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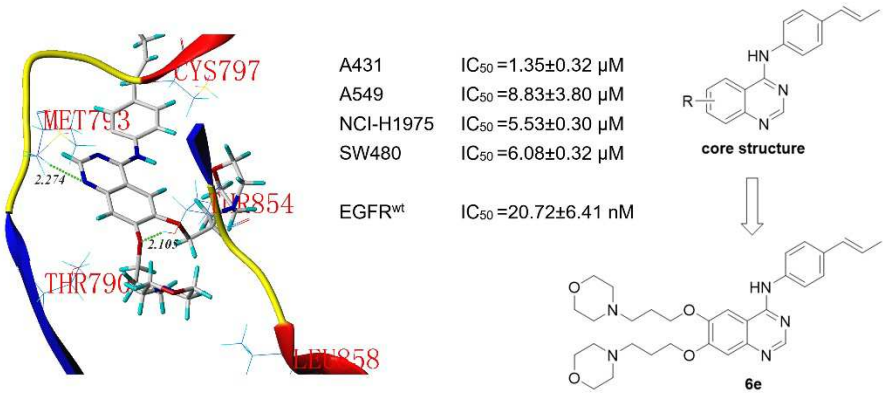
Graphical abstract



Novel 4-arylaminoquinazoline derivatives with (*E*)-propen-1-yl moiety as potent EGFR inhibitors with enhanced antiproliferative activities against tumor cells

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Abstract:

A series of novel 4-anilinoquinazoline derivatives with (*E*)-propen-1-yl moiety were designed, synthesized and evaluated for biological activities *in vitro*. Most compounds exhibited highly antiproliferative activities against all tested tumor cell lines including A431, A549, NCI-H1975 and SW480 cells. Especially, compound **6e** not only presented strong antiproliferative activities against the tested four tumor cell lines (IC_{50} of 1.35, 8.83, 5.53 and 6.08 μM, respectively) which expressed wild type or L858R/T790M double mutant epidermal growth factor receptor (EGFR), but also showed potent inhibitory activity against wild type EGFR (IC_{50} = 20.72 nM). The result of molecular docking with EGFR suggested the binding mode of **6e** was similar to gefitinib, but different from lapatinib. Additionally, western blot analysis showed that **6e** inhibited the phosphorylation of EGFR and its downstream signaling proteins in lung cancer cells. The work could be very useful starting point for developing a new series of tyrosine kinase inhibitors targeting EGFR.

Keywords: 4-anilinoquinazoline, (*E*)-propen-1-yl moiety, antiproliferative activities, epidermal growth factor receptor, tyrosine kinase inhibitors

1. Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase (TK) is a member of the ErbB family [1-4], and regulates essential cellular functions, including proliferation, survival, migration, and differentiation [5]. Overexpression, amplification, and mutation of EGFR occur in a wide range of human cancers [3, 6], and are associated with tumor progression and reduce sensitivity to antitumor agents [7]. Thus EGFR has been considered as one of the main anticancer targets [4, 8]. Some quinazoline derivatives are among the most successful antitumor drugs in the vanguard of targeted therapies [9], such as gefitinib [10], erlotinib [11], lapatinib [12] and afatinib [13, 14] (Fig. 1).

Gefitinib and erlotinib, as ATP-competitive and reversible EGFR inhibitors, have been shown to be particularly beneficial for non-small cell lung cancer (NSCLC) patients harboring common EGFR mutations L858R in exon 21 or del E746-A750 in exon 19 [15-18]. However, the emergence of acquired point mutations, particular T790M mutation, has weakened their therapeutic efficacy, leading to drug resistance to these drugs [4, 19]. Lapatinib, the first dual inhibitor of EGFR and EGFR-2 (HER2) TKs, was approved by FDA in 2007 [12, 20]. Unfortunately, a mandatory black-box warning was released in 2008 because of lapatinib-related hepatotoxicity in clinical trials and post-marketing surveillance, the liver injury was fatal in rare cases [21]. Afatinib, an ATP-competitive anilinoquinazoline derivative and the first irreversible tyrosine kinase inhibitor (TKI), possesses a reactive acrylamide group, which was

designed to covalently bind to a conserved cysteine residue (Cys797) of EGFR and irreversibly block enzymatically active ErbB receptor family members [22, 23]. However, as afatinib, many irreversible TKIs, contain with a reactive "warhead" which can irreversibly bind to proteins other than the target, creating a toxicity burden [23]. Thus there is an urgent demand for new EGFR inhibitors that can effectively treat cancers.

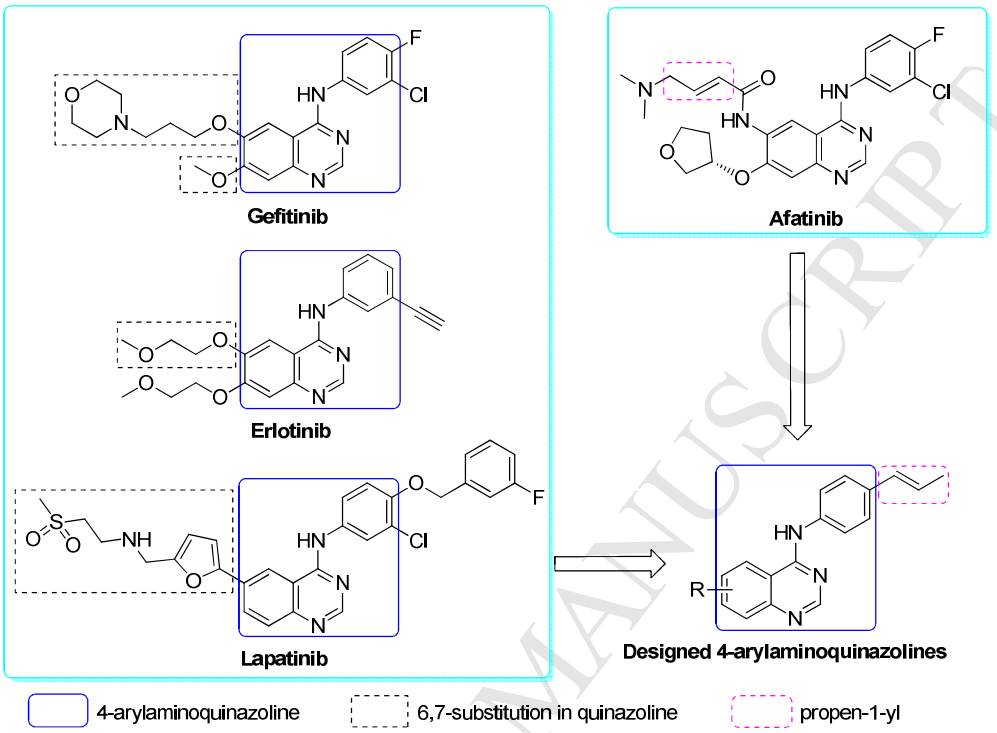


Fig. 1. Structures of some TKIs and design rationale of novel 4-(4-(*E*)-(propen-1-yl)phenylamino)quinazoline derivatives.

Previous studies on the antitumor activity of 4-anilinoquinazolines revealed the relationship of structure and activity as follows: 1) the pyrimidine ring is mandatory, 2) a free NH linker at the 4-position of quinazoline core is clearly optimal, 3) electron-withdrawing lipophilic substituents at the 3-position of the aniline moiety are favorable, and 4) electron-donating groups at the 6- and 7-positions (but not the 5- and 8-positions) of the quinazoline are preferred [4, 24, 25]. As shown in Fig.1, these TKIs have a 4-anilinoquinazoline scaffold [26], and different substituents at 3- or 4-positions of the aniline moiety, and 6- and 7-positions of the quinazoline. In contrast, afatinib has an acrylamide electrophilic moiety as the receptor of Michael addition to form a covalent bond with Cys797 of EGFR. Inspired by the unsaturated feature of afatinib and its analogues neratinib and dacomitinib with acrylamide unit [27], we speculated the introduction of other C=C unsaturated moiety to 4-anilinoquinazoline derivatives (Fig. 1) may lead to a series of new antineoplastic compounds with enhanced antitumor activity and decreased toxicity, which may result from other addition reaction of C=C unit with biomolecules under the catalysis of biological systems. Initially, we introduced a propenyl group to the 4-position of the aniline moiety to yield three compounds, **1a**-**1c** (Fig. 2). The obtained compounds exhibited moderate antiproliferative activities against three human tumor cell lines (IC_{50} in the range of 10.40 – 85.32 μM) [28, 29]. These preliminary results encouraged us to further optimize the structures of these compounds. Thus, we continued to install (*E*)-propenyl substituents to the 4-positions of the aniline moiety, with different hydrophilic fragments introduced to the 6- or 7-positions of quinazoline core (see Fig. 1). These novel 4-(4-(*E*)-(propen-1-yl)phenylamino)quinazoline derivatives were evaluated for their anti-proliferative effects *in vitro* against the $A431^{WT}$ epidermoid carcinoma cells, $A549^{K\text{-}ras\ mutation}$ [30, 31] and

NCI-H1975$^{L858R/T790M}$ NSCLC cells, as well as SW480 colon cancer cells. In order to reveal their antiproliferative mechanism against tumor cells, the most active compound was subjected to the enzymatic inhibition experiment against EGFR^{wt} kinase by ELISA method, and the investigation in its effect on the activation of EGFR and EGFR downstream signaling pathways by western blot.

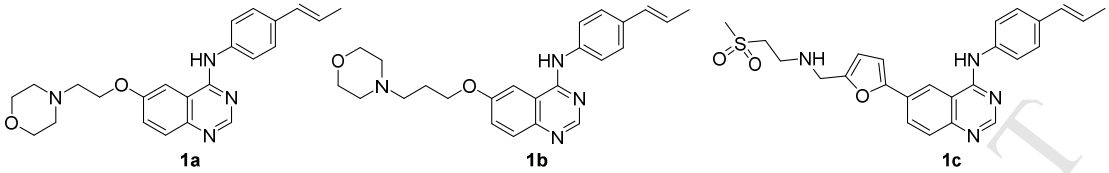
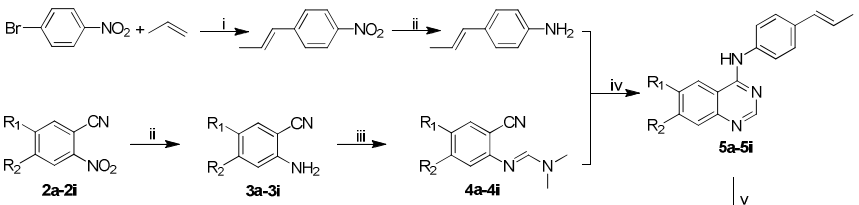


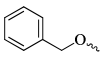
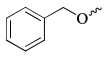
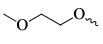
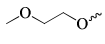
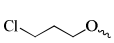
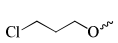
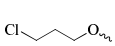
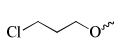
Fig. 2. Previous synthesized compounds **1a**-**1c**.

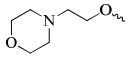
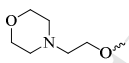
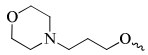
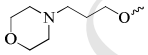
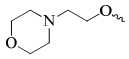
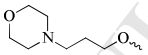
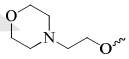
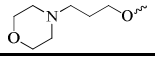
2. Results and discussion

2.1. ***Chemistry***

In this work, a series of new quinazoline derivatives with (*E*)-propenyl were synthesized as shown in Scheme 1. To obtain the 4-(*E*)-(propen-1-yl)aniline building block, Heck reaction of 4-nitro bromobenzene and propene in *N*-methyl pyrrolidone was carried out in the presence of $Pd(OAc)_2$ and Et_3N under 1.6 MPa, 110 °C for 2 h. The obtained mixture was concentrated, washed with water, and purified by silica gel column chromatography to give 1-nitro-4-(*E*)-(propen-1-yl)benzene as yellow needle crystals in 87% yield. 4-(*E*)-(Propen-1-yl)aniline was prepared by the reduction of 1-nitro-4-(*E*)-(propen-1-yl)benzene with $Na_2S_2O_4$ in 63% yield. The configuration of C=C was identified as *E* form deducing from the coupling constant ^3J of hydrogen atoms at double bond in 15.7 Hz in its ^1H NMR. On the other hand, 4,5-disubstituted-2-nitrobenzonitriles (**2**) as starting materials were reduced with $Na_2S_2O_4$ to yield 4,5-disubstituted-2-aminobenzonitriles (**3**) in 66% – 89%. Then, compound **4** was obtained from the reaction of **3** with dimethylformamide-dimethylacetal (DMF-DMA) in toluene in the presence of acetic acid. The target compounds **5a**-**5c** and intermediates **5d**-**5i** were prepared through a Dimroth rearrangement [32] of **4** and 4-(*E*)-(propen-1-yl)aniline in acetic acid. Finally, **5d**-**5i** were treated with morpholine in the presence of potassium iodide to give the desired quinazoline derivatives **6d**-**6i**.



Comp.	R_1	R_2
2a-5a		
2b-5b		
2c-5c		
2d-5d		
2e-5e		
2f-5f		
2g-5g		
2h-5h		
2i-5i		

Comp.	R_3	R_4
6d		
6e		
6f		
6g		
6h		
6i		

Scheme 1. Synthetic route of the target compounds **5a-5c**, **6d-6i**.

Reagents and conditions. i. $Pd(OAc)_2$, Et_3N, *N*-methyl pyrrolidone, 1.6 MPa, 110 °C, 2 h, 87%; ii. (1) $Na_2S_2O_4$, $EtOH/H_2O$, 50 °C, 20 – 30 min, (2) HCl, 70 – 80 °C, 2 h, (3) $NaOH/H_2O$, 63% – 89%; iii. DMF-DMA, AcOH, 35 – 40 °C, 15min, 61% – 94%; iv. 4-(*E*)-(propen-1-yl)aniline, AcOH, 130 °C, 15 min, 63% – 92%; v. KI, morpholine, 120 °C, 0.5 – 1.5 h, 50% – 76%.

2.2. Biological activities

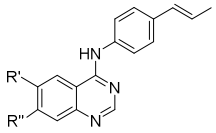
2.2.1. In vitro antiproliferative activity

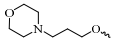
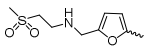
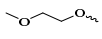
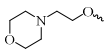
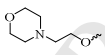
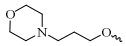
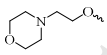
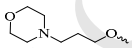
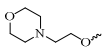
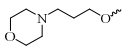
All the target compounds were evaluated for their ability to inhibit the proliferation of the four different human tumor cell lines, including $A431^{WT}$ epidermoid carcinoma cells, $A549^{K\text{-ras mutation}}$ and $NCI\text{-}H1975^{L858R/T790M}$ NSCLC cells, as well as SW480 colon cancer cells. To measure the IC_{50} for tumor cell proliferative inhibition, the indicated compound was serially diluted with dimethyl sulfoxide, and then diluted with the cell culture medium. Finally they were added to 96 well plates, which was pre-seeding cells at certain cell density, to produce an 8-point dose-response curve, and the cell proliferation was measured by MTT assay. Gefitinib and lapatinib were used as reference compounds. The results are listed in Table 1. For comparison, the bioactive data of the previously identified compounds **1a-1c** are also showed in Table 1. Except compound **5a**, most synthesized compounds showed significant antiproliferative activities with IC_{50} values in the range of 1.35 - 57.71 μM against A431 cells, which has a high expression of wild type EGFR. Compounds **6e** and **6i** were the most potent agents (with IC_{50} values of 1.35 and 6.34 μM, respectively) against A431 cells. Assays with A549 NSCLC cells, harbored wild type EGFR and K-ras mutation, were also performed. As shown in Table 1, all the synthesized compounds have antiproliferative activity against A549 cells. Compounds **1b**, **6d**, **6e**, **6g** and **6i** (with IC_{50} of 17.43, 14.09, 8.83, 10.32 and 10.32 μM, respectively) possessed higher activities than gefitinib (IC_{50} of 21.17 μM), whilst, **6d**, **6e**, **6g** and **6i** also showed higher activity than lapatinib

(IC_{50} of 14.90 μM). We then assessed the inhibitory efficacy of these compounds against the drug-resistant NSCLC cell line NCI-H1975, which harbors EGFR-L858R/T790M double mutants. Compounds **6e** and **6i** showed high inhibitory effects. Particularly, compound **6e** exhibited excellent inhibitory activity with IC_{50} of 5.53 μM. The result of antiproliferative test on SW480 cells indicated that the whole series displayed inhibitory effects against SW480 cells. Especially, compounds **1b**, **6e** and **6i** (with IC_{50} values of 10.40, 6.08 and 11.63 μM, respectively) showed higher inhibitory activities than gefitinib and lapatinib (IC_{50} of 12.50 and 12.58 μM, respectively).

Table 1

The antiproliferative effects of the synthesized compounds *in vitro* and the kinase inhibitory activities.[a]



Compounds			IC_{50} (μM)[b]				IC_{50} (nM)[c]
	R′	R″	A431	A549	NCI-H1975	SW480	EGFR-TK
gefitinib			4.45±0.25	21.17±0.47	12.70±2.98	12.50±0.28	3.22±1.48
1a		H	57.71±2.72	85.32±2.07	>100[d]	62.58±14.80	N.D.[e]
1b		H	12.03±0.67	17.43±3.22	23.86±4.57	10.40±0.94	N.D.[e]
1c		H	37.56±2.79	84.50±13.12	>100[d]	56.34±3.03	N.D.[e]
5a			>100[d]	38.18±11.56	>100[d]	34.03±4.08	N.D.[e]
5b			28.11±0.04	36.45±12.95	44.13±2.96	37.04±4.46	N.D.[e]
5c			23.88±1.05	24.74±1.25	27.67±7.04	43.65±4.06	N.D.[e]
6d			10.07±2.41	14.09±1.03	89.34±9.98	29.21±12.13	N.D.[e]
6e			1.35±0.32	8.83±3.80	5.53±0.30	6.08±0.32	20.72±6.41
6f			47.08±2.89	26.47±1.86	>100[d]	28.19±8.66	N.D.[e]
6g			13.91±2.05	10.32±2.68	38.88±14.16	14.66±2.17	N.D.[e]
6h			22.41±9.76	37.53±14.16	>100[d]	27.56±6.30	N.D.[e]
6i			6.34±0.97	10.32±1.23	10.77±2.75	11.63±2.69	N.D.[e]
lapatinib			4.80±0.71	14.90±1.21	9.08±5.82	12.58±1.35	27.06±3.77

[a] The values are mean±SD of at least three independent experiments. [b] The antiproliferative effects of the synthesized compounds against human cancer cells gathered with MTT assay. [c] The inhibitory effects of selected compounds on recombinant $EGFR^{WT}$-TK from ELISA analyses. [d] IC_{50} was not calculated because less than 50% inhibition was observed at the highest concentration (100 μM). [e] N.D. indicated IC_{50} not determined.

For the synthesized 6,7-disubstituted quinazoline derivatives, most compounds containing a morpholino moiety exhibited higher antiproliferative activities against the tested cells than other compounds such as **5a**, **5b** and **5c**. Interestingly the activities of quinazoline derivatives with morpholinopropoxy group were higher than that of compounds with morpholinoethoxyl group. Notably, compound **6e** with bis(morpholinopropoxy) groups showed the highest antiproliferative activities against all tested four tumor cells (IC_{50} of 1.35, 8.83, 5.53 and 6.08 μM). In addition, as shown in Fig. 3, compound **6e** exhibited a good dose-response relationship when four tumor cells were treated with different concentration of **6e** for 72 h.

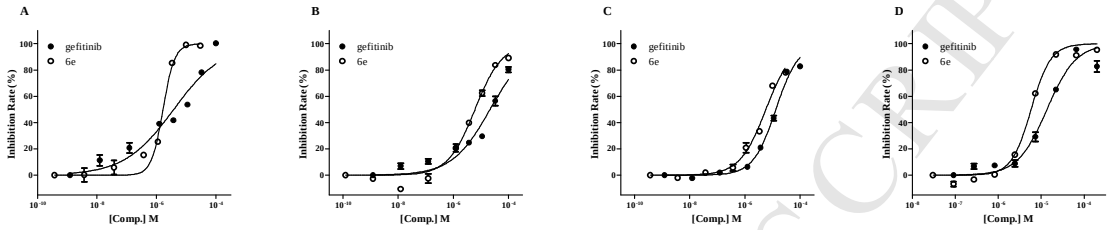


Fig. 3. Dose response curve of inhibition rate to series concentrations of **6e** and gefitinib against the four human tumor cell lines. (A) A431 cells, (B) A549 cells, (C) NCI-H1975 cells, (D) SW480 cells. Cells were treated with **6e** or gefitinib at the series indicated concentrations, and viable cells were measured after 72 h of treatment. All error bars represent in mean±SE.

2.2.2. Kinase inhibitory activity

Compound **6e** presented remarkable antiporliferative activities against the tested four tumor cell lines. Especially, it exhibited strong activity against A431 cells with highly expressed wild type EGFR. We speculated **6e** may display its activity through the inhibition of EGER signaling pathway, and be a potential EGFR inhibitor. Thus, the inhibitory effect of **6e** on $EGFR^{WT}$-TK enzymatic activity was evaluated with recombinant human EGFR protein and anti-phosphotyrosine antibody by ELISA method. Herein, two EGFR inhibitors, gefitinib and lapatinib, were used as reference compounds. The experiment data are listed in Table 1. The results showed that compound **6e** had higher inhibition activity (IC_{50} = 20.72 nM) against EGFR than lapatinib (IC_{50} = 27.06 nM). This undoubtedly demonstrated that **6e** was a potent EGFR inhibitor.

2.3. Molecular modeling

To predict the binding mode of compound **6e** with EGFR, a docking study of **6e** into the active site of the EGFR (PDB ID: 1xkk) were performed using Surflex-Dock module of SYBYL-X 2.1. As our previous work [33], the calculated root-mean-square deviation (RMSD) between the best docked pose and the observed pose of lapatinib in crystal from X-ray diffraction analysis was 1.053 Å. The reference compound gefitinib was also docked into the active site of the EGFR (Fig. 4A). The docking results indicated that compound **6e** has a similar binding mode to that displayed by gefitinib, but in different pose compared with lapatinib as shown in Fig. 4. The three compounds, gefitinib, **6e** and lapatinib, all formed a hydrogen bond from the N1 of quinazoline core to the main chain NH of Met793, and the length of hydrogen bond were 2.461, 2.274 and 1.908 Å, respectively. Comparing with gefitinib and lapatinib, another hydrogen bond existed between **6e** and EGFR from the oxygen atom in the 7-(3-morpholinopropoxy) group of **6e** to the residue Thr854 of EGFR with the length of 2.105 Å

(Fig. 4B). Additionally, a second hydrogen bond between lapatinib and EGFR was seen, from the F atom in 3-fluorobenzyloxy moiety of lapatinib to the residue Thr790 of EGFR with length of 2.676 Å (Fig. 4C). These all indicated that the introduction of (*E*)-propen-1-yl group and two 3-morpholinopropoxy to the 4-anilinoquinazoline core of **6e** lead to a new binding interactions with the EGFR-TK domain, which contributes to its inhibitory activity against EGFR.

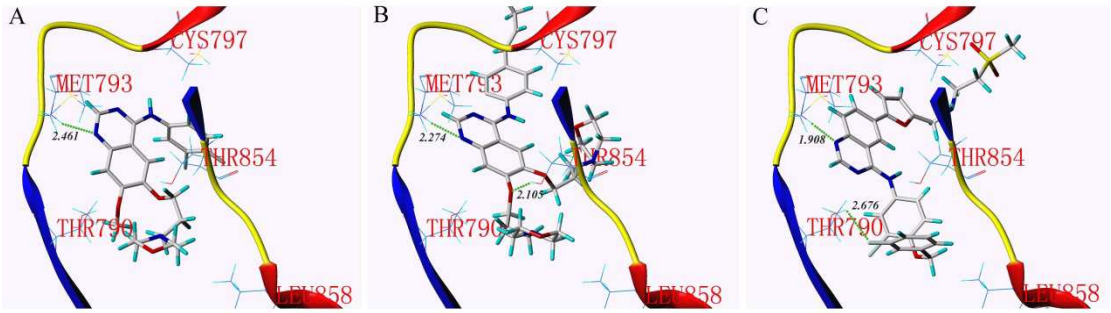


Fig. 4. Binding modes between EGFR-TK and representative compounds predicted by Surflex-Dock program. (A) gefitinib within 1xkk; (B) **6e** within 1xkk; (C) lapatinib within 1xkk. The hydrogen bonds were illustrated as green dashed lines and the length of hydrogen bonds was illustrated in numbers (unit in Å).

2.4. The effect of 6e on EGFR signaling pathways

In order to further investigate the antiproliferative mechanism of compound **6e** against tumor cells, we examined the effects of **6e** on the activation of EGFR and its downstream signaling proteins in human lung cancer cell lines A549 and NCI-H1975 by western blot analysis. As shown in Fig. 5A, when A549 cells were treated with 25 μM of **6e** or gefitinib, it was found that the phosphorylations of EGFR and its downstream signaling proteins induced by epidermal growth factor (EGF) were significantly inhibited by **6e** or gefitinib resulting from the decreased levels of p-Tyr/EGFR (p-Tyr), p-EGFR (Y1086), p-EGFR (Y992), p-Akt (S473), p-Erk1/2 (T202/Y204). Even when the concentration of **6e** was at 5 μM, the above phosphorylated proteins could also be effectively down-regulated. Importantly, the total levels of EGFR, Akt, and Erk1/2 proteins remained unchanged, and the Stat3/p-Stat3 pathway was not influenced by **6e**. These results revealed that compound **6e** exerted its antiproliferative effect against A549 tumor cells via the inhibition of the EGF/EGFR signaling pathway and its downstream Akt and Erk1/2 signaling pathways.

Compound **6e** also exhibited antiproliferative activity against NCI-H1975 cells (IC_{50} = 5.53μM). When NCI-H1975 cells were treated with different concentrations of **6e**, the western blot analysis showed that the levels of p-Tyr/EGFR, p-EGFR (Y1068) and p-EGFR (Y992) were decreased by **6e** in a dose-dependent manner (see Fig. 5B). However the levels of p-Akt (S473) and p-Erk1/2 (T202/Y204) were only slightly down-regulated at the high concentration of **6e** (25 μM). Interestingly, the level of p-Stat3 (Y705) was also decreased at the high concentration of **6e**, which was not observed in the presence of gefitinib (25 μM). This indicated that the antiproliferative mechanism of compound **6e** against NCI-H1975 cells was different from that of gefitinib, although they all exhibited the similar inhibition of EGFR activity. Additionally, the above results suggested that compound **6e** mainly block the EGF/EGFR and the downstream p-Stat3/Stat3 pathways to achieve its potent antiproliferative activity against NCI-H1975 cells.

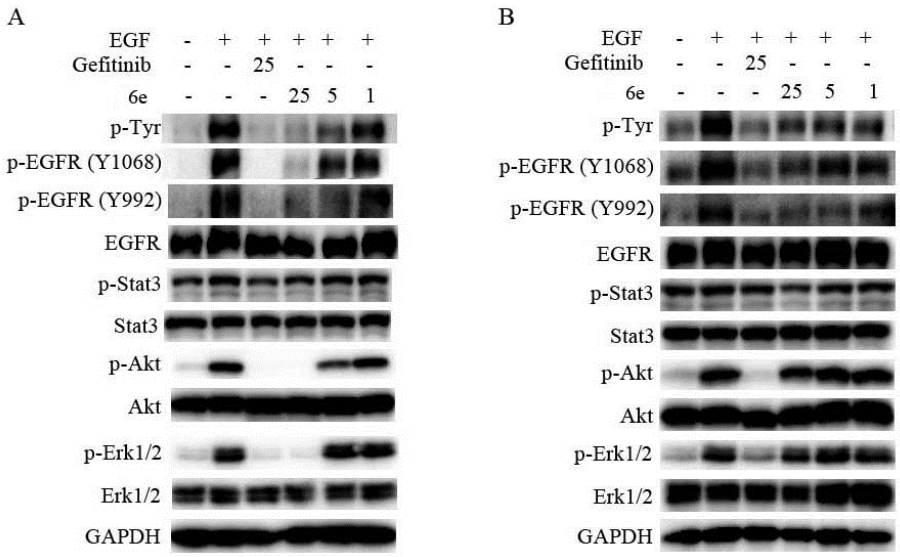


Fig. 5. Western-blot analysis of **6e** against A549 cells (A) and NCI-H1975 cells (B). Cells were cultured in the presence of different concentrations of **6e** or gefitinib (25 μM) for 2 h and stimulated with 50 ng/mL EGF for 10min, then harvested. Whole-cell lysate was analyzed for total EGFR, State, Akt and Erk1/2, as well as p-Tyr/EGFR, p-EGFR (Y1068), p-EGFR (Y992), p-Stat3 (Y705), p-Akt (S473) and p-Erk1/2 (T202/Y204) by immunoblotting. A representative anti-GAPDH immunoblot was showed as loading control.

3. Conclusions

In summary, we have designed and synthesized a series of novel quinazoline derivatives with (*E*)-propen-1-yl substituents at the aniline moiety. These compounds were subjected to biological activities evaluation including antiproliferative effects against four tumor cell lines (A431, A549, NCI-H1975 and SW480). Most of the synthesized compounds exhibited moderate to excellent antiproliferative activities against the tested tumor cell lines. Notably, compound **6e** not only exhibited excellent antiproliferative activities against the tumor cells which expressed wild type or mutant EGFR (with the IC_{50} values in the range of 1.35 - 8.83 μM), but also showed potent inhibitory activity toward $EGFR^{WT}$ (IC_{50} = 20.72 nM). Molecular docking showed that **6e** could form two hydrogen bonds with EGFR, and the binding pose of **6e** was similar to that of gefitinib, while different from that of lapatinib. In addition, the results of western blot analysis indicated that **6e** could inhibit EGF-induced EGFR activation in human lung cancer A549 and NCI-H1975 cells. However, EGF-induced EGFR downstream phosphorylation proteins, such as p-Akt (S473), p-Erk1/2 (T202/Y204) and p-Stat3 (Y705), displayed different levels on the two tested cell lines respectively under specified concentration of **6e**, which indicated affirmatively that **6e** interacted with the signaling proteins in different manner in A549 and NCI-$H1975^{L858R/T790M}$ cells. The above facts suggested compound **6e** is a potent EGFR TKI, and may possess inhibitory effect against drug resistance tumor. This work would be very useful for developing a new series of TKIs targeting EGFR.

4. Materials and methods

4.1. Chemistry

All commercially available starting materials, reagents and solvents were used without further purification unless otherwise noted. Melting point (m.p) was determined on X-6 micro melting point

apparatus, and was uncorrected. High-resolution mass spectra (HRMS) were obtained on a BrukerEspuire 3000plus instrument. ^{1}H NMR and ^{13}C NMR spectra were recorded on 300, 400, 600 MHz (Bruker Avance) instruments, and chemical shifts were reported in parts per million (ppm) relative to the internal standard tetramethylsilane (TMS). Coupling constants (*J*) were reported in Hz. Spin multiplicities were described as s (singlet), brs (broad singlet), d (double), t (triplet), q (quartet), or m (multiplet). Infrared Spectroscopy (IR) was measured on Nicolet 170SXFT-IR instrument. Chromatographic separations were performed on silica gel columns by flash column chromatography. Reactions were followed by thin layer chromatography (TLC) on pre-coated silica gel F254 plates and then visualized in an iodine chamber or with a UV lamp.

4.1.1. General procedure for the synthesis of **3a-3i**

A mixture of **2** (0.01 mol) in water (15 mL) and ethanol (15 mL) was stirred and heated up to 50 °C. $Na_2S_2O_4$ (0.03 mol) was added within an hour. The resultant mixture was stirred for about 30 min. Then concentrated HCl (8 mL) was added slowly in 2 h at 70 – 80°C. The mixture was cooled to 25 °C and adjusted pH to 9 – 11 with 20% sodium hydroxide (100 mL). The obtained mixture was extracted with ethyl acetate (3 × 60 mL) and the combined organic extracts were washed with water (2 × 100 mL) and brine (1 × 100 mL), then dried over anhydrous Na_2SO_4. The organic solvent was evaporated to give compound **3** as green yellow solid (yield 66 – 89%).

4.1.2. General procedure for the synthesis of **4a-4i**

A mixture of **3** (2.00 mmol), toluene (5 mL), and DMF-DMA (4.00 mmol) were heated up to 35 – 40 °C and acetic acid (0.10 mL) was added. After 15 min, the resultant mixture was cooled to approximately 25 °C. The solvent was completely evaporated. The residue was added to water and basified pH to 13 by the addition of 20% sodium hydroxide. The obtained mixture was extracted with methylene chloride (2 × 15 mL) and the combined organic extracts were washed with water (2 × 20 mL) and brine (1 × 70 mL), then dried over anhydrous $MgSO_4$. The organic solvent was evaporated to give **4** as oil (Yield 61 – 94%).

4.1.3. General procedure for the synthesis of **5a-5i**

Compound **4** (1.00 mmol) was added to the mixture of acetic acid (2.0 mL) and 4-(*E*)-(propen-1-yl)aniline (1.10 mmol). The reaction mixture was heated to 130 °C with stirring for 15 min. The mixture was then cooled to 25 °C. The acetic acid was evaporated under reduced pressure. The residue was added to ice-water (10 mL), and adjusted pH to 9 with an ammonia solution. Then it was stirred for 0.5 h. The precipitated product was filtered, and the filtered cake was washed with water (3 × 10 mL) to give crude product. The crude product was purified by silica gel chromatography (eluting with EtOAc/petroleum ether (1/4, v/v)) to afford **5** (yield 63 – 92%).

4.1.3.1. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-7,8-dihydro-[1,4]dioxino[2,3-*g*]quinazoline (**5a**)

Yield: 91%; yellow solid; m.p.: 249.5 – 251.3 °C; HRMS ($C_{19}H_{17}N_3O_2$) *m/z* $[M+H]^+$: calculated: 320.1399, found: 320.1386; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 9.47 (s, 1H), 8.43 (s, 1H), 8.09 (s, 1H), 7.82 (d, *J* = 8.1 Hz, 2H), 7.38 (d, *J* = 8.1 Hz, 2H), 7.17 (s, 1H), 6.39 (d, *J* = 15.7 Hz, 1H), 6.28 - 6.20 (m, 1H), 4.41 (br s, 2H), 4.40 (br s, 2H),1.85 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 156.9, 153.5, 149.4, 146.5, 144.0, 138.7, 133.0, 131.0, 126.2, 124.5, 122.4, 113.0, 110.5, 108.9, 65.0, 64.6, 18.8; IR ν_{max}(KBr) cm^{-1}: 3440, 3023, 2924, 1634, 1608, 1569, 1509, 1459, 1236, 1067, 961, 903.

4.1.3.2. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-6,7- bis(benzyloxy)quinazoline (**5b**)

Yield: 71%; yellow solid; m.p.: 164.8 – 165.5 °C; HRMS ($C_{31}H_{27}N_3O_2$) *m/z* $[M+H]^+$: calculated: 474.2182, found: 474.2186; ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 9.49 (s, 1H), 8.46 (s, 1H), 8.08 (s, 1H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.56 (d, *J* = 7.0 Hz, 2H), 7.50 (d, *J* = 7.0 Hz, 2H), 7.44 - 7.33 (m, 8H), 7.31 (s, 1H), 6.41 (d, *J* = 16.0 Hz, 1H), 6.32 - 6.10 (m, 1H), 5.34 (s, 2H), 5.30 (s, 2H), 1.86 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (151 MHz, DMSO-d_6) δ(ppm): 172.0, 156.3, 153.4, 153.0, 148.1, 146.9, 138.2, 136.6, 136.5, 132.6, 130.5, 128.5, 128.5, 128.1, 127.9, 127.4, 125.7, 124.1, 122.2, 109.0, 109.0, 104.1, 70.6, 69.8, 18.2; IR ν_{max}(KBr) cm^{-1}: 3297, 3036, 2916, 1680, 1626, 1605, 1587, 1515, 1452, 1252, 1021, 965, 837.

4.1.3.3. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-6,7-bis(2-methoxyethoxy)quinazoline (**5c**)

Yield: 74%; faint yellow solid. m.p.: 151.3 – 152.8 °C; HRMS ($C_{23}H_{27}N_3O_4$) *m/z* $[M+H]^+$: calculated: 410.2080, found: 410.2085; ^{1}H NMR (600 MHz, $CDCl_3$) δ(ppm): 8.62 (s, 1H), 7.69 (s, 1H), 7.60 (d, *J* = 8.4 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 2H), 7.26 (s, 1H), 7.16 (s, 1H), 6.37 (dd, *J* =1.6, 15.7 Hz, 1H, -CH=CH-), 6.18 (dq, *J* = 15.7, 6.6 Hz, 1H, -CH=CH-), 4.20 - 4.17 (m, 4H), 3.79 - 3.78 (m, 2H), 3.75 (t, *J* = 4.8 Hz, 2H), 3.41 (s, 3H), 3.40 (s, 3H), 1.88 (dd, *J* =1.6, 6.6 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ(ppm): 156.5, 154.5, 153.8, 148.7, 147.4, 137.3, 134.2, 130.4, 126.4, 125.0, 122.0, 109.2, 108.7, 102.9, 70.9, 70.4, 69.2, 68.3, 59.3, 59.2, 18.5; IR ν_{max}(KBr) cm^{-1}: 3426, 3020, 2926, 2883, 1620, 1576, 1514, 1427, 1244, 1123, 966, 856.

4.1.4. General procedure for the synthesis of **6d-6i**

A mixture of **5** (0.11 mmol), potassium iodide (0.06 mmol) and morpholine (1 mL) was heated to 120 °C for 0.5 – 1.5 h, then filtered, and washed with CH_2Cl_2. The combined mixture of filtering liquor and washing liquor was dried ($MgSO_4$) and evaporated. The residue was purified by silica gel column chromatography with MeOH/EtOAc (1:10) to give the titled compounds (yield 50 – 76%).

4.1.4.1. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-6,7-bis(2-morpholinoethoxy)quinazoline (**6d**)

Yield: 73%; m.p.: 197.4 – 198.5 °C; HRMS ($C_{29}H_{37}N_5O_4$) *m/z* $[M+H]^+$: calculated: 520.2924, found: 520.2926; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 9.43 (s, 1H), 8.46 (s, 1H), 7.87 (s, 1H), 7.76 (d, *J* = 8.5 Hz, 2H), 7.38 (d, *J* = 8.5 Hz, 2H), 7.20 (s, 1H), 6.39 (d, *J* = 15.8 Hz, 1H), 6.31 - 6.09 (m, 1H), 4.27 - 4.23 (m, 4H), 3.71 - 3.50 (m, 8H), 2.81 - 2.75 (m, 4H), 2.60 - 2.53 (m, 8H), 1.85 (d, *J* = 6.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 158.1, 155.4, 154.8, 149.9, 148.8, 140.1, 134.4, 132.4, 127.6, 125.9, 124.1, 110.8, 110.0, 105.1, 69.1, 68.8, 68.1, 61.6, 58.8, 58.6, 55.7, 55.7, 20.2; IR ν_{max}(KBr)cm^{-1}: 3296, 3077, 2964, 1648, 1514, 1454, 1427, 1262, 1038, 969, 855.

4.1.4.2. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-6,7-bis(3-morpholinopropoxy)quinazoline (**6e**)

Yield: 50 %; m.p.: 91.3 – 92.2 °C; HRMS ($C_{31}H_{41}N_5O_4$) *m/z* $[M+H]^+$: calculated: 548.3237, found: 548.3246; ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 9.47 (s, 1H), 8.44 (s, 1H), 7.86 (s, 1H), 7.76 (d, *J* = 8.2 Hz, 2H), 7.39 (d, *J* = 8.2 Hz, 2H), 7.17 (s, 1H), 6.40 (d, *J* = 16.0 Hz, 1H), 6.31 - 6.12 (m, 1H), 4.25 - 4.10 (m, 4H), 3.34 - 3.55 (m, 12H), 2.45 - 2.31 (m, 8H), 2.05 - 1.90 (m, 4H), 1.85 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ(ppm): 156.2, 153.7, 152.8, 148.3, 146.9, 138.2, 132.5, 130.5, 125.7, 124.0, 122.2, 108.9, 107.9, 103.2, 67.1, 66.5, 66.1, 66.1, 54.9, 54.8, 53.4, 53.3, 25.8, 25.6, 18.2; IR ν_{max}(KBr) cm^{-1}: 3435, 3026, 2935, 2839, 1613, 1500, 1435, 1371, 1226, 1113,984.

4.1.4.3. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-7-methoxy-6-(2-morpholinoethoxy)quinazoline (**6f**)

Yield: 71%; m.p.: 204.9 – 205.9 °C; HRMS ($C_{24}H_{28}N_4O_3$) *m/z* $[M+H]^+$: calculated: 421.2240, found: 421.2243; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 9.43 (s, 1H), 8.47 (s, 1H), 7.86 (s, 1H), 7.75 (d, *J* = 8.4 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 2H), 7.19 (s, 1H), 6.40 (dd, *J* =1.1, 15.7 Hz, 1H, -CH=CH-), 6.23 (dq, *J* = 15.7, 6.4 Hz, 1H, -CH=CH-), 4.26 (t, *J* = 6.0 Hz, 2H), 3.94 (s, 3H), 3.63 - 3.58 (m, 4H), 2.81 (t, *J* = 6.0 Hz, 2H), 2.57 - 2.52 (m, 4H), 1.85 (dd, *J* =1.1, 6.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 156.2, 154.2, 152.9, 148.0, 146.9, 138.2, 132.6, 130.5, 125.7, 124.0, 122.2, 108.9, 107.3, 102.8, 66.6, 66.2, 56.9, 55.8, 53.7, 18.2; IR ν_{max}(KBr) cm^{-1}: 3418, 3020, 2932, 2850, 1624, 1578, 1514, 1117, 964, 847.

4.1.4.4. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-7-methoxy-6-(3-morpholinopropoxy)quinazoline (**6g**)

Yield: 76%; m.p.: 186.9 – 187.4 °C; HRMS ($C_{25}H_{30}N_4O_3$) *m/z* $[M+H]^+$: calculated: 435.2396, found: 435.2395; ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm): 9.47 (s, 1H, NH), 8.46 (s, 1H), 7.84 (s, 1H), 7.74 (d, *J* = 8.0 Hz, 2H), 7.39 (d, *J* = 8.0 Hz, 2H), 7.18 (s, 1H), 6.37 (d, *J* = 16.0 Hz, 1H, -CH=CH-), 6.27 - 6.18 (m, 1H), 4.18 (t, *J* = 5.9 Hz, 2H), 3.93 (s, 3H), 3.58 (brs, 4H), 2.47 (t, *J* = 7.0 Hz, 2H), 2.38 (brs, 4H), 2.00 - 1.97 (m, 2H), 1.85 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ(ppm): 156.2, 154.3, 152.8, 148.2, 146.9, 138.2, 132.5, 130.5, 125.7, 124.0, 122.2, 108.9, 107.2, 102.7, 67.1, 66.1, 55.8, 54.9, 53.4, 25.9, 18.2; IR ν_{max}(KBr) cm^{-1}: 3418, 3022, 2953, 2870, 1622, 1512, 1427, 1240, 1113, 955, 858.

4.1.4.5. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-6-methoxy-7-(2-morpholinoethoxy)quinazoline (**6h**)

Yield: 55%; m.p.: 215.1 – 216.8 °C; HRMS ($C_{24}H_{28}N_4O_3$) *m/z* $[M+H]^+$: calculated: 421.2240, found: 421.2227; ^{1}H NMR (600 MHz, $CDCl_3$) δ(ppm): 8.64 (s, 1H), 7.70 (s, 1H), 7.59 (d, *J* = 8.4 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 2H), 7.21 (s, 1H), 7.15 (s, 1H), 6.36 (d, *J* = 15.6 Hz, 1H, -CH=CH-), 6.22 - 6.13 (m, 1H), 4.23 (t, *J* = 5.8 Hz, 2H), 3.90 (s, 3H), 3.73 – 3.70 (m, 4H), 2.88 (t, *J* = 5.8 Hz, 2H), 2.58 (brs, 4H), 1.89 (d, *J* = 5.7 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ(ppm): 156.4, 153.9, 153.6, 149.6, 147.1, 137.1, 134.3, 130.4, 126.4, 125.1, 122.1, 109.2, 108.5, 100.0, 66.9, 66.8, 57.0, 56.3, 54.1, 18.5; IR ν_{max}(KBr) cm^{-1}: 3418, 3026, 2947, 2856, 1622, 1512, 1420, 1240, 1105, 970, 851, 787.

4.1.4.6. 4-(4-(*E*)-(Propen-1-yl)phenylamino)-6-methoxy-7-(3-morpholinopropoxy)quinazoline (**6i**)

Yield: 50%;. m.p.: 170.3 – 171.9 °C; HRMS ($C_{25}H_{30}N_4O_3$) *m/z* $[M+H]^+$: calculated: 435.2396, found: 435.2382; ^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 8.64 (s, 1H), 7.64 (s, 1H), 7.58 (d, *J* = 8.4 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 2H), 7.23 (s, 1H), 7.13 (s, 1H), 6.38 (d, *J* = 15.7 Hz, 1H, -CH=CH-), 6.23 - 6.14 (m, 1H), 4.17 (t, *J* = 6.6 Hz, 2H), 3.92 (s, 3H), 3.75 – 3.68 (m, 4H), 2.53 (t, *J* = 7.2 Hz, 2H), 2.46 (brs, 4H), 2.10 - 2.03 (m, 2H), 1.88 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ(ppm): 156.4, 154.1, 153.6, 149.6, 147.3, 137.1, 134.3, 130.4, 126.4, 125.1, 122.1, 109.0, 108.4, 99.8, 67.3, 66.9, 56.3, 55.3, 53.7, 25.9, 18.5; IR ν_{max}(KBr) cm^{-1}: 3379, 3024, 2957, 2932,2 851, 1624, 1512, 1421, 1244, 1117, 964, 862, 787.

4.2. Biology

4.2.1. *In vitro* antiproliferative activity assay

Dulbecco's modified Eagle's medium (DMEM), 1640 and fetal bovine serum (FBS) were obtained from Gibco. Penicillin, streptomycin and L-glutamine were purchased from Merck Millipore. A431 (epidermoid carcinoma, EGFR wild type and overexpression), A549 (NSCLC, EGFR wild type, K-ras mutation), NCI-H1975 (NSCLC, EGFR L858R/T790M mutation) and SW480 (colon cancer, EGFR

wild type) cells were kindly provided by Cell Bank, Chinese Academy of Sciences. Cells were plated in 96-well plates in regular DMEM or 1640 supplemented with 10% (v/v) FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 mM L-glutamine. After 24 h incubation at 37 °C in a humidified incubator containing 5% CO_2, cells were exposed to different concentrations of each compounds or 0.5% DMSO, then incubated for further 72 h. Cell viability was determined by MTT assay. Briefly, following drug treatment, cells were incubated with 20 μl MTT (5 mg/ml) for 4 h at 37 °C. Next, the medium was discarded and 150 μL DMSO were added to dissolve the formazan product. The absorbance was measured using a multi-well spectrophotometer (PerkinElmer Enspire) at 570 nm, and 630 nm as reference wavelength. The IC_{50} values were calculated from the inhibition curves by nonlinear regression analysis using GraphPad Prism5 (GraphPad Software, Inc.).

4.2.2. *In vitro* EGFR tyrosine kinase assay

Recombinant EGFR was purchased from Sino Biology Inc. Anti-phosphotyrosine mouse mAb was purchased from PTM Bio. The effects of compounds on the activity of EGFR kinase were determined by enzyme-linked immunosorbent assays (ELISAs) with recombinant EGFR according to reported methods [33, 34].

4.2.3. Molecular docking

All the calculations were carried out on a Lenovo PC with Windows 8.1 system using the Tripos Sybyl-X 2.1 (TriposInc, St Louis, MO, USA) molecular modeling package. The crystal structural data of EGFR kinase domain complexed with lapatinib (PDB code: 1xkk) was obtained from RCSB Protein Data Bank [35]. Molecular docking was carried out as previous reported [33].

4.2.4. Western blot analysis

Western blot analysis was carried out according to a reported method [36]. Cells were grown in 6-well plates in media containing with 10% (v/v) FBS until reached 90% confluence, the cultured cells were serum-starved for 24 h, after which they were exposed to a dose range of each drug for 2 h and subsequently treated with 50 ng/mL EGF for 10 min at 37 °C. After washing 3 times with cold PBS, they were resuspended in 100 μL ice-cold RIPA lysis buffer, together with protease and phosphatase inhibitor tablet. The lysates were kept on ice for 30 min with occasional agitation and collected by centrifugation at 12,000 ×g for 20 min at 4 °C. The protein concentration in the supernatant was determined using a BCA protein assay kit. The samples were prepared in SDS-PAGE loading buffer, and then boiled for 10 min at 95 °C. Equal amounts of protein (20 μg) were added to a 10% SDS-polyacrylamide gel (PAGE) electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Non-specific binding on the membrane was minimized with a blocking buffer containing non-fat dry milk (5%) in TBS. Thereafter, immunoblotting was performed according to the antibody manufacturers' recommendations. The primary antibody-bound membranes were washed for 5 min × 5 times with a washing buffer (TBS solution containing 0.1% Tween-20), then incubated with corresponding secondary antibodies conjugated with horseradish peroxidase. After a 30 min washing, immune reactive signals were visualized by enhanced chemiluminescence using ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA). All antibodies were obtained from Cell Signaling Technology.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.

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Highlights

1. A series of novel 4-(4-(*E*)-(propen-1-yl)phenylamino)quinazoline derivatives were designed and synthesized.
2. Most synthesized compounds exhibited good antiproliferative activities against human tumor cells harboring wild type or L858R/T790M double mutant EGFR.
3. Molecular docking suggested that the binding mode of compound **6e** with EGFR was similar to gefitinib, but different from lapatinib.
4. Compound **6e** can significantly inhibit EGFR activity, and suppress the phosphorylation of EGFR and its downstream signaling proteins in tumor cells.