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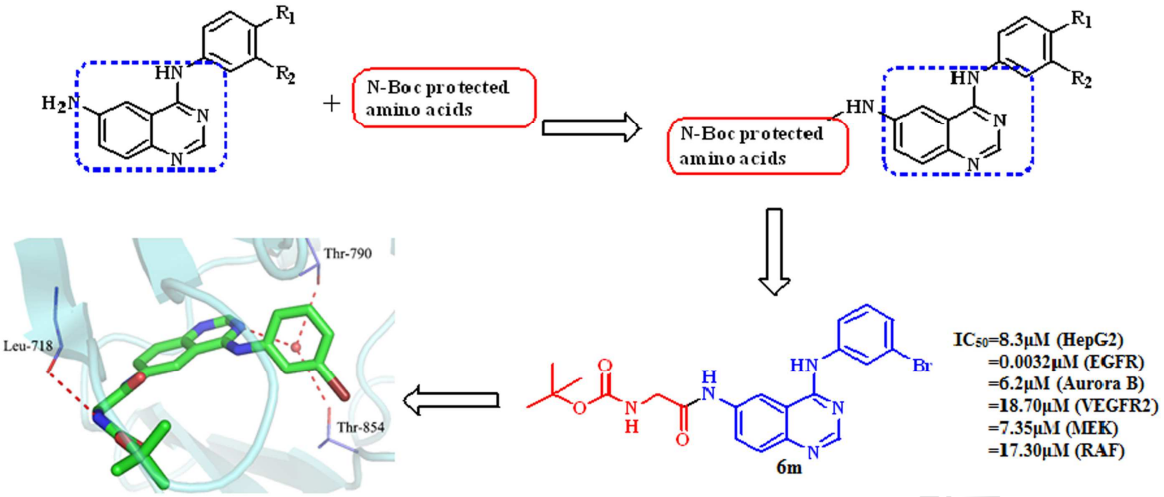


Design, synthesis, and biological evaluation of novel 4-anilinoquinazoline derivatives bearing amino acid moiety as potential EGFR kinase inhibitors

You-Guang Zheng[a, b, c]*, Jun Su[a, b], Cai-Yun Gao[a, b], Ping Jiang[a, b], Lin An[a, b], Yun-Sheng Xue[a, b], Jian Gao[a, b], Yi Liu[a, b]*

[a]College of Pharmacy, Xuzhou Medical University, Xuzhou 221004, PR China;

[b]Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, Xuzhou Medical University, Xuzhou 221004, PR China;

[c]Jiangsu Key Laborotary of Brain Disease Bioinformation, Xuzhou Medical University, Xuzhou 221004, PR China;

You-Guang Zheng and Yi Liu are co-corresponding authors.

ABSTRACT

In this study, a series of 4-anilinoquinazoline derivatives bearing amino acid moiety were designed, synthesized and evaluated for biological activities. The synthesized compounds were screened for anticancer activity against human hepatocellular carcinoma cell HepG2 using SRB assay. *In vitro* cell growth inhibition assays indicated that compound **6m** exhibited moderate inhibitory activities only against human hepatocellular carcinoma cells HepG2 with IC_{50} of 8.3μM. Synthetic derivatives showed excellent selectivity, such as compound **6m** demonstrated a strong inhibition of EGFR (IC_{50}=0.0032μM), with selectivity of over 2000-fold over other kinases. Apoptosis analysis revealed that compound **6m** caused obvious induction of cell apoptosis. **6m** significantly down-regulated the expression of Bcl-2 and up-regulated the expression of Bax, decreased mitochondrial membrane potential (ΔΨm), promoted the mitochondrial cytochrome c release into the cytoplasm, activated caspase-3, and finally induced apoptosis of HepG2 cells. Molecular docking indicated that compound **6m** could bind well with EGFR. Therefore, compound **6m** may be a potential agent for cancer therapy deserving further research.

Keywords: 4-anilinoquinazoline derivatives, amino acid moiety, HepG2, apoptosis

1. Introduction

The heterocyclic compounds play a very important role in medicinal chemistry due to their wide spectrum of biological properties. One class of the most important heterocyclic compounds is quinazolines which have wide range of biological properties such as the anticancer [1], antibacterial [2], anticonvulsant [3], antifungal [4], anti-HIV [5], antihypertensive [6], anti-inflammatory [7] and antimalarial [8] activities. In the past 15 years, FDA has approved several anticancer drugs which have quinazoline skeleton, such as gefitinib [9], erlotinib [10] and lapatinib [11]. In addition, there are some quinazoline derivatives have been studied in clinical trials, such as ZM447439 [12], AZD1152 [13], canertinib and so on [14] (Fig.1). Perusal of literatures also revealed that quinazoline derivatives exhibited remarkable *in vitro* anti-proliferative activity against the tumor cell lines，such as HepG2, A549, MCF-7 and so on[15-18]. Our research group has been focusing on the discovery of 4-anilinoquinazoline derivatives as anticancer agents since 2006. Previously, we had reported several 4-anilinoquinazoline derivatives [19, 20], which show significant activity against cancer cells.

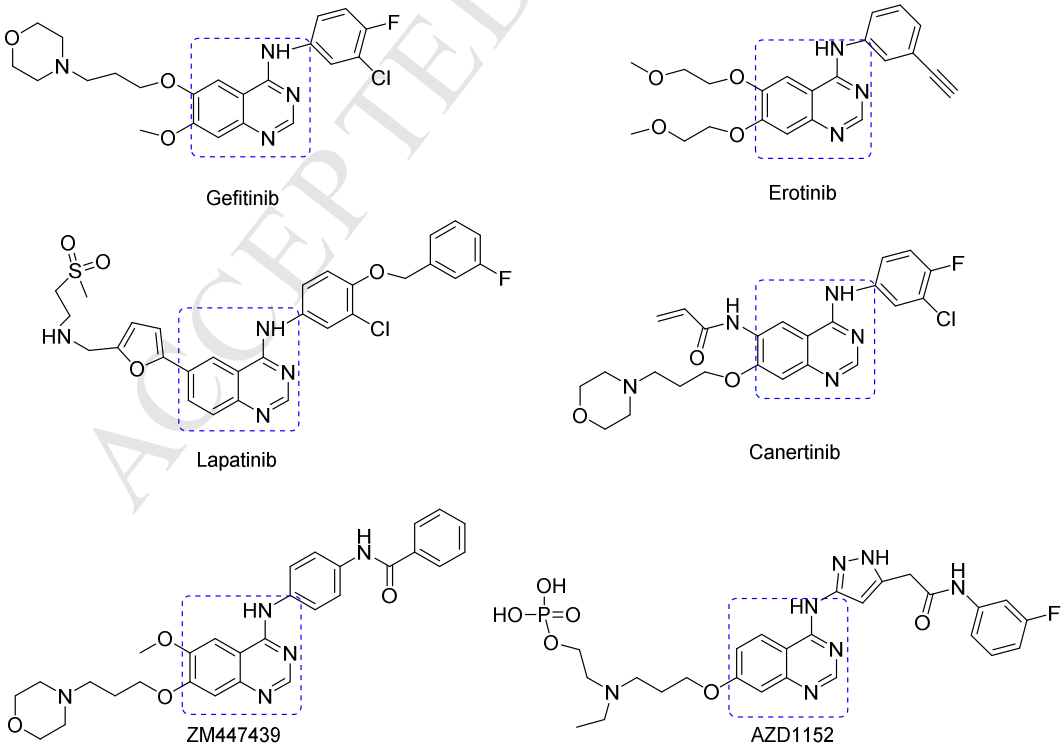


Fig.1. Quinazoline derivatives as anticancer agents

As endogenous substance, amino acid could be used to modification of drug skeletons to promote absorption of the drugs [21]. Furthermore, toxicity of drugs also could be reduced via the introduction of amino acid [22].

In the light of our previous studies, we designed and synthesized a series of novel 4-anilinoquinazoline derivatives through addition of various N-Boc protected amino acids on position 6 of quinazoline ring (Fig.2)

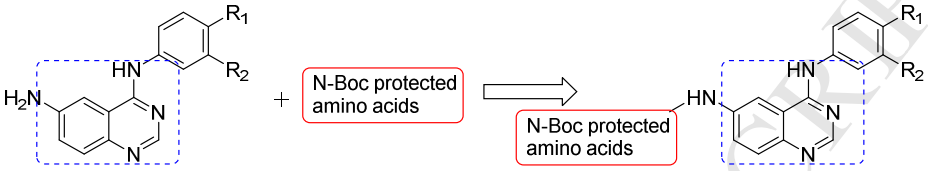
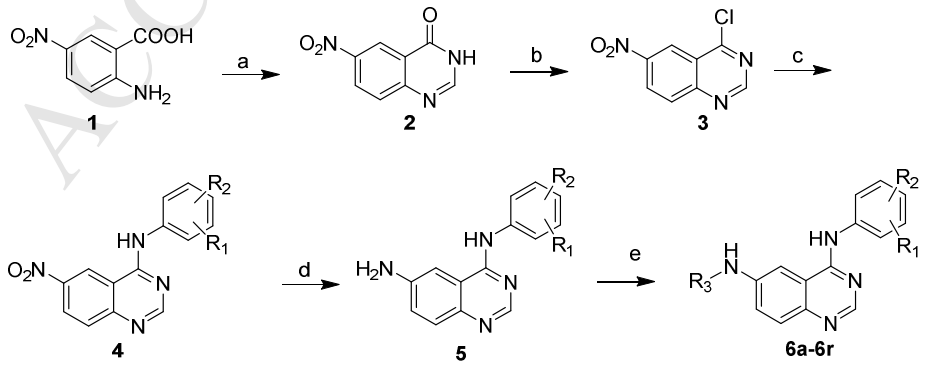


Fig.2. design of novel 4-aminoquinazoline-amino acid derivatives

2. Results and discussion

2.1. Chemistry

The general synthetic strategy to obtain the target compounds is shown in Scheme 1 according to the literatures being reported [23-25]. The route began with 2-amino-5-nitrobenzoic acid (**1**) as raw material, which was treated with formamidine acetate in 2-methoxyethanol to obtain 6-nitro-3H-quinazolin-4-one (**2**). Compound (**3**), which was prepared through chlorination of 6-nitro-3H-quinazolin-4-one **(2**), was subjected to reflux with various substituted aniline in isopropanol to give the products (**4**). Reduction of the nitro group on compound (**4**) using iron powder gave intermediates (**5**). The target compounds (**6a-6r**) were prepared by reacting compound (**5**) with various N-Boc protected amino acids.



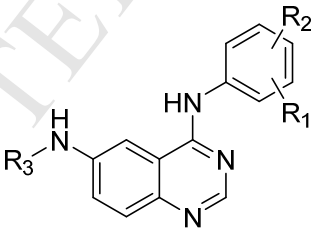
Scheme 1. The synthesis of 4-anilinoquinazoline-amino acid derivatives. Reagents and conditions: (a)formamidine acetate, 2-methoxyethanol, reflux; (b)$SOCl_2$, DMF, reflux; (c)substituted aniline, isopropanol, reflux; (d) iron powder, acetic acid/ethanol, refiux; (e) N-Boc protected amino acids, HATU/DMF/ triethylamine, rt.

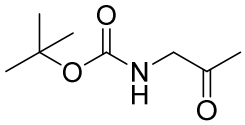
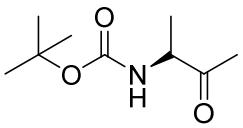
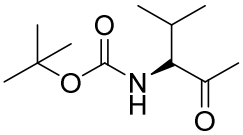
2.2. Biological evaluation

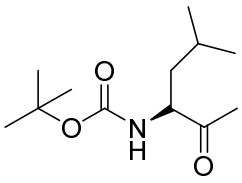
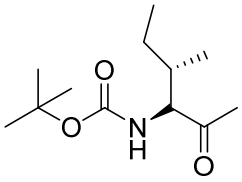
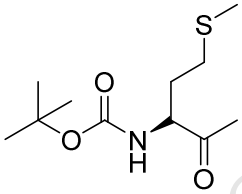
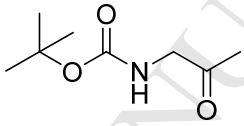
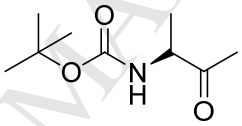
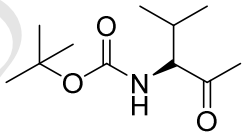
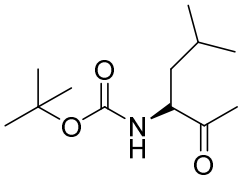
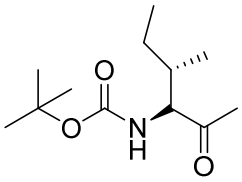
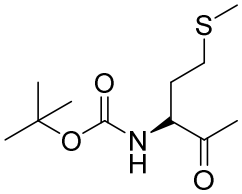
2.2.1. Anti-proliferation assay

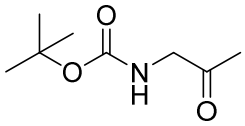
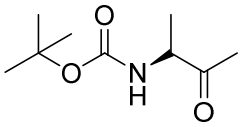
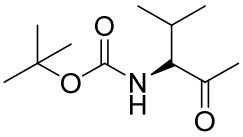
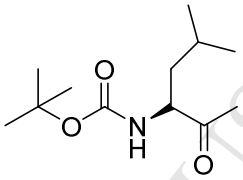
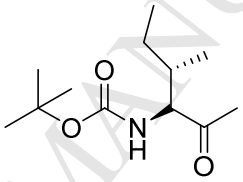
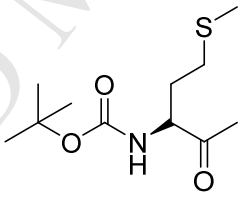
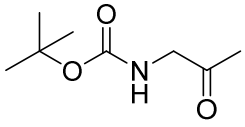
To explore the potential antitumor effect, the synthetic derivatives were evaluated for their *in vitro* anti-proliferative activities against human hepatocellular carcinoma cell HepG2 by SRB assay. As shown in Table 1, several compounds demonstrated moderate anti-proliferative activities against HepG2 (IC_{50} ranging from 4.3 to 34.8μM). Most of the target compounds with (3-bromophenyl) amino group on the 4-position of quinazoline ring showed moderate potency against the cancer cell line, such as compounds **6m**, **6p** and **6q** (IC_{50}=8.3, 25.0 and 34.0μM, respectively). It is worthy to note that compound **6i** exhibited significant anti-proliferative effects against HepG2 cell (IC_{50}=4.3μM) compared to other compounds bearing (3-(trifluoromethyl) phenyl) amino moiety at C-4 position of quinazoline ring.

Table 1 *In vitro* anti-proliferative activity in tumor cell lines of target compounds



Compd	R_1	R_2	R3	HepG2 IC_{50} (μM)
6a	3-Cl	4-F		33±14.9
6b	3-Cl	4-F		>50
6c	3-Cl	4-F		>50

6d	3-Cl	4-F		13.8±5.5
6e	3-Cl	4-F		>50
6f	3-Cl	4-F		>50
6g	3-CF_3	4-H		>50
6h	3-CF_3	4-H		>50
6i	3-CF_3	4-H		4.3±1.8
6j	3-CF_3	4-H		>50
6k	3-CF_3	4-H		>50
6l	3-CF_3	4-H		>50

6m	3-Br	4-H		8.3±0.6
6n	3-Br	4-H		>50
6o	3-Br	4-H		>50
6p	3-Br	4-H		25±13.5
6q	3-Br	4-H		34±16.1
6r	3-Br	4-H		>50
6s	2-CH_3	6-CH_3		>50
ZM447439				1.4±0.2

2.2.2. Kinases inhibitory activity of 4-anilinoquinazoline-amino acid derivatives

Several target compounds were assayed with the enzymatic activities against the kinases that related to liver cancer, such as EGFR, Aurora B, VEGF2, MEK, RAF and c-MET. Unfortunately, the result of kinases inhibitory activity indicated that most of the target compounds exhibited poor activity against Aurora B, VEGF2, MEK, RAF and c-MET, except compound **6m** showed weak IC_{50} value at concentration of 6.2μM (Aurora B), 18.7μM (VEGF2), 7.35μM (MEK), 17.30μM (RAF) and >500μM

(c-MET)(Table 2). Meanwhile, several of the compounds were also tested for their EGFR inhibitory activity. As shown in Table 2, all the tested compounds presented stronger EGFR inhibitory activity (IC_{50} ranging from 0.0032 to 1.02μM) compared to the other kinases. Especially, compound **6m** was the most potent inhibitor of EGFR (IC_{50}= 0.0032μM), with selectivity of 1937-fold over aurora B (IC_{50}= 6.2μM), 5843-fold over VEGF2 (IC_{50}= 18.7μM), 2296-fold over MEK (IC_{50}=7.25μM) and 5406-fold over RAF (IC_{50}=17.30μM). Among the tested compounds, compound **6a** also exhibited potent EGFR inhibitory activity with IC_{50} of 0.017μM. It seems that compounds (**6a** and **6m**) bearing N-Boc protected glycine group at 6-position of quinazoline skeleton have increased enzymatic inhibitory activity compared to the compounds contained other N-Boc protected amino acids, such as N-Boc protected L-alanine (**6b**, **6h** and **6n**). Based on the results of the anti-proliferative activity and enzyme inhibition activity, compound **6m** was chosen for further biological evaluation in HepG2 cells.

Table2 The kinases enzymatic inhibition activity of target compounds

Compd	IC_{50} (μM)					
	EGFR	Aurora B	VEGF2	MEK	RAF	c-MET
6a	0.017±0.0014	23.7±0.8	NT[a]	NT[a]	NT[a]	NT[a]
6b	>100	>100	NT[a]	NT[a]	NT[a]	NT[a]
6c	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]
6d	0.14±0.0057	>100	NT[a]	NT[a]	NT[a]	NT[a]
6e	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]
6f	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]
6g	NT[a]	51.0±3.7	NT[a]	NT[a]	NT[a]	NT[a]
6h	>100	>100	NT[a]	NT[a]	NT[a]	NT[a]
6i	1.021±0.0891	>100	>100	>100	>100	>100
6j	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]
6k	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]
6l	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]
6m	0.0032±0.0002	6.2 ±0.3	18.70±1.80	7.35±0.55	17.30±2.80	>100
6n	>100	>100	NT[a]	NT[a]	NT[a]	NT[a]
6o	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]
6p	0.145±0.0127	>100	NT[a]	NT[a]	NT[a]	NT[a]
6q	0.0555± 0.0049	>100	NT[a]	NT[a]	NT[a]	NT[a]
6r	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]
6s	NT[a]	>100	NT[a]	NT[a]	NT[a]	NT[a]

ZM447439	0.11±0.0113	0.156±0.0007	NT[a]	NT[a]	NT[a]	NT[a]

[a] Not Test

2.2.3. Apoptosis analysis

The morphology of apoptosis was observed using hoechst 33258 staining. As shown in Fig. 3, when treated with 2.0, 5.0, and 10.0 μM of compound **6m** for 24 h, nuclei appeared typical morphology of apoptosis, as condensed chromatin state, a high degree of nuclear chromatin condensation and appearance of apoptotic bodies. This result suggested that compound **6m** induced apoptosis of HepG2 cells effectively.

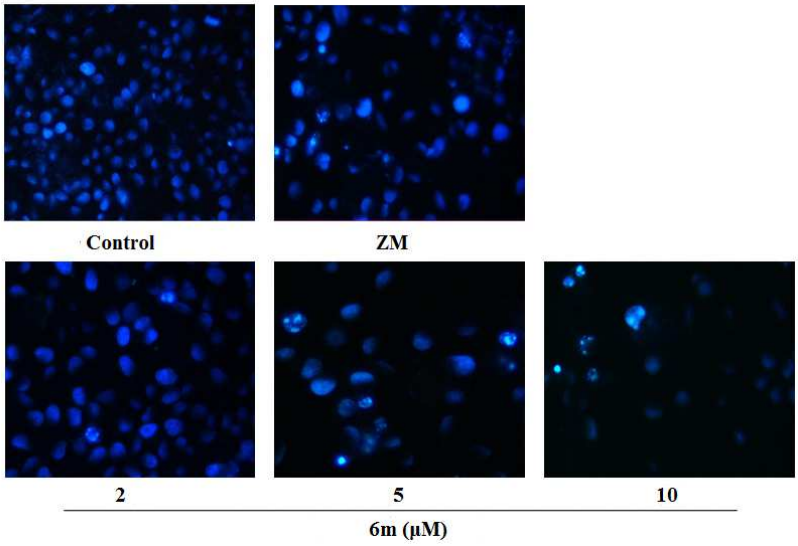


Fig.3. Fluorescent staining of nuclei by Hoechst 33258 in ZM447439 (ZM) and **6m** treated or untreated cells followed by observation using fluorescence microscope (×400).

To further confirm the induction of apoptosis by **6m**, the HepG2 cells were treated with various concentrations of **6m** (2, 5, 10μM), then harvested and stained with Annexin V, followed by PI staining in the dark. The samples were analyzed by flow cytometry. As shown in Fig. 4, the percentages of apoptotic HepG2 cells was increased from 4.86% (control) to 35.74% after 6m treatment, which suggested that compound **6m** caused obvious induction of cell apoptosis in a concentration-dependent manner.

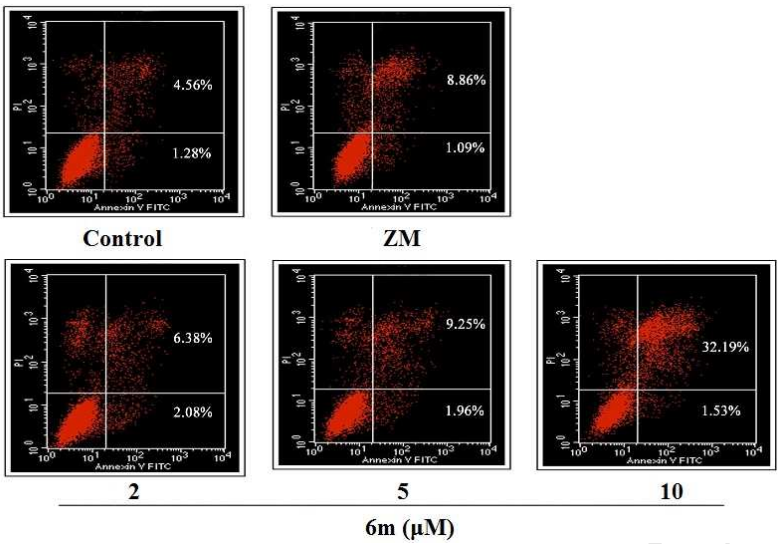


Fig.4. Apoptosis rates of HepG2 cells of the control and treated by ZM447439 (10 μM), **6m** (2 μM, 5 μM, 10 μM) groups, measured using flow cytometry with double staining of Annexin V and PI.

To explore the mechanism of compound **6m** induced HepG2 cells death, the change of mitochondrial membrane potential was investigated. After treatment of HepG2 cells with **6m** at the concentrations of 2, 5, 10 μM, JC-1 staining solution was then added, followed by photographing using fluorescence microscope. As shown in Fig. 5, after **6m** treatment for 24h, mitochondrial membrane potential of HepG2 cells was decreased. These results suggested that **6m** effectively decreased cells mitochondrial membrane potential in a concentration-dependent manner.

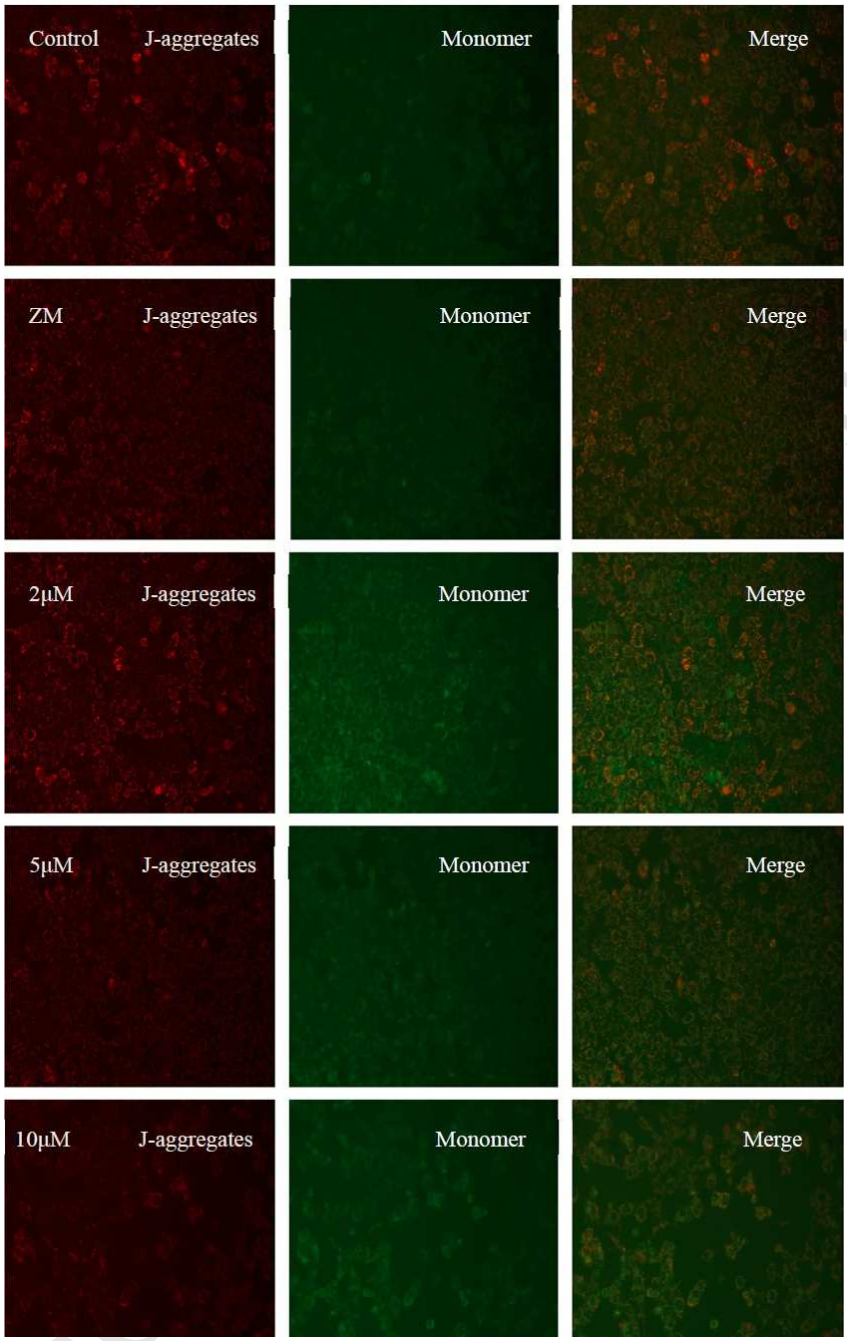


Fig.5. Effects of **6m** induced a decrease in mitochondrial membrane potential. Mitochondrial membrane potential of HepG2 cells was measured by JC-1, red fluorescence represents the mitochondrial aggregate JC-1 and green fluorescence indicates the monomeric JC-1(×200).

In order to further investigate the mechanism of the induction of cellular apoptosis, we examined the expressions of proteins related to apoptotic pathway such as Bax, Bcl-2, cyt c and cleaved-caspase-3 protein. Bcl-2 protein family (including anti-apoptosis protein Bcl-2 and pro-apoptotic protein Bax), which locates on cell

mitochondria and is associated with mitochondrial membrane, plays critical roles in apoptosis of cells [26]. The HepG2 cells were treated with various concentrations of **6m** (2, 5, 10μM) for 24h, and then western blot was used to detect the expression of Bax and Bcl-2. As shown in **Fig. 7**, 10μM of **6m** treatment significantly increased the expression of Bax (Fig. 6A, 6B), and decreased the levels of Bcl-2 (Fig. 6C, 6D).

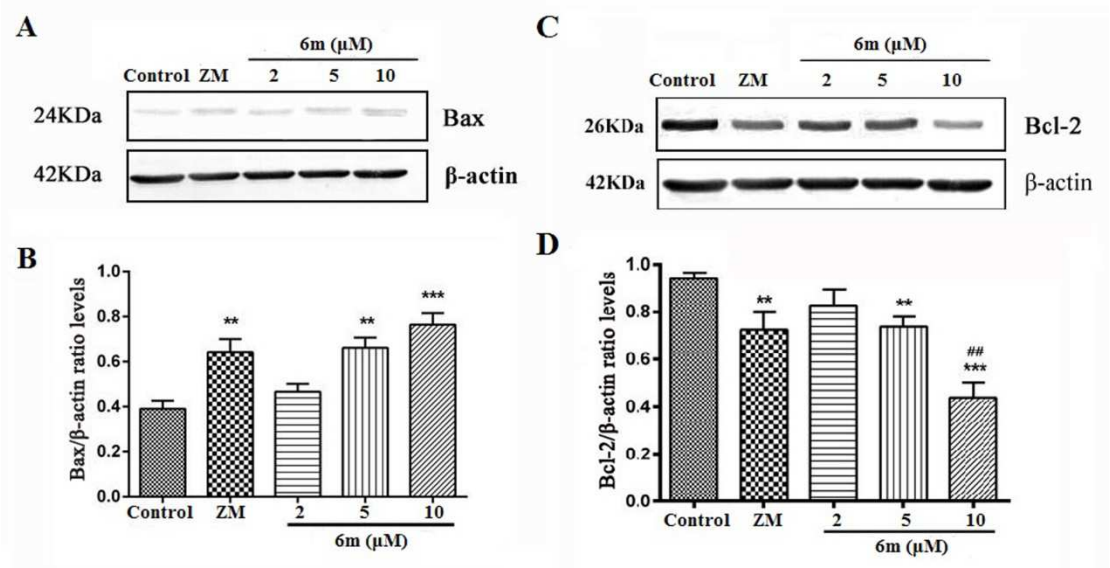


Fig.6. Effect of **6m** on the levels of Bax and Bcl-2 in HepG2 cells. 0.1% DMSO was used as solvent control and 10μM ZM447439 (ZM) used as positive control (A) Western blot analysis for Bax in HepG2 cell; (B) Densitometic analysis was performed after normalization with β-actin.; (C) Western blot analysis for Bcl-2 in HepG2 cell. (D) Densitometic analysis was performed after normalization with β-actin.

These findings demonstrated that compound **6m** decreased mitochondrial membrane potential by decreasing the expression of Bcl-2 and increasing the expression of Bax. We then examined the activation of mitochondrial cytochrome c (cyt c) to study the effect of mitochondrial membrane potential on mitochondrial membrane channel. As shown in Fig. 7, the treatment with 10μM of **6m** increased the expression of cyt c, which suggested that compound **6m** induced cellular apoptosis by the mitochondrial pathway.

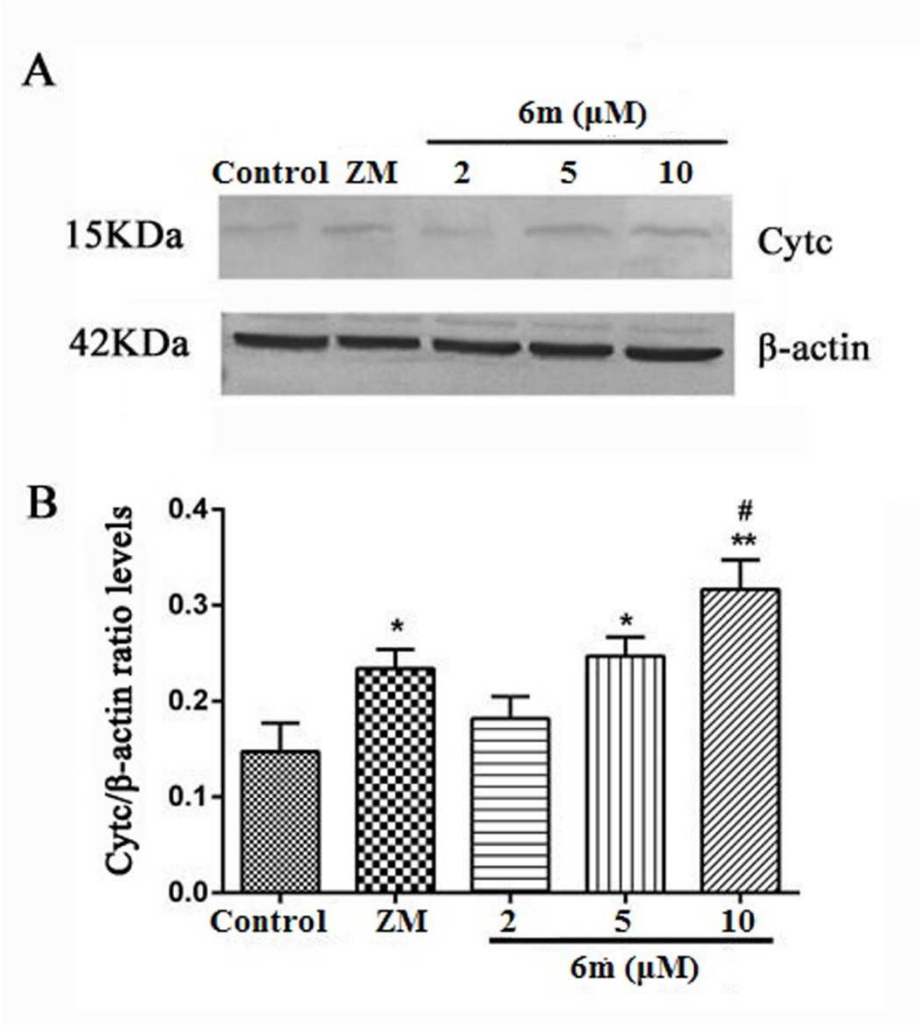


Fig.7. Effect of **6m** on the levels of cytochrome c (cyt c) in HepG2 cells. 0.1% DMSO as control group, 10 μM ZM447439 (ZM) group. (A) Western blot analysis for cyt c in HepG2 cell. (B) Densitometic analysis was performed after normalization with β-actin.

It has been recognized that caspase-3 expression is a critical initiator and executioner of apoptosis [27]. By 24 h treatment with **6m** at the concentrations of 2, 5, 10 μM, and then the functional activity of cleaved-caspase-3 was monitored using western blot. As shown in Fig. 8, the treatment with 5 and 10μM of **6m** increased the expression levels of cleaved-caspase-3, indicating that 6m induced apoptosis in HepG2 cells via decreasing activation of caspase-3.

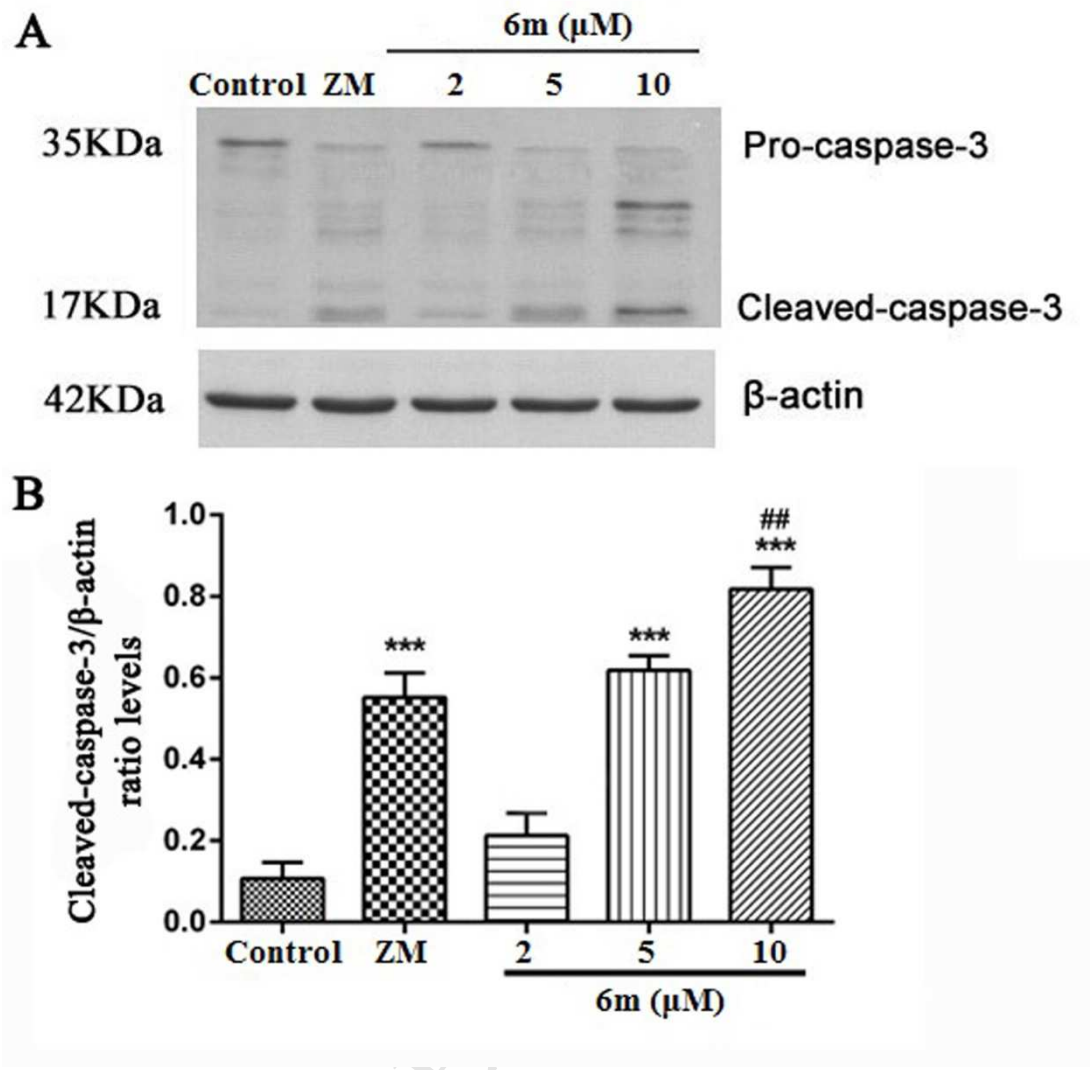


Fig.8. Effect of **6m** on the levels of caspase-3 in HepG2 cells. 0.1% DMSO as control group, 10μM ZM447439 (ZM) group. (A) Western blot analysis for caspase-3 and cleaved-caspase-3 in HepG2 cells. (B) Densitometic analysis was performed after normalization with β-actin.

2.3. Molecular docking studies

To understand the interaction between compound and kinases, the possible binding modes of compound **6m** on EGFR (PDB code: 2ITY) was explored using the AutoDock 4.2. As shown in Fig 9, the quinazoline ring with the 3-N is oriented in the back of the ATP-binding pocket, where its hydrogen bonds with the main chain amide of Thr790 and Thr 854 through a well-defined water molecule. Besides, the amino nitrogen atoms in the side chain amino acid of compound **6m** forms another hydrogen bond with the carbonyl oxygen atom of Leu718.

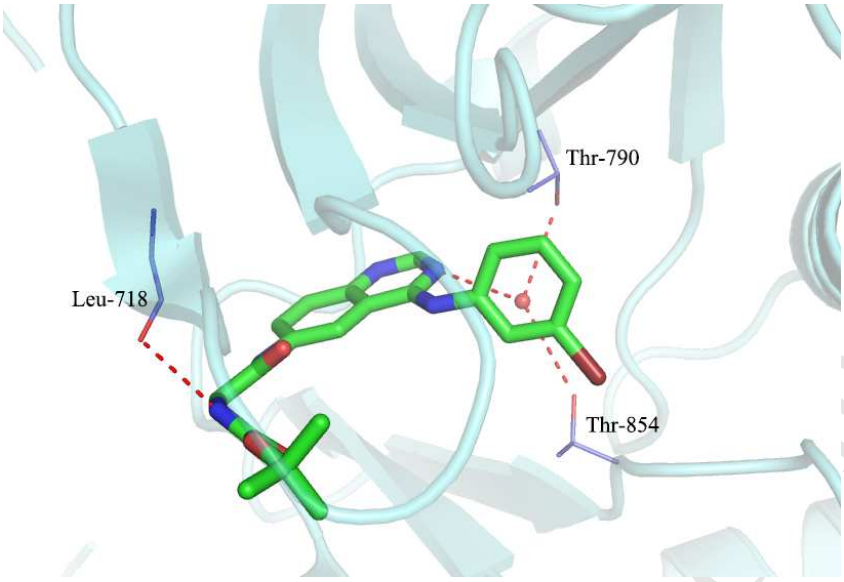


Fig.9. Binding models of compound **6m** target into active site of EGFR.

3. Conclusions

In summary, a series of 4-anilinoquinazoline-amino acid derivatives, which exhibited anti-proliferation potency against human hepatocellular carcinoma cell HepG2, were synthesized. *In vitro* cell growth inhibition assays indicated that compound **6m** exhibited moderate inhibitory activities against a human hepatocellular carcinoma cell HepG2 with IC_{50} of 8.3μM. Compound **6m** demonstrated a strong inhibition of EGFR (IC_{50}= 0.0032μM), with selectivity of over 2000-fold over other kinases. Apoptosis analysis revealed that compound **6m** down-regulated the expression of Bcl-2 and up-regulated the expression of Bax, decreased mitochondrial membrane potential (ΔΨm), and then promoted the mitochondrial cytochrome c release into the cytoplasm, activated caspase-3, induced apoptosis of HepG2 cells. Molecular docking of the compound **6m** into the active binding sites of EGFR, and the result indicated that compound **6m** could bind with EGFR.

4. Experimental

4.1. Chemistry

Unless otherwise noted, all reagents were purchased from commercial sources and used without further purification. All compounds were routinely monitored by thin-layer chromatography with silica gel GF-254 glass plates and viewed under UV light at 254 nm. The ^{1}H-NMR and ^{13}C-NMR spectra were determined in DMSO-d_6 or

$CDCl_3$ on a Bruker Avance 400 MHz spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) relative to tetramethylsilane (TMS), which was used as an internal standard. The mass spectra (MS) were obtained from Agilent 1100LC/MS Spectrometry Services. HR-MS was obtained using a Q-tof high resolution mass spectrometer.

*4.1.1. Synthesis of compounds (**2-5**)*

Compounds **2**, **3**, **4** and **5** were prepared according to a similar procedure in reference [23-25].

*4.1.2 General procedure for the preparation of the compound **6***

To a solution of N-Boc protected amino acids (1.2 mmol) in DMF (20 mL) was added HATU (1.2 mmol) and triethylamine (1.2 mmol). The mixture was stirred at rt for 1.0 h, then compound **5** was added, and was monitored by thin layer chromatography. After completion of the reaction, the reaction mixture was poured into ice water (200 mL) and extracted with ethyl acetate (3×50 mL). The combined organic extract was washed with brine, dried over Na_2SO_4, filtered, concentrated and purified by a silica gel column chromatography (8:1, EtOAc/petroleum ether) to afford compound **6**.

*4.1.2.1 tert-butyl (2-((4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)amino)-2-oxoethyl)carbamate (**6a**)*

Off-white powder, yield: 43.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.76 (s, 1H, NH), 8.57 (s, 1H, ArH), 8.40 (s, 1H, NH), 8.12 (s, 1H, ArH), 7.76 (d, *J* = 8.0 Hz, 2H, ArH), 7.55 (s, 1H, ArH), 7.48 (s, 1H, NH), 7.29 (s, 1H, ArH), 7.18 (t, *J* = 8.0 Hz, 1H, ArH), 4.14 (s, 2H, CH_2), 1.46 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.06, 157.78, 156.43, 153.47, 147.02, 137.15, 128.98, 127.56, 124.25, 123.09, 119.27, 119.09, 117.03, 116.81, 115.81, 112.15, 78.58, 44.19, 28.69; LC-MS *m/z*: 446.1 $[M+H]^+$; HRMS(ESI) Calcd for $C_{21}H_{21}ClFN_5NaO_3$ ($[M+Na]^+$):468.1215, found:468.1255.

4.1.2.2 *(R)-tert-butyl (1-((4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)amino) -1-oxopropan-2-yl)carbamate(**6b**)*

Off-white powder, yield: 47.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.99 (s, 1H, NH), 8.54 (s, 1H, ArH), 8.34 (s, 1H, NH), 8.20 (d, *J* = 4.0 Hz, 1H, ArH), 7.83 (d, *J* = 8.0

Hz, 2H, ArH), 7.38 (d, *J* = 12.0 Hz, 1H, ArH), 7.31 (s, 1H, NH), 7.29 (s, 1H, ArH), 7.20 (t, *J* = 8.0 Hz, 1H, ArH), 4.62(t, *J* = 8.0 Hz, 1H, CH), 1.62 (d, *J* = 8.0 Hz, 3H, CH_3), 1.44 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.64, 170.79, 157.80, 155.70, 153.46, 147.03, 137.32, 128.93, 124.19, 119.27, 119.09, 117.04, 116.82, 115.81, 112.16, 78.58, 50.88, 28.70, 18.41; LC-MS *m/z*: 461.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{22}H_{23}ClFN_5NaO_3$ ($[M+Na]^+$):482.1371, found:482.1428.

*4.1.2.3 (R)-tert-butyl (1-((4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)amino) -3-methyl-1-oxobutan-2-yl)carbamate(**6c**)*

Off-white powder, yield: 36.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.05 (s, 1H, NH), 8.52 (s, 1H, ArH), 8.49 (s, 1H, NH), 8.20 (d, *J* = 4.0 Hz, 1H, ArH), 7.90-7.87 (m, 2H, ArH), 7.37 (d, *J* = 8.0 Hz, 1H, ArH), 7.29 (s, 1H, NH), 7.22-7.18 (m, 2H, ArH), 4.36 (t, *J* = 8.0 Hz, 1H, CH), 2.28-2.23 (m, 1H, CH), 1.42 (s, 9H, CH_3), 1.25 (dd, *J* = 8.0 Hz, 6H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.52, 157.82, 156.08, 154.99, 153.50, 147.12, 137.08, 128.98, 127.70, 124.30, 119.28, 119.09, 117.03, 116.82, 115.82, 78.59, 61.12, 30.79, 28.68, 19.72; LC-MS *m/z*: 489.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{24}H_{27}ClFN_5NaO_3$ ($[M+Na]^+$): 510.1684, found: 510.1765.

*4.1.2.4 (R)-tert-butyl (1-((4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)amino) -4-methyl-1-oxopentan-2-yl)carbamate(**6d**)*

Off-white powder, yield: 28.0 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.32 (s, 1H, NH), 9.97 (s, 1H, ArH), 8.69 (s, 1H, NH), 8.57 (s, 1H, ArH), 8.13 (d, *J* = 8.0 Hz, 1H, ArH), 7.92 (d, *J* = 12.0 Hz, 1H, ArH), 7.81 (s, 1H, NH), 7.79 (s, 1H, ArH), 7.44 (t, *J* = 8.0 Hz, 1H, ArH), 7.14 (d, *J* = 8.0 Hz, 1H, ArH), 4.26-4.20 (m, 1H, CH), 2.51 (s, 2H, CH_2), 1.72-1.68 (m, 1H, CH), 1.40 (s, 9H, CH_3), 0.94 (dd, *J* = 4.0 Hz, 6H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.55, 157.80, 155.96, 153.48, 147.06, 137.30, 128.94, 127.75, 124.21, 119.28, 119.09, 117.05, 116.83, 115.80, 112.29, 78.55, 53.96, 41.06, 28.69, 23.46, 22.01; LC-MS *m/z*: 502.2 $[M+H]^+$; LC-MS *m/z*: 518.3 $[M+H]^+$; HRMS(ESI) Calcd for $C_{25}H_{29}ClFN_5NaO_3$ ($[M+Na]^+$): 524.1841, found: 524.1883.

4.1.2.5 (2R, 3S)-tert-butyl (1-((4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl) amino)-3-methyl-1-oxopentan-2-yl)carbamate(**6e**)

Off-white powder, yield: 30.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.83 (s, 1H, NH), 10.19 (s, 1H, ArH), 8.94 (s, 1H, ArH), 8.56 (s, 1H, ArH), 8.24 (s, 1H, NH), 8.11 (d, *J* = 8.0 Hz, 1H, ArH), 7.90 (s, 1H, NH), 7.77 (d, *J* = 8.0 Hz, 1H, ArH), 7.46-7.41 (m, 1H, ArH), 7.20 (d, *J* = 8.0 Hz, 1H, ArH), 4.05 (t, *J* = 8.0 Hz, 1H, CH), 2.04-1.95 (m, 1H, CH), 1.56-1.51 (m, 2H, CH_2), 1.40 (s, 9H, CH_3), 0.91 (d, *J* = 8.0 Hz, 3H, CH_3), 0.86 (t, *J* = 8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.66, 159.91, 146.76, 131.40, 126.29, 123.97, 123.52, 121.19, 115.19, 99.99, 79.72, 63.48, 37.07, 28.67, 25.14, 15.87, 11.27; LC-MS *m/z*: 502.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{25}H_{29}ClFN_5NaO_3$ ($[M+Na]^+$): 524.1841, found: 524.1830.

*4.1.2.6 (R)-tert-butyl (1-((4-((3-chloro-4-fluorophenyl)amino)quinazolin-6-yl)amino) -4-(methylthio)-1-oxobutan-2-yl)carbamate(**6f**)*

Off-white powder, yield: 46.7%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.35 (s, 1H, NH), 9.97 (s, 1H, ArH), 8.69 (s, 1H, NH), 8.57 (s, 1H, ArH), 8.13 (d, *J* = 4.0 Hz, 1H, ArH), 7.92 (d, *J* = 12.0 Hz, 1H, ArH), 7.81 (s, 1H, NH), 7.79 (s, 1H, ArH), 7.44 (t, *J* = 8.0 Hz, 1H, ArH), 7.26 (d, *J* = 8.0 Hz, 1H, ArH), 4.26 (t, *J* = 12.0 Hz, 1H, CH), 2.57 (m, 2H, CH_2), 2.53-2.50 (m, 2H, CH_2), 2.08 (s, 3H, CH_3), 1.40 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.67, 157.80, 156.03, 153.52, 147.09, 137.20, 128.93, 124.23, 119.28, 119.10, 117.05, 116.83, 115.78, 112.35, 78.71, 54.84, 31.98, 30.35, 28.68, 15.16; LC-MS *m/z*: 521.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{24}H_{27}ClFN_5NaO_3S$ ($[M+Na]^+$):542.1405, found:542.1414.

*4.1.2.7 tert-butyl (2-oxo-2-((4-((3-(trifluoromethyl)phenyl)amino)quinazolin-6-yl) amino)ethyl)carbamate(**6g**)*

Off-white powder, yield: 38.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.79 (s, 1H, NH), 8.59 (s, 1H, ArH), 8.24 (s, 1H, ArH), 8.16 (d, *J* = 8.0 Hz, 1H, ArH), 7.77 (d, *J* = 4.0 Hz, 1H, ArH), 7.64 (s, 1H, NH), 7.50 (t, *J* = 8.0 Hz, 2H, ArH), 7.45 (s, 1H, NH), 7.38 (d, *J* = 8.0 Hz, 1H, ArH), 7.28 (s, 1H, NH), 4.13 (d, *J* = 4.0 Hz, 2H, CH_2), 1.45 (s, 9H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.05, 153.83, 146.62, 139.60, 135.31, 131.34, 131.03, 129.31, 128.51, 126.90, 125.45, 124.37, 122.74, 120.27, 118.11, 115.17, 81.42, 45.15, 28.29; LC-MS *m/z*: 462.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{22}H_{22}F_3N_5NaO_3$ ($[M+Na]^+$):484.1572, found:484.1615.

*4.1.2.8 (R)-tert-butyl (1-oxo-1-((4-((3-(trifluoromethyl)phenyl)amino)quinazolin-6-yl) amino)propan-2-yl)carbamate(**6h**)*

Off-white powder, yield: 36.5%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.31(s, 1H, CONH), 10.10(s, 1H, CONH), 8.75(d, 1H, *J*=2.00Hz, NH), 8.59(s, 1H, Ar-H), 8.27(s, 1H, Ar-H), 8.20(d, 1H, *J*=8.00Hz, Ar-H), 7.92(d, 1H, *J*=8.80Hz, Ar-H), 7.82(d, 1H, *J*=8.80Hz, Ar-H), 7.62(t, 1H, *J*=8.00Hz, Ar-H), 7.46(d, 1H, *J*=8.00Hz, Ar-H), 7.20(d, 1H, *J*=7.20Hz, Ar-H), 4.22(t, 1H, *J*=7.60Hz, CH), 1.40(s, 9H, CH_3), 1.23(t, 3H, *J*=7.20Hz, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.61, 156.96, 153.74, 146.69, 139.89, 135.80, 131.23, 130.91, 129.26, 128.55, 126.76, 125.52, 124.26, 122.81, 119.96, 117.98, 115.19, 111.45, 81.03, 52.78, 51.23, 45.88, 28.30, 18.08, 8.96; ESI-MS 476.2 $[M+1]^+$. HRMS (ESI) Calcd for $C_{23}H_{25}F_3N_5O_3$ ($[M+Na]^+$):476.1904, found:476.1913.

*4.1.2.9 (R)-tert-butyl (3-methyl-1-oxo-1-((4-((3-(trifluoromethyl)phenyl)amino) quinazolin-6-yl)amino)butan-2-yl)carbamate(**6i**)*

Off-white powder, yield: 36. 0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.03 (s, 1H, NH), 8.70 (s, 1H, ArH), 8.57 (s, 1H, ArH), 8.38 (d, *J* = 8.0 Hz, 1H, ArH), 8.31 (s, 1H, NH), 7.87 (d, *J* = 8.0 Hz, 1H, ArH), 7.54 (t, *J* = 8.0 Hz, 1H, ArH), 7.40 (d, *J* = 12.0 Hz, 2H, ArH), 7.33 (s, 1H, NH), 7.28 (s, 1H, ArH), 4.39 (t, *J* = 8.0 Hz, 1H, CH), 2.30-2.22 (m, 1H, CH), 1.41 (s, 9H, CH_3), 1.23-1.20 (m, 6H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.51, 156.90, 153.76, 146.42, 139.97, 135.20, 131.28, 130.96, 129.32, 128.22, 126.84, 125.05, 124.09, 119.91, 115.07, 100.00, 81.27, 61.12, 30.82, 28.31, 19.28; LC-MS *m/z*: 504.3 $[M+H]^+$; HRMS(ESI) Calcd for $C_{25}H_{28}F_3N_5NaO_3$ ($[M+Na]^+$): 526.2024, found:526.2101.

*4.1.2.10 (R)-tert-butyl (4-methyl-1-oxo-1-((4-((3-(trifluoromethyl)phenyl)amino) quinazolin-6-yl)amino)pentan-2-yl)carbamate(**6j**)*

Off-white powder, yield: 40.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.01 (s, 1H, NH), 8.55 (d, *J* = 16.0 Hz, 2H, ArH), 8.36 (s, 1H, NH), 8.29 (d, *J* = 8.0 Hz, 1H, ArH), 7.86 (d, *J* = 8.0 Hz, 1H, ArH), 7.54 (t, *J* = 8.0 Hz, 1H, ArH), 7.39 (d, *J* = 8.0 Hz, 2H, ArH), 7.31 (s, 1H, NH), 7.28 (s, 1H, ArH), 4.63-4.58 (m, 1H, CH), 2.06 (t, *J* = 8.0 Hz, 2H, CH_2), 1.87-1.84 (m,1H, CH), 1.41 (s, 9H, CH_3), 1.06 (dd, *J* = 8.0Hz, 6H, CH_3); ^{13}C

NMR (100 MHz, $CDCl_3$) δ 157.46, 156.78, 153.86, 146.76, 140.03, 135.29, 129.31, 126.81, 123.94, 119.84, 117.76, 115.06, 81.36, 54.38, 41.22, 28.33, 25.01, 21.42; LC-MS *m/z*: 518.3 $[M+H]^+$; HRMS(ESI) Calcd for $C_{26}H_{30}F_3N_5NaO_3$ ($[M+Na]^+$):540.2198, found:540.2285.

*4.1.2.11 (2R, 3S) -tert-butyl (3-methyl-1-oxo-1-((4-((3-(trifluoromethyl)phenyl)amino) quinazolin-6-yl)amino)pentan-2-yl)carbamate(**6k**)*

Off-white powder, yield: 37.7%. ^1H NMR (400 MHz, $CDCl_3$) δ 10.01 (s, 1H, NH), 8.65 (s, 1H, ArH), 8.57 (s, 1H, ArH), 8.37 (d, *J* = 8.0 Hz, 1H, ArH), 8.30 (s, 1H, NH), 7.87 (d, *J* = 8.0 Hz, 1H, ArH), 7.55 (t, *J* = 8.0 Hz, 1H, ArH), 7.43-7.40 (m, 2H, ArH), 7.36 (s, 1H, NH), 7.28 (s, 1H, ArH), 4.43 (t, *J* = 8.0 Hz, 1H, CH), 2.01-1.99 (m, 1H, CH), 1.86-1.82 (m, 2H, CH_2), 1.41 (s, 9H, CH_3), 1.18 (d, *J* = 8.0 Hz, 3H, CH_3), 1.04 (t, *J* = 8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.44, 156.94, 153.74, 146.35, 139.91, 135.20, 131.29, 130.97, 129.32, 128.19, 126.82, 125.54, 125.05, 124.16, 122.84, 117.95, 115.03, 99.99, 81.27, 60.23, 37.29, 28.30, 25.60, 15.66, 11.22; LC-MS *m/z*: 518.3 $[M+H]^+$; HRMS(ESI) Calcd for $C_{26}H_{31}F_3N_5O_3$ ($[M+H]^+$):518.2379, found:518.2372.

*4.1.2.12 (R)-tert-butyl (4-(methylthio)-1-oxo-1-((4-((3-(trifluoromethyl)phenyl)amino) quinazolin-6-yl)amino)butan-2-yl)carbamate(**6l**)*

Off-white powder, yield: 42.0%. ^1H NMR (400 MHz, $CDCl_3$) δ 9.92 (s, 1H, NH), 8.60 (s, 1H, ArH), 8.31-8.23 (m, 2H, ArH), 7.81 (d, *J* = 8.0 Hz, 1H, ArH), 7.54 (t, *J* = 8.0 Hz, 2H, ArH), 7.47 (s, 1H, NH), 7.45 (s, 1H, NH), 7.40 (d, *J* = 8.0 Hz, 1H, ArH), 7.28 (s, 1H, ArH), 4.70 (d, *J* = 4.0 Hz, 1H, CH), 2.78-2.73 (m, 2H, CH_2), 2.32-2.27 (m, 2H, CH_2), 2.18 (s, 3H, CH_3), 1.43 (s, 9H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.25, 156.88, 153.92, 146.79, 139.77, 135.20, 131.02, 129.34, 128.69, 126.70, 125.04, 124.18, 122.79, 120.08, 117.90, 115.09, 81.50, 55.11, 32.21, 30.48, 28.35, 15.57; LC-MS *m/z*: 536.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{25}H_{28}F_3N_5NaO_3S$ ($[M+Na]^+$):558.1763, found:558.1780.

*4.1.2.13 tert-butyl (2-((4-((3-bromophenyl)amino)quinazolin-6-yl)amino)-2-oxoethyl) carbamate(**6m**)*

Off-white powder, yield: 41.2%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.27(s, 1H, CONH), 9.94(s, 1H, CONH), 8.77(s, 1H, NH), 8.58(s, 1H, Ar-H), 8.16(t, 1H, *J*=1.60Hz, Ar-H), 7.84-7.87(m, 2H, Ar-H), 7.81(d, 1H, *J*=9.20Hz, Ar-H), 7.28-7.37(m, 2H, Ar-H), 7.15(t, 1H, *J*=6.00Hz, Ar-H), 3.81(d, 2H, *J*=6.40Hz, CH_2), 1.41(s, 9H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 153.91, 146.79, 135.28, 130.08, 128.64, 126.74, 124.24, 122.45, 119.89, 115.21, 81.35, 28.33; ESI-MS 472.1 $[M+1]^+$; HRMS(ESI) Calcd for $C_{21}H_{23}BrN_5O_3$ ($[M+H]^+$):472.0979, found:472.0990.

*4.1.2.14 (R)-tert-butyl (1-((4-((3-bromophenyl)amino)quinazolin-6-yl)amino)-1-oxopropan-2-yl)carbamate(**6n**)*

Off-white powder, yield: 36.5%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.35(s, 1H, CONH), 10.27(s, 1H, CONH), 8.77(d, 1H, *J*=1.20Hz, NH), 8.15(s, 1H, Ar-H), 8.12(s, 1H, Ar-H), 7.92(t, 1H, *J*=8.80Hz, Ar-H), 7.83(d, 2H, *J*=8.80Hz, Ar-H), 7.33-7.39(m, 2H, Ar-H), 7.20(d, 1H, *J*=7.20Hz,Ar-H), 3.08-3.11(m, 1H CH), 1.40(s, 9H, CH_3), 1.32(d, 3H, *J*=7.20Hz, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.64, 156.89, 153.61, 146.19. 240.47, 135.66, 130.07, 128.24, 126.76, 126.69, 124.24, 122.43, 119.87, 115.04, 111.43, 81.23, 51.31, 28.33, 17.89, 8.60; ESI-MS 487.1 $[M+1]^+$; HRMS(ESI) Calcd for $C_{22}H_{25}BrN_5O_3$ ($[M+Na]^+$):486.1135, found:486.1148.

*4.1.2.15 (R)-tert-butyl (1-((4-((3-bromophenyl)amino)quinazolin-6-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate(**6o**)*

Off-white powder, yield: 36.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H, NH), 9.96 (s, 1H, ArH), 8.71 (s, 1H, NH), 8.59 (s, 1H, ArH), 8.16 (s, 1H, NH), 7.91 (d, *J* = 8.0 Hz, 1H, ArH), 7.85 (d, *J* = 8.0 Hz, 1H, ArH), 7.80 (d, *J* = 8.0 Hz, 1H, ArH), 7.35 (t, *J* = 8.0 Hz, 1H, ArH), 7.29 (d, *J* = 8.0 Hz, 1H, ArH), 6.99 (d, *J* = 8.0 Hz, 1H, ArH), 4.02-3.98 (m, 1H, CH), 2.10-2.04 (m, 1H, CH), 1.41 (s, 9H, CH_3), 0.96 (d, *J* = 4.0 Hz, 6H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.46, 171.52, 157.80, 156.08, 153.49, 147.14, 141.67, 137.09, 130.76, 128.94, 127.74, 126.34, 124.82, 121.59, 115.96, 112.38, 78.60, 63.95, 30.80, 28.69, 19.73; LC-MS *m/z*: 515.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{24}H_{28}BrN_5NaO_3$ ($[M+Na]^+$):538.1253, found:538.1260.

*4.1.2.16 (R)-tert-butyl (1-((4-((3-bromophenyl)amino)quinazolin-6-yl)amino)-4-methyl-1-oxopentan-2-yl)carbamate(**6p**)*

Off-white powder, yield: 32.6%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.31 (s, 1H, NH), 9.95 (s, 1H, ArH), 8.69 (s, 1H, NH), 8.59 (s, 1H, ArH), 8.17 (s, 1H, NH), 7.94 (d, *J* = 8.0 Hz, 1H, ArH), 7.86 (d, *J* = 8.0 Hz, 1H, ArH), 7.80 (d, *J* = 8.0 Hz, 1H, ArH), 7.35 (t, *J* = 8.0 Hz, 1H, ArH), 7.29 (d, *J* = 8.0 Hz, 1H, ArH), 7.13 (d, *J* = 8.0 Hz, 1H, ArH), 4.23 (t, *J* = 12.0 Hz, 1H, CH), 2.52-2.50 (m, 2H, CH_2), 1.73-1.68 (m, 1H, CH), 1.40 (s, 9H, CH_3), 0.94 (d, *J* = 8.0 Hz, 6H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.46, 157.79, 155.96, 153.47, 137.32, 130.77, 126.31, 124.75, 121.59, 121.33, 115.95, 78.55, 53.96, 28.69, 23.46, 21.51; LC-MS *m/z*: 529.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{25}H_{30}BrN_5NaO_3$ ($[M+Na]^+$):550.1430, found:550.1423.

*4.1.2.17 (2R, 3S)-tert-butyl (1-((4-((3-bromophenyl)amino)quinazolin-6-yl)amino)-3-methyl-1-oxopentan-2-yl)carbamate(**6q**)*

Off-white powder, yield: 28.0%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.35 (s, 1H, NH), 9.96 (s, 1H, ArH), 8.72 (s, 1H, NH), 8.59 (s, 1H, ArH), 8.16 (s, 1H, NH), 7.92 (d, *J* = 8.0 Hz, 1H, ArH), 7.85 (d, *J* = 8.0 Hz, 1H, ArH), 7.80 (d, *J* = 8.0 Hz, 1H, ArH), 7.35 (t, *J* = 8.0 Hz, 1H, ArH), 7.29 (d, *J* = 8.0 Hz, 1H, ArH), 7.03 (d, *J* = 8.0 Hz, 1H, ArH), 4.05 (t, *J* = 8.0 Hz, 1H, CH), 2.20-2.17 (m, 1H, CH), 1.56-1.53 (m, 2H, CH_2), 1.40 (s, 9H, CH_3), 0.93 (d, *J* = 4.0 Hz, 3H, CH_3), 0.87 (t, *J* = 8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.66, 157.81, 155.98, 153.49, 141.68, 137.10, 130.76, 128.94, 126.33, 124.83, 121.59, 121.41, 115.97, 112.32, 78.57, 59.94, 36.74, 28.69, 25.13, 15.86, 11.23; LC-MS *m/z*: 529.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{25}H_{30}BrN_5NaO_3$ ($[M+Na]^+$):550.1430, found:550.1427.

*4.1.2.18 (R)-tert-butyl (1-((4-((3-bromophenyl)amino)quinazolin-6-yl)amino)-4-(methylthio)-1-oxobutan-2-yl)carbamate(**6r**)*

Off-white powder, yield: 38.3%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H, NH), 9.95 (s, 1H, ArH), 8.69 (s, 1H, NH), 8.59 (s, 1H, ArH), 8.17 (s, 1H, NH), 7.93 (d, *J* = 12.0 Hz, 1H, ArH), 7.86 (d, *J* = 8.0 Hz, 1H, ArH), 7.81 (d, *J* = 12.0 Hz, 1H, ArH), 7.35 (t, *J* = 8.0 Hz, 1H, ArH), 7.29 (d, *J* = 8.0 Hz, 1H, ArH), 7.25 (d, *J* = 8.0 Hz, 1H,

ArH) 4.29-4.26 (m, 1H, CH), 2.60-2.56 (m, 2H, CH_2), 2.52-2.50 (m, 2H, CH_2), 2.09 (s, 3H, CH_3), 1.40 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.46, 157.79, 156.03, 153.52, 147.14, 141.68, 137.21, 130.78, 128.90, 126.32, 124.76, 121.60, 115.93, 112.50, 99.99, 78.71, 63.95, 32.00, 30.36, 28.69, 15.16; LC-MS *m/z*: 547.2 $[M+H]^+$; HRMS(ESI) Calcd for $C_{24}H_{28}BrN_5NaO_3S$ ($[M+Na]^+$):570.0973, found:570.0965.

*4.1.2.19 tert-butyl (2-((4-((2,6-dimethylphenyl)amino)quinazolin-6-yl)amino)-2-oxoethyl)carbamate(**6s**)*

Off-white powder, yield: 50.9%. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.30(s, 1H, CONH), 10.10(s, 1H, CONH), 9.54(s, 1H, NH), 8.67(s, 1H, Ar-H), 8.26(s, 1H, Ar-H), 7.86(d, 1H, *J*=9.20Hz, Ar-H), 7.73(d, 1H, *J*=9.20Hz, Ar-H), 7.14(s, 4H, Ar-H), 3.83(d, 2H, *J*=9.60Hz, CH_2), 2.12(s, 6H, CH_3), 1.40(s, 9H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.81, 154.26, 136.31, 128.26, 127.49, 126.51, 114.85, 45.97, 28.31, 18.42, 8.64; ESI-MS 422.2 $[M+1]^+$; HRMS(ESI) Calcd for $C_{23}H_{28}N_5O_3$ ($[M+Na]^+$): 422.2187, found: 422.2180.

4.2 Cytotoxicity assay

The cytotoxicity of each compound was determined using sulforhodamine B assay. Cells were seeded in 96-well plates and then treated with different concentrations of drugs. After 72 h of incubation, cells were fixed with 10% trichloroacetic acid for 1 h at 4^{o}C, washed 5 times with tap water and air-dried. Cells that survived were stained with 0.4% (w/v) sulforhodamine B (SRB) for 20 min at room temperature and washed 5 times with 1% acetic acid. Bound SRB was solubilized with 10mM Tris and absorbance was measured at 540 nm.

4.3 Kinase inhibition assays

Theses assays were carried out as described previously. All of the enzymatic reactions were conducted at 30 ^{o}C for 40 minutes. The 50μL reaction mixture contains 40mM Tris, pH 7.4, 10mM $MgCl_2$, 0.1 mg/ml BSA, 1mM DTT, 10μM ATP, Kinase and the enzyme substrate. The compounds were diluted in 10% DMSO and 5μl of the dilution was added to a 50μL reaction so that the final concentration of DMSO is 1% in all of

reactions. The assay was performed using Kinase-Glo Plus luminescence kinase assay kit. It measures kinase activity by quantitating the amount of ATP remaining in solution following a kinase reaction. The luminescent signal from the assay is correlated with the amount of ATP present and is inversely correlated with the amount of kinase activity. The IC_{50} values were calculated using nonlinear regression with normalized dose-response fit using Prism GraphPad sofeware.

4.4 Immunofluorescence assay

HepG2 cells were grown on glass cover slips sterilized and coated with poly-L-lysine. The cells were treated with compound, and then were fixed with 4% paraformaldehyde buffered in phosphate buffer solution (PBS) (15 min). After adding 500μL Hoechst 33258 staining solution, the cells were placed at room temperature for 5 min, washed by PBS. Fluorescence was determined using fluorescence microscopy.

4.5 Flow cytometry analysis

Cells (5×10^5cells/mL) were seeded in six-well plates and treated with compounds at different concentrations for 72 h. The cells were then harvested by trypsinization and washed twice with cold PBS. After centrifugation and removal of the supernatants, cells were resuspended in 400μL of 1×binding buffer which was then added to 5μL of annexin V-FITC and incubated at room temperature for 15 min. After adding 10μL of PI the cells were incubated at room temperature for another 15 min in the dark. The stained cells were analyzed by a flow cytometer.

4.6 Mitochondrial membrane potential was observed under fluorescence microscopy

Cells were seeded in six-well plates and treated with compounds at different concentrations. The cells were then harvested for 24h, and then were washed twice with PBS (2 mL×2). After adding 1 mL JC-1 staining solution, the cells were incubated at 37°C for 20 min, washed twice with JC-1 buffer solution, added 2mL culture medium, and then were observed under fluorescence microscopy.

4.7 Western blotting analysis

HepG2 cells were treated with compounds at different concentrations. Cells were then harvested and washed in ice-cold PBS, cold RIPA buffer (contain 1% PMSF) for 30 min followed by sonication. After sonication cells were centrifuged at 14000 rpm at

4 °C for 10 min and the supernatant was collected as whole cell lysate. Protein concentration was determined by BCA method. Cell lysates containing equal amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter membrane. Membranes were blocked for 2 h at room temperature, were incubated overnight at °C with primary antibodies in the same buffer, and were then washed with washing buffer for 15 min. Membranes were added secondary antibody, incubated for 2h, and then were then washed with washing buffer for 15 min. Signal detection was accomplished by using chemiluminescence (NBT/BCIP) kit.

4.8 Docking studies

The X-ray crystal structure of EGFR (PDB code: 2ITY) was retrieved from protein data bank (www.pdb.org). We used the AutoDock 4.2 Tools version 1.5.6 software packages to perform the molecular docking experiment. Compound 6m was energetically minimized by Amber 12 software before docking. Ligand docking was carried out applying the Lamarckian genetic algorithm implemented in AutoDock 4.2. The grid size was set to 40, 40 and 40 along the X-, Y- and Z-axis to recognize the binding site. Spacing was set as 0.375 Å. Number of GA runs was set to 20. Population size was set to 300. Maximum number of evals (medium) was set to 5000000. The lowest binding energy conformers were selected out of 20 different conformers for each docking simulation and resultant data was further analyzed. Other miscellaneous parameters were assigned to the default values obtained from the AutoDock 4.2 program.

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Highlights

- We have devised and synthesized a series of novel 4-anilinoquinazoline derivatives bearing amino acid moiety;
- The synthesized compounds were screened for anticancer activity against human hepatocellular carcinoma cell HepG2 using SRB assay. The target compounds exhibited moderate inhibitory activities against human hepatocellular carcinoma cells HepG2.
- Synthetic derivatives showed excellent selectivity, such as compound **6m** demonstrated a strong inhibition of EGFR (IC_{50}=0.0032μM), with selectivity of over 2000-fold over other kinases, such as Aurora B, VEGF2, MEK, RAF and c-MET;
- Apoptosis analysis revealed that compound **6m** caused obvious induction of cell apoptosis, decreased mitochondrial membrane potential through decreasing the expression of Bcl-2 and increasing the expression of Bax, promoted the mitochondrial cytochrome c release into the cytoplasm, activated caspase-3, induced apoptosis of HepG2 cells..