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NH | EGFR^{L8} EGFR^W NCI-H19 A431 73

EGFR^{L858R/T790M} 4.1 nM EGFR^{WT} 350 nM NCI-H1975 59 nM A431 7368 nM

Discovery of Novel 2,4-Diarylaminopyrimidine Derivatives as Potent and Selective Epidermal Growth Factor Receptor (EGFR) inhibitors against

L858R/T790M Resistance Mutation

Qi Yan^{1,#}, Yuzhe Chen^{2,3,#}, Baiyou Tang^{2,4}, Qiang Xiao¹, Rong Qu^{2,3}, Linjiang Tong²,

Jian Liu¹, Jian Ding², Yi Chen², Ning Ding¹, Wenfu Tan^{1*}, Hua Xie^{2,*}, Yingxia Li^{1,*}

¹School of Pharmacy, Fudan University, Shanghai 201203, China

²Division of Antitumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China ³University of Chinese Academy of Sciences, No.19A Yuquan Road, Beijing 100049, China ⁴Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China

Abstract

A series of novel 2,4-diarylaminopyrimidine derivatives of AZD9291 were discovered as L858R/T790M mutant selective epidermal growth factor receptor (EGFR) inhibitors. The majority of these compounds exhibited moderate to excellent EGFR T790M/L858R inhibitory activities and comparable anti-proliferative activities against double mutant over-expressed NCI-H1975 cells to that of AZD9291. The most promising compounds **8a** displayed an IC₅₀ of 4.1 nM against EGFR L858R/T790M mutants. **8a** also showed excellent cytotoxic effect against NCI-H1975 cells with an IC₅₀ of 59 nM and 100-fold selectivity over wide-type EGFR over-expressed A431

^{*} Corresponding author. Tel./Fax.: +86 21 51980039 (W.T.), Tel.: +86 21 50805897; fax: +86 21 50806722 (H.X.), Tel/Fax.: +86 21 51980127 (Y.L.)

[#] These authors contributed equally to this work.

E-mail address: wftan@fudan.edu.cn (W. Tan), hxie@simm.ac.cn (H. Xie), liyx417@fudan.edu.cn (Y. Li).

cells. Compound **8a** significantly inhibited tumor growth in NCI-H1975 xenograft models at a non-toxic dose. Docking study performed for **8a** with ATP binding site of EGFR-TK showed the similar binding mode to that of AZD9291. All these results suggested that compound **8a** was a potential mutant-selective EGFR inhibitor.

Keywords

EGFR inhibitors, Pyrimidine derivatives, Mutant selective inhibitors, AZD9291, T790M

1. Introduction

The epidermal growth factor receptor (EGFR) protein belongs to the ErbB family of receptor tyrosine kinases (RTKs), which play an important role in the regulation of cell growth, differentiation, and survival [1]. EGFR over-expression is observed in many human malignancies, including non-small cell lung cancer (NSCLC), ovarian cancer, and colon cancer [2]. Therefore, EGFR has become one of the targets in anticancer drug research. In recent years, several EGFR inhibitors have been approved for the treatment of advanced NSCLC by FDA, such as gefitinib (1) and erlotinib (2) (Fig. 1). EGFR tyrosine kinase inhibitors (TKIs) have been emerging as one of the effective and targetable treatment strategies for NSCLC [3].



Fig. 1. First- and second- generation EGFR inhibitors

First-generation EGFR TKIs provide significant clinical benefit in patients with activating EGFR mutations [4, 5]. However, patients ultimately develop disease progression, often accompanied with the EGFR T790M mutation [6]. To overcome the T790M mutation-related resistance, subsequent identification of irreversible EGFR inhibitors (second-generation EGFR inhibitors) containing a Michael acceptor functional group such as dacomitinib (**3**) and afatinib (**4**) have been developed. These agents inhibit mutant EGFR as well as the wild type EGFR (EGFR wt), offering a potential therapeutic option for T790M positive patients [7, 8]. However, less selectivity between EGFR wt and EGFR T790M mutation eventually limits their utility due to the severe toxicities such as diarrhea and skin rash [9]. Therefore, discovery of mutant selective third-generation EGFR inhibitors, has become the focus of more attention recently [3].

AZD9291 (5) (Fig. 2) is a novel oral, potent and selective third-generation irreversible inhibitor, developed for the treatment of advanced non-small cell lung cancer. It has been designed to target the EGFR T790M mutation, while sparing wild-type EGFR [10]. This compound is structurally distinct from other third-generation EGFR TKIs, such as WZ4002 (6) and CO-1686 (7) [11], which offers a pharmacologically differentiated profile from earlier generation EGFR TKIs [12]. In November 2015, AZD9291 was granted accelerated approval in the USA for the treatment of patients with metastatic EGFR T790M mutation-positive NSCLC who have progressed on or after EGFR TKI therapy [13].



Fig. 2. Third generation EGFR inhibitors

2. Design

As depicted in the modeled structure for AZD9291 with EGFR (Fig. 3), the orientation of the indole group is adjacent to the gatekeeper residue (Met790), indicating the selectivity over the wild-type form of the receptor [12].



Fig. 3. AZD9291 binding mode

To exploit novel irreversible EGFR inhibitors to overcome the problem of drug resistance with mutant selectivity, we focused on the replacement of the indole residue of AZD9291 with a *o*-heterocycle substituted aniline as shown in compound **8a**. The more flexible and bulky motif was excepted to occupy the binding pocket better than indole residue, therefore higher selectivity and potency might be achieved (Fig. 4). A similar ring expansion strategy was utilized by Zhu et al [14]. Based on this strategy, a series of novel 2,4-diarylaminopyrimidine analogues (**8a-m**) with different heterocycle substituents and polar fragments were designed and synthesized. Their EGFR kinase inhibitory activity, *in vitro* antiproliferative effects and *in vivo* antitumor efficacy were evaluated. Some of these compounds were identified with greater potency.



Fig. 4. Design strategy of pseudo-ring replacement

3. Chemistry

The synthetic routine of target compounds 8a-m was displayed in Scheme 1.

Compounds **13a-d** were synthesized as reported in the literature [15, 16]. Condensation of commercially available 2,4-dichloropyrimidine with **13a-d** in 2-butyl alcohol containing DIPEA provided intermediates **15a-d**. Adding 4-methylbenzenesulfonic acid hydrate in one portion to the mixture of **15** and 4-fluoro-2-methoxy-5-nitroaniline [10] in 2-pentanol at a high temperature gave compounds **16a-d**. **17a-m** were synthesized through nucleophilic reaction of **16a-d** with different amines in moderate to good yields. The target compounds were generated from compounds **17a-m** via reduction and the subsequent acylation.



Scheme 1. Reagent and conditions: (a) DMF-DMA, 110 °C, 100%; (b) NH₂NH₂, AcOH (for **11a**) or EtOH (for **11b**), 100 °C, 87%; (c) MeI, NaH, THF, r.t., 63% (**12a**), 54% (**12b**); (d) Fe, NH₄Cl, EtOH/H₂O, 80 °C, 93% (**13a**), 95% (**13b**); (e) pyrazole (for **12c**) or 1,2,3-trizole (for **12d**), CuI, DMF, 110 °C, 87% (**12c**), 75% (**12d**); (f) 2,4-dichloropyrimidine, 2-BuOH, DIPEA, 110 °C, 76-89%; (g) 4-fluoro-2-methoxy-5-nitroaniline, 2-pentanol, 110 °C, 56-77%; (h) amine, DMF, 110 °C, 78-90%; (i) H₂, Pd/C, MeOH, 70-95%; (j) acryloyl chloride, DIPEA, CH₂Cl₂, r.t., 35-45%.

4. Results and discussion

4.1 Kinase inhibitory activity

All the synthesized compounds were evaluated in terms of *in vitro* inhibitory activities on EGFR wt and EGFR T790M/L858R mutants (Table 1). As shown in Table 1, compounds 8a, 8b, 8f, and 8m showed good or modest inhibition against EGFR wt and EGFR T790M/L858R. 1-Methyl-1H-pyrazole and pyrazole derivative (8a and 8f) showed the best activity (IC₅₀ = 4.1 nM), while other heterocycle such as 1-methyl-1*H*-1,2,4-triazole was well tolerated (8h and 8m). The hydrophilic motif R showed a great impact on activities. The ether linked analogue 8b was a replacement that was tolerated which resulted in a very similar profile with a slight erosion in kinase inhibition potency (EGFR T790M/L858R IC_{50} = 16.4 nM) and a loss in selectivity. When the R was replaced with other amines such as piperazine and morpholine, the inhibitory activities against EGFR wt and EGFR T790M/L858R were diminished significantly (8c, 8g), in some cases resulted in a complete loss of potency (8d, 8e and 8h). As mentioned in a previous study [17], they employed a glutathione (GSH) reaction assay to determine the relative reactivity of inhibitors, results showed a dependence of GSH half-life on the pKa of the basic group. The intramolecular base catalysis would be a explanation for the observed loss of activity in some cases.

Compd.	EGFR wt	EGFR T790M/L858R	Enzyme selectivity ^a
8a	349.9±252.6	4.1±2.2	85
8b	316.8±295.3	16.4±5.8	19
8c	>1000	38.5±16.5	ND^b
8d	>1000	>1000	ND
8e	>1000	>1000	ND
8f	648.2±208.7	4.1±0.2	158

Table 1. EGFR kinase inhibition (IC₅₀, nM) by the pseudo-ring compounds

8g	>1000	142.7±59.2	ND
8h	>1000	>1000	ND
8 i	280.6±160.4	33.1±26.0	8
8j	>1000	202.3±84.7	ND
8k	>1000	174.5±78.6	ND
81	>1000	838.5±98.4	ND
8m	660.0±165.3	7.7±2.5	86
AZD9291	270.4±53.5	12.4±4.3	22

^{*a*} WT:DM; ^{*b*}Not determined

4.2 In vitro anti-proliferative activity

Compounds **8a**, **8b**, **8f**, **8j** and **8m** were chosen to further evaluate their anti-proliferative activities against the A431 epidermoid carcinoma cell line and the NCI-H1975 non-small cell lung cancer cell line, which expressed high levels of EGFR wt or EGFR T790M/L858R, respectively. As shown in Table 2, all compounds demonstrated comparable anti-proliferative activities against NCI-H1975 cells to that of AZD9291, and displayed less inhibition of the growth of A431 cells. Intriguingly, compounds **8a** and **8f** showed 124-fold and 148-fold selectivity over A431 cells, which were much better than AZD9291.

	A 421	NCI-H1975	Cellular
Compa.	A431		selectivity ^b
8a	7.368±2.491	0.059±0.007	124
8b	1.583±0.492	0.085±0.015	18
8f	10.071±6.103	0.068±0.018	148

Table 2. NCI-H1975 and A431 cancer cell proliferation inhibition $(IC_{50}, \mu M)^{a}$

8j	2.760±0.607	0.070±0.015	39
8m	1.499±1.112	0.074±0.016	20
AZD9291	1.260±0.032	0.041±0.031	30

^{*a*}IC₅₀ data are averages of at least three independent experiments; ^{*b*}A431:H1975

4.3. Compound 8a blocks the phosphorylation of EGFR and downstream signaling transduction in human cancer cells

We further examined the ability of the representative compound **8a** to inhibit the phosphorylation of EGFR T790M/L858R and EGFR wt in NCI-H1975 cells and A431 cells, respectively. The results demonstrated that compound **8a** potently and dose-dependently inhibited the phosphorylation of EGFR T790M/L858R and downstream molecules indicated by p-Akt and p-Erk in NCI-H1975 cells (Fig. 5A), whilst showing less effective inhibition against the phosphorylation of EGFR wt in A431 cells (Fig. 5B).





Fig. 5. Effect of compound **8a** on the phosphorylation of EGFR T790M/L858R and EGFR wt in cancer cells induced by EGF. Representative Western blot analysis for the EGFR T790M/L858R inhibition by **8a** in the NCI-H1975 cells (A) and the EGFR wt inhibition in A431 cells (B).

4.4. In vivo anti-tumor activity of 8a

In vivo anti-tumor activity of 8a

We further evaluated the *in vitro* antitumor activity of **8a** in xenograft models of NCI-H1975 and A431, respectively. **8a** was administrated orally twice daily (10

mg/kg or 20 mg/kg) and AZD9291 (10 mg/kg) was used for comparison. As illustrated in Fig. 6A, **8a** significantly and dose-dependently suppressed the tumor growth in NCI-H1975 xenograft model, which was comparable with AZD9291. Whereas, in A431 xenograft mouse model (Fig. 6C), **8a** only exhibited moderate tumor growth inhibition, with TGI less than 20% even at 20 mg/kg dose treatment. During the course of experiment, no obvious body weight loss of mice was observed in **8a** treatment group both in NCI-H1975 model (Fig. 6B) and in A431 models (Fig. 6D), indicating this compound was well tolerated.

The target inhibition of **8a** in NCI-H1975 and A431 tumor was investigated by Western blot analysis. As shown in Fig. 6E and 6F, **8a** (10 mg/kg) treatment significantly blocked the phosphorylation of EGFR T790M/L858R and the activation of downstream molecule Akt in NCI-H1975 cells ($p^* < 0.05$). While in A431 xenograft tumor, the inhibition of phosphorylation about EGFR and Akt was not significant by **8a** which was consistent with the weak inhibition of **8a** on A431 tumor growth (Fig. 6G and 6H). These results clearly indicated that **8a** might serve as a promising third-generation EGFR–TKI for further development to overcome EGFR T790M mutation-related lung cancer.



Fig. 6. *In vivo* antitumor activity of compound **8a** against NCI-H1975 and A431 xenograft mouse models. NCI-H1975 tumor growth (A) and A431 tumor growth (C) were inhibited by **8a**. Body weight changes of control and **8a** treatment group were monitored in NCI-H1975 model (B) and A431model (D). The phosphorylation of EGFR and Akt in NCI-H1975 tumor was inhibited by **8a** treatment compared with vehicles control group (E). (F) Qutification data of Fig. 6E. In **8a** treatment tumor, the phosphorylation EGFR T790M/L858R and the activation of Akt was significantly less than that of vehicle control group (*p < 0.05).

4.5 Binding mode of compound 8a

In order to provide deeper insights into the structure-activity relationship of the new series, compound **8a** was selected based on the potency and scaffold type and

docked with the ATP-binding pocket of EGFR (PDB code: 3IKA) [18]. As illustrated in Fig. 7, compound **8a** irreversibly binds to the T790M mutant EGFR by targeting the Cys797 residue in the ATP binding site via the formation of a covalent bond. The pyrimidine core forms an expected bidentate hydrogen-bonded interaction with the hinge Met793 residue, the orientation of the heterocycle is adjacent to the gatekeeper residue Met790; the amine motif positions in the solvent exposed region. (Fig. 7).



Fig. 7. Docking model for the binding of **8a** in the ATP-pocket of EGFR T790M. The result was calculated by Maestro 10.1 (Schrödinger Release 2015-3). The figure was generated using Pymol.

Conclusions

We have described the structural optimization of AZD9291aimed at discovery of mutant-selective EGFR inhibitors. A novel series of 2,4-diarylaminopyrimidine derivatives based on the pseudo-ring replacement strategy was designed, synthesized and evaluated as EGFR inhibitors. Compounds **8a** and **8f** showed good inhibitory activities against EGFR T790M/L858R *in vitro*, with IC₅₀ values of 4.1 nM and comparable antiproliferation potency to AZD9291 in H1975 cells (59 nM for **8a** and 68 nM for **8f**). Meanwhile, **8a** and **8f** exhibited 124-fold and 148-fold selectivity over A431 cells, which are better than AZD9291. **8a** also effectively blocked the

phosphorylation of EGFR and downstream signaling pathway. In NCI-H1975 xenograft model, oral dosing of compound **8a** at 10 and 20mg/kg/day led to significant tumor growth inhibition with no observed loss in body weight. The improved biological activities of **8a** indicate that the design strategy could be useful optimization for better activity and selectivity. This inhibition results in antiproliferative effects in cancer cell lines harboring mutant forms of EGFR, and translates into pronounced antitumor activity in corresponding *in vivo* xenograft models. These compounds show much less activities against wild-type EGFR. In conclusion, the findings presented herein show that compounds **8a** has potential as promising anticancer agents.

5. Experimental section

5.1 Chemistry

All commercially available starting materials, reagents and solvents were used without further purification. All the solid compounds are recrystallized from methanol. Melting points were determined with an Electro thermal melting point apparatus, and are uncorrected. High resolution mass spectra (HRMS) were recorded on QSTAR XL Hybrid MS/MS mass spectrometer. ¹H NMR and ¹³C NMR spectra on a Bruker AV 400 or 600 MHz spectrometer were recorded in CDCl₃. Chemical shifts are reported in δ (ppm) units relative to the internal standard tetramethylsilane (TMS).

5.1.1. General method to synthesize compounds

General method to synthesize compounds **16a-d**: Compounds **13a-d** were synthesized as reported in the literature [14-15]. A solution of commercially available

2-nitroacetophenone and N,N-dimethylformamide dimethyl acetal in DMF was heated at 100 °C for 2 h. The reaction mixture was concentrated and the resultant solid was washed with Et2O and collected by filtration to afford 10a. 10a and hydrazine monohydrate in ethanol was refluxed for 10 h. The mixture was concentrated and the resultant was subjected to column chlomatography on silica gel to give 11a. 2-nitrobenzamide was dissolved in dimethylformamide dimethylacetal and heated to 100 °C for 1 h. The reaction mixture was cooled and a white precipitate was collected by filtration and rinsed with hexane to provide 10b. 10b in acetic acid and *n*-butanol was treated with hydrazine monohydrate, and heated to reflux for 2 h. The solvent was removed in vacuo, and the residue was subjected to column chlomatography on silica gel to give 11b. 13a-b could be obtained through methylation and reduction. A cooper-catalized coupling was used to synthesize 12c and 12d and followed by a reduction, 13c and 13d were obtained in good yields. Condensation of commercially available 2,4-dichloropyrimidine with 13a-d in 2-BuOH containing DIPEA provided intermediates with structure 15a-d. 4-methylbenzenesulfonic acid hydrate was added in one portion to 15 and 4-fluoro-2-methoxy-5-nitroaniline in 2-pentanol. The resulting mixture was stirred at 105 °C for 2.5 h. The mixture was cooled to room temperature. The precipitate was collected by filtration, washed with 2-pentanol, and dried under vacuum to afford 16a-16d as a yellow solid, which were used in the subsequent reaction without purification.

General method to synthesize compounds **17a-m**: different amines (0.1 mmol) was added to a solution of **16a-d** (0.9 mmol) and DIPEA (0.1 mmol) in DMF. The

mixture was heated at 110 °C for 2 hours. The solvent was removed in vacuo, and the crude product was purified by flash silica gel chromatography (4% 7 N NH₃/MeOH in CH_2Cl_2) to obtain the title product.

General method to synthesize compounds **18a-m**: A solution of compound **17a-m** (0.10 mmol) in enthanol (20 mL) was treated with 10% palladium on charcoal (10 wt %) and hydrogenated at 30 psi for 12 h. The catalyst was filtered through Celite, and the filtrate was concentrated under reduced pressure. The resulting crude product was purified by flash silica gel chromatography (dichloromethane/methanol = 15:1, v/v) to obtain the title product.

General method to synthesize compounds **8a-m**: Acryloyl chloride (34.5 mg, 0.38 mmol) in CH₂Cl₂ (1 mL) was added dropwise to a stirred solution of **18a-m** (170 mg, 0.38 mmol) and DIPEA (0.073 mL, 0.42 mmol) in DCM (5 mL), cooled in an ice/water bath. The mixture was stirred for 90 min and then diluted with CH₂Cl₂ (25 mL) and washed with saturated aqueous NaHCO₃ solution (50 mL). The organics were removed, and the aqueous portion was further extracted with CH₂Cl₂ (2 × 25 mL). The combined organics were dried over MgSO4, filtered, and concentrated onto silica for purification. The crude product was purified by flash silica chromatography, with elution gradient 0 –4% 7 N NH₃/MeOH in DCM to afford **8a-m**.

5.1.2.

N-(2-((2-(Dimethylamino)ethyl)(methyl)amino)-4-methoxy-5-((4-((2-(1-methyl-1
H-pyrazol-3-yl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)acrylamide (8a)
According to general method to synthesize compounds 8a-m, compound 8a was

synthesized in 45% yield as a yellow solid. mp: 63-65 °C; ¹H NMR (400 MHz, CDCl₃) δ 10.37 (s, 1H), 10.07 (s, 1H), 9.49 (s, 1H), 8.40 (d, J = 8.3 Hz, 1H), 8.11 (d, J = 5.7 Hz, 1H), 7.62–7.59 (m, 1H), 7.39 (s, 1H), 7.35 (s, 1H), 7.26-7.23 (m, 2H), 7.02 (t, J = 7.6 Hz, 1H), 6.76 (s, 1H), 6.57 (d, J = 2.4 Hz, 1H), 6.37–6.31 (m, 3H), 6.22 (d, J = 5.8 Hz, 1H), 5.66 (dd, J = 9.5, 2.4 Hz, 1H), 3.99 (s, 3H), 3.85 (s, 3H), 2.88 (t, J = 5.6 Hz, 2H), 2.69 (s, 3H), 2.27 (s, 6H); ¹³C NMR (150 MHz, CDCl₃) δ 163.24, 161.09, 159.92, 156.61, 151.11, 145.25, 137.22, 135.68, 132.57, 130.95, 129.47, 128.10, 128.05, 127.15, 125.92, 122.20, 121.71, 121.63, 112.36, 104.68, 104.48, 99.48, 57.51, 56.16, 45.56, 43.80, 39.27, 29.84; HRMS (ESI) (m/z): [M+H]⁺ calcd for C₂₉H₃₆N₉O₂ 542.2992; found 542.2990.

5.1.3.

N-(2-(2-(Dimethylamino)ethoxy)-4-methoxy-5-((4-((2-(1-methyl-1H-pyrazol-3-yl) phenyl)amino)pyrimidin-2-yl)amino)phenyl)acrylamide (8b)

According to general method to synthesize compounds **8a-m**, compound **8b** was synthesized in 35% yield as a solid. mp:125-128 °C; ¹H NMR (400 MHz, CDCl₃) δ 10.39 (s, 1H), 9.78 (s, 1H), 9.34 (s, 1H), 8.41 (d, J = 8.3 Hz, 1H), 8.09 (d, J = 5.8 Hz, 1H), 7.61 (dd, J = 7.8, 1.6 Hz, 1H), 7.39 (d, J = 2.4 Hz, 1H), 7.24 (t, J = 4.0 Hz, 2H), 7.02 (t, J = 7.6 Hz, 1H), 6.61 (s, 1H), 6.57 (d, J = 2.3 Hz, 1H), 6.42 (d, J = 1.8 Hz, 1H), 6.38 (d, J = 1.8 Hz, 1H), 6.29 (d, J = 10.1 Hz, 1H), 6.24 (d, J = 10.0 Hz, 1H), 6.20 (d, J = 5.8 Hz, 1H), 5.68 (dd, J = 10.0, 1.8 Hz, 1H), 4.11 (t, J = 5.2 Hz, 2H), 4.00 (s, 3H), 3.85 (s, 3H), 2.62–2.50 (m, 2H), 2.36 (s, 6H); ¹³C NMR (150 MHz, CDCl₃) δ 163.3, 161.1, 160.0, 156.6, 151.1, 145.7, 142.8, 137.2, 132.1, 130.9, 128.1, 128.0,

126.3, 125.0, 124.40, 122.1, 121.3, 113.9, 104.4, 101.8, 99.4, 70.3, 58.1, 56.2, 45.3, 39.2, 29.8; HRMS (ESI) (m/z): $[M + H]^+$ calcd for $C_{28}H_{31}N_8O_3$ 529.2676; found 529.2680

5.1.4.

N-(2-(4-(Dimethylamino)piperidin-1-yl)-4-methoxy-5-((4-((2-(1-methyl-1H-pyraz ol-3-yl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)acrylamide (8c)

According to general method to synthesize compounds **8a-m**, compound **8c** was synthesized in 50% yield as a solid. mp:80-83 °C; ¹H NMR (400 MHz, CDCl₃) δ 10.37 (s, 1H), 9.41 (s, 1H), 8.51 (s, 1H), 8.38 (d, *J* = 8.3 Hz, 1H), 8.10 (d, *J* = 5.8 Hz, 1H), 7.62 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.40 (d, *J* = 2.3 Hz, 1H), 7.32 (s, 1H), 7.25-7.23 (m, 2H), 7.04 (t, *J* = 7.6 Hz, 1H), 6.72 (s, 1H), 6.58 (d, *J* = 2.3 Hz, 1H), 6.38–6.25 (m, 2H), 6.23 (d, *J* = 6.0 Hz, 1H), 5.72 (d, *J* = 9.8 Hz, 1H), 3.99 (s, 3H), 3.86 (s, 3H), 3.04 (d, *J* = 11.6 Hz, 2H), 2.71 (t, *J* = 11.4 Hz, 2H), 2.38 (s, 6H), 2.30-2.28 (m, 2H), 2.20-2.15 (m, 1H); ¹³C NMR (150 MHz, CDCl₃) δ 162.8, 161.1, 159.8, 156.5, 151.0, 145.2, 137.1, 135.9, 132.3, 130.9, 128.1, 128.0, 126.7, 126.6, 126.4, 122.3, 121.7, 112.1, 104.4, 103.2, 99.5, 62.1, 56.1, 52.4, 42.0, 39.2, 30.0; HRMS (ESI) (m/z): [M+H]⁺ calcd for C₃₁H₃₈N₉O₂, 568.3134, found 568.3137.

5.1.5.

N-(4-Methoxy-5-((4-((2-(1-methyl-1H-pyrazol-3-yl)phenyl)amino)pyrimidin-2-yl) amino)-2-(4-methylpiperazin-1-yl)phenyl)acrylamide (8d)

According to general method to synthesize compounds **8a-m**, compound **8d** was synthesized in 60% yield as a solid. mp:66-70 $^{\circ}$ C; ¹H NMR (400 MHz, CDCl₃) δ

10.70 (s, 1H), 9.21 (s, 1H), 8.21-8.18 (m, 2H), 8.07 (d, J = 8.2 Hz, 1H), 7.41-7.37 (m, 1H), 7.14-7.09 (m, 1H), 6.60 (s, 1H), 6.41 (d, J = 5.8 Hz, 1H), 4.01 (s, 3H), 3.85 (s, 3H), 2.95-2.89 (m, 4H), 2.60-2.58 (m, 4H), 2.38 (s, 3H); ¹³C NMR (150 MHz, CDCl₃) δ 162.1, 160.7, 159.8, 156.6, 143.3, 141.3, 138.4, 135.4, 132.2, 129.7, 128.5, 126.8, 122.1, 121.7, 118.4, 107.3, 104.3, 99.4, 56.9, 56.0, 51.3, 46.2, 36.5; HRMS (ESI) (m/z): [M+H]⁺ calcd for C₂₉H₃₄N₉O₂, 540.2830; found 540.2835.

5.1.6.

N-(4-Methoxy-5-((4-((2-(1-methyl-1H-pyrazol-3-yl)phenyl)amino)pyrimidin-2-yl) amino)-2-morpholinophenyl)acrylamide (8e)

According to general method to synthesize compounds **8a-m**, compound **8e** was synthesized in 44% yield as a solid. mp:98-101 °C; ¹H NMR (400 MHz, CDCl₃) δ 10.40 (s, 1H), 9.46 (s, 1H), 8.53 (s, 1H), 8.39 (d, J = 8.3 Hz, 1H), 8.10 (d, J = 5.7 Hz, 1H), 8.06 (s, 1H), 7.64–7.61 (m, 1H), 7.40 (d, J = 2.5 Hz, 2H), 7.25 (d, J = 7.4 Hz, 1H), 7.04 (t, J = 7.5 Hz, 1H), 6.74 (s, 1H), 6.58 (d, J = 2.3 Hz, 1H), 6.41–6.26 (m, 2H), 6.23 (d, J = 5.9 Hz, 1H), 5.74 (d, J = 9.9 Hz, 1H), 4.00 (s, 3H), 3.88-3.85 (m, 7H), 2.88 (t, J = 4.5 Hz, 4H); ¹³C NMR (150 MHz, CDCl₃) δ 162.7, 161.0, 160.9, 159.8, 156.4, 151.0, 145.3, 137.1, 135.0, 132.2, 130.9, 128.1, 128.0, 127.2, 126.8, 126.6, 122.3, 121.6, 112.2, 104.4, 103.8, 99.6, 67.8, 67.3, 66.5, 56.2, 52.9, 45.5, 40.7, 39.2; HRMS (ESI) (m/z): [M + H]+ calcd for C₂₈H₃₁N₈O₃, 527.2514, found 527.2516. **51.7**.

N-(5-((4-((2-(1H-pyrazol-1-yl)phenyl)amino)pyrimidin-2-yl)amino)-2-((2-(dimeth ylamino)ethyl)(methyl)amino)-4-methoxyphenyl)acrylamide (8f)

According to general method to synthesize compounds **8a-m**, compound **8f** was synthesized in 43% yield as a solid. mp:90-94 °C; ¹H NMR (400 MHz, CDCl₃) δ 10.07 (s, 1H), 9.42 (s, 1H), 9.20 (s, 1H), 8.25 (d, *J* = 8.1 Hz, 1H), 8.09 (d, *J* = 5.7 Hz, 1H), 7.2 (d, *J* = 1.9 Hz, 1H), 7.78 (d, *J* = 2.5 Hz, 1H), 7.32 (q, *J* = 3.5, 3.0 Hz, 3H), 7.1 –7.06 (m, 1H), 6.75 (s, 1H), 6.50–6.42 (m, 1H), 6.42–6.28 (m, 2H), 6.11 (d, *J* = 5.8 Hz, 1H), 5.68 (dd, *J* = 9.2, 2.6 Hz, 1H), 3.84 (s, 3H), 2.88 (t, *J* = 5.6 Hz, 2H), 2.69 (s, 3H), 2.29 (s, 9H); ¹³C NMR (150 MHz, CDCl₃) δ 163.2, 160.6, 159.8, 157.2, 145.2, 141.2, 135.8, 132.9, 132.5, 130.6, 130.3, 129.3, 127.9, 126.8, 126.0, 123.8, 123.7, 123.2, 112.3, 107.1, 104.6, 98.6, 57.4, 56.4, 56.1, 45.5, 43.7; HRMS (ESI) (m/z): [M+H]⁺ calcd for C₂₈H₃₄N₉O₂, 528.2830; found 528.2828.

5.1.8.

N-(5-((4-((2-(1H-pyrazol-1-yl)phenyl)amino)pyrimidin-2-yl)amino)-4-methoxy-2-(4-methylpiperazin-1-yl)phenyl)acrylamide (8g)

According to general method to synthesize compounds **8a-m**, compound **8g** was synthesized in 66% yield as a solid. mp:93-95 °C; ¹H NMR (400 MHz, CDCl₃) δ 9.69 (s, 1H), 9.43 (s, 1H), 8.49 (s, 1H), 8.21 (d, *J* = 8.4 Hz, 1H), 8.25–8.12 (m, 4H), 8.04 (s, 1H), 7.37 (d, *J* = 8.0 Hz, 2H), 7.08 (t, *J* = 7.4 Hz, 1H), 6.79 (s, 1H), 6.39–6.29 (m, 2H), 5.73 (d, *J* = 9.7 Hz, 1H), 3.85 (s, 3H), 2.94 (s, 4H), 2.46 (s, 3H); ¹³C NMR (150 MHz, CDCl₃) δ 162.7, 160.6, 159.8, 157.2, 145.1, 141.2, 135.3, 132.9, 132.3, 130.5, 130.2, 127.9, 126.8, 126.7, 126.4, 123.6, 123.3, 111.9, 107.1, 107.1, 103.5, 98.7, 56.1, 56.1, 52.5, 46.2; HRMS (ESI) (m/z): [M+H]⁺ calcd for C₂₈H₃₂N₉O₂, 526.2673; found 526.2669.

5.1.10.

N-(5-((4-((2-(1H-pyrazol-1-yl)phenyl)amino)pyrimidin-2-yl)amino)-4-methoxy-2morpholinophenyl)acrylamide (8h)

According to general method to synthesize compounds **8a-m**, compound **8h** was synthesized in 45% yield as a solid. mp:123-126 °C; ¹H NMR (400 MHz, CDCl₃) δ 9.41 (s, 1H), 9.27 (s, 1H), 8.50 (s, 1H), 8.23 (d, *J* = 8.2 Hz, 1H), 8.09 (d, *J* = 5.8 Hz, 1H), 7.82 (d, *J* = 1.9 Hz, 1H), 7.78 (d, *J* = 2.4 Hz, 1H), 7.38–7.27 (m, 3H), 7.10 (t, *J* = 7.6 Hz, 1H), 6.72 (s, 1H), 6.46 (t, *J* = 2.2 Hz, 1H), 6.42 – 6.24 (m, 2H), 6.14 (d, *J* = 5.8 Hz, 1H), 5.80–5.71 (m, 1H), 3.86-3.79 (m, 7H), 2.86 (t, *J* = 4.5 Hz, 4H); ¹³C NMR (150 MHz, CDCl₃) δ 162.7, 160.6, 159.7, 157.1, 145.1, 141.2, 135.0, 132.8, 132.2, 130.5, 130.2, 127.8, 126.9, 126.7, 126.5, 123.6, 123.3, 112.1, 107.1, 103.4, 98.8, 67.8, 56.1, 52.9; HRMS (ESI) (m/z): [M + H] + calcd for C₂₇H₂₉N₈O₃, 513.2363; found 513.2360.

5.1.11.

N-(5-((4-((2-(2H-1,2,3-triazol-2-yl)phenyl)amino)pyrimidin-2-yl)amino)-2-((2-(di methylamino)ethyl)(methyl)amino)-4-methoxyphenyl)acrylamide (8i)

According to general method to synthesize compounds **8a-m**, compound **8i** was synthesized in 33% yield as a solid. mp:66-68 °C; ¹H NMR (400 MHz, CDCl₃) δ 10.09 (s, 1H), 9.67 (s, 1H), 9.48 (s, 1H), 8.24 (d, J = 8.2 Hz, 1H), 8.13 (d, J = 5.6 Hz, 1H), 7.95 (dd, J = 8.2, 1.6 Hz, 1H), 7.91 (s, 2H), 7.39–7.31 (m, 2H), 7.17–7.11 (m, 1H), 6.76 (s, 1H), 6.26 (d, J = 5.8 Hz, 1H), 5.71–5.66 (m, 1H), 3.85 (s, 3H), 2.88 (t, J = 5.6 Hz, 2H), 2.69 (s, 3H), 2.33 – 2.26 (m, 9H); ¹³C NMR (150 MHz, CDCl₃) δ

163.2, 160.6, 159.9, 157.5, 145.1, 135.8, 135.0, 134.9, 132.6, 131.4, 129.9, 129.4,
128.4, 126.8, 125.9, 123.3, 123.3, 123.1, 112.3, 104.6, 98.4, 57.5, 56.1, 45.5, 43.7;
HRMS (ESI) (m/z): [M+H]⁺ calcd for C₂₇H₃₃N₁₀O₂, 529.2782; found 529.2780.
5.1.12.

N-(5-((4-((2-(2H-1,2,3-triazol-2-yl)phenyl)amino)pyrimidin-2-yl)amino)-2-(4-(di methylamino)piperidin-1-yl)-4-methoxyphenyl)acrylamide (8j)

According to general method to synthesize compounds **8a-m**, compound **8j** was synthesized in 40% yield as a solid. mp:69-73 °C; ¹H NMR (400 MHz, CDCl₃) δ 9.67 (s, 1H), 9.41 (s, 1H), 8.49 (s, 1H), 8.22 (d, *J* = 8.2 Hz, 1H), 8.12 (d, *J* = 5.7 Hz, 1H), 7.96 (d, *J* = 9.3 Hz, 1H), 7.91 (s, 2H), 7.38–7.31 (m, 2H), 7.16 (t, *J* = 7.7 Hz, 1H), 6.71 (s, 1H), 6.39–6.29 (m, 1H), 6.27 (d, *J* = 5.7 Hz, 1H), 5.77–5.72 (m, 1H), 3.85 (s, 3H), 3.05 (d, *J* = 11.4 Hz, 2H), 2.78 – 2.68 (m, 2H), 2.43 (s, 6H), 2.39-2.35 (m, 2H), 2.09-2.01 (m, 2H), 1.79–1.65 (m, 2H); ¹³C NMR (150 MHz, CDCl₃) δ 162.8, 160.6, 159.9, 157.4, 135.1, 132.3, 131.4, 129.9, 128.2, 126.7, 126.4, 123.4, 123.3, 112.0, 103.2, 98.4, 62.2, 56.2, 52.3, 41.8, 29.8; HRMS (ESI) (m/z): [M+H]⁺ calcd for C₂₉H₃₅N₁₀O₂, 555.2939; found 555.2928.

5.1.13.

N-(5-((4-((2-(2H-1,2,3-triazol-2-yl)phenyl)amino)pyrimidin-2-yl)amino)-4-metho xy-2-(4-methylpiperazin-1-yl)phenyl)acrylamide (8k)

According to general method to synthesize compounds **8a-m**, compound **8k** was synthesized in 51% yield as a solid. mp:84-86 °C; ¹H NMR (400 MHz, CDCl₃) δ 9.69 (s, 1H), 9.43 (s, 1H), 8.50 (s, 1H), 8.22 (d, *J* = 8.3 Hz, 1H), 8.12 (d, *J* = 5.8 Hz, 1H),

7.96 (dd, J = 8.1, 1.5 Hz, 1H), 7.91 (s, 2H), 7.45 (s, 1H), 7.34 (t, J = 7.8 Hz, 1H), 7.16 (t, J = 7.7 Hz, 1H), 6.76 (s, 1H), 6.37 (d, J = 1.7 Hz, 1H), 6.32 (d, J = 9.9 Hz, 1H), 6.27 (d, J = 5.8 Hz, 1H), 5.75 (d, J = 10.0 Hz, 1H), 3.85 (s, 3H), 2.95-2.90 (m, 4H), 2.74-2.63 (m, 4H)2.46 (s, 3H); ¹³C NMR (150 MHz, CDCl₃) δ 162.7, 160.7, 159.7, 157.1, 135.1, 132.3, 131.3, 129.9, 128.4, 126.8, 126.5, 123.4, 123.3, 112.0, 103.6, 98.5, 56.1, 55.9, 52.2, 45.9; HRMS (ESI) (m/z): [M+H]+ calcd for C₂₇H₃₁N₁₀O₂, 527.2626; found 527.2626.

5.1.14.

N-(5-((4-((2-(2H-1,2,3-triazol-2-yl)phenyl)amino)pyrimidin-2-yl)amino)-4-metho xy-2-morpholinophenyl)acrylamide (8l)

According to general method to synthesize compounds **8a-m**, compound **8l** was synthesized in 45% yield as a solid. mp:117-170 °C; ¹H NMR (400 MHz, CDCl₃) δ 9.69 (s, 1H), 9.47 (s, 1H), 8.53 (s, 1H), 8.21 (d, *J* = 8.4 Hz, 11H), 8.13 (d, *J* = 5.8 Hz, 11H), 7.97 (dd, *J* = 8.1, 1.4 Hz, 10H), 7.92 (d, *J* = 0.9 Hz, 19H), 7.34 (t, *J* = 7.6 Hz, 9H), 7.16 (t, *J* = 7.7 Hz, 11H), 6.74 (s, 11H), 6.43–6.29 (m, 18H), 6.28 (d, *J* = 6.0 Hz, 14H), 5.76 (d, *J* = 10.0 Hz, 10H), 5.30 (s, 10H), 2.88 (t, *J* = 4.5 Hz, 47H); ¹³C NMR (150 MHz, CDCl₃) δ 160.7, 135.1, 132.3, 128.4, 126.5, 123.4, 103.5, 98.5, 67.8, 56.2, 53.5, 53.0, 29.8, 29.4; HRMS (ESI) (m/z): [M+H]⁺ calcd for C₂₆H₂₈N₉O₃, 514.2310; found 514.2314.

5.1.15.

N-(2-((2-(Dimethylamino)ethyl)(methyl)amino)-4-methoxy-5-((4-((2-(1-methyl-1 H-1,2,4-triazol-3-yl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)acrylamide (8m) According to general method to synthesize compounds **8a-m**, compound **8m** was synthesized in 33% yield as a solid. ¹H NMR (400 MHz, CDCl₃) δ 10.52 (s, 1H), 10.03 (s, 1H), 9.45 (s, 1H), 8.44 (d, *J* = 8.3 Hz, 1H), 8.16 (d, *J* = 7.8 Hz, 1H), 8.13 (s, 1H), 8.13 (s, 2H), 7.54 (d, *J* = 8.6 Hz, 1H), 7.37 (d, *J* = 12.3 Hz, 2H), 7.33 (t, *J* = 7.8 Hz, 1H), 7.14–7.12 (m, 1H), 7.05 (t, *J* = 7.6 Hz, 1H), 6.76 (s, 1H), 4.00 (s, 3H), 3.86 (s, 3H), 2.89 (t, *J* = 5.6 Hz, 2H), 2.69 (s, 3H), 2.32 (t, *J* = 5.8 Hz, 2H), 2.28 (s, 6H); ¹³C NMR (150 MHz, CDCl₃) δ 163.3, 162.1, 160.9, 159.9, 156.8, 145.3, 143.4, 138.3, 135.8, 132.6, 129.8, 129.3, 128.4, 127.0, 125.9, 124.8, 121.9, 121.1, 118.3, 104.6, 99.5, 56.1, 45.4, 43.8, 36.5; HRMS (ESI) (m/z): [M+H]⁺ calcd for C₂₈H₃₅N₁₀O₂ 543.2944; found 543.2950.

6.1. Pharmacology

6.1.1. In vitro enzymatic activity assay

The kinases domain of EGFR wt and EGFR T790M/L858R 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). 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. Kinases were incubated with indicated test compounds or reference compound in kinase reaction buffer (50 mM HEPES pH 7.4, 20 mM MgCl₂, 0.1 mM MnCl₂, 0.2 mM Na₃VO₄, 1 mM DTT) containing 5 µmol/L ATP at 37 °C for 1 h. After incubation, the wells were washed with PBS and then incubated with an anti-phosphotyrosine (PY99) antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. The wells were visualized using *o*-phenylenediamine (OPD) and read with a multiwell spectrophotometer (VERSAmaxTM, Molecular Devices, Sunnyvale, CA, USA) at 492 nM.

6.1.2. Cytotoxicity assay

The cytotoxic activity *in vitro* was measured using sulforhodamine B (SRB) assay. All the compounds were dissolved in DMSO at the concentrations 10.0 mg/ml and were then diluted to appropriate concentrations. Cells were plated in 96-well plates $(5*10^3 \text{ per well})$ for 24 h and subsequently treated with different concentrations of all tested compounds for 72 h. Cells were then fixed with 10% (v/v) trichloroacetic acid at 4 °C for 1 h. After washing, the cells were stained for 15 min with 4 mg/mL SRB (Sigma) dissolved in 1% acetic acid. Before measured, 100 µL Tris base was added per well. The absorbance was measured at 515 nm using a multiscan spectrum (VERSAmax, Molecular Devices, Sunnyvale, CA, USA). The inhibition rate was calculated as $[1-(A515_{treated}/A515_{control})] \times 100\%$. The IC₅₀ value was obtained using the logit method.

6.1.3. In vitro EGFR phosphorylation assays

NCI-H1975 cells or A431 cells were treated with each compound or without for 2h and then stimulated with 50 ng/ml of EGF for 10 minutes. Cell samples were lysed in SDS lysis buffer and the Western blot analysis was subsequently performed using standard procedures. Antibodies directed against the following proteins were used: p-EGFR, EGFR, p-ERK, ERK, p-AKT, AKT, which were obtained from Cell

Signaling Technologies (Cambridge, MA, USA), and tubulin, which was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

6.1.4. Molecular modeling

A published structure of the EGFR T790M mutant (PDB code 3IKA) was used as template. Crystal structures were prepared using the protein preparation wizard in Maestro (Schrödinger Release 2015-3) which optimizes hydrogen placements. The active site was defined by using the bound ligand, and the covalent docking protocol was used to model potential binding modes. These were ranked using the assigned scores and manually inspected for the retention of the key hinge interactions to the hinge region residue M793.

6.1.5. In vivo antitumor activity assay

Pathogen-free, 6-8 week-old, female BALB/c nude mice (Shanghai Institute of Materia Medica, Chinese Academy of Sciences) were maintained under clean-room conditions in sterile filter-top cages with Aspen Chip bedding. NCI-H1975 xenograft model was established by inoculated 5×10^6 cancer cells subcutaneous in the right flanks of athymic mice. When the tumor reached a volume of 100-300 mm³, the mice were randomly assigned into control and treatment groups (n = 6 per group). Control groups were given vehicle alone, and treatment groups received different drugs. Health conditions of mice were observed daily. The size of tumor and body weight was measured on the indicated days during the treatment. Tumor volume (V) was calculated as V = (length×width²)/2. The body weight change (%) was calculated as follows: body weight change (%)= (Wt/W0-1)\times 100, where Wt represented the body

weight measured on the indicated days and W_0 represented the predosing body weight. Results are presented as mean for each group. Error bars represent the standard error of the mean (SEM). Animal experiments were carried out in compliance with the Animal Management Rules of the Ministy of Health of the People's Republic of China (document no. 55, 2001) and in the guidelines for the Care and Use of Laboratory Animals of Shanghai Institute of Materia Medica.

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27

Highlights

- A series of novel 2,4-diarylaminopyrimidine derivatives were designed as EGFR-TKIs.
- Most of the compounds displayed moderate to good inhibition toward EGFR L858R/T790M kinase and low anti-proliferative potencies against NCI-H1975 cells.
- Compound **8a** significantly inhibited tumor growth in NCI-H1975 xenograft models at a non-toxic dose.