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Synthesis and anti-proliferation activity evaluation of novel 2-chloroquinazoline as potential EGFR-TK inhibitors

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A novel series of 2-chloroquinazoline derivatives had been synthesized and their anti-proliferation activities against the four EGFR high-expressing cells A549, NCI-H1975, AGS and HepG2 cell lines were evaluated. The preliminary SAR study of the scaffold of new compounds showed that the compounds with a chlorine substituent on R^3 had a better anti-proliferation activity than those substituted by hydrogen atom or vinyl group. Among them, 2-chloro-*N*-(2-chloro-4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)acetamide **(10b)** had the best activity, and the corresponding IC_{50} were 3.68, 10.06, 1.73 and 2.04 μM, respectively. And compound **10b** had better or equivalent activity against four cell lines than Gefitinib. The activity of the compound **10b** on the EGFR enzyme was subsequently tested. The Wound Healing of A549, AGS and HepG2 cells by this compound showed that the compound can inhibit the migration of cancer cells. Finally, the action channel of the compound **10b** was supported by western blotting experiments. It provides useful information for the design of EGFR-TK inhibitors.

Keywords: Antiproliferative, EGFR inhibitors, Quinazoline, Western blot.

Introduction

Cancer, as a potential cause of death, continues being a global killer commonly all over the world.[1,2] Under this situation, many studies related to anticancer compounds which possess high specificity to tumor cells and low toxicity as well as side effects to other normal cells were gradually emerging. Targeted compounds, which were distinct from the traditional chemotherapeutic drugs, can selectively target cancer cells with higher efficacy and lower side effects. In fact, the mechanism of this kind of compounds may in relation to their selective actions on intracellular signaling pathways which show close connections with the tumor cell cycles and

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its microenvironment triggering the biological activities including cell apoptosis, cell proliferation, tumor growth,[3,4] angiogenesis and so on.

The epidermal growth factor receptor (EGFR),[5] as a member of the ErbB family of receptor tyrosine kinases (RTKs),[6-10] plays an essential role in regulating cell proliferation, differentiation and apoptosis. The over expression of the EGFR was reported associated with a number of the most lethal cancers in the world,[11,12] such as breast, ovarian, colon and non-small cell lung cancer (NSCLC).[13] EGFR may be activated by binding to endogenous ligands including the epidermal growth factor (EGF),[14-16] transforming growth factor-α (TGF-α), amphiregullin, betacellulin and epiregulin which result in the sensitization of intracellular signaling transduction pathways. Moreover, the downstream signaling transduction may be triggered by the mechanism of auto-phosphorylation of several tyrosine residues after EGFR dimerization. Thereby, developing a kind of small molecule EGFR tyrosine kinase inhibitors (EGFR-TKIs) appears to be a feasible approach.

As shown in **Fig. 1**, the first-generation EGFR-TKIs such as gefitinib and erlotinib were successfully developed and had been used as the front-line standard therapy in the treatment of NSCLC patients with sensitizing EGFR mutations currently.[17,18] However, cancer cells were found to develop resistance rapidly to EGFR-TKIs[19] through several unique mechanisms and the phenomenons of recrudescence as well as some serious side effects were also emerging at the same time.[20] Consequently, to overcome the drug resistance towards the first-generation EGFR-TKIs, the second-generation irreversible EGFR-TKIs such as dacomitinib and afatinib were developed. Subsequently, it was identified that the irreversible EGFR inhibitors have the covalent bond with Cys797, and they mostly contain a Michael acceptor functional group targeting the Cys797 residue in the ATP binding cleft.[21] Nevertheless, their clinical efficacy has also been limited eventually by corresponding skin rash and gastrointestinal toxicity due to their less selectivity between $EGFR^{wt}$ and $EGFR^{T790M}$ mutation (the 790th amino acid of the EGFR protein is converted from threonine to methionine).[22] Thus, the third-generation EGFR-TKIs such as AZD9291 and CO-1686 (**Fig. 1**), as covalent inhibitors similar with the second-generation EGFR-TKIs have been identified recently, and their selectivity on $EGFR^{T790M}$ mutant over $EGFR^{wt}$ have been demonstrated as well.[23] But it is well known that irreversible inhibitors have potential safety concerns due to their poor selectivity and the reason that they cannot be

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transferred by ATP. Therefore, their possible toxicity may be inevitable owing to the prolonged off-target inhibition of $EGFR^{wt}$.

In conclusion, the first-generation inhibitor such as gefitinib can solve the $EGFR^{L858R}$ mutation (the 858th amino acid of the EGFR protein is mutated from leucine to arginine), but after long-term clinical use, EGFR will develop T790M resistance Mutation. Although the second-generation inhibitor such as afatinib can better inhibit the $EGFR^{T790M}$ mutation, its clinical use has been greatly restricted due to its severe side effects. Hence, there is an ultimate need for us to develop a type of novel, potent EGFR inhibitors that targets both $EGFR^{L858R}$ and $EGFR^{T790M}$ to overcome the persistent resistance emerged in currently used ones and thus provide better treatment options.[24]

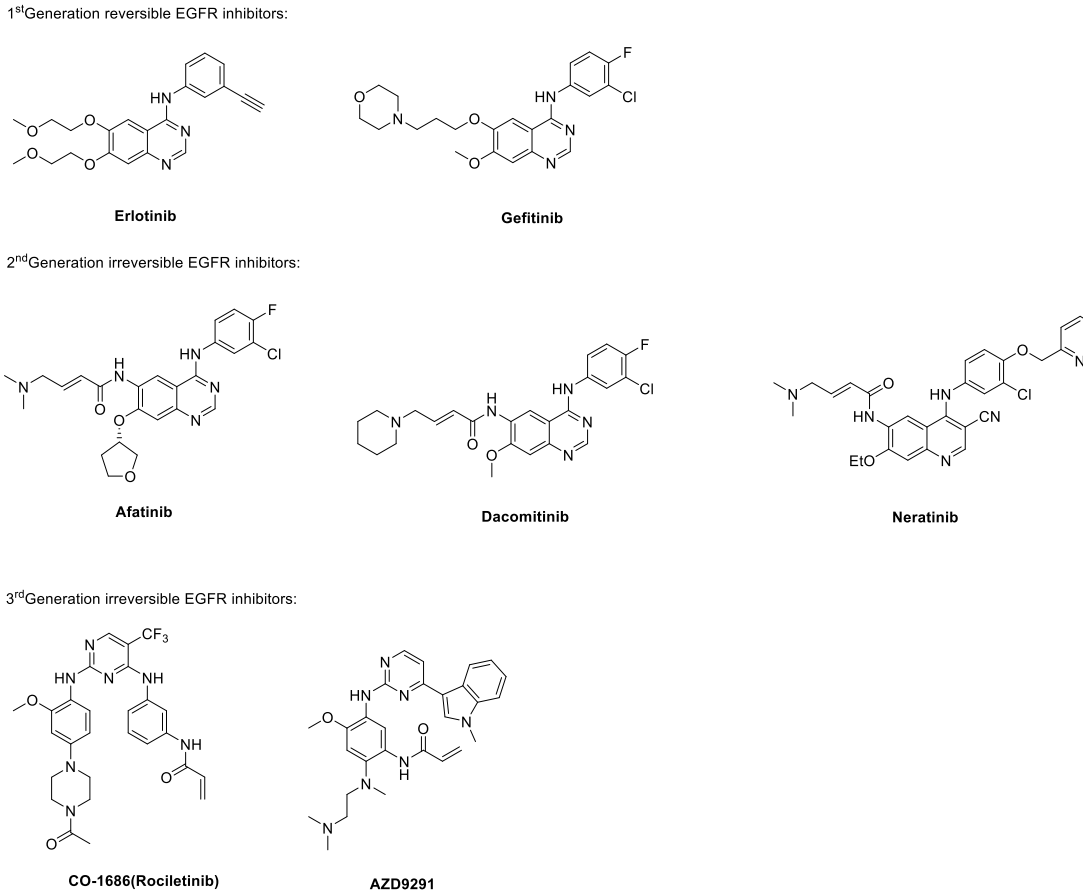

Fig. 1. The first-, second- and third-generation of EGFR inhibitors.

Rational Drug Design

In the past decade, researchers have studied the structure-activity relationship (SAR) of quinazoline derivatives and found that most EGFR-TKI inhibitors have four common pharmacophoric groups structures, including quinazoline heterocyclic scaffold, NH linker,

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hydrophobic head of various substituted phenyl groups, and hydrophobic tail **(Fig. 2)**.[25] Therefore, it is completely in line with the rational drug design concept that a novel type of EGFR inhibitor is designed by purposeful structural modification and transformation in these four positions.

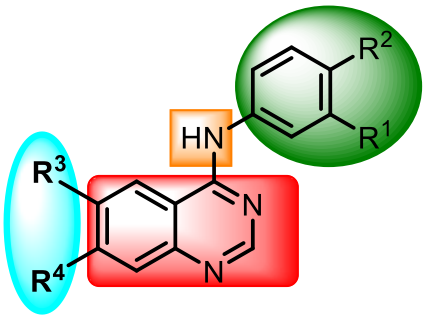

Fig. 2. Four common pharmacodynamic structures of most EGFR-TKI inhibitors.

Previously, the SAR study of gefitinib showed that compounds with aniline substitution at the 4-position of the quinazoline scaffold had better activity, while the SAR of afatinib showed that the introduction of amide group at the 6-position of quinazoline scaffold could also improve the inhibitory activity of compounds against EGFR.[26] Barbosa et al.[27] found that it had good biological activity after replacing it with a chlorine atom at the 2-position of the quinazoline scaffold. After docking the compounds to the EGFR protein, it was found that the presence of the chlorine atom at 2-position facilitates the binding of the compounds to EGFR, and at the same time, the chlorine atom was beneficial to improve the fat solubility, electron absorption and metabolic hindrance of the compounds. In short, we designed a series of novel 2-chloroquinazoline compounds by introducing a chlorine atom at the 2-position, aniline groups at the 4-position, and amide groups at the 6-position of the quinazoline scaffold. The entire rational drug design idea for the novel EGFR inhibitor is illustrated in **Fig. 3**.

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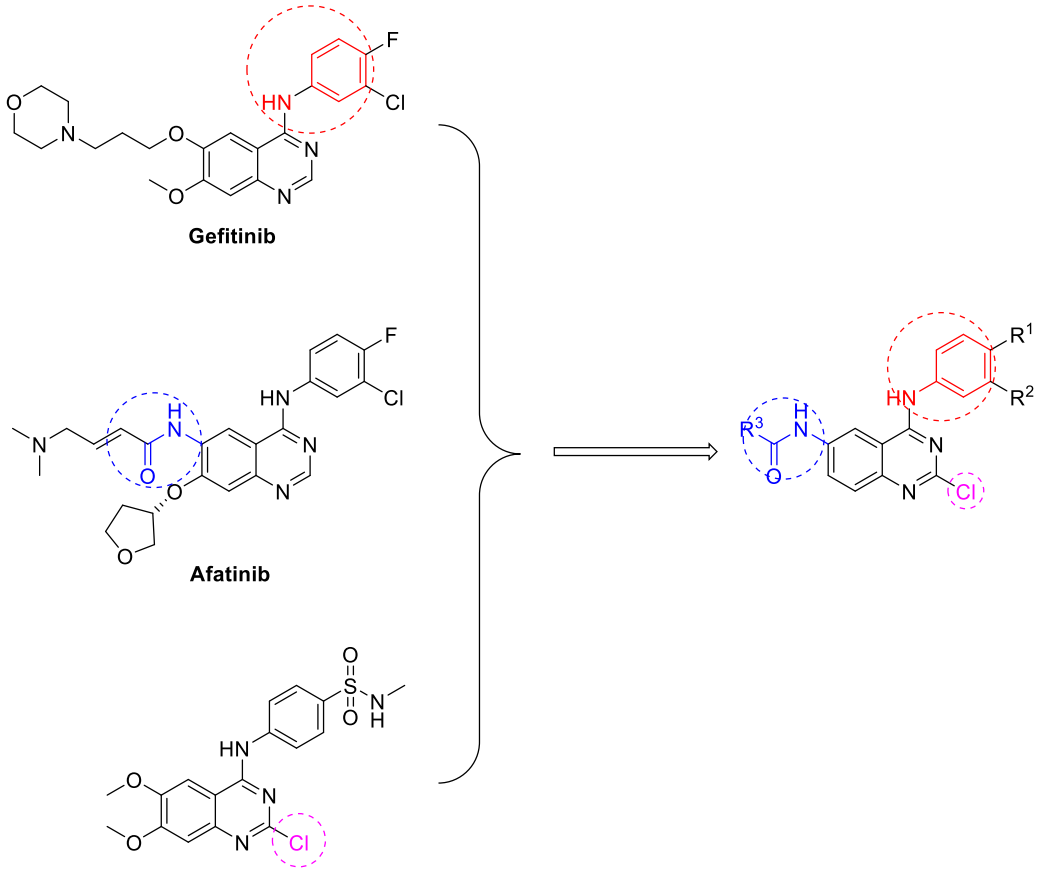

Fig. 3. Design idea of novel 2-chloroquinazolines as new EGFR inhibitors.

Results and Discussion

Chemistry

The synthesis of compounds **10a-l** was described in **Scheme 1**. Target compounds **10a-l** were achieved in seven steps using **1** as starting materials, which were coupled with KCNO in acetic acid at ambient temperature. Cyclization of **2** was done at reflux in NaOH methanol solution (pH = 10).[28] **4** was achieved by regioselective nitration of **3** at -10 ^{o}C.[29] Chlorination of **4** was achieved by refluxing with $POCl_3$ at the presence of *N*,*N*-Diisopropylethylamine in toluene. Then the corresponding phenylamines **6a-f** were attached to the 4-position of **5** through nucleophilic addition under basic condition in THF at room temperature in a dropwise way.[30] Finally, the nitro groups of compounds **7a-f** were reduced using iron in acetic acid,[21] followed by acylation of the resulting amines **8a-f** with the corresponding acid chlorides **9a-c** to obtain the quinazolinederivatives **10a-l**.[31] These compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, IR spectroscopy, HR-MS, and HPLC. (All the Spectroscopic data of **10a-l** can be found in the Supporting Information).

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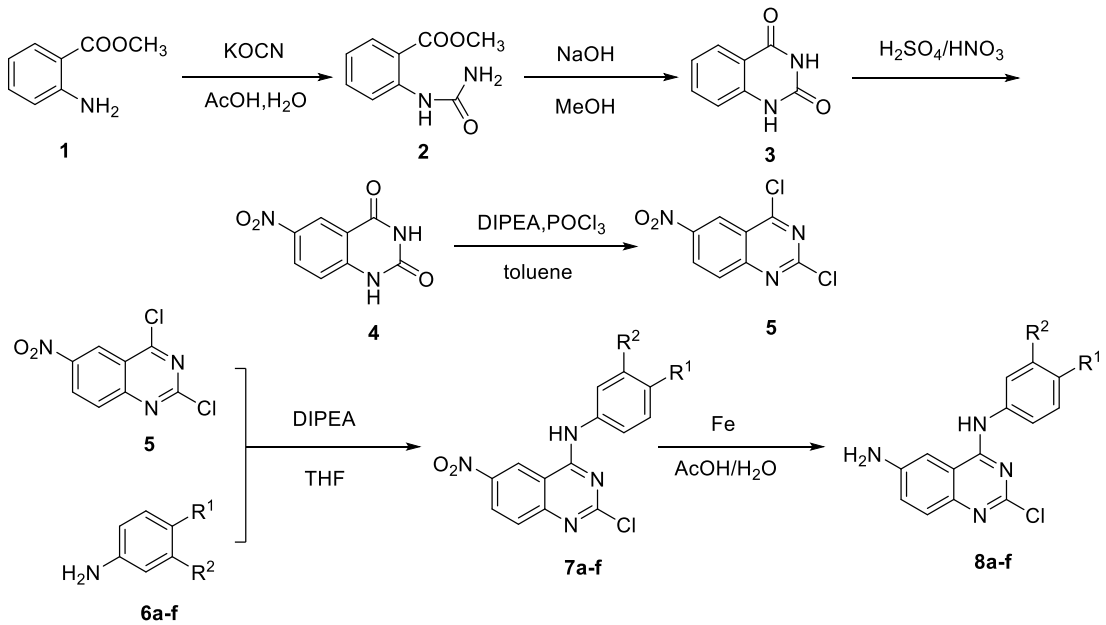
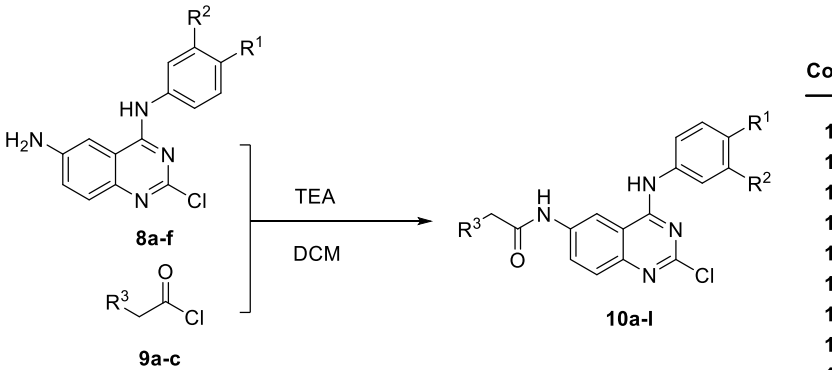


Compd	R^1	R^2
6a	Cl	Cl
6b	F	Cl
6c	Cl	CF_3
6d	Cl	H
6e	H	Cl
6f	OCH_3	H

Compd	R^1	R^2
7a	Cl	Cl
7b	F	Cl
7c	Cl	CF_3
7d	Cl	H
7e	H	Cl
7f	OCH_3	H

Compd	R^1	R^2
8a	Cl	Cl
8b	F	Cl
8c	Cl	CF_3
8d	Cl	H
8e	H	Cl
8f	OCH_3	H



Compd	R^3
9a	H
9b	Cl
9c	vinyl

Compd	R^1	R^2	R^3
10a	Cl	Cl	Cl
10b	F	Cl	Cl
10c	Cl	CF_3	Cl
10d	Cl	H	H
10e	H	Cl	H
10f	Cl	Cl	H
10g	OCH_3	H	H
10h	F	Cl	H
10i	H	Cl	Cl
10j	Cl	H	Cl
10k	Cl	H	vinyl
10l	H	Cl	vinyl

Scheme 1. Synthesis methods of Compounds **10a-l.**

Biological activity

Anti-proliferation assay

As shown in **Table 1**, the anti-proliferation activities of all synthesized compounds against the four EGFR high-expressing cells A549,[32,33] NCI-H1975,[33] AGS[34] and HepG2[35,36] cell lines had been evaluated *in vitro* by the MTT assay. Gefitinib was used as the positive control. Results

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were expressed as median growth inhibition concentration (IC_{50}) values, which represent the concentration of compound required to produce 50% inhibition of cell growth after 24 hours of incubation, compared to untreated controls. The results of anti-proliferation activities for the compounds were illustrated in **Table 1**. The results showed that most of the compounds exhibited potent anti-proliferation effects. The compounds with a chlorine substituent on R^3 had better anti-proliferation activities than those substituted by hydrogen atom or vinyl group. Compound **10b** had better or equivalent activity against four cell lines than Gefitinib.

Table 1

In vitro anti-proliferation activity of target compounds (Mean ± SD, n = 3).[a]

Compd	Cell proliferation assays IC_{50} (μM) [b]			
	A549	NCI-H1975	AGS	HepG2
10a	19.67±1.84	15.05±0.84	13.16±1.36	6.82±1.21
10b	**3.68±0.34**	**10.06±1.11**	**1.73±0.42**	**2.04±0.81**
10c	10.65±1.34	19.19±2.16	2.00±0.84	10.33±1.33
10d	>20	>20	>20	>20
10e	>20	>20	>20	>20
10f	>20	16.95±1.68	>20	>20
10g	>20	>20	>20	>20
10h	>20	20.14±2.57	>20	>20
10i	6.87±0.86	16.34±1.67	2.24±0.64	5.76±0.64
10j	5.37±0.34	17.87±1.37	3.53±0.54	5.13±0.42
10k	12.58±1.36	>20	19.63±2.01	9.03±1.63
10l	8.11±0.58	>20	4.47±1.01	5.72±0.87
Gefitinib[c]	14.53±0.48	12.48±0.22	7.65±0.73	3.15±0.91

[a] Data shown represent the mean ± SD of three independent experiments, each performed in triplicate.
[b] IC_{50}: compounds concentration that reduces 50% cell proliferation evaluated by the MTT assay.
[c] A positive control.

In order to explore the SAR of the novel scaffold, hydrogen atom was introduced to R^3 (compounds **10d-h**) in the first. It was found that the anti-proliferation activities of compounds against the four EGFR high-expressing cells were not good *in vitro*, basically >20 μM. Only when R^2 and R^1 were both halogen atoms, just compounds **10f** and **10h** showed certain anti-proliferation

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activities against NCI-H1975 in the four EGFR high-expressing cells, which were 16.95 μM and 20.14 μM, respectively. When R^2 was replaced by hydrogen atom (compounds **10d**, **10g**) or R^1 was replaced by hydrogen atom (compound **10e**), the compounds showed no anti-proliferation activity against the four EGFR high-expressing cells. It may be that the radius of the hydrogen atom is too small to fill the pocket of EGFR kinase.

To find a more effective EGFR inhibitor, chlorine atom was introduced as R^3 (compounds **10a-c, 10i-j**) in the subsequent synthesis to explore the effect of the halogen atom at the R^3 position on the overall scaffold SAR. By comparing **10a** and **10f**, **10b** and **10h**, **10d** and **10j**, **10e** and **10i**, it was easy to find that compounds **10a**, **10b**, **10i**, and **10j** had a significant increase in the inhibitory activity of the four EGFR high-expressing cells *in vitro*. Obviously, the introduction of a chlorine atom at the R^3 position had a direct effect on the activity of the compounds. Compared with hydrogen atoms, the larger atomic radius of chlorine atoms may better fill the pocket of EGFR kinase.

To further verify the effect of the size of the atom or atomic group at the R^3 position on the SAR of the scaffold, a larger vinyl group was introduced in the subsequent synthesis, thereby obtaining compounds **10k-l**. By comparing **10i** and **10l**, **10j** and **10k**, that **10k** and **10l** with a vinyl group introduced at the R^3 position were slightly weaker activity than **10i** and **10j** with a chlorine atom at the position. All in all, the preliminary SAR study of the scaffold of the novel compounds showed that the introduction of a suitable atom or atomic group at the R^3 position had a significant effect on the activity of the compounds.

Enzyme assay

The compounds **10b**, **10i**, and **10j**, which were synthesized through the SAR research of the new scaffold in the early stage and showed the best in vitro inhibitory activity for the four EGFR high-expressing cells, were further studied for enzymatic activity to explore the inhibition of the new compounds scaffold on EGFR kinase. The results were shown in **Table 2**, the compound **10b** had the best effect, its IC_{50} for $EGFR^{wt}$ and $EGFR^{L858R}$ was 35.7 nM and 217.6 nM, respectively.

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Table 2

Compounds tested for their inhibitory effects on both $EGFR^{wt}$, $EGFR^{L858R}$, $EGFR^{L858R/T790M}$ and $EGFR^{T790M}$ (Mean ± SD, n = 3).[a]

Compd	IC_{50} (nM)[b]			
	$EGFR^{wt}$-TK	$EGFR^{L858R}$-TK	$EGFR^{L858R/T790M}$-TK	$EGFR^{T790M}$-TK
10b	35.7±3.7	217.6±8.6	N. D.[c]	>1000
10i	153.8±5.8	427.3±16.7	>1000	>1000
10j	352.4±10.9	N. D.	>1000	>1000
Gefitinib[d]	12.4±0.6	3.1±0.4	>1000	187.4±6.4

[a] Data shown represent the mean ± SD of three independent experiments, each performed in triplicate.
[b] IC_{50}: compounds concentration that reduces 50% enzyme activity evaluated by the enzyme assay.
[c] N. D.: not determined.
[d] A positive control.

Cell migration assay

In order to test whether compound **10b** had an inhibition effect on the migration progression of gastric cancer cells (AGS), lung cancer cells (A549) and liver cancer cells (HepG2), AGS cells, A549 and HepG2 cells were treated with compound **10b** (0.5 μM and 1.0 μM) or not for 0 to 48 h. AGS cells, A549 and HepG2 cells were allowed to migrate in scratch-wound cultures, and the migration of AGS cells, A549 and HepG2 cells in scratch-wound cultures was inhibited by the addition of compound **10b**. The ability of compound **10b** to inhibit the migration of AGS, A549 and HepG2 cells increased with the increasing of concentration **(Fig. 4).**

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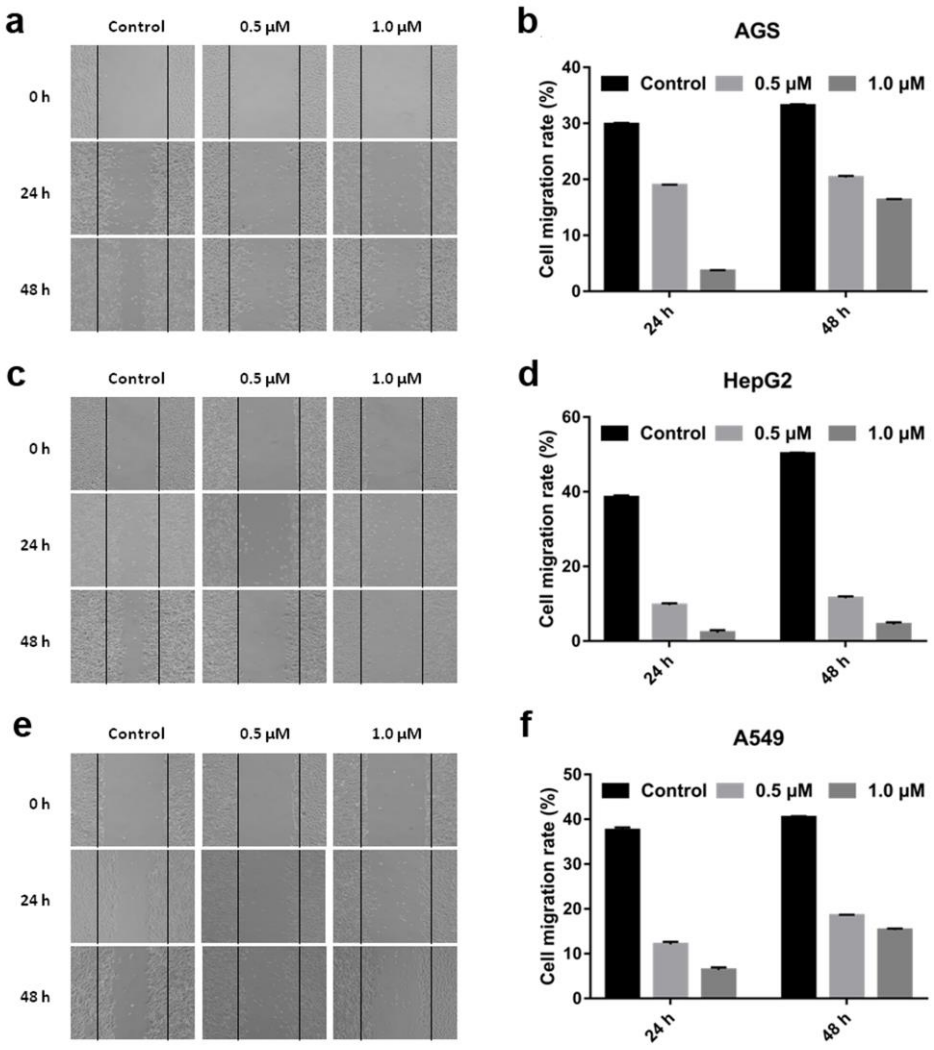

Fig. 4. Cell mobility inhibition for the migration of AGS, HepG2 and A549 treated with compound **10b** for 0 to 48 h. Figures **4a** and **4b** show the effect of compound **10b** on the migration of AGS cells; Figures **4c** and **4d** show the effect of compound **10b** on the migration of HepG2 cells; Figures **4e** and **4f** show the effect of compound **10b** on the migration of A549 cells.

Western blot assay

To further explore the anti-proliferative mechanism of compound **10b**. Western blot assay was used to evaluate the effect of compound **10b** on the expression of related proteins in A549 cells, including EGFR/p-EGFR, Akt/p-Akt, Erk/p-Erk. As shown in **Fig. 5**, the expressions of EGFR, Akt and Erk in A549 cells were not affected, while the expressions of p-EGFR, p-Akt and p-Erk decreased in a dose-dependent manner. Western blot assay showed that compound **10b** inhibited the proliferation of A549 cells by inhibiting the phosphorylation of EGFR and its downstream Akt and Erk.

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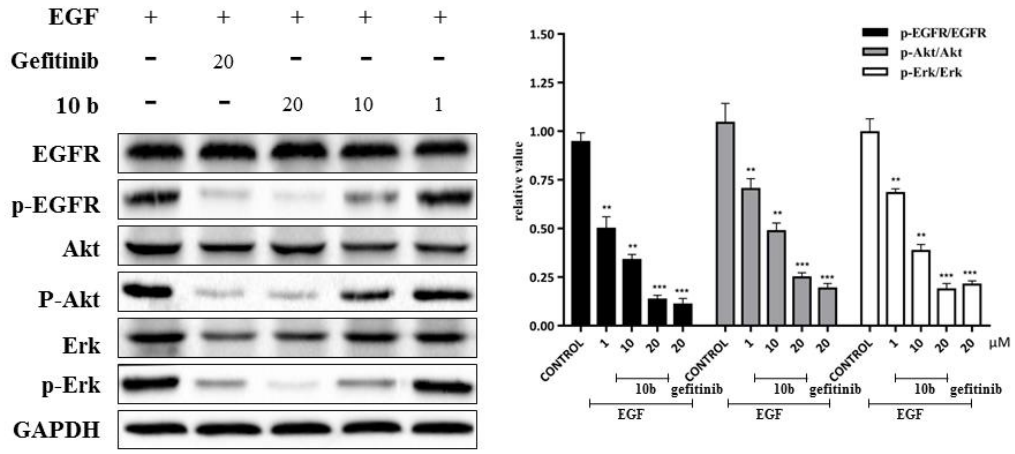

Fig. 5. Effects of compound **10b** on the expression of related proteins in A549 cells, including EGFR/p-EGFR, Akt/p-Akt, Erk/p-Erk. A549 cells were treated with different concentrations of compound **10b** or gefitinib for 12 h.

Docking study

To explore the binding mode of compound **10b** with EGFR (**PDB: 2ITY**), a docking study was carried out employing Discovery Studio 4.0 software. Before docking the designed compound molecule, the reliability of the model had been verified. The proposed binding mode of **10b** in the allostericbinding pocket was depicted in **Fig. 6**. It was observed that **10b** fitted to the hydrophobic environment composed with Leu788 and Leu844. The *N*-1 of the quinazoline scaffold forming a H-bond with the backbone NH of Lys728 side chain, and the aniline lying in an adjacent hydrophobic pocket. Moreover, the chlorine at the 2-position of quinazoline forming a hydrogen bond with Ala743, at the same time, the fluorine on the benzene ring and lys745 also having a hydrogen bond. This helps explain why compound **10b** had better biological activity.

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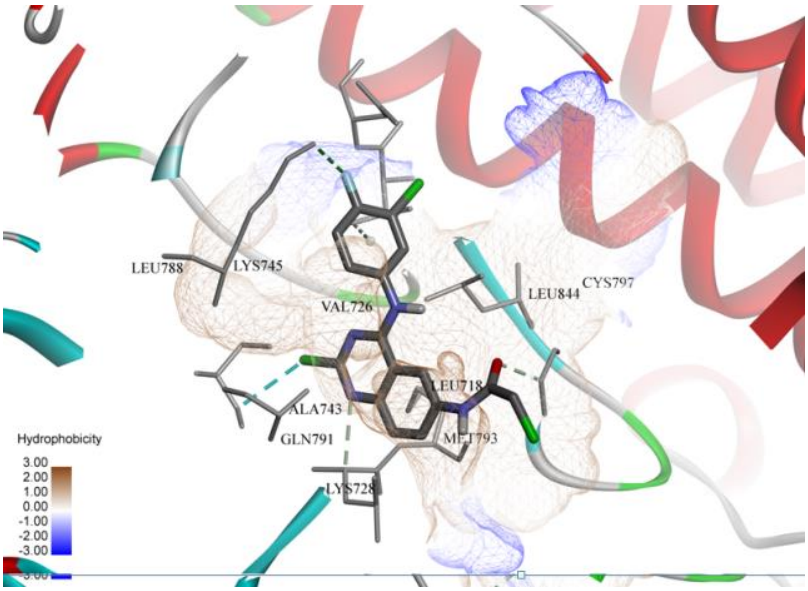

Fig. 6. Molecular docking between compound **10b** and EGFR kinase (**PDB: 2ITY**)

Conclusions

In conclusion, a series of novel *N*-(2-chloro-4-(phenylamine) quinazolin-6-yl) acetamide derivatives were synthesized, and their biological activities were evaluated *in vitro*. Most of new designed compounds had potential anti-proliferation activities against the A549, NCI-H1975, AGS and HepG2 four cell lines. The preliminary SAR study of the scaffold of new compounds showed that the compounds with a chlorine substituent on R^3 had a better anti-proliferation activity than those substituted by hydrogen atom or vinyl group. Among them, compound **10b** had the best activity. **10b** had a certain inhibitory effect on enzymes and the migration of tumor cells. Finally, the action channel of the compound **10b** was proved by western blotting experiments. It provides useful information for the design of EGFR-TK inhibitors.

Experimental Section

Chemistry

All reagents and solvents were purchased from commercial sources without further purification, and had been dried according to standard procedures. Reactions were monitored by using thin-layer chromatography (TLC) on silica gel 60 F_{254}. Melting points were determined on a X-4 melting point apparatus and uncorrected. IR spectra were taken on a Thermo Nicolet Avatar 370 FT-IR spectrophotometer (KBr pellets). NMR spectra were recorded at 500 MHz on a Bruker Anance III spectrometer. Mass spectra were obtained on a Bruker micrOTOF-Q mass spectrometer using ESI.

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Synthesis

Methyl-2-ureidobenzoate (2)

To a solution of methyl 2-aminobenzoate (**1**) (5 g, 33.08 mM) in acetic acid (25 ml) was treated with a solution of KNCO (3.88 g, 59.54 mM) in water (15 ml). After 15 mins mechanical stirring at room temperature, the resulting precipitate was collected by filtration, then washed with water twice and dried to yield Methyl-2-ureidobenzoate (**2**) (6 g, yield: 93%).

(1H)-Quinazolin-2,4-dione (3)

A solution of 15% NaOH solution was added to a mechanically stirred suspension of Methyl-2-ureidobenzoate (**2**) (6 g, 30.9 mM) in methanol (50 ml) adjusting the pH to 10. After 3 h at reflux, the mixture was cooled to room temperature and diluted with water (50 ml). The pH was adjusted to 1 with HCl. The precipitate was collected by filtration to yield a white solid (4.5 g, 90%).

6-Nitroquinazolin-2,4-dione (4)

The compound 3 (2.0 g, 12.3 mM) was dissolved in concentrated H_2SO_4 (6 mL), and this solution was added to a mixture of concentrated H_2SO_4 (2 mL) and HNO_3 (5.6 mL) at -10 ^oC. The mixture was stirred for 1 h at -5 ^oC.The reaction mixture was poured onto crushed ice (50 g). A crystalline solid was collected and washed with water. The precipitate was collected by filtration to yield a light yellow solid (1.8 g, 70%) mp 352-354 ^oC; MS (EI) m/z 207.

2,4-Dichloro-6-nitroquinazoline (5)

The reaction mixture of compound 4 (0.5 g, 2.41 mmol), *N*,*N*-Diisopropyl ethylamine (0.62 g, 4.4 mM) and $POCl_3$ (3 mL) was stirred at reflux for 6.0 h. The residue was dissolved in ice water. The water phase was extracted with EtOAc (60 mL) and the organic layer was dried over anhydrous $MgSO_4$, concentrated to give the crude product which was purified by column chromatography on silica gel (petroleum ether/DCM = 10:1) to afford compound 5 as white solid (0.36 g, 61%); mp 122-124 ^oC; ^1H NMR ($CDCl_3$) d: 8.18 (d, *J* = 9.0 Hz, 1H), 8.76 (dd, *J* = 9.3, 2.1 Hz, 1H), 9.18 (d, *J* = 1.8 Hz, 1H).

General procedure for the synthesis of 2-chloro-*N*-phenyl-6-nitroquinazolin-4-amine derivatives (7)

To a suspension of 2,4-dichloro-6-nitroquinazoline 5 (0.30 g, 1.23 mmol) in THF (30 mL) was added aniline analogs (1.48 mmol) and DIPEA (0.32 g, 2.46 mmol). The resulting mixture was stirred at room temperature for 8 h and concentrated under vacuum. The residue was dissolved in EtOAc. The crude product obtained after concentration was purified with column chromatography to afford product 7 as red solid.

General procedure for the synthesis of 2-chloro-*N*-4-phenylquinazoline-4,6-diamine derivatives (8)

To a dichloromethane solution of appropriate compound 7 (0.67 mmol), Fe (0.185 g, 3.33 mM) was added under the presence of acetic acid at the reflux temperature for 1 h. Thereafter, the

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catalyst was filtered over a celite bed and the filtrate was evaporated to yield a solid residue. The residue was recrystallized from MeOH to afford the pure compounds 8 as yellow solids.

General procedure for the synthesis of *N*-(2-chloro-4-(phenylamino)quinazolin-6-yl) acetamide derivatives (10)

The compound was synthesized from 2-chloro-N^4-phenylquinazoline-4,6-diamine derivatives 8 (0.37 mmol), corresponding acid chloride (0.44 mmol), and *N*,*N*-diisopropylethylamine (0.44 mmol) in 35 mL of THF. The resulting mixture was stirred at room temperature for 12 h and concentrated under vacuum. The residue was dissolved in EtOAc. The crude product obtained after concentration was purified with column chromatography to afford product 10.

Biological

Cell viability assay

The MTT assay was used to evaluate cell viability. Cells were seeded in a 96-well plate (4×10^3 cells/well). After 24 h of drug treatment, MTT was added to each well to a final concentration of 0.5 mg/ml, followed by incubation at 37 °C for 3 h. Then, the medium was removed, and 100 µl of dimethyl sufoxide (DMSO) was added per well. The absorbance in each well was measured at 570 nm using a Flexs tation 3 microplate reader (Molecular Devices). To determine the IC_{50} of 20A, a non-linear regression curve was fit using GraphPad Prism software.

In vitro kinase assay

The effects of compounds on the activity of EGFR kinase were determined using enzyme-linked immunosorbent assays (ELISAs) with purified recombinant proteins (Sino Biological Inc.). Briefly, 50 µg/mL poly (Glu,Tyr) 4:1 (Sigma) was pre-coated in 96-well plates at 37 °C as a substrate. The enzyme reaction was carried in kinase reaction buffer (50 mmol/L HEPES (pH 7.4), 0.01% BRIJ-35, 10 mmol/L $MgCl_2$, 4 mmol/L $MnCl_2$, 0.1 mmol/L EGTA, and 2 mmol/L DTT). 10 µL of various concentrations of indicated compounds and 20 µL of purified tyrosine kinase proteins were added to each reaction well. DMSO (0.5%, v/v) was used as the negative control. The kinase reaction was initiated by addition of a 50 µL of 5µmol/L ATP solution which diluted in kinase reaction buffer. After incubation for 60 min at 37 °C, the plates was washed 6 times with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST). Anti-phosphotyrosine mouse mAb (PTM Bio) antibody was then added. After a 60min incubation at 37 °C, the plate was washed 3 times with PBST, and horseradish peroxidase-conjugated goat anti-mouse IgG was added. The plate was then incubated at 37 °C for 40 min and washed 5 times with PBST. A 100 µL aliquot of a solution containing 0.03% H_2O_2 and 2 mg/ml TMB in 0.1 mol/L citrate buffer (pH 5.5)

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was added. The reaction was terminated by addition of 100 µL of 2 M H_2SO_4 as the color changed, and the plate was analyzed using a multi-well spectrophotometer (PE Enspire) at 450 nm. The IC_{50} values were calculated from the inhibition curves using GraphPad Prism5 (GraphPad Software, Inc.) in at least three separate experiments.

The emission ration was calculated using the following equation:

$$\text{Emission Ration} = OD_{450}/OD_{450control}$$

Wound healing

Cells were seeded in 6-well plates to form a cell monolayer (near 90% confluence), and a wound was created by scratching the length of the well with a 10 µL pipette tip. Then, the plate was washed three times with PBS to remove floating cells and photographed (time 0 h). The cells were incubated in serum lacking medium (0% FBS), then the corresponding concentration of compound was added. Next, the wounded cultures were incubated in conditioned medium at 37 ºC. At 0, 1 and 2 day, images were captured using an inverted microscope to assess wound closure and then compared to determine differences in cell migration. Each assay was replicated 3 times.

Western blotting assay

Cells were Collected and lysed in 1X RIPA buffer with phosphatase inhibitors. Cell lysates were cleared by centrifugation at 4 ºC for 15 min at 13200 xg. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking in 5% BSA/TBST buffer for 2 h, membranes were incubated in primary antibodies overnight at 4 ℃. The following primary antibodies were used in the dilution as suggested by the supplier: EGFR, P-EGFR, Akt, P-Akt, Erk, P-Erk, GAPDH (1:1,000; Cell Signal Technology). Then, blots were incubated in secondary antibodies for 2h at room temperature. Immersed in BeyoECL Star (Beyotime) for 2 min, and then photographed using a Bio-Rad gel imaging analyzer.

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Author Contribution Statement

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Quan Zheng and Xuan-Bo Xu designed and synthesized these compounds and wrote the article. Xuan-Bo Xu and Hao Jin performed the experiments, analyzed the data. Wen Zhang and Guo-Wu Rao designed and conceived the experiments.

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