**). We observed that compounds **8k** (IC_{50} = 0.024 μM) and **8l** (IC_{50} = 0.032 μM) demonstrated potent EGFR inhibition. This suggested that increasing the C-chain of the N-terminal of the urea, and containing a hydrogen-bonded atom (such as O or N), increased the inhibitory activity. We then investigated the influence of the di-substituted N-terminal of the urea on the inhibitory activity of the compounds. As shown in Table 1, compounds **8m**–**z** (IC_{50} = 0.265–1.715 μM) displayed sharply decreased EGFR inhibition. In particular, the cyclic-substitution on the N-terminal of the urea (**8p**–**z**) displayed decreased inhibition. In addition, we investigated the compounds containing the Michael acceptor (compounds **9a**–**b**), and found that inhibition was not as potent as expected. This could be because the urea-linked side chains are too long and cannot form covalent bonds.

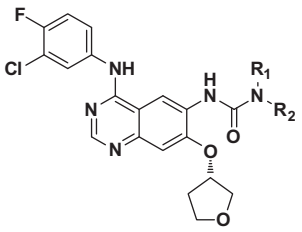
4.2. Binding mode of compounds 8a, 8d, 8k and 8o

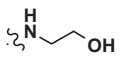
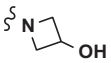
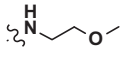
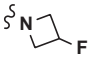
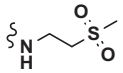
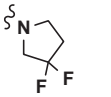
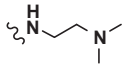
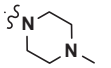
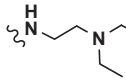
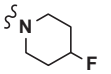
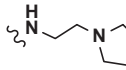
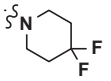
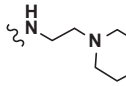
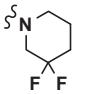
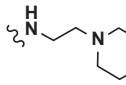
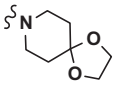
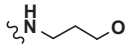
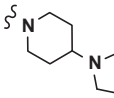
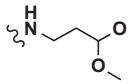
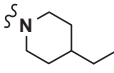
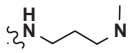
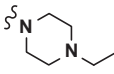
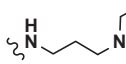
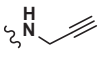
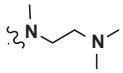
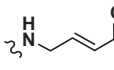
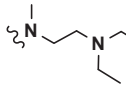
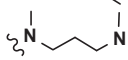
Compared to compound **8a** (IC_{50} = 0.027 μM), the inhibitory activity of compound **8d** (IC_{50} = 0.635 μM) was reduced 23.5-fold, while the inhibitory activity of compound **8k** (IC_{50} = 0.024 μM) was increased 19.3-fold compared to compound **8o** (IC_{50} = 0.464 - μM). To explore the reasons for these differences and provide structural information for further optimization and SAR studies, the 3D proposed binding mode of compounds **8a**, **8d**, **8k** and **8o** were generated by docking simulation (Fig. 3). The docking model showed the quinazoline core of compounds **8a**, **8d**, **8k** and **8o** bound to the active site of EGFR in a similar manner to Gefitinib. The N-1 nitrogen of the quinazoline scaffold was hydrogen-bonded to the main chain NH of the hinge region Met793, whereas the N-3 nitrogen formed a water-mediated hydrogen bond network with the side chain of Thr790. The 4-fluoro-3-chloroaniline moiety occupied the hydrophobic pocket formed by the side chains of amino acids near the DFG motif. The C-6 urea-linked side chains were positioned at the solvent interface of EGFR. The O atom of the urea moiety of compound **8a** (IC_{50} = 0.027 μM) may form a hydrogen bond with Cys797, and the hydroxyl group of the N-terminal of the urea may form a hydrogen bond with Asp800 (Fig. 3a). However, compound **8d** (IC_{50} = 0.635 μM), only the dimethylamino group of the N-terminal of the urea may form hydrogen bond with Asp800, the urea moiety did not form the hydrogen bond with Cys797 (Fig. 3b). This could suggest a reason that the inhibition of compound **8a** was superior to that of compound **8d**. Due to the increased length of the C-chain, the dimethylamino group of the N-terminal of the urea of compound **8k** (IC_{50} = 0.024 μM) could form a hydrogen bond with Asp800 (Fig. 3c). Therefore, compound **8k** demonstrated improved inhibition over compound **8d**. However, the introduction of the methyl group (compound **8o**) changed the chain configuration of the N-terminal of the urea and could no longer form a hydrogen bond with Asp800, even if the O atom of the urea moiety may form the hydrogen bond with Cys797 (Fig. 3d). This potentially explains why inhibition by compound **8o** was decreased (IC_{50} = 0.464 μM).

4.3. Inhibitory activities of compounds on the EGF-induced activation of EGFR in cancer cells

As the above results demonstrated, most of the compounds display inhibitory effects on EGFR activity in vitro, so we further examined their anti-proliferative activities in A431 human epithelial carcinoma cells, which express high-levels of EGFR wt. As shown in Table 1, all of the compounds (**8a**–**z**, and **9a**–**b**) moderately inhibited the proliferation of A431 cells (IC_{50} = 0.116–22.008 μM). In comparison with these compounds, we found that compounds with chain substituted of the N-terminal of the urea (mono-substituted or di-substituted) showed improved inhibition in A431 cells cyclic substitution on the N-terminal of the urea. Among these compounds, compounds **8g** (IC_{50} = 0.116 μM), **8k** (IC_{50} = 0.226 μM), and **8l** (IC_{50} = 0.196 μM) demonstrated the best inhibition of A431 cell proliferation.

Table 1
Enzymatic and cellular inhibitory activities of compounds **8a–z** and **9a–b** against EGFR/WT and A431[a]



Compd	R_1/R_2	EGFR wt (IC_{50}, μM)	A431 (IC_{50}, μM)	Compd	R_1/R_2	EGFR wt (IC_{50}, μM)	A431 (IC_{50}, μM)
8a		0.027 ± 0.004	0.598 ± 0.485	**8p**		0.543 ± 0.079	1.930 ± 1.817
8b		0.479 ± 0.050	0.387 ± 0.179	**8q**		0.967 ± 0.107	5.784 ± 3.046
8c		0.041 ± 0.033	1.569 ± 0.223	**8r**		1.581 ± 0.081	1.484 ± 0.302
8d		0.635 ± 0.114	0.806 ± 0.000	**8s**		0.464 ± 0.015	0.675 ± 0.337
8e		0.365 ± 0.276	0.249 ± 0.118	**8t**		0.699 ± 0.274	3.043 ± 2.528
8f		0.068 ± 0.008	0.247 ± 0.126	**8u**		0.859 ± 0.099	1.855 ± 1.371
8g		0.165 ± 0.011	0.116 ± 0.030	**8v**		0.884 ± 0.047	0.935 ± 0.591
8h		0.082 ± 0.021	0.307 ± 0.205	**8w**		1.715 ± 0.256	2.179 ± 0.400
8i		0.197 ± 0.081	4.100 ± 0.515	**8x**		0.265 ± 0.002	0.317 ± 0.267
8j		0.544 ± 0.210	22.008 ± 11.580	**8y**		0.304 ± 0.248	0.759 ± 0.120
8k		0.024 ± 0.015	0.226 ± 0.0.07	**8z**		0.331 ± 0.033	1.13 ± 0.829
8l		0.032 ± 0.020	0.196 ± 0.016	**9a**		0.232 ± 0.069	3.734 ± 0.000
8m		0.281 ± 0.015	0.450 ± 0.443	**9b**		0.404 ± 0.161	1.410 ± 0.817
8n		0.505 ± 0.035	0.481 ± 0.332	Erlotinib	—	2.600 ± 2.300 (nM)	0.409 ± 0.230
8o		0.464 ± 0.125	0.200 ± 0.045	Afatinib	—	0.190 ± 0.014 (nM)	0.023 ± 0.009 (nM)

[a] IC_{50} values were obtained by logit method based on the data obtained from three separate experiments and expressed as means ± SD.

In addition to the kinase and cellular inhibitory activities, we further determined whether the compounds inhibit the phosphorylation of EGFR and the activation of downstream signaling pathways in A431 cells. Compounds **8k** and **8l** were selected for further experiments. A431 cells were incubated with **8k** and **8l** for 1 h, followed by exposure to EGF (Fig. 4). Potent and concentration-dependent inhibition of EGFR phosphorylation was observed after treatment with **8k** and **8l**, with almost complete inhibition observed at 0.01 μM. In addition, **8k** and **8l** dramatically inhibited EGF-induced activation of downstream signaling

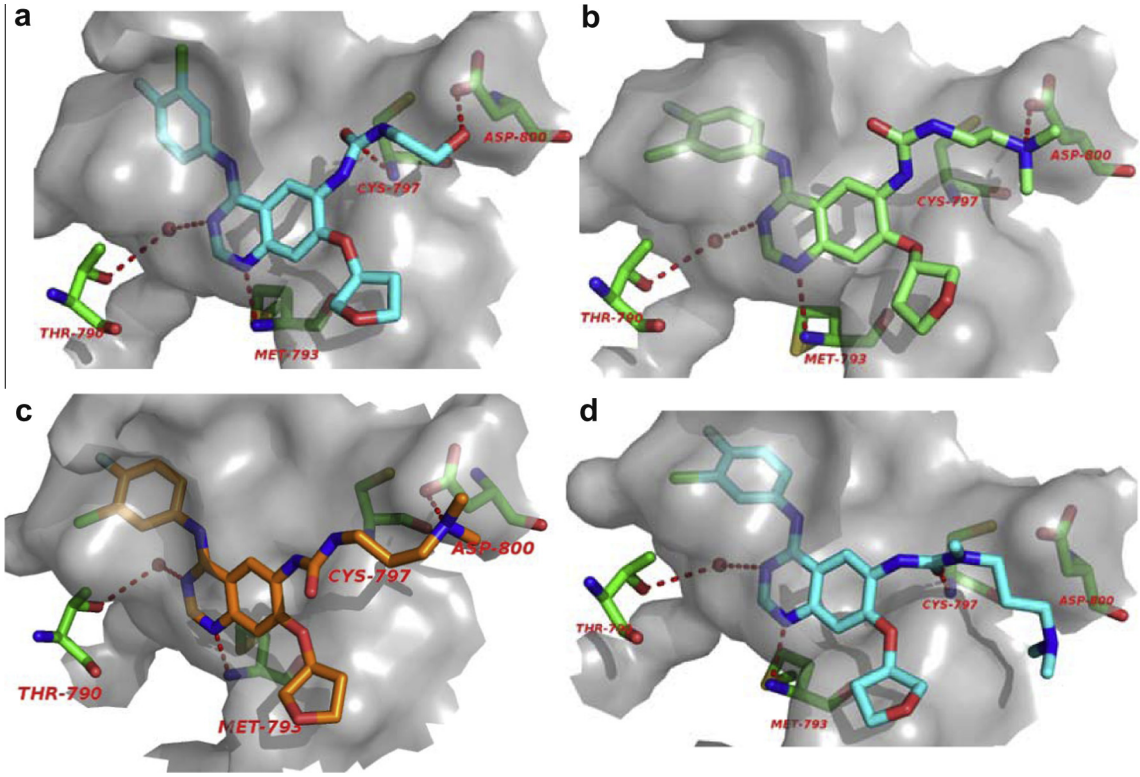


Figure 3. Proposed binding mode of compounds **8a** (a), **8d** (b), **8k** (c) and **8o** (d) (N and O atoms in blue and red, respectively). These four images were generated using the PYMOL program.

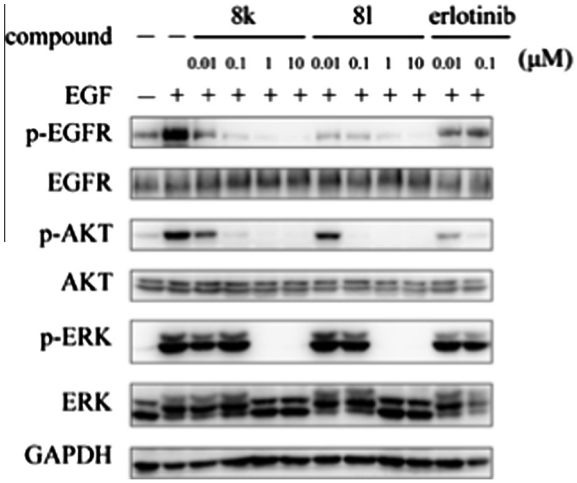


Figure 4. Effect of compounds **8k** and **8l** on EGF-induced EGFR phosphorylation. Representative Western blot analysis for EGFR inhibition by **8k** and **8l** in the A431 cell line. Analysis was done using monoclonal antibodies directed to p-Tyr1068 (see the Section 6). Total EGFR is shown as the loading control.

molecules, as shown by the decrease of the ERK and AKT phosphorylation.

4.4. Inhibition of EGFR mutant T790M/L858R activity in cell-free and and intact cell assays

Drug resistance is a critical issue in cancer therapy, and the T790M mutation in EGFR is a key factor in this regard. Therefore, inhibitors that can overcome drug resistance are needed. The compounds we designed inhibited EGFR wt. We then wondered whether these compounds could inhibit the EGFR/T790M/L858R mutant in vitro kinase assay. Intriguingly, all of the compounds moderately inhibited EGFR/T790M/L858R (IC_{50} = 0.049–5.578 μM) (Table 2). Moreover, compounds **8f** (IC_{50} = 0.061 μM) and **8h** (IC_{50} = 0.049 μM) showed excellent inhibition. Furthermore, we examined the activities of these compounds on the proliferation of NCI-H1975 cells, which harbors the T790M mutation in EGFR. The results (as shown in Table 2) demonstrate that most of them possess anti-proliferative activity, specifically compounds **8d** (IC_{50} = 4.7 μM), **8e** (IC_{50} = 5.2 μM), **8f** (IC_{50} = 5.3 μM), **8g** (IC_{50} = 3.2 μM), **8h** (IC_{50} = 5.3 μM), **8l** (IC_{50} = 4.3 μM), **8o** (IC_{50} = 3.3 μM), and **8r** (IC_{50} = 4.5 μM) inhibit the proliferation of NCI-H1975 cells.

We further examined the ability of compounds **8f** and **8h** to inhibit EGFR T790M phosphorylation in NCI-H1975 cells. (Fig. 5) NCI-H1975 cells were incubated with compounds **8f** or **8h** for 1 h followed by exposure to EGF. Compounds **8f** and **8h** blocked EGFR autophosphorylation at high concentrations (10 μM), indicating reduced inhibition of EGFR T790M phosphorylation in NCI-H1975 cells compared to phosphorylation of EGFR wt in A431 cells (Fig. 4). Moreover, the compounds did not show inhibition of downstream signaling, such as the phosphorylation of Erk and AKT at concentrations lower than 10 μM.

4.5. Irreversible inhibition

To further explore the intrinsic properties of compound **8f**, we used a dilution method to assess whether it binds irreversibly to its target.[21] In this approach, the enzyme and compound were pre-incubated at high concentrations for 30 min to form an enzyme-inhibitor complex, which was then diluted 100-fold in reaction buffer with ATP and peptide. The phosphorylated of the peptide was used as a read-out for enzyme activity, which in turn reflects the dissociation rate of the enzyme. If the compound is a reversible inhibitor, EGFR activity will quickly recover and will coincide with

Table 2
Enzymatic and cellular inhibitory activities of compounds **8a–z** and **9a–b** against EGFR T790M/L858R and NCI-H1975[a]

Compd	EGFR T790M/L858R (IC_{50}, μM)	NCI-H1975 (IC_{50}, μM)	Compd	EGFR T790M/L858R (IC_{50}, μM)	NCI-H1975 (IC_{50}, μM)
8a	0.432 ± 0.028	17.6 ± 4.9	**8p**	1.419 ± 0.526	32.2 ± 5.2
8b	3.564 ± 0.939	8.3 ± 6.1	**8q**	2.165 ± 0.789	13.4 ± 6.2
8c	0.672 ± 0.377	35.2 ± 8.6	**8r**	3.121 ± 0.536	4.5 ± 0.9
8d	2.077 ± 0.510	4.7 ± 0.4	**8s**	5.578 ± 0.211	12.3 ± 3.0
8e	2.235 ± 1.090	5.2 ± 0.9	**8t**	1.824 ± 0.834	33.3 ± 10.6
8f	0.061 ± 0.024	5.3 ± 1.2	**8u**	5.311 ± 1.760	32.3 ± 5.4
8g	0.447 ± 0.153	3.2 ± 0.9	**8v**	1.891 ± 0.534	43.2 ± 12.3
8h	0.049 ± 0.021	5.3 ± 0.4	**8w**	5.187 ± 1.200	32.2 ± 5.2
8i	1.659 ± 0.471	22.7 ± 6.2	**8x**	1.594 ± 0.783	9.6 ± 2.0
8j	0.400 ± 0.206	>100	**8y**	2.93781.132	25.3 ± 11.1
8k	0.276 ± 0.130	6.3 ± 1.7	**8z**	4.212 ± 2.633	18.2 ± 8.0
8l	3.058 ± 1.600	4.3 ± 1.2	**9a**	0.374 ± 0.049	14.8 ± 4.5
8m	0.677 ± 0.255	8.2 ± 3.4	**9b**	0.265 ± 0.076	9.6 ± 2.0
8n	0.392 ± 0.088	8.3 ± 1.2	Erlotinib	1.024 ± 0.090	6.9 ± 1.2
8o	5.261 ± 2.026	3.3 ± 0.8	Afatinib	0.780 ± 0.003 (nM)	0.148 ± 0.058

[a] IC_{50} values were obtained by logit method based on the data obtained from three separate experiments and expressed as means ± SD.

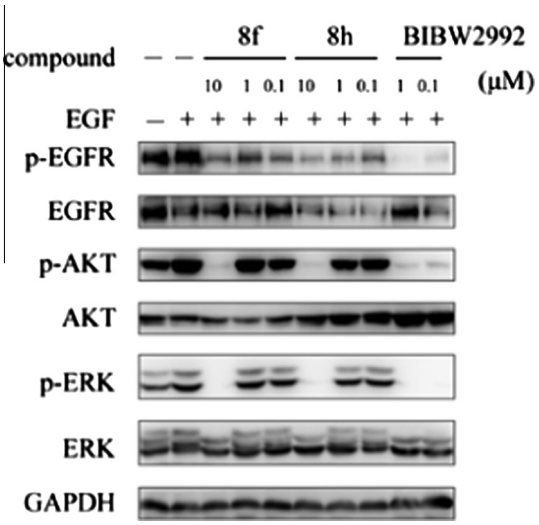


Figure 5. Effect of compounds **8f** and **8h** on EGF-induced EGFR phosphorylation. Representative Western blot analysis for EGFR inhibition by **8f** and **8h** in the H1975 cell line. Analysis was done using monoclonal antibodies directed to p-Tyr1068 (see the Section 6). Total EGFR is shown as the loading control.

the curve from reactions without preincubation, similar to Erlotinib, which is a reversible inhibitor (Fig. 6A). However, if the compound is an irreversible inhibitor, such as BIBW2992 (Fig. 6B), the recovery is much slower. As shown in Figure 6C, preincubation of **8f** caused no obvious recovery compared with that of the control, clearly suggesting that **8f** is an irreversible inhibitor of EGFR.

Importantly, compounds with C-6 urea-linked side chains that did not contain a Michael addition site demonstrated moderate to good irreversible inhibition of EGFR. Moreover, compounds **8f** (IC_{50} = 0.061 μM) and **8h** (IC_{50} = 0.049 μM) showed excellent inhibitory activities. We suggest that the C-6 urea moiety may form hydrogen bonds with amino acid residues of EGFR. This result is consistent with Sogabe et al.,[22] in which reversible inhibitors were active against the EGFR T790M/L858R mutant. We also confirmed that compound **8f** was an irreversible inhibitor of EGFR through the dilution method, although this compound merits further study.

4.6. Kinase profiling

To investigate whether **8f** and **8l** were multiple kinase inhibitors, the effect of the compounds on other EGFR family members, including ErbB2, ErbB4, and 11 different tyrosine kinases were evaluated by enzyme-linked immunosorbent assay (ELISA). As shown in Table 3, compound **8f** was a potent inhibitor of EGFR (wt), EGFR (T790M/L858R), ErbB2, ErbB4 with IC_{50} values of 0.068, 0.061, 0.112, and 0.012 μM, respectively. Compound **8f** also inhibited the activity of RET and RON, with an IC_{50} of 0.223 and 6.097 μM, respectively. However, **8f** showed little effect on other tyrosine kinases, including ABL, Flt-1, KDR, c-Kit, PDGFR-α, PDGFR-β, EPH-A2, IGF1R, FGFR1 (Table 3). Compound **8l**, inhibited EGFR (T790M/L858R) with an IC_{50} of inhibition 3.058 μM, which was 100-fold lower than that of compound **8f**, and was also a selective inhibitor of EGFR family members. It inhibited the kinase activities of EGFR (wt), ErbB2 and ErbB4 with IC_{50} values of 0.032, 0.078 and 0.008 μM, respectively. In addition, **8l** inhibited RET and ABL with IC_{50} values of 0.347 and 0.566 μM, respectively.

5. Conclusions

A novel series of 4-anilinoquinazolines with C-6 urea-linked side chains was designed, synthesized and evaluated as potent irreversible EGFR inhibitors. Compounds **8f**, **8h**, **8k**, and **8l** inhibited EGFR activity and EGFR autophosphorylation in the A431 human epithelial carcinoma cell line. Furthermore, compounds **8f** and **8h** successfully inhibited EGFR/T790M/L858R activity and EGFR autophosphorylation in the NCI-H1975 human lung cancer cell line. Compound **8f** was confirmed to be an irreversible inhibitor, although further investigation of the mechanism of inhibition of compounds **8f** and **8h** is required.

6. Experimental section

6.1. Chemistry

The reagents (chemicals) were purchased and used without further purification. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AMX-400 (IS as TMS). Chemical shifts were reported in parts per million (ppm, δ) downfield from tetramethylsilane. Proton coupling patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad (br). Low- and high-resolution mass spectra (LRMS and HRMS) were given with electric, electrospray, and matrix-assisted laser desorption ionization (EI, and ESI) produced by a Finnigan MAT-95, LCQ-DECA spectrometer and IonSpec 4.7 T.

All of the compounds were synthesized as shown in Scheme 1. Compound **17** was disclosed in WO2005037824 as an important

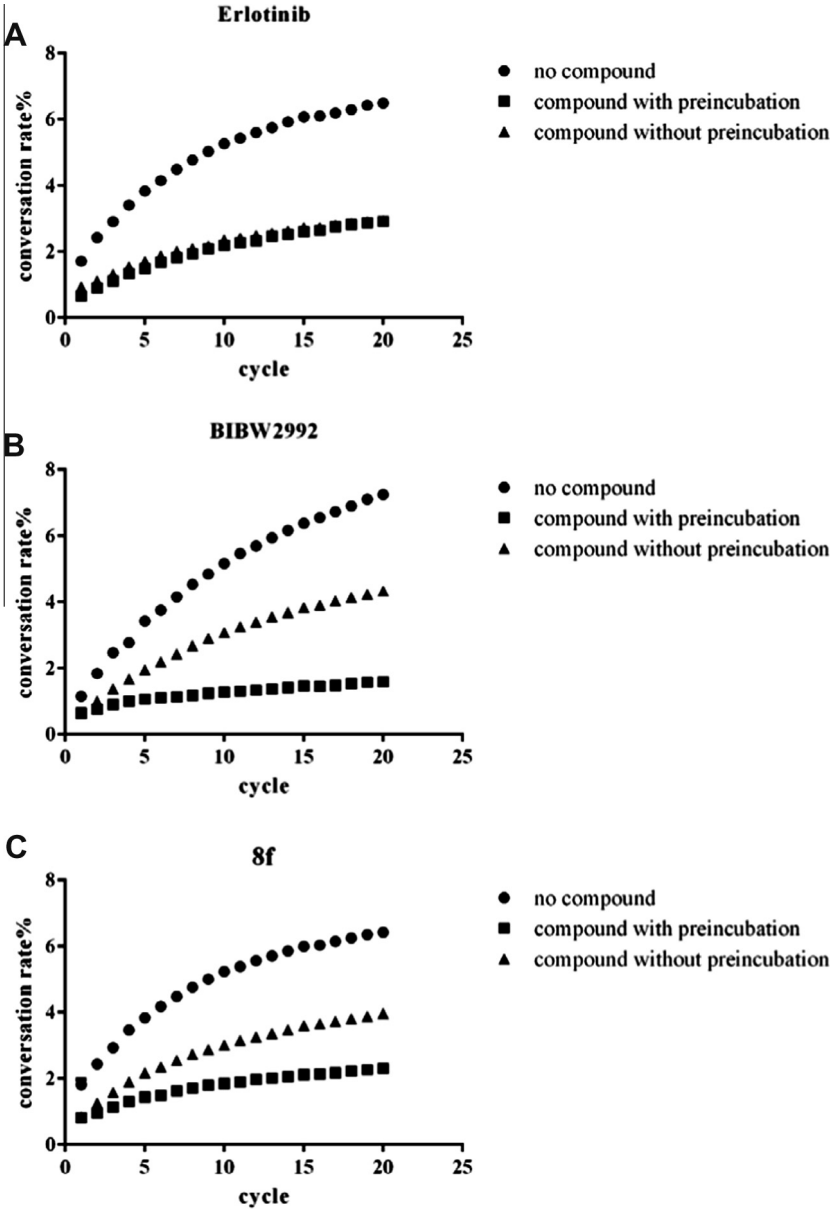


Figure 6. Compound **8f** irreversibly binds EGFR. Using Caliper EZ Reader to assay the enzyme activity of EGFR under three different conditions: without compound, with compound and preincubated with compound, and the enzyme activity was assessed by the percent of converge of substrate peptide (5-FAM-EEPLYWSFPAKKK-CONH2).

Table 3
Selectivity profile of compounds **8f** and **8l** against 15 kinases[a]

Kinases	**8f** (IC_{50}, μM)	**8l** (IC_{50}, μM)	Kinases	**8f** (IC_{50}, μM)	**8l** (IC_{50}, μM)
EGFR(wt)	0.068 ± 0.008	0.032 ± 0.002	KDR	>10	>10
EGFR(T790M/L858R)	0.061 ± 0.024	3.058 ± 1.600	c-Kit	>10	>10
ErbB2	0.112 ± 0.006	0.078 ± 0.021	PDGFR-α	>10	>10
ErbB4	0.012 ± 0.002	0.008 ± 0.001	PDGFR-β	>10	>10
RET	0.223 ± 0.064	0.347 ± 0.101	EPH-A2	>10	>10
RON	6.097 ± 5.189	>10	IGF1R	>10	>10
ABL	>10	0.566 ± 0.222	FGFR1	>10	>10
Flt-1	>10	>10			

[a] The kinase profiling assay was conducted as described in the Section 6 by using ELISA kinases assay. IC_{50} values were obtained by logit method based on the data obtained from three separate experiments and expressed as means ± SD.

intermediate for preparing EGFR inhibitor BIBW2992. Finally, phenylcarbamate was obtained which underwent nucleophilic reaction with various amines to give the desired compounds **8a**–**8z**, **9a** and **9b**.

6.2. General methods

To a solution of compound **17** (10.0 g, 26.7 mmol) in THF (100 mL) containing DIPEA (9.3 mL, 53.4 mmol) was added phenyl

chloroformate (3.7 mL, 29.4 mmol) in dropwise at 5 °C for 1 h. The reaction was completely after 3 h (monitored by TLC), and the reaction mixture was diluted with ethyl acetate (EA) and washed with 1 M HCl solution, saturated sodium carbonate solution, brine, and dried with Na_2SO_4 and concentrated. The crude solid was recrystallizated by the mixture of petroleum ether and ethyl acetate (1:1) to get the compound 18.

6.1.1. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(2-hydroxyethyl)urea (8a)

A mixture of compound **18** (200 mg, 0.404 mmol) and 2-aminoethanol (72 μL, 1.21 mmol) was stirred in THF (10 mL) and the reaction mixture was heated to reflux for 4 h. After the reaction was cooled to ambient temperature, the crude reaction mixture was extracted three times with EA (30 mL × 3). The combined organic phase was wash with saturated water, brine, dried with Na_2SO_4 and concentrated. The residue was purified by flash chromatography on silica gel to provide the desired product **8a** in 71% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.13–2.70 (m, 2H), 3.46–3.52 (m, 4H), 3.88–4.19 (m, 4H), 5.11 (s, 1H), 5.81 (s, 1H), 7.05–7.11 (m, 2H), 7.50–7.54 (m, 1H), 7.60 (s, 1H), 7.87–7.89 (m, 1H), 8.06 (s, 1H), 8.57 (s, 1H), 8.76 (s, 1H). ESI-MS *m*/*z* 462 $[M+H]^+$.

6.1.2. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(2-methoxyethyl)urea (8b)

Compound **8b** was synthesized by following the procedure described for **8a** using 2-methoxyethanamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8b** in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.17–2.72 (m, 2H), 3.40 (s, 3H), 3.48–3.56 (m, 4H), 3.88–4.19 (m, 4H), 5.11 (s, 1H), 5.81 (s, 1H), 7.05–7.11 (m, 2H), 7.50–7.54 (m, 1H), 7.60 (s, 1H), 7.87–7.89 (m, 1H), 8.06 (s, 1H), 8.57 (s, 1H), 8.76 (s, 1H). ESI-MS *m*/*z* 476 $[M+H]^+$.

6.1.3. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(2-(methylsulfonyl)ethyl)urea (8c)

Compound **8c** was synthesized by following the procedure described for **8a** using 2-(methylsulfonyl)ethanamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8c** in 86% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.17–2.36 (m, 2H), 3.03 (s, 1H), 3.37–3.38 (m, 2H), 3.89–3.95 (m, 3H), 4.06–4.20 (m, 3H), 5.12 (s, 1H), 6.40 (s, 1H), 7.10–7.14 (m, 2H), 7.58–7.60 (m, 1H), 7.74 (s, 1H), 7.90–7.91 (m, 1H), 7.97 (s, 1H), 8.59–8.61 (m, 1H). ESI-MS *m*/*z* 524 $[M+H]^+$.

6.1.4. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(2-(dimethylamino)ethyl)urea (8d)

Compound **8d** was synthesized by following the procedure described for **8a** using N^1,N^1-dimethylethane-1,2-diamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8d** in 72% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.17–2.32 (m, 2H), 2.40 (s, 6H), 2.59–2.61 (m, 2H), 3.40–3.44 (m, 2H), 3.85–4.12 (m, 4H), 5.06 (s, 1H), 6.35 (s, 1H), 7.00–7.05 (m, 2H), 7.49–7.52 (m, 1H), 7.72 (s, 1H), 7.83–7.85 (m, 1H), 8.27–8.28 (m, 1H), 8.54 (s, 1H), 8.71 (s, 1H). ESI-MS *m*/*z* 489 $[M+H]^+$.

6.1.5. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(2-(diethylamino)ethyl)urea (8e)

Compound **8e** was synthesized by following the procedure described for **8a** using N^1,N^1-diethylethane-1,2-diamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8e** in 76% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (t, *J* = 6.8 Hz, 6H), 2.03 (s, 2H), 3.10–3.16 (m, 4H), 3.61–3.62 (m, 2H), 3.86–4.21 (m, 4H), 5.03 (s, 1H), 6.99–7.04 (m, 2H), 7.49–7.52 (m, 1H), 7.59 (s, 1H), 7.78–7.79 (m, 1H), 8.23 (s, 1H), 8.52 (s, 1H), 8.64 (s, 1H). ESI-MS *m*/*z* 517 $[M+H]^+$.

6.1.6. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(2-(pyrrolidin-1-yl)ethyl)urea (8f)

Compound **8f** was synthesized by following the procedure described for **8a** using 2-(pyrrolidin-1-yl)ethanamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8f** in 82% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 2.12 (br s, 4H), 2.20–2.26 (m, 1H), 2.31–2.40 (m, 1H), 3.42–3.45 (m, 2H), 3.51 (br s, 4H), 3.65–3.68 (m, 2H), 3.84–4.10 (m, 4H), 5.11 (s, 1H), 7.01 (d, *J* = 6.0 Hz, 1H), 7.14–7.20 (m, 1H), 7.60–7.64 (m, 1H), 7.94–7.97 (m, 1H), 8.38 (s, 1H), 8.51 (d, *J* = 8.4 Hz, 1H). ESI-MS *m*/*z* 515 $[M+H]^+$.

6.1.7. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(2-(piperidin-1-yl)ethyl)urea (8g)

Compound **8g** was synthesized by following the procedure described for **8a** using 2-(piperidin-1-yl)ethanamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8g** in 84% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 1.68 (br s, 2H), 1.88–1.92 (m, 4H), 2.24–2.42 (m, 2H), 3.29–3.33 (m, 6H), 3.68–3.71 (m, 2H), 3.86–4.14 (m, 4H), 5.17 (s, 1H), 6.97–6.99 (m, 1H), 7.14–7.19 (m, 1H), 7.60–7.63 (m, 1H), 7.93–7.95 (m, 1H), 8.36–8.37 (m, 1H), 8.53–8.56 (m, 1H). ESI-MS *m*/*z* 529 $[M+H]^+$.

6.1.8. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(2-morpholinoethyl)urea (8h)

Compound **8h** was synthesized by following the procedure described for **8a** using 2-(piperidin-1-yl)ethanamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8h** in 84% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.14–2.31 (m, 2H), 2.39–2.62 (m, 5H), 3.58–4.14 (m, 11H), 5.04 (s, 1H), 6.21 (s, 1H), 6.97–7.13 (m, 2H), 7.48 (s, 1H), 7.70–7.78 (m, 2H), 8.51–8.67 (m, 3H). ESI-MS *m*/*z* 531 $[M+H]^+$.

6.1.9. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(3-methoxypropyl)urea (8i)

Compound **8i** was synthesized by following the procedure described for **8a** using 3-methoxypropan-1-amine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8i** in 82% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.76–1.82 (m, 2H), 2.06–2.30 (m, 2H), 3.03 (s, 3H), 3.32–3.83 (m, 2H), 3.46 (t, *J* = 5.6 Hz, 2H), 3.80–4.08 (m, 4H), 5.00 (s, 1H), 6.13 (s, 1H), 6.91–6.96 (m, 1H), 7.00 (s, 1H), 7.41–7.45 (m, 1H), 7.64 (s, 1H), 7.72–7.74 (m, 1H), 8.49 (s, 2H), 8.67 (s, 1H). ESI-MS *m*/*z* 490 $[M+H]^+$.

6.1.10. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(3,3-dimethoxypropyl)urea (8j)

Compound **8j** was synthesized by following the procedure described for **8a** using 3,3-dimethoxypropan-1-amine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8j** in 79% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.18–2.22 (m, 1H), 2.40–2.45 (m, 1H), 3.42 (s, 6H), 3.53–3.63 (m, 5H), 3.86–4.11 (m, 4H), 5.11 (s, 1H),

7.02–7.06 (m, 1H), 7.52–7.54 (m, 1H), 7.81–7.82 (m, 1H), 8.55 (s, 1H), 8.64 (s, 1H), 8.68 (s, 1H). ESI-MS *m*/*z* 520 $[M+H]^+$.

6.1.11. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(3-(dimethylamino)propyl)urea (8k)

Compound **8k** was synthesized by following the procedure described for **8a** using N^1,N^1-dimethylpropane-1,3-diamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8k** in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.12–2.21 (m, 2H), 2.33–2.40 (m, 2H), 2.77 (s, 6H), 3.04–3.15 (m, 2H), 3.31–3.35 (m, 1H), 3.44–3.48 (m, 1H), 3.90–3.95 (m, 2H), 4.17–4.22 (m, 2H), 5.11 (s, 1H), 6.95–6.99 (m, 2H), 7.38–7.40 (m, 1H), 7.54–7.57 (m, 1H), 8.21 (s, 1H), 8.44 (s, 1H), 8.53 (s, 1H), 8.75 (s, 1H). ESI-MS *m*/*z* 503$[M+H]^+$.

6.1.12. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(3-(diethylamino)propyl)urea (8l)

Compound **8l** was synthesized by following the procedure described for **8a** using N^1,N^1-diethylpropane-1,3-diamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8l** in 73% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.27 (t, *J* = 7.2 Hz, 6H), 1.33–2.26 (m, 2H), 2.26–2.39 (m, 2H), 2.94–3.12 (m, 6H), 3.19–3.39 (m, 2H), 3.86–3.95 (m, 2H), 4.11–4.17 (m, 2H), 5.02 (s, 1H), 6.85–6.89 (m, 2H), 7.29–7.33 (m, 1H), 7.42–7.44 (s, 1H), 7.77–7.80 (m, 1H), 8.29 (s, 1H), 8.46 (s, 1H), 8.73 (s, 1H), 8.77 (s, 1H). ESI-MS *m*/*z* 531 $[M+H]^+$.

6.1.13. (*S*)-3-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-1-(2-(dimethylamino)ethyl)-1-methylurea (8m)

Compound **8m** was synthesized by following the procedure described for **8a** using N^1,N^1,N^2-trimethylethane-1,2-diamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8m** in 81% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.05–2.11 (m, 1H), 2.28–2.34 (m, 1H), 2.37 (s, 6H), 2.54–2.64 (m, 2H), 3.04 (s, 3H), 3.40–3.59 (m, 2H), 3.82–4.05 (m, 4H), 4.88 (s, 1H), 6.88–6.95 (m, 2H), 7.33–7.35 (m, 1H), 7.58 (s, 1H), 8.44 (s, H), 8.59 (s, 1H), 8.73 (s, 1H). ESI-MS *m*/*z* 503 $[M+H]^+$.

6.1.14. (*S*)-3-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-1-(2-(diethylamino)ethyl)-1-methylurea (8n)

Compound **8n** was synthesized by following the procedure described for **8a** using N^1,N^1-diethyl-N^2-methylethane-1,2-diamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8n** in 77% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.08 (t, *J* = 6.8 Hz, 6H), 2.07–2.10 (m, 1H), 2.35–2.40 (m, 1H), 2.63–2.76 (m, 6H), 3.09 (s, 3H), 3.39–3.64 (m, 2H), 3.83–4.08 (m, 4H), 4.87 (s, 1H), 6.90 (s, 1H), 6.95–6.99 (m, 1H), 7.34–7.35 (m, 1H), 7.56–7.57 (m, 1H), 8.48 (s, 1H), 8.57 (s, 1H), 8.68 (s, 1H). ESI-MS *m*/*z* 531 $[M+H]^+$.

6.1.15. (*S*)-3-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-1-(3-(dimethylamino)propyl)-1-methylurea (8o)

Compound **8o** was synthesized by following the procedure described for **8a** using N^1,N^1,N^3-trimethylpropane-1,3-diamine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8o** in 71% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 1.94–2.04 (m, 2H), 2.15–2.23 (m, 1H), 2.36–2.54 (m, 1H), 2.69 (s, 6H), 2.90–2.95 (m, 2H), 3.12 (s, 3H), 3.50–3.54 (m, 2H), 3.85–4.08 (m, 4H), 5.21 (m, 1H), 7.06 (s, 1H), 7.16–7.22 (m, 1H), 7.60–7.66 (m, 1H), 7.96–7.99 (m, 1H), 8.40 (s, 1H), 8.50 (s,1H). ESI-MS *m*/*z* 517 $[M+H]^+$.

6.1.16. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-hydroxyazetidine-1-carboxamide (8p)

Compound **8p** was synthesized by following the procedure described for **8a** using azetidin-3-ol instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8p** in 81% yield. ^{1}H NMR (300 MHz, CD_3OD) δ 2.15–2.22 (m, 1H), 2.36–2.40 (m, 1H), 2.78 (br s, 1H), 3.88–4.07 (m, 4H), 4.39–4.47 (m, 4H), 5.08 (m, 1H), 6.96–7.07 (m, 3H), 7.43–7.47 (m, 1H), 7.72–7.75 (m, 1H), 8.15 (s, 1H), 8.55 (s, 1H), 8.64 (s, 1H). ESI-MS *m*/*z* 474 $[M+H]^+$.

6.1.17. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-fluoroazetidine-1-carboxamide (8q)

Compound **8q** was synthesized by following the procedure described for **8a** using 3-fluoroazetidine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8q** in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.18–2.40 (m, 2H), 3.92–4.04 (m, 4H), 4.14–4.36 (m, 4H), 5.08 (s, 1H), 5.28 (d, *J* = 57.2 Hz, 1H), 6.83 (s, 1H), 6.98–7.08 (m, 2H), 7.45 (s, 1H), 7.73–7.74 (m, 1H), 8.36 (s, 1H), 8.53 (s, 1H), 8.65 (s, 1H). ESI-MS *m*/*z* 476 $[M+H]^+$.

6.1.18. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3,3-difluoropyrrolidine-1-carboxamide (8r)

Compound **8r** was synthesized by following the procedure described for **8a** using 3,3-difluoropyrrolidine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8r** in 87% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.13–2.20 (m, 1H), 2.33–2.40 (m, 1H), 2.42–3.52 (m, 2H), 3.64–3.68 (m, 2H), 3.77–3.83 (m, 2H), 3.89–4.06 (m, 4H), 5.05 (m, 1H), 6.92–6.96 (m, 1H), 7.00–7.01 (m, 2H), 7.43–7.47 (m, 1H), 7.68–7.71 (m, 1H), 8.37 (s, 1H), 8.49 (s, 1H), 8.63 (s, 1H). ESI-MS *m*/*z* 508 $[M+H]^+$.

6.1.19. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-4-methylpiperazine-1-carboxamide (8s)

Compound **8s** was synthesized by following the procedure described for **8a** using 1-methylpiperazine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8s** in 76% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.33 (s, 6H), 2.36–2.46 (m, 4H), 3.53 (s, 3H), 3.88–4.07 (m, 4H), 5.05 (s, 1H), 6.93–6.98 (m, 1H), 7.02 (s, 1H), 7.30 (s, 1H), 7.42–7.46 (m, 1H), 8.48–8.51 (m, 2H), 8.69 (s, 1H). ESI-MS *m*/*z* 501 $[M+H]^+$.

6.1.20. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-4-fluoropiperidine-1-carboxamide (8t)

Compound **8t** was synthesized by following the procedure described for **8a** using 4-fluoropiperidine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8t** in 88% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.86–1.95 (m, 4H), 2.15–2.36 (m, 2H), 3.58–3.62 (m, 4H), 3.89–4.05 (m, 4H), 5.00 (s, 1H), 6.89–6.93 (m, 1H), 6.97 (s, 1H), 7.29 (s, 1H), 7.38–7.39 (m, 1H), 7.57–7.58 (m, 1H), 8.48 (s, 1H), 8.58 (s, 1H), 8.65 (s, 1H). ESI-MS *m*/*z* 504 $[M+H]^+$.

6.1.21. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-4,4-difluoropiperidine-1-carboxamide (8u)

Compound **8u** was synthesized by following the procedure described for **8a** using 4,4-difluoropiperidine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8u** in 85% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.01–2.11 (m, 4H), 2.14–2.41 (m, 2H), 3.64–3.66 (m, 4H), 3.87–4.06 (m, 4H), 5.02 (s, 1H), 6.93–6.97 (m, 1H), 7.02 (s, 1H), 7.34–7.38 (m, 1H), 7.53–7.55 (m, 1H), 8.44 (s, 1H), 8.50 (s, 1H), 8.65 (s, 1H). ESI-MS *m*/*z* 522 $[M+H]^+$.

6.1.22. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3,3-difluoropiperidine-1-carboxamide (8v)

Compound **8v** was synthesized by following the procedure described for **8a** using 3,3-difluoropiperidine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8v** in 89% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.85–1.87 (m, 2H), 2.06–2.38 (m, 2H), 3.64–3.66 (m, 4H), 3.87–4.06 (m, 4H), 5.02 (s, 1H), 6.93–6.97 (m, 1H), 7.02 (s, 1H), 7.34–7.38 (m, 1H), 7.53–7.55 (m, 1H), 8.44 (s, 1H), 8.50 (s, 1H), 8.65 (s, 1H). ESI-MS *m*/*z* 522 $[M+H]^+$.

6.1.23. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-1,4-dioxa-8-azaspiro[4.5]decane-8-carboxamide (8w)

Compound **8w** was synthesized by following the procedure described for **8a** using 1,4-dioxa-8-azaspiro[4.5]decane instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8w** in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.75–1.77 (m, 4H), 2.11–2.16 (m, 1H), 2.30–2.39 (m, 1H), 3.59–3.61 (m, 4H), 3.87–4.04 (m, 8H), 5.01 (s, 1H), 6.90–6.94 (m, 1H), 6.98 (s, 1H), 7.30 (s, 1H), 7.37–7.39 (m, 1H), 7.59–7.60 (m, 1H), 8.48 (s, 1H), 8.61–8.64 (m, 2H). ESI-MS *m*/*z* 544 $[M+H]^+$.

6.1.24. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-4-(pyrrolidin-1-yl)piperidine-1-carboxamide (8x)

Compound **8x** was synthesized by following the procedure described for **8a** using 4-(pyrrolidin-1-yl)piperidine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8x** in 84% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.61–1.69 (m, 2H), 1.81–1.85 (m, 4H), 1.96–1.99 (m, 2H), 2.11–2.17 (m, 1H), 2.30–2.38 (m, 2H), 2.68–2.71 (m, 4H), 2.93–2.99 (m, 2H), 3.86–4.14 (m, 4H), 5.02 (s, 1H), 6.94–6.99 (m, 2H), 7.32 (s, 1H), 7.47–7.51 (m, 1H), 7.76–7.79 (m, 1H), 8.50 (s, 1H), 8.63–8.66 (m, 2H). ESI-MS *m*/*z* 555 $[M+H]^+$.

6.1.25. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-4-(2-hydroxyethyl)piperidine-1-carboxamide (8y)

Compound **8y** was synthesized by following the procedure described for **8a** using 2-(piperidin-4-yl)ethanol instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8y** in 86% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.06–1.14 (m, 2H), 1.37–1.41 (m, 2H), 1.64–1.72 (m, 2H), 2.08–2.12 (m, 1H), 2.28–2.33 (m, 1H), 2.82–2.88 (m, 2H), 3.47 (br s, 2H), 3.78–4.10 (m, 6H), 4.39 (s, 1H), 5.28 (s, 1H), 7.22 (s, 1H), 7.39–7.43 (m, 1H), 7.79–7.81 (m, 1H), 7.89 (s, 1H), 8.13–8.15 (m, 1H), 8.52 (s, 1H), 8.59 (s, 1H), 9.81 (s, 1H). ESI-MS *m*/*z* 530 $[M+H]^+$.

6.1.26. (*S*)-*N*-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-4-(2-hydroxyethyl)piperazine-1-carboxamide (8z)

Compound **8z** was synthesized by following the procedure described for **8a** using 2-(piperazin-1-yl)ethanol instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **8z** in 81% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.58–2.63 (m, 6H), 2.77–2.81 (m, 3H), 3.53–3.56 (m, 4H), 3.67–3.70 (m, 2H), 3.92–4.09 (m, 4H), 5.10–5.12 (m, 1H), 7.02–7.06 (m, 1H), 7.10 (s, 1H), 7.36 (s, 1H), 7.47–7.51 (m, 1H), 7.76–7.79 (m, 1H), 8.26 (s, 1H), 8.56 (s, 1H), 8.73 (s, 1H). ESI-MS *m*/*z* 531$[M+H]^+$.

6.1.27. (*S*)-1-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-3-(prop-2-ynyl)urea (9a)

Compound **9a** was synthesized by following the procedure described for **8a** using prop-2-yn-1-amine instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **9a** in 78% yield. ^{1}H NMR (400 MHz, CD_3OD) δ 2.18–2.24 (m, 1H), 2.35–2.44 (m, 1H), 2.66–2.67 (m, 1H), 3.87–4.12 (m, 6H), 5.17 (d, J = 5.2 Hz, 1H), 7.00 (s, 1H), 7.15–7.19 (m, 1H), 7.59–7.63 (m, 1H), 7.95–7.97 (m, 1H), 8.37 (s, 1H), 8.59 (m, 1H). ESI-MS *m*/*z* 456 $[M+H]^+$.

6.1.28. (*S*,*E*)-Methyl-4-(3-(4-(3-chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)ureido)but-2-enoate (9b)

Compound **9b** was synthesized by following the procedure described for **8a** using (*E*)-methyl 4-aminobut-2-enoate instead of 2-aminoethanol. The crude was purified by flash chromatography on silica gel to provide the desired product **9b** in 82% yield. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.13–1.14 (m, 1H), 2.29–2.33 (m, 1H), 3.80–4.00 (m, 9H), 4.21–4.22 (m, 2H), 5.27 (s, 1H), 7.21 (s, 1H), 7.38–7.42 (m, 1H), 7.60–7.66 (m, 2H), 7.78 (s, 1H), 8.12 (s, 1H), 8.50 (s, 1H), 8.67 (s, 1H), 9.79 (s, 1H). ESI-MS *m*/*z* 516 $[M+H]^+$.

6.2. Biological evaluations

6.2.1. ELISA kinase assay

The kinases domain of EGFR (WT), EGFR (T790M/L858R), ErbB2, IGF1R, FGFR1, KDR was expressed using the Bac-to-Bac™ baculovirus expression system (Invitrogen, Carlsbad, CA, USA) and purified on Ni-NTA columns (QIAGEN Inc., Valencia, CA, USA). Recombinant ErbB4, RET, RON, ABL, flt-1, c-kit, EPH-A2, PDGFRα and PDGFRβ proteins were obtained from Upstate Biotechnology. The screening of tyrosine kinase inhibitors was based on enzyme-linked-immunosorbent assay (ELISA). Briefly, 20 μg/mL Poly (Glu, Tyr)$_{4:1}$ (Sigma, St. Louis, MO) was precoated in 96-well ELISA plates as substrate. Each well was treated with 50 μL of 10 μmol/L ATP solution which was diluted in kinase reaction buffer (50 mM HEPES pH 7.4, 20 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.2 mM Na_3VO_4, 1 mM DTT). Then 1 μL of various concentrations of test compounds or reference compound dissolved in DMSO were added to each reaction well. Experiments at each concentration were performed in duplicate. The reaction was initiated by tyrosine kinase diluted in kinase reaction buffer. After incubation at 37 °C for 60 min, the wells were washed three times with phosphate buffered saline (PBS) containing 0.1% Tween 20 (T-PBS). Next, 100 μL anti-phosphotyrosine (PY99) antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in T-PBS containing 5 mg/mL BSA was added and the plate was incubated at 37 °C for 30 min. After the plate was washed three times, 100 μL horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000, Calbiochem, SanDiego, CA) diluted in T-PBS containing 5 mg/mL BSA was added and the plate was incubated at 37 °C for 30 min. The plate was washed, then 100 μL citrate buffer (0.1 M, pH 5.5) containing 0.03% H_2O_2 and 2 mg/mL *o*-phenylenediamine

was added and samples were incubated at room temperature until color emerged. The reaction was terminated by adding 50 μL of 2 M H_2SO_4, and the plate was read using a multiwell spectrophotometer (VERSAmax™, Molecular Devices, Sunnyvale, CA, USA) at 492 nm. The inhibitory rate (%) was calculated with the formula: $[1 - (A_{492}$ treated/A_{492} control)] × 100%. IC_{50} values were calculated from the inhibitory curves.

6.2.2. Cell proliferation assay

Cell proliferation was evaluated using the SRB (Sulforhodamine B) assay as previously described. Briefly, cells were seeded into 96-well plates and grown for 24 h. The cells were then treated with various concentrations of test compounds and grown for a further 72 h. The medium remained unchanged until the completion of the experiment. The cells were then fixed with 10% precooled trichloroacetic acid (TCA) for 1 h at 4 °C and stained for 15 min at room temperature with 100 μL of 4 mg/mL SRB solution (Sigma) in 1% acetic acid. The SRB was then removed, and the cells were quickly rinsed five times with 1% acetic acid. After cells were air-dried, protein-bound dye was dissolved in 150 μL of 10 mmol/L Tris base for 5 min and measured at 515 nm using a multiwell spectrophotometer (VERSAmax, Molecular Devices). The inhibition rate on cell proliferation was calculated as $[1 - (A_{515}$ treated/A_{515} control)] × 100%. The IC_{50} value was obtained by the Logit method.

6.2.3. Western blot analysis

Cells were grown to half confluence in six-well plates, starved in serum-free medium for 24 h, and then exposed to indicated concentrations of compounds for 1 h. Cells were stimulated with 50 ng/mL EGF (R&D Systems, Minneapolis, MN, USA) for 10 min and lysed in 1× SDS sample buffer. Western blot analyses were subsequently performed with standard procedures. Antibodies against the following were used: p-EGFR (Tyr1068), EGFR, phospho-ERK, ERK, p-AKT (Ser473), AKT were obtained from (Cell Signaling Technologies, Cambridge, MA), and GAPDH was from (Santa Cruz Biotechnology, Santa Cruz, CA).

6.2.4. Irreversible assay

Rapid dilution experiment was used to demonstrate reversible binding of **8f** to EGFR. EGFR kinase (800 nM) was pre-incubated with excessive **8f** at a final concentration of 100-fold IC_{50} under room temperature for 30 min, then the mix solution were diluted with reaction solution containing substrate peptide (5-FAM-EEPLYWSFPAKKK-CONH2) and ATP (2.3 μM) to a hundred times. Then the enzyme activity of this mixture was assayed by the EZ Reader (Caliper Life Sciences, MA). Wells were repeatedly read for 2 h.

6.3. Docking studies

The EGFR kinase domain was extracted from RCSB Protein Data Bank (PDB ID: 2ITY). Compounds were generated using SYBYL program.[23] Gasteiger–Hückel charge was used and the conformation of each compound was minimized using default parameters. Docking studies were performed using GLIDE program.[24] The EGFR kinase domain was processed by minimal minimization with OPLS2005 force field. The grid was sized to 15 Å in each direction at the centre of the binding pocket. Compounds were prepared for docking using Ligprep. Ligand docking was performed in SP mode and flexible option, with up to 100 poses saved per molecule. GLIDE score was consulted for results analyzing.

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