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Original article

Synthesis and anticancer activities of 4-(4-substituted piperazin)-5,6,7-trialkoxy quinazoline derivatives

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ABSTRACT

A series of 4-(4-substituted piperazin)-5,6,7-trialkoxy quinazoline was prepared by conventional heating methods. Among these compounds, the crystal structure of compound **10o** (CCDC: 916922) was determined by X-ray crystallography. Bioassay results showed that most target compounds had certain inhibition activities against proliferation of tumor cells, and some compounds even had good broad-spectrum inhibition activities. The ethoxyl series of compounds possessed higher inhibition activities against tumor cells than the methoxyl series of compounds. Bioactivity tests showed that the IC_{50} values of compound **10s** against PC3, MGC803, A375, and A549 cells were 1.8, 2.8, 1.3, and 2.9 μM, respectively, which were much higher than those of commercial gefitinib (7.2, 7.6, 7.2, and 9.8 μM, respectively). Conversely, the IC_{50} values of compound **10s** were very low against NH3T3, indicating only weak effect on normal cells as also proven by lactate dehydrogenase and acridine orange/ethidium bromide staining. Analyses of cell configuration and cell cycle revealed that compound **10s** possibly caused cells to remain at *G*0/*G*1 phase by inhibiting cell proliferation for 24 h. Compound **10s** also inhibited the phosphorylation of ERK1/2 and P38 with obvious concentration dependence. Thus, these compounds can inhibit the proliferation of A549 cells through the interruption of ERK1/2 and P38signaling pathways.

1. Introduction

Protein tyrosine kinases (PTKs) are enzymes involved in many cellular processes, such as cell proliferation, metabolism, survival, and apoptosis. Several protein tyrosine kinases are known to be activated in cancer cells and to drive tumor growth and progression. Thus, blocking tyrosine kinase activity can be a rational approach to cancer therapy. PTKs catalyze the phosphorylation of tyrosine and serine/threonine residues in various proteins involved in the regulation of many functions [1]. Epidermal growth factor receptor (EGFR) belongs to the family of transmembrane growth factor receptor PTKs. EGFR PTKs have been identified as explicit targets for medicinal chemistry programs, particularly cancer therapy [2–6]. Since Fry et al. [7] discovered that the 4-anilinoquinazoline derivative PD153035 possesses specific inhibition activity against EGFR PTKs, 4-anilinoquinazoline derivatives have recently attracted increased attention from chemists and biologists. Indeed, numerous 4-anilinoquinazoline compounds have been synthesized and found to show good anticancer activities [8–20]. Gefitinib (Iressa, ZD-1839) [21,22] and erlotinib (OSI-774, Tarceva) [23,24] are first-generation EGFR-targeting 4-anilinoquinazoline chemotherapeutics used to treat non-small-cell lung cancer. Meanwhile, the second-generation EGFR-targeting chemotherapeutic BIBW2992 is a potent, irreversible EGFR inhibitor that has also been developed into phase-II clinical trials against lung cancer [25,26]. Moreover, 2-chloro-6,7-dimethoxy-4-(4-tosylpiperazin-1-yl)quinazoline reportedly shows good inhibition activity against MCF-7 [27]. In our previews work, a series of 5,6,7-trimethoxy-*N*-phenyl(ethyl)-4-aminoquinazoline has been synthesized and found to show certain anticancer activities. Some of the compounds possess strong inhibition activities against ERK1/2 phosphorylation [28].

Abbreviationlist: AO/EB, acridine orange/ethidium bromide; ^{13}C NMR, ^{13}C Nuclear Magnetic Resonance; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK1/2, extracellular regulated kinase1/2; P38, extracellular regulated kinase; ESI-MS, electrospray ionization mass spectrometry; ^{1}H NMR, Proton Nuclear Magnetic Resonance; IR, Infra-red; MS, mass spectroscopy; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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In the present study, we replaced the aniline moiety with substituted piperazine fragments to determine if these compounds would significantly affect the aforementioned inhibition activity. On the other hand, trimethoxy and triethoxy substitution patterns were synthesized to investigate whether the alkoxy substitution would affect the activities of the target compounds. Thus, twenty-six new 4-(4-substituted piperazin)-5,6,7-trialkoxy quinazoline derivatives were synthesized from 2,3,4-trihydroxybenzoic acid or 2,3,4-trimethoxybenzoic acid by the synthesis route shown in Scheme 1. The structures of the title compounds were characterized by ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and mass spectroscopy (MS). The single-crystal structure of **10o** was confirmed by X-ray crystallography. The antiproliferation activities of the title compounds against PC3, Bcap-37, MGC803, A375, and BGC823 cells *in vitro* were evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Results showed that title compounds **10a**–**10z** possessed weak to strong anticancer activities.

2. Results and discussion

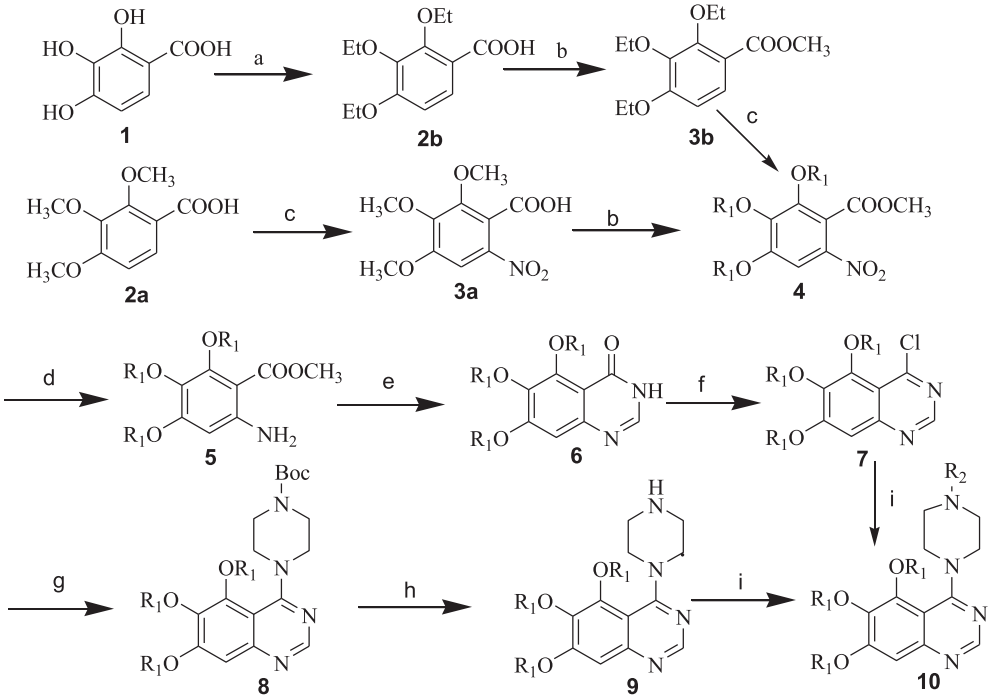
2.1. Chemistry

For the methoxyl series, the starting material 2,3,4-trimethoxybenzoic acid was nitrated with 70% nitric acid, esterified with methanol in the presence of 98% sulfuric acid, hydrogenated with Pd/C as catalyst in ethanol, cyclized with formamide, and chlorinated with phosphorus oxychloride to give 4-choloro-5,6,7-trimethoxy-quinazoline. The chlorinated product was reacted with *N*-Boc piperazine to obtain 4-(*N*-Boc-piperazin)-5,6,7-trimethoxy-quinazoline. Boc was then removed to obtain 4-piperazin-5,6,7-trimethoxy-quinazoline. Target compounds **10a**–**6e** were obtained by the substitution reaction of 4-piperazin-5,6,7-trimethoxy-quinazoline with substituted benzenesulfonyl chloride. Meanwhile, target compounds **10f**–**10m** were obtained by the substitution reaction of 4-chloro-5,6,7-trimethoxyquinazoline with substituted piperazine.

For the ethoxyl series, the starting material 2,3,4-trihydroxybenzoic acid was etherified with diethyl sulfate, esterified with methanol in the presence of 98% sulfuric acid, nitrated with 70% nitric acid, hydrogenated with Pd/C as catalyst in ethanol, cyclized with formamide, and chlorinated with phosphorus oxychloride to give 4-choloro-5,6,7-triethoxy-quinazoline. The chlorinated product was reacted with *N*-Boc piperazine to obtain 4-(*N*-Boc-piperazin)-5,6,7-triethoxy-quinazoline. Boc was then removed to obtain 4-piperazin-5,6,7-triethoxy-quinazoline. Target compounds **10n**–**10r** were obtained by the substitution reaction of 4-piperazin-5,6,7-triethoxy-quinazoline with substituted benzenesulfonyl chloride. Meanwhile, target compounds **10s**–**10z** were obtained by the substitution reaction of 4-chloro-5,6,7-triethoxyquinazoline with substituted piperazine (Scheme 1).

Probably due to the steric hindrance of the 5-ethoxyl group was stronger than that of the methoxyl group, the yields of ethoxyl series intermediates were lower than those of methoxyl series intermediates, and more byproducts were produced. Moreover, the chlorinated product of the ethoxyl series cannot react with *N*-Boc piperazine or substituted piperazine at refluxing temperature, but the methoxyl series can. The ethoxyl series can only react with *N*-Boc piperazine or substituted piperazine in the presence of DMF and NaH.

The crystal structure of compound **10o** (CCDC: 916922) was determined by X-ray crystallography. The details of data collection and structure refinement are listed in Table 1, and the X-ray structures are shown in Fig. 1. Compound **10o** crystallized in the monoclinic space group P2(1)/c.



Scheme 1. Reagents and conditions of the synthesis of title compounds **10**: (a) NaOH, H_2O, $(Et)_2SO_4$, reflux, 5 h; (b) CH_3OH, 98% H_2SO_4, reflux, 12 h; (c) nitric acid, 0 °C, 1 h; (d) H_2, Pd/C, 95% C_2H_5OH, reflux, 12 h; (e) N_2, toluene, formamide, $POCl_3$, 102 °C, 5 h; (f) N_2, toluene, $POCl_3$, 102 °C, 3.5 h; (g) N-Boc piperazine, 95% C_2H_5OH, reflux, 4 h to obtain **8a**; N-Boc piperazine, DMF, NaH, 4 h to obtain **8b**; (h) 18% HCl, 20 °C, 4 h; (i) substituted benzene sulfochloride, 95% C_2H_5OH,Et_3N, reflux, 3–5 h to obtain **10a**–**10e**, **10n**–**10r**; N-substituted piperazine, DMF, NaH, 2–12 h to obtain **10f**–**10m**, **10s**–**10z**.

Table 1
Details of data collection and structure refinement for compound **10o**.

Compound	**10o**
Empirical formula	C_{25} H_{29} F_3 N_4 O_5 S
Formula weight	554.58
Temperature (K)	113 (2)
Wavelength (Å)	0.71073 (MoK\a)
Crystal system, Space group	Monoclinic, P2(1)/c
Crystal	Colorless/prism
a (Å)	15.097 (6)
b (Å)	40.800 (16)
c (Å)	12.921 (5)
α (°)	90
β (°)	92.977 (3)
γ (°)	90
Volume (Å^3)	7948 (5)
Z, D_{calc} (mg m^{-3})	12/1.315
F (000)	3480
Crystal size (mm)	0.20 mm × 0.20 mm × 0.20 mm
θ Range for data	1.44–27.90
Collection (°)	$-19 \le h \le 10$
Limiting indices	$-50 \le k \le 53$, $-16 \le l \le 16$
Reflections collected/unique	18,342/14,776 [R_{int} = 0.0435]
Data/restraints/parameters	14,776/78/1063
Goodness-of-fit on F^2	1.119
Final *R* indices[I > 2σ(*I*)]	R_1 = 0.0679, wR_2 = 0.1423
$R_{indices}$ (all data)	R_1 = 0.0886, wR_2 = 0.1532

2.2. Antiproliferation activities of title compounds against PC3, Bcap-37, MGC803, A375, and BGC823 cells

The antiproliferation activities of title compounds **10a**–**10z** were evaluated against PC3, MGC803, Bcap-37, A375, and BGC823 cells using gefitinib as a positive control. As shown in Table 2, the antiproliferation activity of **10n**, **10o**, **10r**, **10s**, **10y**, and **10z** against PC3 cells at 10 μM were 54.4% ± 6.2%, 59.3% ± 1.7%, 67.6% ± 2.5%, 64.0% ± 2.0%, 78.3% ± 1.8%, and 56.8% ± 3.7%, respectively, which were similar to that of gefitinib (58.2% ± 3.0%). Compounds **10n**, **10p**, **10r**, **10s**, **10v**, **10y**, and **10z** displayed higher inhibition activities against Bcap37 cells at 10 μM, with inhibition rates of 41.5% ± 12.3%, 64.4% ± 10.7%, 43.1% ± 10.3%, 58.0% ± 7.0%, 51.9% ± 7.2%, 40.9% ± 5.9%, and 47.4% ± 5.5%, respectively, which were similar to or even higher than that of gefitinib (31.6% ± 6.9%). The antiproliferation activity of **10n**, **10o**, **10p**, **10r**, **10s**, **10v**, and **10y** against MGC803 cells at 10 μM were 72.6% ± 5.4%, 93.6% ± 0.8%, 65.3% ± 4.8%, 67.3% ± 5.6%, 85.6% ± 1.4%, 84.6% ± 1.4%, and 63.8% ± 3.0%, respectively, similar to or even higher than that of gefitinib (67.4% ± 1.8%). Compounds **10s**, **10v**, and **10y** displayed higher inhibition activities against A375 cells at 10 μM, with inhibition rates of 78.0% ± 2.1%, 73.4% ± 2.5%, and 70.6% ± 3.4%, respectively, which were similar to that of gefitinib (68.9% ± 3.2%). The antiproliferation activities of **10a**, **10h**, **10i**, **10s**, **10u**, and **10v** against BGC823 cells at 10 μM were 47.3% ± 13.7%, 45.1% ± 12.5%, 42.4% ± 7.0%, 61.7% ± 10.7%, 52.8% ± 7.2%, and 42.7% ± 10.7%, respectively, which were similar to or even higher than that of gefitinib (35.0% ± 8.6%). Interestingly, among all title compounds, **10s** showed the strongest and broad-spectrum inhibition activity, as well as antiproliferation activity, against all cell lines (PC3, Bcap37, MGC803, A375, and BGC823 cells) with inhibition rates of 64.0% ± 2.0%, 58.0% ± 7.0%, 85.6% ± 1.4%, 78.0% ± 2.1%, and 61.7% ± 10.7% at 10 μM, respectively. These values were relatively higher than those of gefitinib. Interestingly, it can be seen from Table 2 that the antiproliferation activities of the triethoxy substitution compounds were generally higher than those of the trimethoxy series (10a–10m as compared to **10n**–**10z**, only different at trialkoxy), indicating that the alkoxy substitution pattern did affect the activities.

MTT method was applied to further detect the antiproliferation activities of compounds **10o** and **10s** against A549 *in vitro*. The antiproliferation activities of **10o** and **10s** against A549 cells at 10 μM were 60.6% ± 5.7% and 90.4% ± 7.7%, respectively. Compound **10o** was similar to that of gefitinib (63.3% ± 7.6%), and compound **10s** was higher than that of gefitinib. IC_{50} assays were further carried out on **10o**, **10s**, and gefitinib. As shown in Table 3, the IC_{50} values of **10o** against PC3 and A549 cells were 7.8 ± 0.5 and 7.8 ± 0.9 μM, respectively, similar to those of gefitinib. Moreover, the IC_{50} values of **10s** against PC3, MGC803, A375, and A549 cells were 1.8 ± 0.1, 2.8 ± 0.1, 1.3 ± 0.1, and 2.9 ± 0.1 μM, respectively, which were higher than those of gefitinib (7.2 ± 0.8, 7.6 ± 0.9, 7.2 ± 1.1, and 9.8 ± 1.1 μM, respectively).

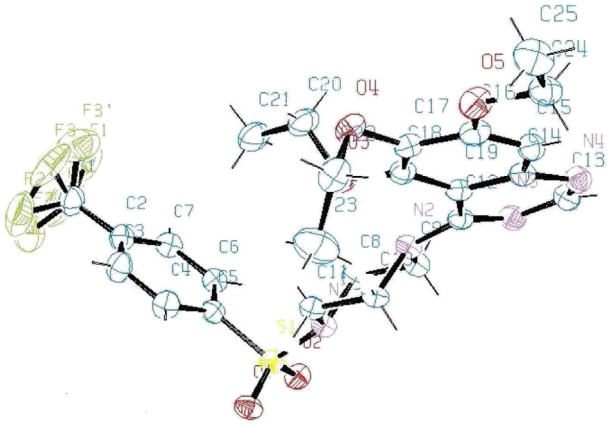


Fig. 1. X-ray crystal structure and atom numbering schemes of compound **10o**.

2.3. Preliminary investigation on the toxicity effect to A549 cells of title compound *10s*

2.3.1. LDH activities detecting the cytotoxicities of compound *10s*

The cells toxicities of compound **10s** were evaluated on A549 cells in terms of LDH activities using gefitinib and ZSC as a positive control for 24 h Table 4 shows the difference analysis values (P1 and P2) of compounds at different concentrations, as well as full (1/100) and natural (DMSO) release groups, on the cytotoxicity of A549 cells. ZSC was found to possess higher cytotoxities on A549 (P2 < 0.05) at ≥1 μM. Cells were treated with **10s** (0.01, 0.1, 1, 20, and 40 μM) for 24 h (all P1 = 0, P2 > 0.05). Results showed that cytotoxities were very low for compound **10s**, similar to that of gefitinib (P1 = 0, P2 > 0.05).

2.3.2. AO/EB staining

AO is a vital green dye that can stain nuclear DNA across an intact cell membrane, whereas EB can stain only cells with lost membrane integrity. Thus, after simultaneous treatment with AO and EB, live cells were uniformly stained green, early apoptotic cells were densely stained as green yellow or show green yellow fragments, late apoptotic cells were densely stained orange or displayed orange fragments, and necrotic cells were stained orange without condensed chromatin. The cytotoxicity of **10s** at 1, 20, and 40 μM against A549 cells within 48 h was detected by AO/EB staining, with DMSO used as a negative control. Results are shown in Fig. 2.

Fig. 2 shows that the cells treated with **10s** from 1 μM to 40 μM for 48 h changed in A549 cells. Nuclei were stained as yellow green or orange, and the morphology showed pycnosis, membrane blebbing, and cell budding. These phenomena were associated with cell apoptosis.

Table 2
Growth inhibition ratios of title compounds against selected cell lines.

Compds[a]	Inhibitory ratio (%)[a]			
	PC3		Bcap37	
	1 μM	10 μM	1 μM	10 μM
10a	5.4 ± 8.1	15.4 ± 4.7	6.8 ± 6.6	8.4 ± 10.9
10b	10.2 ± 2.9	32.4 ± 6.3	0.9 ± 5.8	11.3 ± 8.8
10c	24.8 ± 10.6	32.1 ± 5.0	4.8 ± 8.7	5.1 ± 9.3
10d	24.6 ± 14.5	38.0 ± 5.0	16.4 ± 7.8	12.9 ± 4.8
10e	8.1 ± 4.8	39.1 ± 3.0	7.3 ± 7.5	3.7 ± 7.2
10f	−3.5 ± 9.7	1.4 ± 10.5	1.1 ± 5.9	11.7 ± 11.9
10g	7.7 ± 6.6	12.9 ± 5.8	5.0 ± 8.7	10.2 ± 7.4
10h	15.8 ± 7.8	35.9 ± 3.3	20.3 ± 9.6	28.0 ± 8.8
10i	7.6 ± 8.8	5.4 ± 7.8	26.9 ± 12.1	16.7 ± 11.3
10j	−11.4 ± 6.4	16.5 ± 10.4	3.6 ± 5.5	14.7 ± 12.0
10k	−12.1 ± 6.3	6.7 ± 7.7	9.2 ± 15.1	4.7 ± 14.0
10l	−11.1 ± 6.9	22.2 ± 5.5	−6.7 ± 9.2	4.3 ± 10.9
10m	4.8 ± 3.3	17.4 ± 7.0	19.9 ± 6.3	25.7 ± 8.8
10n	14.9 ± 2.0	54.4 ± 6.2	0.3 ± 11.0	41.5 ± 12.3
10o	18.9 ± 4.5	59.3 ± 1.7	5.0 ± 6.4	34.3 ± 11.8
10p	6.6 ± 3.1	26.9 ± 2.8	18.5 ± 14.5	64.4 ± 10.7
10q	6.4 ± 7.7	17.1 ± 3.1	19.6 ± 14.2	5.4 ± 10.4
10r	15.3 ± 11.7	67.6 ± 2.5	26.7 ± 9.6	43.1 ± 10.3
10s	13.6 ± 6.6	64.0 ± 2.0	1.7 ± 3.4	58.0 ± 7.0
10t	11.6 ± 7.1	40.4 ± 4.2	10.8 ± 12.2	16.9 ± 14.5
10u	−8.8 ± 7.7	21.9 ± 8.0	23.7 ± 10.6	23.7 ± 12.9
10v	−2.3 ± 9.6	42.3 ± 2.4	13.6 ± 9.1	51.9 ± 7.2
10w	−26.5 ± 4.7	23.7 ± 8.8	11.7 ± 9.8	18.0 ± 2.8
10x	3.9 ± 4.0	9.0 ± 4.4	29.2 ± 14.3	32.0 ± 10.7
10y	2.6 ± 5.5	78.3 ± 1.8	18.2 ± 8.9	40.9 ± 5.9
10z	−8.0 ± 6.8	56.8 ± 3.7	27.7 ± 6.0	47.4 ± 5.5
Gefitinib	22.8 ± 6.6	58.2 ± 3.0	18.4 ± 6.8	31.6 ± 6.9

Compds[a]	Inhibitory ratio (%)[a]					
	MGC803		A375		BGC823	
	1 μM	10 μM	1 μM	10 μM	1 μM	10 μM
10a	6.6 ± 9.1	20.8 ± 10.8	8.7 ± 6.1	13.6 ± 7.0	35.4 ± 8.4	47.3 ± 13.7
10b	10.6 ± 6.2	15.8 ± 2.0	15.0 ± 6.9	26.7 ± 8.7	−3.0 ± 8.7	−6.2 ± 7.8
10c	−1.9 ± 3.6	13.0 ± 3.4	16.1 ± 6.8	25.4 ± 2.9	−6.5 ± 11.0	−7.5 ± 10.6
10d	−7.6 ± 6.5	4.1 ± 4.2	0.6 ± 8.0	7.1 ± 7.5	−2.0 ± 8.9	19.1 ± 7.1
10e	−1.0 ± 4.1	17.5 ± 6.8	7.5 ± 6.7	12.8 ± 5.8	21.5 ± 8.0	22.8 ± 3.3
10f	13.4 ± 5.8	35.6 ± 5.4	−1.1 ± 4.3	3.4 ± 3.8	28.9 ± 7.6	25.8 ± 6.7
10g	28.9 ± 13.5	29.3 ± 11.9	10.4 ± 7.9	19.1 ± 8.1	16.0 ± 7.1	30.4 ± 6.0
10h	39.6 ± 8.9	55.2 ± 6.6	23.3 ± 6.4	29.7 ± 5.5	15.8 ± 7.3	45.1 ± 12.5
10i	27.7 ± 10.3	36.7 ± 12.9	1.0 ± 8.9	0.3 ± 4.4	30.0 ± 5.7	42.4 ± 7.0
10j	37.0 ± 14.3	39.1 ± 13.8	11.2 ± 3.9	7.7 ± 3.9	16.6 ± 4.2	39.6 ± 7.4
10k	18.3 ± 8.7	23.6 ± 6.2	2.9 ± 9.5	0.6 ± 5.2	5.1 ± 12.5	28.2 ± 14.0
10l	2.3 ± 5.5	39.6 ± 8.4	9.9 ± 6.0	14.1 ± 8.6	1.6 ± 11.5	38.4 ± 7.6
10m	5.6 ± 15.0	19.3 ± 7.1	15.3 ± 5.0	37.7 ± 2.3	6.8 ± 4.2	13.8 ± 3.1
10n	15.6 ± 7.1	72.6 ± 5.4	0.4 ± 13.2	23.0 ± 3.9	NT[b]	NT
10o	18.5 ± 8.2	93.6 ± 0.8	−10.5 ± 10.4	41.3 ± 8.1	NT	NT
10p	9.8 ± 9.4	65.3 ± 4.8	5.0 ± 15.1	40.3 ± 5.6	NT	NT
10q	5.3 ± 9.2	33.0 ± 8.2	1.6 ± 7.7	30.3 ± 5.7	NT	NT
10r	11.8 ± 6.4	67.3 ± 5.6	1.9 ± 1.3	23.5 ± 3.1	NT	NT
10s	3.7 ± 12.6	85.6 ± 1.4	19.4 ± 2.2	78.0 ± 2.1	27.1 ± 6.0	61.7 ± 10.7
10t	3.9 ± 11.9	56.1 ± 5.5	14.1 ± 11.8	23.3 ± 5.8	6.5 ± 14.6	33.5 ± 10.3
10u	12.7 ± 5.6	28.4 ± 9.7	21.4 ± 6.7	29.4 ± 9.2	24.5 ± 9.3	52.8 ± 7.2
10v	21.3 ± 3.4	84.6 ± 1.4	4.0 ± 3.5	73.4 ± 2.5	17.6 ± 14.3	42.7 ± 10.7
10w	23.7 ± 4.0	30.3 ± 9.7	3.0 ± 3.3	16.9 ± 7.3	−10.3 ± 4.3	−1.6 ± 4.6
10x	14.2 ± 5.5	29.7 ± 7.5	23.0 ± 8.2	20.3 ± 9.4	NT	NT
10y	8.6 ± 11.2	63.8 ± 3.0	8.7 ± 7.6	70.6 ± 3.4	−2.8 ± 5.8	27.7 ± 4.2
10z	−2.7 ± 8.6	26.3 ± 7.1	4.3 ± 6.5	35.8 ± 4.8	6.0 ± 6.7	30.2 ± 4.7
Gefitinib	12.3 ± 7.8	67.4 ± 1.8	13.2 ± 6.2	68.9 ± 3.2	10.3 ± 2.5	35.2 ± 8.6

[a] Cells were treated with the title compounds or gefitinib at 1 and 10 μM for 72 h and assayed by the MTT method. The data shown are the mean ± SD of triplicate experiments.
[b] NT mean not tested.

Green live A549 cells with normal morphology were observed in the negative control group. Cells treated with **10s** displayed orange dots and cell budding in A549 cells at 1 μM and were stained light yellow at 20 μM after treatment. The emerging yellow dots at 40 μM showed late apoptotic cells.

The results above indicated that the cells had apoptotic morphology. The almost complete absence of red cells in **10s** indicated very low cytotoxicity. These findings showed that **10s** was likely to induce apoptosis with low cytotoxicity.

2.4. Flow cytometry

Flow cytometry was used to evaluate the effects of the active compound **10s** at a given concentration on the growth and division

Table 3
IC_{50} values of **10o** and **10s** against a variety of cells for 72 h *in vitro*.

Compound	IC_{50} (μM)[a]			
	PC3	MGC803	A375	A549
10s	1.8 ± 0.1	2.8 ± 0.1	1.3 ± 0.1	2.9 ± 0.2
10o	7.8 ± 0.5	NT[b]	NT	7.8 ± 0.9
Gefitinib	7.2 ± 0.8	7.6 ± 0.9	7.2 ± 1.1	9.8 ± 1.1

[a] IC_{50} means the concentration required to inhibit cell growth by 50% as determined from the dose–response curve. Each experiment was performed in triplicate.
[b] NT mean not tested.

of A549 cells using gefitinib as a positive control for 24 h, by measuring the DNA content of eukaryotic cells. Results showed the proportion of cells emitting a given fluorescent level proportional to the DNA content, as shown in Fig. 3.

Fig. 4 shows that changes occurred in A549 cell cycle distribution after treatment with **10s** at 2, 5, and 10 μM for 24 h. Unlike antimitotic agents that accumulate their DNA content at the G2/M phase of the cell cycle, the results in Table 5 showed that compound **10s** caused significant cell cycle arrest at *G*0/*G*1 phase with increased compound concentration. As shown in Table 5, compound **10s** caused significant cell cycle arrest at *G*0/*G*1 phase (the proportion of cells increased from 33.9% to 90.7%), with a corresponding decrease in the proportion of cells at G2/M phase compared with control cultures (the proportion of cells decreased from 63.2% to 3.1%). This result was consistent with the behavior of gefitinib (the proportion of cells increased from 84.2% to 87.0% at *G*0/*G*1 phase, and proportion of cells decreased from 12.0% to 3.7% at G2/M).

2.5. Inhibition activity of title compounds against ERK1/2 and P38 phosphorylation

Western blot analysis demonstrated that compound **10s** had significant inhibition activities against EGF-induced ERK1/2 and P38 phosphorylation in A549 cells. Fig. 4 shows that **10s** inhibited ERK1/2 and P38 phosphorylation in A549 cells induced by EGF at 5, 10, 20, and 40 μM. Compound **10s** possessed obvious inhibition activities against ERK1/2 and p38 phosphorylation at 20 and 40 μM, respectively, with obvious concentration dependency. Thus, these compounds can inhibit the proliferation of A549 cells through the mediated signaling pathway of ERK1/2 and P38.

3. Conclusion

A series of 4-(4-substituted piperazin)-5,6,7-trialkoxy quinazoline was prepared by conventional methods. The ethoxyl series of compounds were more difficult to prepare because the effect of steric hindrance of the ethoxyl group at the 5-position was stronger than that of the methoxyl group. The structure of compound **10o** (CCDC: 916922) was determined by X-ray crystallography. Compound **10o** crystallized in the monoclinic space group P2(1)/c. Bioassay results showed that most target compounds had certain inhibition activities, and some compounds even displayed better broad-spectrum inhibition activities (e.g., **10n**, **10o**, **10p**, **10r**, **10s**, **10v**, **10y**, and **10z**). Notably, the ethoxyl series of compounds possessed higher inhibition activities than the methoxyl series of compounds against tumor cells. Further bioactivity test showed that the IC_{50} values of compound **10s** against PC3, MGC803, A375, and A549 cells were 1.8, 2.8, 1.3, and 2.9 μM, respectively, which were much higher than those of gefitinib (7.2, 7.6, 7.2, and 9.8 μM, respectively). Moreover, the IC_{50} values of compound **10o** against PC3 nd A549 cells were 7.8 and 7.8 μM, respectively, which were similar to those of gefitinib. The inhibition activities of compound **10s** were lower against NH3T3, indicating a weaker effect on normal cells. Thus, compound **10s** was used to study the antitumor (A549) mechanism of action. LDH and AO/EB staining proved that the toxicity to normal cells was weak. Analyses of cell configuration and cell cycle revealed that the compound possibly caused cells to remain at *G*0/*G*1 phase by inhibiting cell proliferation for 24 h. The inhibition activities of Erk1/2 and p38 protein phosphorylation were also tested by Western blot analysis. Results showed that the compound inhibited the phosphorylation of ERK1/2 and P38 with obvious concentration dependence. Thus, these compounds can inhibit the proliferation of A549 cells through the mediated signaling pathway of ERK1/2 and P38.

4. Experimental

4.1. Materials and instruments

Unless specifically stated, all reagents and solvents used were obtained from commercial suppliers without further purification. All melting points of the products were determined on an XT-4 binocular microscope (Beijing Tech Instrument Co., China) and were not corrected. The IR spectra were recorded on a Bruker VECTOR22 spectrometer in KBr disks. ^{1}HNMRand ^{13}CNMR spectra (solvent CD_3OD, or $CDCl_3$) were recorded on a JEOL-ECX 500NMR spectrometer at room temperature using tetramethylsilane as an internal standard. Elemental analysis was performed with an Elementar Vario-III CHN analyzer.

4.2. Chemistry

4.2.1. Preparation of methoxyl series intermediate

4.2.1.1. Preparation of 2,3,4-trimethoxy-6-nitrobenzoic acid (*3a*) [29].

2,3,4-Trimethoxybenzoic acid was added in portions to 45 mL of

Table 4
Cell toxicity of compounds tested on A549 cells by LDH at different concentrations within 24 h.

Concentration (μM)	0.01	0.1	1	20	40	Natural release	Full release
Gefitinib (u/L)	1277.2 ± 268.2	1054.1 ± 78.4	689.0 ± 231.0	1182.7 ± 217.2	941.7 ± 124.9	821.1 ± 112.4	3611.8 ± 231.8
P1	0	0	0	0	0	0	
P2	0.3	0.5	0.7	0.2	0.9		0
ZSC (u/L)	1014.7 ± 163.8	1159.1 ± 142.8	1163.9 ± 113.4	1589.0 ± 112.8	1989.8 ± 121.4	821.1 ± 112.4	3611.8 ± 231.8
P1	0	0	0	0	0	0	
P2	0.4	0.2	0.03	0.01	0		0
10s (u/L)	868.2 ± 108.3	644.2 ± 126.8	1211.2 ± 139.1	896.2 ± 134.8	1225.2 ± 121.8	804.4 ± 102.1	3608.8 ± 145.7
P1	0	0	0	0	0	0	
P2	0.9	0.8	0.2	0.8	0.3		0

"u/L" enzyme activity unit.
"P1" Difference analysis of compounds at different concentration and full release (1/100) group on the cytotoxicity of A549 cells.
"P2" Difference analysis of compound**s** at different concentration and natural release (DMSO) group on the cytotoxicity of A549 cells.
"$P < 0.05$" significant difference.

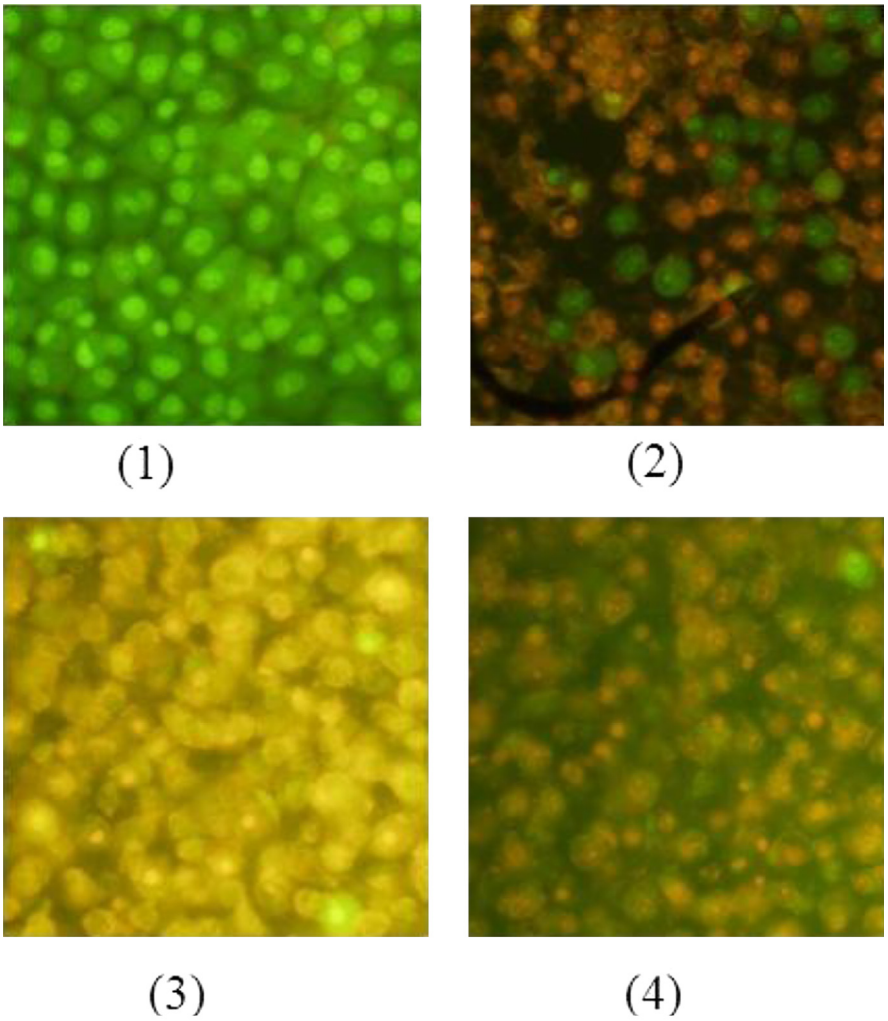


Fig. 2. AO/EB staining of **10s** in A549 cells. (1) Cells were treated with 0.1% DMSO for 48. (2–4) Cells were treated with **10s** (1, 20, and 40 μM) for 48 h.

concentrated 70% nitric acid at 0 °C (5.0 g, 0.02 mol). After stirring at 0 °C for 10 min, the mixture was diluted with 500 mL of cold water. Stirring was continued at 0 °C for 1 h, and the resulting light orange precipitate was filtered and washed well with water to produce 3.60 g of **1**. An additional 0.58 g of product was obtained by extracting the aqueous filtrate with methylene chloride and then evaporating the organic solvent. Total yield: 69.0%; mp: 150–152 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.98–4.15 (3s, 9H, $3OCH_3$), 7.55

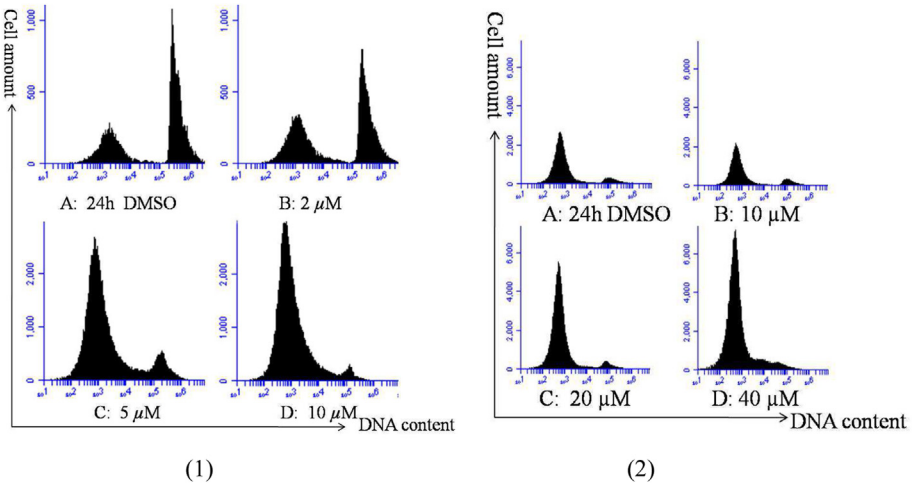


Fig. 3. Cell cycle analysis histogram. (1) Cells were treated with **10s** at 2, 5, and 10 μM for 24 h (2) Cells were treated with gefitinib (10, 20, and 40 μM) for 24 h.

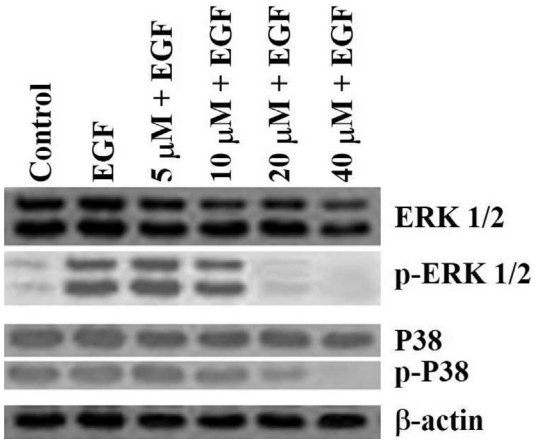


Fig. 4. Dose effect of compound **10s** (different concentrations, 60 min treatment) on their inhibition activities against ERK 1/2 and p38 phosphorylation in A549 cells treated by EGF (10 ng/mL, 10 min).

(s, 1H), 9.52 (s, 1H). IR (KBr) ν: 735.6, 1112.9, 1305.8, 1334.7, 1496.8, 1523.8 1710.9, 2953.0, 3010.8 cm^{-1}. Anal. calcd. for $C_{10}H_{11}NO_7$ (%): C, 46.70; H, 4.31; N, 5.45. Found: C, 47.02; H, 4.64; N, 5.13.

*4.2.1.2. Preparation of methyl 2,3,4-trimethoxy-6-nitrobenzoate (**4a**).* 2,3,4-Trimethoxy-6-nitrobenzoic acid (5.0 g, 0.02 mol) was added to 30 mL of methanol and 2.5 mL of 98% sulfuric acid with stirring at refluxing temperature for 8 h. Another 2 mL of 98% sulfuric acid was added, the temperature was rapidly increased to 110 °C, and the water generated in the reaction was separated by azeotropic distillation. When the mixture became a thick brown liquid, the mixture was cooled down and placed in a refrigerator at 0 °C for 12 h. The resulting solid was then filtered and washed well with water to give 3.25 g of **2**. Yield: 60.4%; mp: 66–69 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.63 (s, 3H), 3.83–3.89 (3s, 9H, $3OCH_3$), 7.53 (s, 1H). IR (KBr) ν: 852.6, 1116.8, 1312.8, 1352.1, 1683.4, 2943.4, 3003.3 cm^{-1}. Anal. calcd. for $C_{11}H_{13}NO_7$ (%): C, 48.71; H, 4.83; N, 5.16. Found: C, 48.43; H, 4.57; N, 5.32.

*4.2.1.3. Preparation of methyl 6-amino-2,3,4-trimethoxybenzoate (**5a**).* Pd/C (10%, 0.54 g) was added to a solution of methyl 2,3,4-trimethoxy-6-nitrobenzoate (5.4 g, 0.02 mol) in 50 mL of 95% ethanol, and the mixture was stirred in the presence of H_2 at room temperature for 12 h. Then, the mixture was filtered and the obtained solids were purified by silica gel column chromatography to produce 4.0 g of white solid **3**. Yield: 83.2%; mp 93–95 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.76 (s, 3H), 3.98–4.02 (3s, 9H, $3OCH_3$), 5.34 (s, 2H), 5.95 (s, 1H). IR (KBr) ν: 771.5, 1130.3, 1273.0, 1452.4, 1500.6, 1616.4, 1685.8, 2980.0, 3005.7, 3373.5, 3477.7 cm^{-1}. Anal. calcd. for $C_{11}H_{15}NO_5$ (%): C, 54.77; H, 6.27; N, 5.81. Found: C, 54.84; H, 6.02; N, 5.96.

Table 5
Cell cycle distribution by flow cytometry in A549 cells treated for 24 h.

Compound	Concentration (μM)	*G2*/M (%)	*G1*/*G0* (%)	*S* (%)
Control		63.2	33.9	2.9
10s	2	43.5	52.8	3.7
	5	10.1	81.3	8.6
	10	3.1	90.7	6.2
Control		12.0	84.2	3.8
Gefitinib	10	13.9	83.0	3.1
	20	6.2	90.0	3.8
	40	3.7	87.0	9.3

*4.2.1.4. Preparation of 5,6,7-trimethoxyquinazoline-4-one (**6a**).* Methanol sodium (0.45 g, 0.01 mol) and 4 mL of methanol were added to a solution of methyl 6-amino-2,3,4-trimethoxybenzoate (2.0 g, 0.008 mol) in 12 mL of *N*,*N*-dimethylformamide (DMF) and 8 mL of formamide with stirring at 150 °C for 24 h. Then, the mixture was cooled to room temperature and purified by silica gel column chromatography to produce1.05 g of white solid **4**. Yield: 53.6%; mp 182–183 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.95–4.01 (3s, 9H, $3OCH_3$), 7.02 (s, 1H), 8.02 (s, 1H), 11.84 (s, 1H, NH). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 56.3, 61.6, 62.3, 105.2, 110.9, 142.3, 143.9, 147.9, 152.9, 159.0, 161.2. IR (KBr) ν: 800.5, 1141.8, 1263.3, 1427.5, 1620.2, 1684.6, 2881.6, 3200.0, 3302.1. Anal. calcd. for $C_{11}H_{12}N_2O_4$ (%): C, 55.93; H, 5.12; N, 11.86. Found: C, 55.76; H, 5.48; N, 11.67.

*4.2.1.5. Preparation of 4-chloro-5,6,7-trimethoxyquinazoline (**7a**).* A suspension of trimethoxyquinazolin-4-one (0.5 g, 0.002 mol) and $POCl_3$ (2 mL) in toluene (12 mL) were added dropwise to triethylamine (6.0 mL). The resulting solution was heated at 102 °C under N_2 atmosphere for 3.5 h, cooled, and concentrated. The resulting solid was then diluted with CH_2Cl_2. The solution was neutralized with ammonia water and extracted with methylene chloride. The obtained organic layer was then passed through a short column of silica gel and then sequentially washed with CH_2Cl_2 and CH_2Cl_2/EtOAc (3:1) to produce the chlorinated product 0.395 g. Yield: 73.3%; mp 113–115 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.00–4.05 (3s, 9H, $3OCH_3$), 7.23 (s, 1H), 8.84 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 56.5, 61.3, 62.0, 114.9, 104.2, 144.2, 148.3, 150.4, 153.1, 157.8, 160.0. IR (KBr) ν: 783.1, 1140.0, 1249.9, 1427.3, 1604.8, 2845.0, 2945.3, 3100.2 cm^{-1}. Anal. calcd. for $C_{11}H_{11}ClN_2O_3$ (%): C, 51.88; H, 4.35; N, 11.00. Found: C, 52.05; H, 4.63; N, 11.28.

*4.2.1.6. Preparation of tert-butyl 4-(5,6,7-trimethoxyquinazolin-4-yl) piperazine-1-carboxylate (**8a**).* A solution of 4-chloro-5,6,7-trimethoxyquinazoline (1.0 mmol) and tert-butyl piperazine-1-carboxylate (1.2 mmol) in ethanol (8 mL) and triethylamine (1.2 mmol) was stirred under reflux for 4 h. Upon reaction completion as monitored by TLC, the solvent was removed under reduced pressure. The resulting oil matter was purified by silica gel to give 0.3 g the intermediate **8a**. Yield: 75.0%; mp: 121–132 °C. ^{1}H NMR ($CDCl_3$, MHz): δ 1.47 (s, 9H, $COOC(CH_3)_3$), 3.80, 3.96, 3.98 (3s, 9H, $3OCH_3$), 3.49 (s, 4H, H-3,5 of piperazin), 3.58 (s, 4H, H-2,6 of piperazin), 7.08 (s, 1H, H-8 of quinazoline), 8.53 (s, 1H, H-2 of quinazoline);^{13}C NMR($CDCl_3$, MHz): δ 28.5, 28.5, 28.5, 50.3, 50.3, 56.3, 56.3, 56.3, 61.8, 62.7, 80.1, 103.9, 106.3, 142.2, 148.2, 150.4, 153.0, 154.9, 158.3, 162.7. MS(ESI): m/z $(M + H)^+$ 405.2.

*4.2.1.7. Preparation of 4 piperazin-5,6,7-trimethoxyquinazoline (**9a**).* A solution of *tert-butyl 4-(5,6,7-trimethoxyquinazolin-4-yl)piperazine-1-carboxylate* (1.0 mmol) and 18% HCl (8 mL) was stirred at 25 °C for 2 h. Upon reaction completion as monitored by TLC, the solution was neutralized with ammonia water and extracted with methylene chloride. The obtained organic layer was purified by silica gel to give 0.23 g white solid **9a**. Yield: 75.9%; mp: 158–159 °C. ^{1}H NMR ($CDCl_3$, MHz): δ 3.79, 3.95, 3.97 (3s, 9H, $3OCH_3$), 3.04 (s, 4H, H-2,6 of piperazine), 3.53 (s, 4H, H-3,5 of piperazine), 7.05 (s, 1H, H-8 of quinazoline), 8.51 (s, 1H, H-2 of quinazoline), 2.92 (s, 1H, NH of piperazine). ^{13}C NMR ($CDCl_3$, MHz): δ 46.0, 46.0, 51.4, 51.4, 56.2, 61.8, 62.8, 103.9, 106.1, 142.0, 148.2, 150.6, 153.1, 158.1, 162.6; MS(ESI): m/z $(M + H)^+$ 305.2.

4.2.2. Preparation of ethoxyl series intermediate

*4.2.2.1. Preparation of 2,3,4-triethoxybenzoic acid (**2b**).* 2,3,4-Trihydroxybenzoic acid (5.0 g, 29.4 mmol) was added in portions to 20 mL water with stirring at normal temperatures, 25 mL NaOH solution (4 mol/L) and diethyl sulfate were added

dropwise to above solution. After refluxing temperature for 6 h, cooled and neutralized by diluted HCl until 7 of PH. The resulting solid was then separated out and filtered. The obtained solid was then passed through a column of silica gel and then sequentially washed with $CHCl_3$ to produce the etherified product 4.0 g. Yield: 53.5%; mp 128–130 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 11.40 (s, 1H, Ph-COOH), 6.77 (d, 1H, Ph-H-5, J = 8.0 Hz), 7.86 (d, 1H, Ph-H-6, J = 8.0 Hz), 4.04, 4.15, 4.46 (3q, 6H, 3O*CH*$_2$CH$_3$ J = 7.4 Hz, 6.9 Hz, 7.4 Hz); 1.36, 1.46 (2t, 9H, 3OCH$_2$*CH*$_3$, J = 6.8 Hz, 7.4 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 14.8, 15.6, 15.7, 64.7, 69.5, 71.8, 108.8, 114.6, 128.2, 140.3, 152.0, 157.9, 165.4. MS(ESI): m/z $(M + Na)^+$ 277.2.

*4.2.2.2. Preparation of methyl 2,3,4-triethoxybenzoate (**3b**).* 2,3,4-Triethoxybenzoic acid (1.2 g, 4.7 mmol) was added to 5 mL of methanol and 0.5 mL of 98% sulfuric acid with stirring at refluxing temperature for 10 h. The mixture was cooled down and extracted with methylene chloride. The obtained organic layer was purified by silica gel column chromatography to produce the oil product 0.9 g of **2**. Yield: 71.8%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 3.86 (s, 3H, COO*CH*$_3$), 6.66 (d, 1H, Ph-H-5, J = 6.8 Hz), 7.57 (d, 1H, Ph-H-6, J = 7.0 Hz), 4.04, 4.39, 4.45 (3q, 6H, 3O*CH*$_2$CH$_3$, J = 6.8 Hz, 6.8 Hz, 6.9 Hz); 1.36, 1.45, 1.48 (3t, 9H, 3OCH$_2$*CH*$_3$, J = 7.4 Hz, 6.2 Hz, 6.9 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 14.8, 15.7, 15.8, 51.5, 65.0, 69.5, 69.9, 98.5, 104.6, 127.2, 140.8, 153.1, 157.8, 166.8. MS(ESI): m/z $(M + Na)^+$ 291.2.

*4.2.2.3. Preparation of 2,3,4-triethoxy-6-nitrobenzoic acid (**4b**) [28]. Methyl 2,3,4-triethoxybenzoate* was added in portions to 10 mL of concentrated 70% nitric acid at 0 °C (1.2 g, 4.5 mmol). After stirring at 0 °C for 10 min, the mixture was diluted with 50 mL of cold water. Stirring was continued at 0 °C for 1 h, and the resulting light orange oil matter was extracted with methylene chloride. The obtained organic layer was purified by silica gel column chromatography to produce the oil product 0.9 g of **3**. Yield: 64.2%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 3.87 (s, 3H, COO*CH*$_3$), 7.41 (s, 1H, Ph-H-5), 3.98 (q, 2H, O*CH*$_2$CH$_3$, J = 6.8 Hz), 4.06–4.11 (m, 4H, 2O*CH*$_2$CH$_3$); 1.23, 1.37, 1.44 (3t, 9H, 3OCH$_2$*CH*$_3$, J = 6.8 Hz, 6.8 Hz, 7.4 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 14.5, 15.5, 15.6, 53.0, 65.1, 69.7, 70.8, 104.3, 119.3, 140.0, 147.2, 150.2, 153.3. MS(ESI): m/z $(M + H)^+$ 314.2.

*4.2.2.4. Preparation of methyl 6-amino-2,3,4-trimethoxybenzoate (**5b**).* Pd/C (10%, 0.12 g) was added to a solution of methyl 2,3,4-triethoxy-6-nitrobenzoate (1.3 g, 4.2 mmol) in 5 mL of 95% ethanol, and the mixture was stirred in the presence of H_2 at room temperature for 24 h. Then, the mixture was filtered and the obtained solids were purified by silica gel column chromatography to produce 0.8 g of oil matter **4**. Yield: 68.1%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 5.90 (s, 1H, Ph-H), 5.30 (s, 2H, NH_2), 3.91, 3.96, 4.05 (3q, 6H, 3O*CH*$_2$CH$_3$, J = 7.4 Hz, 7.4 Hz, 7.4 Hz); 1.30, 1.34, 1.40 (3t, 9H, 3OCH$_2$*CH*$_3$, J = 6.9 Hz, 6.8 Hz, 6.9 Hz), 3.84 (s, 3H, COOCH$_3$). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 14.7, 15.7, 15.8, 51.6, 64.0, 69.2, 69.9, 95.6, 101.2, 133.6, 147.4, 154.3, 157.2, 168.6. MS(ESI): m/z $(M + H)^+$ 284.2.

*4.2.2.5. Preparation of 5,6,7-triethoxyquinazoline-4-one (**6b**).* Formamide (10 mL) and 3 mL of $POCl_3$ were added dropwise to a solution of methyl 6-amino-2,3,4-triethoxybenzoate (5.0 g, 0.008 mol) in 25 mL of toluene with stirring at 100 °C under N_2 atmosphere for 5 h. Then, the mixture was cooled to room temperature and concentrated. The resulting solid was then diluted with water. The solution was neutralized with ammonia water and extracted with methylene chloride. The obtained organic layer was then purified by silica gel column chromatography to produce 3.3 g of white solid **5**. Yield: 67.2%; mp 192–195 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 11.69 (s, 1H, NH), 7.98 (s, 1H, quinazoline H-2), 6.98 (s, 1H, quinazoline H-8), 4.13 (q, 2H, O*CH*$_2$CH$_3$, J = 6.8 Hz), 4.19–4.23 (m, 4H, 3O*CH*$_2$CH$_3$); 1.39, 1.48, 1.54 (3t, 9H, 3OCH$_2$*CH*$_3$, J = 6.9 Hz, 6.8 Hz, 6.8 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 14.6, 15.8, 15.9, 64.6, 69.8, 71.0, 105.6, 111.0, 141.7, 143.5, 147.7, 152.4, 158.8, 161.2; MS(ESI): m/z $(M + H)^+$ 279.2.

*4.2.2.6. Preparation of 4-chloro-5,6,7-triethoxyquinazoline (**7b**).* A suspension of triethoxyquinazolin-4-one (0.5 g, 1.8 mmol) and $POCl_3$ (1 mL) in toluene (8 mL) were added dropwise to triethylamine (3.0 mL). The resulting solution was heated at 102 °C under N_2 atmosphere for 3.5 h, cooled, and concentrated. The resulting solid was then diluted with CH_2Cl_2. The solution was neutralized with ammonia water and extracted with methylene chloride. The obtained organic layer was then passed through a short column of silica gel and then sequentially washed with CH_2Cl_2 and CH_2Cl_2/EtOAc (5:1) to produce the chlorinated product 0.4 g. Yield: 75.1%; mp 83–84 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 8.79 (s, 1H, quinazoline H-2), 7.17 (s, 1H, quinazoline H-8), 4.15 (q, 2H, O*CH*$_2$CH$_3$, J = 6.9 Hz), 4.21–4.26 (m, 4H, 3O*CH*$_2$CH$_3$); 1.41, 1.47, 1.55 (3t, 9H, 3OCH$_2$*CH*$_3$, J = 7.4 Hz, 6.8 Hz, 6.9 Hz); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 14.5, 15.8, 15.9, 65.0, 69.8, 70.8, 104.5, 115.1, 143.5, 147.7, 150.5, 152.9, 157.9, 159.7. MS(ESI): m/z $(M + H)^+$ 297.1.

*4.2.2.7. Preparation of tert-butyl 4-(5,6,7-triethoxyquinazolin-4-yl) piperazine-1-carboxylate (**8b**).* A solution of 4-chloro-5,6,7-triethoxyquinazoline (1.2 mmol) and tert-butyl piperazine-1-carboxylate (1.8 mmol) in DMF (8 mL) and NaH (1.8 mmol) was stirred at 25 °C for 4 h. Upon reaction completion as monitored by TLC, washed by saturated ammonia chloride solution, and extracted with acetic ether, the solvent was removed under reduced pressure. The resulting oil matter was purified by silica gel to give 0.38 g the intermediate **8b**. Yield: 71.4%. ^{1}H NMR (CD_3OD, MHz): δ 1.46 (s, 9H, COOC(CH$_3$)$_3$), 1.28–1.50 (3t, 9H, 3OCH$_2$*CH*$_3$), 3.56 (s, 8H, H-2,3,5,6 of piperazin), 3.95–4.20 (m, 6H, 3O*CH*$_2$CH$_3$), 6.98 (s, 1H, H-8 of quinazoline), 8.37 (s, 1H, H-2 of quinazoline); ^{13}C NMR($CDCl_3$, MHz): δ 13.5, 14.7, 14.7, 27.3, 27.3, 27.3, 47.5, 47.5, 49.8, 49.8, 64.5, 69.5, 71.0, 80.2, 102.6, 105.9, 141.6, 147.6, 149.3, 152.0, 155.1, 158.5, 162.6. MS(ESI): m/z $(M + H)^+$ 447.3.

*4.2.2.8. Preparation of 4-piperazin-5,6,7-triethoxyquinazoline (**9b**).* A solution of *tert-butyl 4-(5,6,7-triethoxyquinazolin-4-yl)piperazine-1-carboxylate* (1.0 mmol) and 18% HCl (8 mL) was stirred at 25 °C for 2 h. Upon reaction completion as monitored by TLC, the solution was neutralized with ammonia water and extracted with methylene chloride. The obtained organic layer was purified by silica gel to give 0.25 g oil matter **9b**. Yield: 72.7%. ^{1}H NMR($CDCl_3$, MHz): δ 1.30–1.48 (3t, 9H, 3OCH$_2$*CH*$_3$), 3.90–4.16 (m, 6H, 3O*CH*$_2$CH$_3$), 2.99 (s, 4H, H-2,6 of piperazine), 3.52 (s, 4H, H-3,5 of piperazine), 6.98 (s, 1H, H-8 of quinazoline), 8.44 (s, 1H, H-2 of quinazoline), 2.54 (s, 1H, NH of piperazine). ^{13}C NMR($CDCl_3$, MHz): δ 14.6, 15.8, 15.8, 45.9, 45.9, 51.2, 51.2, 64.5, 69.8, 71.2, 104.4, 106.3, 141.1, 147.6, 150.4, 152.8, 157.8, 162.6. MS(ESI): m/z $(M + H)^+$ 347.3.

*4.2.3. General procedure for the synthesis of 5,6,7-trimethoxy-N-aryl-4-aminoquinazolines (**10a**–**10z**)*

A solution of 4-piperazin-5,6,7-trialkoxyquinazoline (1.0 mmol) and substituted benzene sulfochloride (1.2 mmol) in ethanol (8 mL) and triethylamine (1.2 mmol) was stirred under reflux for 3–5 h. Upon reaction completion as monitored by TLC, the solvent was removed under reduced pressure. The resulting oil matter was purified by silica gel to give the title compounds (**10a**–**10e**, **10n**–**10r**).

A solution of 4-chloro-5,6,7-trialkoxyquinazoline (1.0 mmol) and substituted piperazine (1.2 mmol) in DMF (8 mL) and NaH (1.5 mmol) was stirred at 25 °C for 4–8 h. Upon reaction completion as monitored by TLC, washed by saturated ammonia chloride

solution, and extracted with acetic ether, the solvent was removed under reduced pressure. The resulting oil matter was purified by silica gel to give the title compounds (**10f**–**10m**, **10s**–**10z**).

*4.2.3.1. 5,6,7-Trimethoxy-4-(4-(2-trifluoromethylphenylsulfonyl) piperazin-1-yl)quinazoline (**10a**).* Yield: 67.2%; yellow oil matter. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.73, 3.89, 3.97 (3s, 9H, $3OCH_3$), 3.39 (t, 4H, J = 4.9 Hz, piperazine-H), 3.59 (s, 4H, piperazine-H), 7.07 (s, 1H, quinazoline-H), 8.15 (t, 1H, J = 6.9 Hz, Ar–H), 8.51 (s, 1H, quinazoline-H), 7.97 (t, 1H, J = 6.9 Hz, Ar–H), 7.72–7.74 (m, 2H, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 45.7, 45.7, 50.0, 50.0, 56.3, 61.8, 62.7, 104.1, 106.2, 121.5, 123.6, 128.1, 128.8, 132.3, 133.0, 137.4, 142.3, 147.9, 150.5, 153.0, 158.4, 162.3. MS(ESI): m/z $(M + H)^+$ 513.2.

*4.2.3.2. 5,6,7-Trimethoxy-4-(4-(4-trifluoromethylphenylsulfonyl) piperazin-1-yl)quinazoline (**10b**).* Yield: 74.1%; white solid; mp: 211–213 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.60, 3.94, 3.97 (3s, 9H, $3OCH_3$), 3.20 (t, 4H, J = 5.4 Hz, piperazine-H), 3.64 (s, 4H, piperazine-H), 7.06 (s, 1H, quinazoline-H), 7.83 (d, 2H, J = 8.0 Hz, Ar–H), 8.50 (s, 1H, quinazoline-H), 7.92 (d, 2H, J = 8.6 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 46.1, 46.1, 49.6, 49.6, 56.3, 61.8, 62.6, 104.2, 106.1, 124.3, 126.4, 126.4, 128.3, 128.3, 134.9, 139.5, 142.3, 147.7, 150.6, 153.0, 158.4, 162.1. MS(ESI): m/z $(M + H)^+$ 513.2.

*4.2.3.3. 5,6,7-Trimethoxy-4-(4-(4-methylphenylsulfonyl)piperazin-1-yl)quinazoline (**10c**).* Yield: 74.1%; white solid; mp: 175–177 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.59, 3.94, 3.96 (3s, 9H, $3OCH_3$), 3.14 (t, 4H, J = 5.4 Hz, piperazine-H), 3.63 (s, 4H, piperazine-H), 2.43 (s, 3H, CH_3-Ph), 7.34 (d, 2H, J = 8.0 Hz, Ar–H), 8.49 (s, 1H, quinazoline-H), 7.67 (d, 2H, J = 8.0 Hz, Ar–H), 7.05 (s, 1H, quinazoline-H). ^{13}C NMR ($CDCl_3$, MHz): δ 21.6, 46.1, 46.1, 49.5, 49.5, 56.2, 61.8, 62.6, 104.1, 106.0, 127.9, 127.9, 129.8, 129.8, 132.5, 142.2, 143.9, 147.8, 150.6, 153.0, 162.0. MS(ESI): m/z $(M + H)^+$ 459.2.

*4.2.3.4. 5,6,7-Trimethoxy-4-(4-(4-methoxyphenylsulfonyl)piperazin-1-yl)quinazoline (**10d**).* Yield: 75.0%; white solid; mp: 176–178 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.59, 3.94, 3.96 (3s, 9H, $3OCH_3$), 3.14 (t, 4H, J = 5.4 Hz, piperazine-H), 3.63 (s, 4H, piperazine-H), 3.60 (s, 3H, OCH_3-Ph), 7.01 (d, 2H, J = 8.6 Hz, Ar–H), 8.49 (s, 1H, quinazoline-H), 7.72 (d, 2H, J = 9.2 Hz, Ar–H), 7.05 (s, 1H, quinazoline-H). ^{13}C NMR ($CDCl_3$, MHz): δ 46.2, 46.2, 49.5, 49.5, 55.7, 56.3, 61.8, 62.6, 104.1, 106.1, 114.4, 114.4, 127.1, 130.0, 130.0, 142.2, 147.8, 150.6153.0, 158.3, 162.1, 163.2. MS(ESI): m/z $(M + H)^+$ 475.2.

*4.2.3.5. 5,6,7-Trimethoxy-4-(4-(phenylsulfonyl)piperazin-1-yl)quinazoline (**10e**).* Yield: 68.4%; yellow solid; mp: 112–114 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.57, 3.94, 3.96 (3s, 9H, $3OCH_3$), 3.17 (t, 4H, J = 5.9 Hz, piperazine-H), 3.62 (s, 4H, piperazine-H), 7.06 (s, 1H, quinazoline-H), 7.79 (d, 2H, J = 8.0 Hz, Ar–H), 8.49 (s, 1H, quinazoline-H), 7.62 (t, 1H, J = 7.4 Hz, Ar–H), 7.56 (t, 2H, J = 8.0 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 46.2, 46.2, 49.6, 49.6, 56.3, 61.8, 62.6, 104.1, 106.1, 127.9, 127.9, 129.2, 129.2, 133.1, 135.5, 142.4, 147.8, 150.6, 153.0, 158.4, 162.1. MS(ESI): m/z $(M + H)^+$ 445.2.

*4.2.3.6. 5,6,7-Trimethoxy-4-(4-(3-methoxyphenyl)piperazin-1-yl) quinazoline (**10f**).* Yield: 75.0%; yellow oil matter. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.80, 3.98, 3.99 (3s, 9H, $3OCH_3$), 3.36 (t, 4H, J = 5.0 Hz, piperazine-H), 3.70 (s, 4H, piperazine-H), 3.80 (s, 3H, OCH_3–Ar), 7.09 (s, 1H, quinazoline-H), 7.20 (t, 1H, J = 8.0 Hz, H-3 of Ar–H), 8.55 (s, 1H, quinazoline-H), 6.45, 6.44 (dd, 1H, J = 8.0 Hz, 2.0 Hz, Ar–H), 6.60, 6.59 (dd, 1H, J = 8.0 Hz, 2.0 Hz, Ar–H), 6.52 (t, 1H, J = 2.5 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 49.4, 49.4, 50.2, 50.2, 55.3, 56.3, 61.8, 62.8, 102.7, 104.0, 104.9, 106.3, 109.0, 130.0, 142.1, 148.2, 150.6, 152.6, 153.1, 158.2, 160.7, 162.6. MS(ESI): m/z $(M + H)^+$ 411.3.

*4.2.3.7. 4-(4-(4-Fluorophenyl)piperazin-1-yl)-5,6,7-trimethoxyquinazoline (**10g**).* Yield: 73.8%; brown solid; mp: 122–124 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.80, 3.98, 3.99 (3s, 9H, $3OCH_3$), 3.27 (t, 4H, J = 5.6 Hz, piperazine-H), 3.70 (s, 4H, piperazine-H), 7.09 (s, 1H, quinazoline-H), 6.92–6.94 (m, 2H, Ar–H), 8.56 (s, 1H, quinazoline-H), 6.97–7.00 (m, 2H, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 50.3, 50.3, 50.5, 50.5, 56.3, 61.8, 62.8, 104.0, 106.2, 115.6, 115.8, 118.0, 118.1, 142.2, 147.9, 148.2, 150.5, 153.1, 156.5, 158.2, 162.6. MS(ESI): m/z $(M + H)^+$ 399.3.

*4.2.3.8. 4-(4-(4-Nitrophenyl)piperazin-1-yl)-5,6,7-trimethoxyquinazoline (**10h**).* Yield: 77.5%; brown solid; mp: 210–213 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.81, 3.98, 3.99 (3s, 9H, $3OCH_3$), 3.58 (t, 4H, J = 5.8 Hz, piperazine-H), 3.70 (s, 4H, piperazine-H), 7.10 (s, 1H, quinazoline-H), 6.87 (d, 2H, J = 9.7 Hz, Ar–H), 8.56 (s, 1H, quinazoline-H), 8.13 (d, 2H, J = 9.2 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 47.2, 47.2, 49.7, 49.7, 56.3, 61.8, 62.8, 104.2, 106.4, 112.9, 112.9, 126.0, 126.0, 138.9, 142.3, 148.1, 150.6, 153.1, 154.9, 158.4, 162.5. MS(ESI): m/z $(M + H)^+$ 426.3.

*4.2.3.9. 5,6,7-Trimethoxy-4-(4-(pyridin-2-yl)piperazin-1-yl)quinazoline (**10i**).* Yield 66.7%; yellow solid; mp: 134–136 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.81, 3.98, 3.99 (3s, 9H, $3OCH_3$), 3.67 (t, 4H, J = 4.0 Hz, piperazine-H), 3.71 (s, 4H, piperazine-H), 7.08 (s, 1H, quinazoline-H), 8.22, 8.21 (dd, 1H, J = 2.9 Hz, 2.0 Hz, pyridine-H), 8.56 (s, 1H, quinazoline-H), 7.50–7.53 (m, 1H, pyridine-H), 6.72 (d, 1H, J = 8.6 Hz, pyridine-H), 6.66–6.68 (m, 1H, pyridine-H). ^{13}C NMR ($CDCl_3$, MHz): δ 45.6, 45.6, 50.1, 50.1, 56.3, 61.8, 62.7, 104.0, 106.3, 107.4, 113.8, 137.7, 142.1, 148.1, 148.3, 150.5, 153.1, 158.2, 159.6, 162.7. MS(ESI): m/z $(M + H)^+$ 382.3.

*4.2.3.10. 4-(4-Benzylpiperazin-1-yl)-5,6,7-trimethoxyquinazoline (**10j**).* Yield 79.6%; brown solid; mp: 86–89 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.77, 3.93, 3.96 (3s, 9H, $3OCH_3$), 2.60 (t, 4H, J = 4.8 Hz, piperazine-H), 3.59 (s, 4H, piperazine-H), 3.57 (s, 2H, CH_2–Ar), 7.04 (s, 1H, quinazoline-H), 8.49 (s, 1H, quinazoline-H), 7.30–7.33 (m, 5H, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 50.1, 50.1, 53.3, 53.3, 56.2, 61.8, 62.7, 63.2, 103.7, 105.9, 127.3, 128.4, 128.4, 129.4, 129.4, 137.8, 141.9, 148.3, 150.5, 153.1, 158.1, 162.3. MS(ESI): m/z $(M + H)^+$ 395.2.

*4.2.3.11. 5,6,7-Trimethoxy-4-(4-(3-morpholinopropyl)piperazin-1-yl) quinazoline (**10k**).* Yield: 51.6%; brown solid; mp: 102–105 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.71–1.77 (m, 2H, $CH_2CH_2CH_2$), 2.39 (t, 4H, J = 7.6 Hz, $CH_2CH_2CH_2$), 2.44 (t, 4H, J = 4.2 Hz, morpholino-H), 3.71 (t, 4H, J = 8.0 Hz, morpholino-H), 3.78, 3.95, 3.97 (3s, 9H, $3OCH_3$), 2.60 (s, 4H, J = 5.0 Hz, piperazine-H), 3.60 (t, 4H, piperazine-H), 7.05 (s, 1H, quinazoline-H), 8.50 (s, 1H, quinazoline-H). ^{13}C NMR ($CDCl_3$, MHz): δ 23.8, 50.0, 53.4, 53.4, 53.8, 53.8, 56.2, 56.7, 57.1, 57.1, 61.8, 62.7, 67.0, 67.0, 103.8, 106.0, 141.9, 148.3, 150.6, 153.1, 158.1, 162.2. MS(ESI): m/z $(M + H)^+$ 432.4.

*4.2.3.12. 4-(4-(4-Chlorobenzyl)piperazin-1-yl)-5,6,7-trimethoxyquinazoline (**10l**).* Yield: 51.8%; yellow solid; mp: 124–127 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.78, 3.94, 3.98 (3s, 9H, $3OCH_3$), 2.58 (t, 4H, J = 6.4 Hz, piperazine-H), 3.59 (s, 4H, piperazine-H), 3.53 (s, 2H, CH_2-Ar), 7.06 (s, 1H, quinazoline-H), 7.30 (s, 4H, Ar–H), 8.50 (s, 1H, quinazoline-H); ^{13}C NMR ($CDCl_3$, MHz): δ 50.1, 53.3, 53.3, 56.2, 56.2, 61.8, 62.4, 62.7, 103.8, 106.0, 128.5, 128.5, 130.5, 130.5, 133.0, 136.5, 141.9, 148.3, 150.6, 153.1, 158.1, 162.3. MS(ESI): m/z $(M + H)^+$ 429.3.

*4.2.3.13. 5,6,7-Trimethoxy-4-(4-(4-fluorobenzyl)piperazin-1-yl)quinazoline (**10m**).* Yield: 61.5%; yellow solid; mp: 142–145 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.78, 3.94, 3.97 (3s, 9H, $3OCH_3$), 3.53 (s, 2H, CH_2-Ar), 2.57 (t, 4H, J = 5.6 Hz, piperazine-H), 3.58 (s, 4H,

piperazine-H), 7.05 (s, 1H, quinazoline-H), 8.50 (s, 1H, quinazoline-H), 7.00 (t, 2H, J = 8.6 Hz, Ar–H), 7.30 (q, 2H, J = 5.2 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 50.1, 50.1, 53.2, 56.2, 56.2, 61.8, 62.4, 62.7, 103.9, 106.0, 115.1, 115.3, 130.7, 130.8, 133.6, 141.9, 148.3, 150.6, 153.1, 153.1, 158.1, 162.3. MS(ESI): m/z $(M + H)^+$ 413.2.

*4.2.3.14. 5,6,7-Triethoxy-4-(4-(2-(trifluoromethyl)phenylsulfonyl) piperazin-1-yl)quinazoline (**10n**).* Yield: 70.3%; white solid; mp: 154–157 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.39, 1.45, 1.51 (3t, 9H, J = 7.4 Hz, 6.9 Hz, 6.8 Hz, $3OCH_2CH_3$), 3.89 (q, 2H, J = 7.4 Hz, OCH_2CH_3), 4.12–4.18 (m, 4H, $2OCH_2CH_3$), 3.38 (s, 4H, piperazine-H), 3.62 (s, 4H, piperazine-H), 7.02 (s, 1H, quinazoline-H), 8.13 (d, 1H, J = 5.0 Hz, Ar–H), 8.46(s, 1H, quinazoline-H), 7.90 (d, 1H, J = 6.8 Hz, Ar–H), 7.69–7.72 (m, 2H, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.7, 15.8, 45.5, 45.5, 49.8, 49.8, 64.6, 69.9, 71.3, 104.3, 106.4, 128.7, 132.2, 132.2, 132.3, 133.0, 133.0, 137.3, 141.6, 147.3, 150.4, 152.7, 158.1, 162.2. MS(ESI): m/z $(M + H)^+$ 555.2.

*4.2.3.15. 5,6,7-Triethoxy-4-(4-(4-(trifluoromethyl)phenylsulfonyl) piperazin-1-yl)quinazoline (**10o**).* Yield: 62.5%; white solid; mp: 172–174 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.02, 1.41, 1.50 (3t, 9H, J = 6.8 Hz, 6.8 Hz, 7.4 Hz, $3OCH_2CH_3$), 3.76 (q, 2H, J = 6.8 Hz, OCH_2CH_3), 4.10–4.18 (m, 4H, $2OCH_2CH_3$), 3.17 (s, 4H, piperazine-H), 3.63 (s, 4H, piperazine-H), 7.00 (s, 1H, quinazoline-H), 7.82 (d, 2H, J = 8.2 Hz, Ar–H), 8.46 (s, 1H, quinazoline-H), 7.92 (d, 2H, J = 8.6 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.5, 15.8, 46.0, 46.0, 49.3, 49.3, 64.6, 69.8, 71.2, 104.5, 106.4, 124.3, 126.4, 126.4, 128.4, 128.4, 134.7, 139.0, 141.6, 147.3, 150.4, 152.8, 158.1, 162.1. ESI-MS m/z: $(M + H)^+$ 555.2.

*4.2.3.16. 5,6,7-Triethoxy-4-(4-(4-(methyl)phenylsulfonyl)piperazin-1-yl)quinazoline (**10p**).* Yield: 69.2%; white solid; mp: 164–166 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.04, 1.40, 1.50 (3t, 9H, J = 7.4 Hz, 6.9 Hz, 6.8 Hz, $3OCH_2CH_3$), 3.79 (q, 2H, J = 6.9 Hz, OCH_2CH_3), 4.11–4.17 (m, 4H, $2OCH_2CH_3$), 3.12 (s, 4H, piperazine-H), 3.63 (s, 4H, piperazine-H), 2.42 (s, 3H, CH_3-Ph), 7.00 (s, 1H, quinazoline-H), 7.33 (d, 2H, J = 8.0 Hz, Ar–H), 8.44 (s, 1H, quinazoline-H), 7.65 (d, 2H, J = 7.4 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.5, 15.8, 21.6, 46.0, 46.0, 49.4, 49.4, 64.6, 69.8, 71.2, 104.3, 106.3, 128.0, 128.0, 129.8, 129.8, 132.2, 141.5, 144.0, 147.4, 150.4, 152.8, 158.0, 162.1. MS(ESI): m/z $(M + H)^+$ 501.2.

*4.2.3.17. 5,6,7-Triethoxy-4-(4-(4-(methoxyl)phenylsulfonyl)piperazin-1-yl)quinazoline (**10q**).* Yield: 66.7%; yellow solid; mp: 148–151 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.07, 1.41, 1.50 (3t, 9H, J = 6.8 Hz, 6.9 Hz, 6.9 Hz, $3OCH_2CH_3$), 3.78 (q, 2H, J = 7.4 Hz, OCH_2CH_3), 4.10–4.17 (m, 4H, $2OCH_2CH_3$), 3.11 (s, 4H, piperazine-H), 3.63 (s, 4H, piperazine-H), 3.86 (s, 3H, OCH_3-Ph), 7.01 (s, 1H, quinazoline-H), 7.71 (d, 2H, J = 8.6 Hz, Ar–H), 8.45 (s, 1H, quinazoline-H), 6.99 (d, 2H, J = 4.6 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.5, 15.8, 46.0, 46.0, 49.4, 49.4, 55.7, 64.6, 69.8, 71.2, 104.3, 106.3, 114.4, 114.4, 126.8, 130.1, 130.1, 141.5, 147.4, 150.4, 152.8, 158.0, 162.1, 163.3. MS(ESI): m/z $(M + H)^+$ 517.3.

*4.2.3.18. 5,6,7-Triethoxy-4-(4-(phenylsulfonyl)piperazin-1-yl)quinazoline (**10r**).* Yield: 62.3%; white solid, mp: 170–173 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.01, 1.39, 1.48 (3t, 9H, J = 6.8 Hz, 6.8 Hz, 7.4 Hz, $3OCH_2CH_3$), 3.75, 4.10, 4.14 (3q, 6H, J = 7.3 Hz, 6.9 Hz, 6.9 Hz, $3OCH_2CH_3$), 3.14 (s, 4H, piperazine-H), 3.61 (s, 4H, piperazine-H), 6.99 (s, 1H, quinazoline-H), 7.77 (d, 2H, J = 7.4 Hz, Ar–H), 8.44 (s, 1H, quinazoline-H), 7.60 (t, 1H, J = 7.4 Hz, Ar–H), 7.55 (d, 2H, J = 8.0 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.5, 15.8 , 46.0, 46.0, 49.4, 49.4, 64.6, 69.8, 71.2, 104.3, 106.3, 127.9, 127.9, 129.2, 129.2, 133.1, 135.3, 141.5, 147.4, 150.4, 152.8, 158.0, 162.2. MS(ESI): m/z $(M + H)^+$ 487.2.

*4.2.3.19. 5,6,7-Triethoxy-4-(4-(3-methoxyphenyl)piperazin-1-yl)quinazoline (**10s**).* Yield: 57.4%; yellow oil matter. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.32, 1.44, 1.52 (3t, 9H, J = 7.4 Hz, 6.9 Hz, 6.8 Hz, $3OCH_2CH_3$), 4.18 (q, 2H, J = 7.4 Hz, $2OCH_2CH_3$), 3.97 (q, 2H, J = 7.4 Hz, OCH_2CH_3), 3.33 (t, 4H, J = 4.9 Hz, piperazine-H), 3.71 (s, 4H, piperazine-H), 3.79 (s, 3H, OCH_3-Ar), 7.04 (s, 1H, quinazoline-H), 7.19 (t, 1H, J = 8.0 Hz, Ar–H), 8.51 (s, 1H, quinazoline-H), 6.44, 6.43 (dd, H, J = 8.0 Hz, 2.6 Hz, Ar–H), 6.58, 6.57 (dd, H, J = 8.0 Hz, 2.6 Hz, Ar–H), 6.50 (t, H, J = 2.2 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.9, 15.9, 49.2, 49.2, 50.0, 50.0, 55.3, 64.6, 69.8, 71.4, 102.8, 104.1, 104.8, 106.4, 109.1, 130.0, 141.4, 147.6, 150.2, 152.7, 152.8, 157.9, 160.7, 162.5. MS(ESI): m/z $(M + H)^+$ 453.3.

*4.2.3.20. 4-(4-(4-Fluorophenyl)piperazin-1-yl)-5,6,7-triethoxyquinazoline (**10t**).* Yield: 84.0%; yellow solid; mp: 118–121 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.34, 1.44, 1.52 (3t, 9H, J = 7.4 Hz, 6.8 Hz, 6.8 Hz, $3OCH_2CH_3$), 3.98 (q, 2H, J = 6.8 Hz, OCH_2CH_3), 4.15–4.22 (m, 4H, $2OCH_2CH_3$), 3.25 (t, 4H, J = 4.6 Hz, piperazine-H), 3.74 (t, 4H, J = 5.6 Hz, piperazine-H), 7.05 (s, 1H, quinazoline-H), 8.52 (s, 1H, quinazoline-H), 6.91–6.93 (m, 2H, Ar–H), 6.96–7.00 (m, 2H, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.8, 15.8, 50.1, 50.1, 50.4, 50.4, 64.5, 69.8, 71.3, 104.2, 106.5, 115.6, 115.8, 118.1, 118.1, 141.5, 147.6, 148.0, 150.4, 152.9, 156.5, 157.9, 162.5. MS(ESI): m/z $(M + H)^+$ 441.3.

*4.2.3.21. 4-(4-(4-Nitrophenyl)piperazin-1-yl)-5,6,7-triethoxyquinazoline (**10u**).* Yield: 71.4%; yellow solid; mp: 203–206 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.33, 1.42, 1.52 (3t, 9H, J = 6.8 Hz, 6.8 Hz, 6.9 Hz, $3OCH_2CH_3$), 3.98 (q, 2H, J = 7.4 Hz, OCH_2CH_3), 4.13–4.22 (m, 4H, $2OCH_2CH_3$), 3.58 (t, 4H, J = 5.0 Hz, piperazine-H), 3.74 (s, 4H, piperazine-H), 7.06 (s, 1H, quinazoline-H), 8.54 (s, 1H, quinazoline-H), 6.87 (d, 2H, J = 8.0 Hz, Ar–H), 8.14 (d, 2H, J = 7.2 Hz, Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.8, 15.8, 47.1, 47.1, 49.5, 49.5, 64.6, 69.8, 71.3, 104.4, 106.6, 112.9, 112.9, 126.0, 126.0, 138.9, 141.6, 147.4, 150.4, 152.8, 154.9, 158.1, 162.4. MS(ESI): m/z $(M + H)^+$ 468.3.

*4.2.3.22. 5,6,7-Triethoxy-4-(4-(pyridin-2-yl)piperazin-1-yl)quinazoline (**10v**).* Yield 61.2%; yellow oil matter. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.32, 1.43, 1.52 (3t, 9H, J = 7.4 Hz, 7.4 Hz, 6.8 Hz, $3OCH_2CH_3$), 4.18 (q, 2H, J = 7.4 Hz, $2OCH_2CH_3$), 3.98 (q, 2H, J = 7.4 Hz, OCH_2CH_3), 3.69 (s, 8H, piperazine-H), 7.04 (s, 1H, quinazoline-H), 8.21, 8.20 (dd, 1H, J = 3.4 Hz, 2.0 Hz, pyridine-H), 8.52 (s, 1H, quinazoline-H), 7.49–7.52 (m, 1H, pyridine-H), 6.70 (d, 1H, J = 8.6 Hz, pyridine-H), 6.65 (q, 1H, J = 2.2 Hz, pyridine-H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.8, 15.8, 45.3, 45.3, 49.9, 49.9, 64.5, 69.8, 71.3, 104.2, 106.6, 107.3, 113.7, 137.6, 141.4, 147.7, 148.1, 150.4, 152.9, 157.9, 159.6, 162.7. MS(ESI): m/z $(M + H)^+$ 424.3.

*4.2.3.23. 4-(4-Benzylpiperazin-1-yl)-5,6,7-triethoxyquinazoline (**10w**).* Yield 84.9%; brown oil matter. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.31, 1.40, 1.50 (3t, 9H, J = 6.8 Hz, 6.8 Hz, 6.8 Hz, $3OCH_2CH_3$), 3.92, 4.12, 4.16 (3q, 6H, J = 6.8 Hz, 6.8 Hz, 6.8 Hz, $3OCH_2CH_3$), 2.57 (t, 4H, J = 5.3 Hz, piperazine-H), 3.61 (s, 4H, piperazine-H), 3.57 (s, 2H, CH_2-Ar), 7.00 (s, 1H, quinazoline-H), 8.46 (s, 1H, Quinazoline-H), 7.26–7.34 (m, 5H, H-2,3,4,5,6 of Ar–H). ^{13}C NMR ($CDCl_3$, MHz): δ 14.6, 15.8, 15.8, 50.1, 50.1, 53.1, 53.1, 63.2, 64.5, 69.8, 71.2, 104.0, 106.2, 127.2, 128.4, 128.4, 129.3, 129.3, 137.8, 141.2, 147.7, 150.4, 152.9, 157.7, 162.2. MS(ESI): m/z $(M + H)^+$ 437.3.

*4.2.3.24. 5,6,7-Triethoxy-4-(4-(3-morpholinopropyl)piperazin-1-yl) quinazoline (**10x**).* Yield 46.9%; yellow oil matter. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.70–1.76 (m, 2H, $CH_2CH_2CH_2$), 2.34–2.41 (m, 4H, $CH_2CH_2CH_2$), 2.44 (t, 4H, J = 5.0 Hz, morpholino-H), 3.70 (t, 4H, J = 7.6 Hz, morpholino-H), 1.30, 1.40, 1.49 (3t, 9H, J = 6.9 Hz, 6.8 Hz,

7.4 Hz, 3OCH$_2$*CH$_3$*), 3.89 (q, 2H, *J* = 7.4 Hz, OC*H*$_2$CH$_3$), 4.10–4.17 (m, 4H, 2OC*H*$_2$CH$_3$), 2.57 (s, 4H, piperazine-H), 3.62 (s, 4H, piperazine-H), 6.99 (s, 1H, quinazoline-H), 8.44 (s, 1H, quinazoline-H). ^{13}C NMR (CDCl$_3$, MHz): *δ* 14.6, 15.8, 23.7, 29.8, 49.8, 53.2, 53.2, 53.8, 53.8, 56.6, 57.0, 57.0, 64.5, 66.9, 66.9, 69.8, 71.2, 103.9, 106.1, 141.2, 147.6, 150.2, 152.7, 157.8, 162.1. MS(ESI): *m/z* (M + H)$^+$ 474.4.

*4.2.3.25. 4-(4-(4-Chlorobenzyl)piperazin-1-yl)-5,6,7-triethoxyquinazoline (**10y**).* Yield 63.0%; yellow solid; mp: 99–101 °C. ^{1}HNMR (CD$_3$OD, 500 MHz): *δ* 1.29, 1.37, 1.47 (3t, 9H, *J* = 6.8 Hz, 6.8 Hz, 6.9 Hz, 3OCH$_2$*CH$_3$*), 3.91, 4.11, 4.17 (3q, 6H, *J* = 6.9 Hz, 6.9 Hz, 6.8 Hz, 3OC*H*$_2$CH$_3$), 2.58 (s, 4H, piperazine-H), 3.60 (s, 4H, piperazine-H), 3.55 (s, 2H, *CH*$_2$-Ph), 6.95 (s, 1H, quinazoline-H), 7.29–7.33 (m, 4H, Ar–H), 8.32 (s, 1H, quinazoline-H). ^{13}C NMR (CD$_3$OD, MHz): *δ* 13.5, 14.7, 14.7, 49.6, 49.6, 52.5, 61.6, 61.6, 64.4, 69.5, 71.0, 102.5, 105.7, 128.1, 128.1, 130.8, 130.8, 132.9, 135.9, 141.3, 147.7, 149.3, 152.0, 158.4, 162.3. MS(ESI): *m/z* (M + H)$^+$ 471.2.

*4.2.3.26. 5,6,7-Triethoxy-4-(4-(4-fluorobenzyl)piperazin-1-yl)quinazoline (**10z**).* Yield 65.0%; yellow solid; mp: 85–88 °C. ^{1}H NMR (CDCl$_3$, 500 MHz): *δ* 1.32, 1.41, 1.50 (3t, 9H, *J* = 7.4 Hz, 6.8 Hz, 6.8 Hz, 3OCH$_2$*CH$_3$*), 3.92, 4.13, 4.17 (3q, 6H, *J* = 7.4 Hz, 6.8 Hz, 6.9 Hz, 3OC*H*$_2$CH$_3$), 2.55 (s, 4H, piperazine-H), 3.60 (s, 4H, piperazine-H), 3.52 (s, 2H, *CH*$_2$-Ph), 7.00 (s, 1H, quinazoline-H), 6.99 (t, 2H, *J* = 9.2 Hz, Ar–H), 7.28–7.31 (q, 2H, *J* = 2.8 Hz, Ar–H), 8.47 (s, 1H, quinazoline-H). ^{13}C NMR (CDCl$_3$, MHz): *δ* 14.6, 15.8, 15.8, 50.0, 50.0, 53.0, 62.4, 62.4, 64.5, 69.8, 71.2, 104.0, 106.2, 115.1, 115.2, 130.7, 130.8, 133.6, 141.2, 147.7, 150.4, 152.8, 157.8, 161.2, 162.3. MS(ESI): *m/z* (M + H)$^+$ 455.3.

4.3. Anticancer activity bioassay

4.3.1. Cell culture

The human prostate cancer cell line PC3, breast cancer cell line Bcap-37, gastric cancer cell line MGC803 and BGC823, malignant melanoma cell line A375 and nonsmall-cell lung cancer A549 were purchased from the Institute of Biochemistry and Cell Biology, China Academy of Sciences and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). All cell lines were maintained at 37 °C in a humidified 5% carbon dioxide and 95% air incubator.

4.3.2. MTT assay

All tested compounds were dissolved in DMSO (1–100 μM solution) and subsequently diluted in culture medium before being used to treat cultured cells. Tested cells were plated in 96-well plates at a density of 2×10^3 cells per well per 100 μL of the proper culture medium and treated with the compounds at 1–100 μM for 72 h. In parallel, cells treated with 0.1% DMSO served as a control. An MTT assay (Roche Molecular Biochemicals, 1465-007) was performed 30 h later according to the manufacturer's instructions. This assay was based on the cellular cleavage of MTT into formazane, which is soluble in cell culture medium. Any absorbance caused by formazan was measured at 595 nm with a microplate reader (BIO-RAD, model 680), and this absorbance was directly proportional to the number of living cells in culture. The experiment was performed in triplicate. The percentage cytotoxicity was calculated using the formula.

$$\%\ \text{Cytotoxicity} = \frac{(\text{Control abs} - \text{Blank abs}) - (\text{Test abs} - \text{Blank abs})}{(\text{Control abs} - \text{Blank abs})} \times 100$$

4.3.3. LDH release assay

1.0×10^5 cells per well (total volume 1.6 mL) were plated into a 6 well plate and incubated for 24 h at 37 °C in 5% CO_2. Cytotoxicity was assessed based on measuring the release of LDH after 24 h compound treatment. Prior to each assay, the cells were lysed with 2% (V/V) Triton X-100 in culture media for 10 min at 37 °C to obtain a representative maximal LDH release as the positive control with 100% toxicity and cells in culture media alone was the low control. The amounts of LDH in the supernatant were determined and calculated as kit instructions. All tests were performed in triplicate and assay was repeated three times independently with similar results.

4.3.4. AO/EB staining

Cells were seeded at a concentration of 5×10^4 cells/mL in a volume of 0.6 mL on a sterile cover slip in six-well tissue culture plates. After incubation, the culture medium was removed and replaced with fresh medium plus 10% FBS and then supplemented with tested compound. After the treatment period, the cover slip with monolayer cells was inverted on the glass slide with 20 μL of AD/EB stain (100 μg/mL). Fluorescence was read on an IX71SIF-3 fluorescence microscope (OLYMPUS Co., Japan).

4.3.5. Cell cycle analysis

Test substances were dissolved in DMSO and diluted to given concentration. A549 cells (1.6×10^5 cells/mL) were cultured six-well plate for 24 h with test compounds. The cells were harvested, washed with PBS and centrifuged. The fixation of cells was realized through the addition of 0.6 mL ethanol (70% ice-cold) and then keeping cells at −20 °C overnight until DNA staining. The fixed cells were treated with 10 mg/mL Rnase A in PBS for 1 h, followed by staining with 50 mg/mL propidium iodide in PBS in the dark. The DNA content of eukaryotic cells was then measured with flow cytometery.

4.3.6. Western blot analysis

A549 cells were seeded on a six-well plate and incubated in RPMI 1640 medium plus 10% FBS at 37 °C. After incubation for 36–48 h, the medium was removed and the cells were incubated with serum-free medium for 24 h. Then, the cells were treated with the title compounds at different concentrations for 60 min followed by 10 ng/mL EGF for 10 min. The plate was then immediately placed on ice to quench the phosphorylation process. The medium was removed and the cells were rinsed with ice-cold phosphate-buffered saline (PBS) buffer twice. Then, the cells were treated with lysis buffer [1%NP-40, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, 0.6 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 40 μg/mL phenylmethanesulfonyl fluoride]and then with the sample buffer, followed by denaturation at 90 °C water for 5 min.

The previously prepared cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto poly(vinylidenedifluoride) membranes (Bio-Rad). The membrane was blocked with 5% nonfat dried milk freshly made in PBS

plus 0.2% Tween 20 and then incubated with monoclonal antibody (anti-pErk1/2, anti-pp38, Stanta Cruz Biotechnology; or anti-actin) overnight at 4 °C. The membrane was then washed for 3–5 min with PBS plus0.2% Tween 20, incubated again with second antibody for 2–3 h at 25 °C, and washed three times with PBS plus 0.2% Tween 20. The signal was detected by enhanced chemical luminescence detection system (PIERCE) [30].

Conflicts of interest

None.

Author's contributions

Yang ZHANG synthesized the compounds and carried out part of the bioassay experiments. Yin-Jiu HUANG did part of the bioassay experiments. Hong-Mei XIANG also did part of the bioassay experiments. De-Yu HU took part in the compound structural elucidation and bioassay experiments. Wei XUE carried out some structure elucidation experiments. Prof. Bao-An Sopng and Prof. Song Yang are the corresponding authors for this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.036.

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