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Synthesis and anticancer activities of 5,6,7-trimethoxy-*N*-phenyl(ethyl)-4-aminoquinazoline derivatives

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ABSTRACT

A series of 5,6,7-trimethoxy-*N*-phenyl(ethyl)-4-aminoquinazoline compounds was prepared by microwave irradiation and conventional heating methods. Compounds **6p**, **6q**, and **6x** strongly inhibited extracellular regulated kinase1/2 (ERK1/2) phosphorylation induced by epidermal growth factor (EGF) at 1.28 μM in PC3 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed that all compounds had certain anticancer activities, and the IC_{50} values of **6x** were 6.2 ± 0.9, 3.2 ± 0.1, and 3.1 ± 0.1 μM against PC3, BGC823, and Bcap37 cells, respectively. Acridine orange/ethidium bromide staining, Hoechst 33258 staining, DNA ladder, and flow cytometry analyses revealed that **6x** induced cell apoptosis in PC3 cells, with apoptosis ratios of 11.6% at 1 μM and 31.8% at 10 μM after 72 h.

1. Introduction

Protein kinases play diverse and important roles in regulating cellular processes such as cell proliferation, cell cycle, metabolism, survival, apoptosis, and DNA damage/repair. Thus, protein kinases are extensively targeted for the discovery of inhibitors as potential cancer-treatment drugs [1–4]. Epidermal growth factor receptor (EGFR) protein tyrosine kinase is one of the most important kinases that play a fundamental role in signal transduction pathways. The tyrosine kinase activity of EGFR is activated by ligand binding to the receptor, and an inhibitor of EGFR tyrosine kinase may compete with a ligand for EGFR binding or may directly interfere with the catalytic site of EGFR [5]. Fry et al. [6] first discovered that the 4-anilinoquinazoline derivativePD153035 possesses specific inhibitory activity against EGFR tyrosine kinase. Since then, various 4-anilinoquinazoline derivatives have been synthesized based on the 4-anilinoquinazoline framework [7–11]. Modification of the quinazoline structure has been performed in many anticancer studies, such as against NSCLC, PC3, BTC, and MCF-7 cells [12–20]. Gefitinib (Iressa, ZD-1839) [21,22] and erlotinib (OSI-774, Tarceva) [23,24], which are first-generation EGFR-targeting 4-anilino quinazoline chemotherapeutics, have been approved for the treatment of non-small-cell lung cancer. These two small compounds directly act on the ATP binding area of EGFR, interfering with the binding of ATP to EGFR and inhibiting the activity of EGFR-TK. The second-generation EGFR-targeting chemotherapeuticBIB W2992, which is an excellent non-irreversible EGFR inhibitor [25], has also been approved for Phase-II clinical trials against lung cancer [26].

Trimethoxyphenyl is a crucial pharmacophoric group for analogs of the antitumor natural product CA-4((*Z*)-2-methoxy-5-[2-(3,4,5-trimethoxyphenyl)ethenyl]phenol) [27]. In our study on PD153035 (the specific inhibitor for EGFR-TK) as the leading compound, we have successfully produced 6,7,8-trimethoxy-*N*-aryl-4-aminoquina zoline compounds that are potent inhibitors of PC3, Bcap37, BGC823, and A431 cells. We found that methoxy-substituted

Abbreviations: AO/EB, acridine orange/ethidium bromide; ^{13}C NMR, ^{13}C nuclear magnetic resonance; CH, conventional heating; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK1/2, extracellular regulated kinase1/2; ESI-MS, electrospray ionization mass spectrometry; HCPT, 10-hydroxyl camptothecine; ^{1}H NMR, proton nuclear magnetic resonance; IR, infra-red; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MW, microwave irradiation.

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quinazoline compounds possess potent antitumor activity but not antiphosphorylation inhibitory activity, suggesting that cytotoxicity may not result from inhibiting EGFR [28]. Thus, a series of new quinazoline compounds **6a**–**6z** was designed and synthesized with three methoxy (5,6,7-OCH_3) groups on the quinazoline ring to study further the effect of the substitution position of quinazoline methoxy groups on anticancer and antiphosphorylation activities. Twenty-six new 5,6,7-trimethoxy-*N*-aryl-4-aminoquinazoline derivatives were synthesized from 2,3,4-trimethoxybenzoic acid by the synthesis route shown in Scheme 1. The structures of the title compounds were characterized by infrared (IR), ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and elemental analyses. The microwave irradiation (MW) synthesis conditions for the target compounds were also optimized. The antiproliferation activities of the title compounds against PC3, Bcap-37, andBGC823 cells *in vitro* were evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The results showed that title compounds **6a**–**6z** possessed weak to strong anticancer activities, and most of the compounds exerted an inhibitory effect on EGF-induced ERK1/2 phosphorylation in PC3 cells.

2. Results and discussion

2.1. Chemistry

The starting material 2,3,4-trimethoxybenzoic acid was nitrated with70% nitric acid, esterified with methanol in the presence of 98% sulfuric acid, hydrogenated with Pd/C as catalyst in EtOH, cyclized with formamide, and finally chlorinated with phosphorus oxychloride to give the key intermediate 4-choloro-5,6,7-trimethoxyquinazoline. The target compounds **6a**–**6z** were obtained by the substitution reaction of 4-chloro-5,6,7-trimethoxyquinazoline with amine to yield 5,6,7-trimethoxyl-*N*-aryl-4-aminequinazoline (Scheme 1). The yields of the title compounds were relatively low under conventional heating (CH) method. MW method was used to improve the yields of the title compounds, and the reaction conditions under MW were optimized using **6o** as the model compound. Table 1 shows that when the power of MW was optimized from 40 W to 100 W, the yield of **6o** increased from 73.1% to 80.7% (Table 1, entries 1–4) within 30 min at 80 °C. Then, the effect of reaction time on yield was investigated. With increased reaction time from 10 min to 30 min, the yield increased from 72.3% to 80.7% within 30 min under an MW power 100 W (Table 1, entries 4–6). However, the yield did not obviously increase when the reaction time was 40 min (Table 1, entry 7). Thus, the reaction conditions for MW irradiation were as follows: temperature, 80 °C; reactant molar ratio, 1:1; MW power, 100 W; and reaction time, 30 min. For comparison, the yield of **6o** was found to be only 45.0% after 8 h of reaction (Table 1, entry 8).

All title compounds were synthesized under both CH and MW methods, and the results are listed in Table 2. The yields of the title compounds increased from 30.1% to 71.6% under CH to 80.1%–90.0% under MW with decreased reaction time from 2 to 12 h to 30 min. Interestingly, **6f** and **6r** were obtained only under CH method, whereas **6g** and **6m** were obtained only under MW irradiation.

Table 1
Effects of reaction time and MW power on the yield of **6o**.[a]

Entry	Reaction time	Power (Watt)	Yield (%)
1	30 min.	40	73.1
2	30 min.	60	78.5
3	30 min.	80	79.4
4	30 min.	100	80.7
5	10 min.	100	72.3
6	20 min.	100	78.7
7	40 min	100	77.3
8[b]	8 h	0	45.0

[a] Reaction conditions: reactants molar ratio, 1:1; reflux temperature of isopropanol, 80 °C.
[b] Conventional heating.

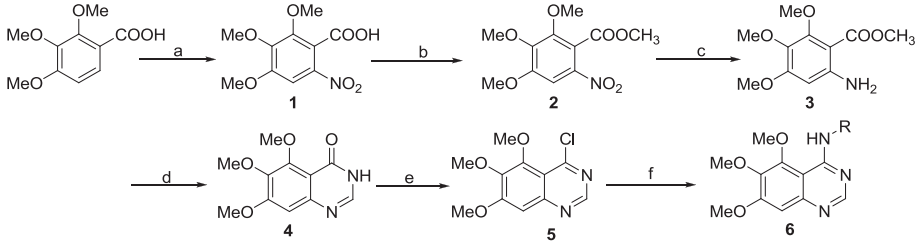
2.2. Inhibition activity of title compounds against ERK1/2 phosphorylation induced by EGF

Western blot analysis demonstrated that the title compounds had significant inhibitory activities against EGF-induced ERK1/2 phosphorylation in PC3 cells. As shown in Fig. 1, **6b**, **6d**–**6g** (Fig. 1A), **6i**, **6m**–**6q** (Fig. 1B), **6r** (Fig. 1C), and **6x** (Fig. 1D) inhibited ERK1/2 phosphorylatin in PC3 cells induced by EGF at 10 μM. The 4-phenyl ring containing a fluorine atom or trifluoromethyl group generally had good antiphosphorylation activities (**6e**, **6f**, **6i**, **6m**, **6o**–**6q**, and **6x**).

Further dose experiments were carried out on **6d**, **6e**, **6f**, **6p**, **6q**, and **6x**. Fig. 2 shows that the inhibition activities of **6d**–**6f** against ERK phosphorylation were weak to moderate at 5.12 μM but strong at 10 μM (Fig. 2A–C). However, **6p**, **6q**, and **6x** showed strong inhibition activities against ERK phosphorylation using low concentration of 1.28 μM (Fig. 2D–F). Furthermore, the 4-aniline moiety bearing a 3- or 4-trifluoromethyl group showed very strong inhibition activities against ERK1/2 phosphorylation in PC3 cells. Interestingly, 4-phenylethylamine substitution also showed very strong inhibition activities against ERK phosphorylation in PC3 cells.

2.3. Antiproliferation activities of title compounds against PC3, BGC823, and Bcap-37 cells

The antiproliferation activities of title compounds **6a**–**6z** were evaluated against PC3, BGC823, and Bcap-37 cells using PD153035as a positive control. As shown in Table 3, the antiproliferation activity of **6z** against PC3 cells at 10 μM was 61.75% ± 12.5%, similar to that of PD153035. The antiproliferation activities of **6l**, **6v**, **6w**, and **6z** against BGC823 cells at 10 μM were



Scheme 1. Reagents and conditions of the synthesis of title compounds **6**: (a) nitric acid, 0 °C, 2 h; (b) CH_3OH, 98% H_2SO_4, reflux, 12 h; (c) H_2, Pd/C, 95% C_2H_5OH, reflux, 12 h; (d) DMF, formamide, CH_3OH, CH_3ONa, 150 °C, 24 h; (e) N_2, toluene, $POCl_3$, reflux, 3.5 h; (f) aryl amine, 2-propanol, MW, 100 W, 80 °C, 30 min to obtain **6a**–**6z**; aryl amine, 2-propanol, reflux, 2–12 h to obtain **6a**–**6z**.

Table 2
Reaction conditions and yields of title compounds **6a**–**6z**.[a]

Product	Microwave irradiation[b]		Conventional heating[c]	
	Reaction time (min)	Yield (%)	Reaction time (h)	Yield (%)
6a	30	85.8	6	51.2
6b	30	89.7	6	70.2
6c	30	86.1	4	32.6
6d	30	84.8	2	71.6
6e	30	89.0	6	50.6
6f	/[c]	/	7	37.2
6g	30	81.9	/	/
6h	30	85.0	6	46.3
6i	30	88.9	6	53.6
6j	30	83.2	6	31.5
6k	30	81.0	8	51.3
6l	30	80.1	8	47.7
6m	30	81.9	/	/
6n	30	82.4	6	41.3
6o	30	80.7	8	45.0
6p	30	81.1	6	30.1
6q	30	85.1	9	40.1
6r	/	/	6	45.8
6s	30	83.6	12	42.8
6t	30	90.0	3	65.0
6u	30	81.2	9	61.4
6v	30	84.8	8	54.2
6w	30	80.5	10	60.2
6x	30	83.9	9	63.0
6y	30	85.4	12	51.5
6z	30	83.7	12	50.8

[a] Reaction conditions: reactants molar ratio 1:1, at 80 °C in isopropanol.
[b] MW power = 100 W.
[c] Not obtained.

60.7% ± 5.4%, 68.4% ± 10.7%, 51.9% ± 7.2%, and 58.9% ± 6.1%, respectively, which were higher than that of PD153035 (32.6% ± 6.5%). Compounds **6h**, **6u**, and **6y** displayed higher inhibition activities against Bcap37 cells at 10 μM, with inhibition rates of 50.0% ± 7.8%, 69.3% ± 10.1%, and 53.7% ± 8.1%, respectively, which were higher than that of PD153035 (43.4% ± 5.7%). Interestingly, among all title compounds, **6x** showed the strongest inhibition and antiproliferation activities against all three cell lines (PC3, BGC823, and PC3 cells), with inhibition rates of 90.6% ± 9.1%, 88.2% ± 6.1%, and 90.4% ± 2.8% at 10 μM, respectively. These values were relatively higher than that of PD153035.

IC_{50} assays were further carried out on **6x** and PD153035. As shown in Table 4, the IC_{50} values of **6x** against PC3, BGC823, and Bcap37 cells were 6.2 ± 0.9, 3.2 ± 0.1, and 3.1 ± 0.1 μM, respectively, which were higher than those of PD153035 (8.3 ± 1.1, 14.4 ± 1.5, and 15.7 ± 1.2 μM, respectively).

2.4. Preliminary investigation on the apoptosis-inducing effect of title compound **6x** on PC3 cells

PD153035 reportedly exerts apoptosis-inducing effects [29,30]. However, studies on the apoptosis-inducing activity of 4-phenylethylaminoquinazoline compounds are limited. Given that **6x** had excellent antiproliferation activities against the three cell lines, further investigation was carried out to evaluate its apoptosis-inducing effect on PC3 cells.

2.4.1. Fluorescence staining

Changes in the morphology of PC3 cells were investigated using acridine orange (AO)/ethidium bromide (EB) and Hoechst 33258 staining under fluorescence microscopy to determine whether the growth–inhibitory activity of the selected compounds was related to apoptosis induction.

2.4.1.1. 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 **6x** at 10 μM against PC3 cells from 24 h to 72 h was detected by AO/EB staining, with 10-hydroxyl camptothecine (HCPT) used as a positive control at 10 μM against PC3 cells for 72 h. The results are shown in Fig. S1, which shows that the cells treated with **6x** from 24 h to 72 h changed in PC3 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.

Green live PC3 cells with normal morphology were observed in the negative control group. Green yellow or orange dots were detected in HCPT after 48 h. Cells treated with **6x** displayed yellow dots and cell budding in PC3 cells at 48 h and were stained green 72 h after treatment. The emerging orange dots at 72 h also showed typical late apoptotic cells.

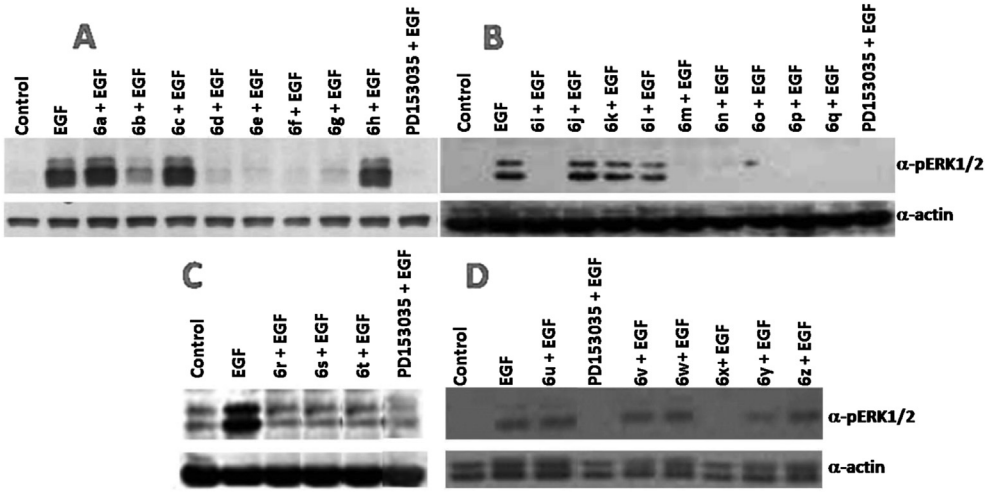


Fig. 1. Effects of title compounds (10 μM, 60 min treatment) on ERK1/2 phosphorylation induced by EGF (10 ng/mL, 10 min treatment).

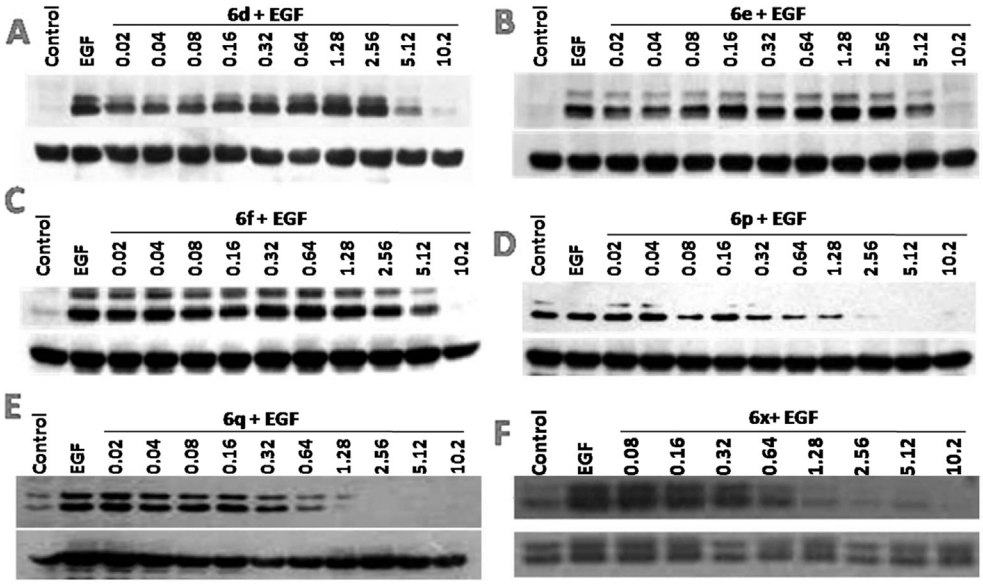


Fig. 2. Dose effect of six title compounds (different concentrations, 60 min treatment) on their inhibition activities against ERK1/2 phosphorylation in PC3 cells induced by EGF (10 ng/mL, 10 min treatment).

The results above indicated that the cells had apoptotic morphology. The almost complete absence of red cells in **6x** indicated that it was associated with very low cytotoxicity. These findings showed that **6x** induced apoptosis with low cytotoxicity.

2.4.1.2. Hoechst 33258 staining. Hoechst 33258 is a membrane-permeable blue fluorescent dye that stains cell nuclei. Live cells with uniformly light blue nuclei were observed under a fluorescence microscope after treatment with Hoechst 33258, whereas apoptotic cells had bright blue nuclei because of karyopyknosis and chromatin condensation. Meanwhile, the nuclei of dead cells were not stained. PC3 cells treated with **6x** at 1 and 10 μM for 72 h were stained with Hoechst 33258. HCPT was used as a positive control at 10 μM for 72 h. The results are shown in Fig. S2, which shows that

Table 3
Growth inhibitory ratios of selected cell lines.

Compds[a]	Inhibitory ratio (%)[a]					
	PC3		BGC823		Bcap37	
	1 μM	10 μM	1 μM	10 μM	1 μM	10 μM
6a	7.9 ± 7.8	24.9 ± 6.7	NT[b]	NT	−12.0 ± 10.0	−16.3 ± 11.6
6b	9.3 ± 6.3	24.6 ± 7.4	NT	NT	−7.5 ± 8.0	3.3 ± 6.0
6c	11.3 ± 8.2	25.5 ± 6.2	NT	NT	−8.3 ± 7.6	15.4 ± 4.3
6d	23.8 ± 5.4	45.6 ± 7.2	1.2 ± 1.3	19.3 ± 6.5	7.3 ± 8.8	11.1 ± 11.0
6e	3.1 ± 7.0	9.1 ± 6.9	NT	NT	7.8 ± 4.0	10.8 ± 6.2
6f	14.9 ± 10.0	38.9 ± 7.9	NT	NT	7.0 ± 2.5	13.5 ± 3.1
6g	−4.5 ± 24.6	11.9 ± 19.6	NT	NT	14.1 ± 7.9	28.8 ± 3.9
6h	14.5 ± 4.9	26.4 ± 9.5	9.0 ± 5.2	16.3 ± 5.3	31.6 ± 5.4	50.0 ± 7.8
6i	0.9 ± 4.6	24.0 ± 5.5	NT	NT	NT	NT
6j	9.7 ± 7.4	19.8 ± 7.7	0.6 ± 9.2	11.4 ± 6.1	NT	NT
6k	−8.4 ± 5.6	49.7 ± 4.8	NT	NT	8.7 ± 4.3	18.6 ± 2.3
6l	12.8 ± 3.8	34.7 ± 2.7	12.4 ± 6.4	60.7 ± 5.4	10.5 ± 7.2	18.9 ± 6.0
6m	14.7 ± 6.0	21.5 ± 10.4	NT	NT	NT	NT
6n	1.0 ± 27.1	22.2 ± 4.4	13.8 ± 6.5	32.6 ± 6.3	10.4 ± 5.8	26.1 ± 3.7
6o	7.6 ± 8.9	29.2 ± 5.4	NT	NT	14.4 ± 6.4	27.6 ± 6.2
6p	7.4 ± 8.1	29.0 ± 8.0	2.4 ± 6.4	17.4 ± 6.5	8.6 ± 3.3	32.3 ± 2.2
6q	14.9 ± 11.4	46.6 ± 4.1	1.0 ± 9.3	24.3 ± 8.8	21.0 ± 8.6	23.5 ± 9.6
6r	16.9 ± 5.6	28.8 ± 7.9	5.9 ± 4.9	23.9 ± 3.2	11.0 ± 8.6	17.5 ± 4.7
6s	−3.9 ± 4.6	25.3 ± 6.4	2.1 ± 4.4	23.2 ± 3.0	NT	NT
6t	6.3 ± 10.2	31.3 ± 7.1	15.5 ± 3.0	43.0 ± 5.9	22.2 ± 10.6	26.4 ± 7.6
6u	9.2 ± 3.0	42.7 ± 5.6	3.9 ± 5.0	41.9 ± 4.9	11.3 ± 4.2	69.3 ± 10.1
6v	8.1 ± 14.6	43.0 ± 9.5	38.5 ± 11.1	68.4 ± 10.7	4.3 ± 9.6	2.2 ± 6.0
6w	14.1 ± 11.5	22.6 ± 7.1	42.2 ± 8.7	51.9 ± 7.2	19.4 ± 5.7	20.7 ± 6.9
6x	22.6 ± 12.2	90.6 ± 9.1	38.7 ± 6.5	88.2 ± 6.1	46.5 ± 12.6	90.4 ± 2.8
6y	12.5 ± 9.1	40.4 ± 10.7	13.2 ± 2.3	34.3 ± 9.2	40.9 ± 10.9	53.7 ± 8.1
6z	35.6 ± 12.4	61.7 ± 8.5	40.8 ± 9.7	58.9 ± 6.1	2.1 ± 14.9	45.8 ± 7.8
PD153035	21.5 ± 7.9	62.8 ± 4.7	9.3 ± 8.2	32.6 ± 6.5	19.8 ± 10.4	43.4 ± 5.7

[a] Cells were treated with the title compounds or PD153035 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.

Table 4
IC_{50} values of **6x** against three kinds of cells for 72 h *in vitro*.

Compound	IC_{50} (μM)[a]		
	PC3	BGC823	Bcap37
6x	6.2 ± 0.9	3.1 ± 0.1	3.2 ± 0.1
PD153035	8.3 ± 1.1	14.4 ± 1.5	15.7 ± 1.2

[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.

cells treated with the negative control dimethyl sulfoxide (DMSO) were normally blue. On the other hand, the nuclei of cells treated with HCPT appeared to be compact and condensed. After treatment with **6x**, the cells exhibited strong blue fluorescence and revealed typical apoptotic morphology after 72 h at 10 μM. These findings demonstrated that **6x** induced apoptosis against PC3 cell lines, consistent with the AO/EB double staining results.

2.4.2. DNA ladder assay

Endonucleases were activated when cells exhibited apoptosis. DNA was selectively degraded to form 50–300 kb fragments and was finally cleaved in the vicinity of the nucleosome in fragments or multiples of 180–200 bp. These DNA fragments were then extracted from cells. DNA fragmentation was detected by agarose gel electrophoresis and Gold View staining. The results are shown in Fig. 3.

Fig. 4 shows that the cells in the negative group did not show any DNA ladder, but those treated with **6x** had the typical DNA ladder.

2.4.3. Flow cytometry

The apoptosis ratios induced by **6x** in tumor cells were quantitatively assessed by flow cytometry. In the early stages of apoptosis, phosphatidylserine was translocated from within the cell membrane to the cell exterior. Annexin V, a calcium-dependent phospholipid-binding protein associated with a high affinity for phosphatidylserine, was used to detect early apoptotic cells. Propidine iodide (PI) is a red fluorescent dye that stains cells with lost their membrane integrity. Cells stained with Annexin V-fluorescein isothiocyanate and PI were classified as necrotic (Q1; $Annexin^-/PI^+$), late apoptotic (Q2; $Annexin^+/PI^+$), intact (Q3; $Annexin^-/PI^-$), orearly apoptotic (Q4; $Annexin^+/PI^-$) cells. The results are shown in Fig. 4.

Fig. 4 shows that **6x** induced apoptosis in PC3 cells at 1 μM. As shown in Table 5, the apoptosis ratios (including the early and late apoptosis ratios) after 72 h of treatment with **6x** were 11.6%, 19.8%, and 31.8% at 1, 5, and 10 μM, respectively. For the positive control HCPT, the apoptosis ratio was only 17.0% after 72 h of treatment at 10 μM. The apoptosis of PC3 cells treated with **6x** gradually increased in a dose-dependent and time-dependent manner. The apoptosis ratios of **6x** at 10 μM for different treatment times (10.9%, 16.4%, and 31.8% at 24, 48, and 72 h, respectively) were markedly higher than those of the positive control HCPT (5.7%, 8.3%, and 17.0% at 24, 48, and 72 h, respectively). Even at 1 μM, **6x** also induced cell apoptosis at a ratio of 11.6% after 72 h of treatment.

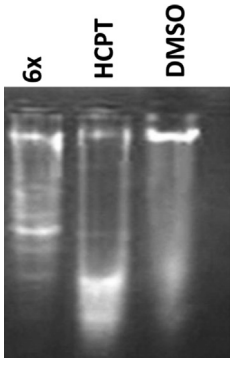


Fig. 3. Effect of **6x** on DNA fragmentation in PC3 cells at10 μM.

3. Conclusion

A series of 5,6,7-trimethoxy-*N*-phenyl(ethyl)-4-aminoquinazoline was prepared by MW and CH methods. The yields of the title compounds increased from 30.1% to 71.6% under CH to 80.1%–90.0% under MW with decreased reaction time from 2 to 12 h to 30 min. Some of the title compounds were found to inhibit EGF-induced ERK1/2 phosphorylation in PC3 cells effectively, which indicated that the position of trimethoxy groups on the phenyl ring affected the antiphosphorylation activities of 4-phenyl(ethyl)quinazoline compounds. Preliminary structure–activity relationship analysis showed that the 4-phenyl ring containing a fluorine atom or trifluoromethyl group generally had good antiphosphorylation activities (**6e**, **6f**, **6i**, **6m**, **6o**–**6q**, and **6x**). Further investigation showed that **6p**, **6q**, and **6x** showed strong inhibition activities against ERK1/2 phosphorylation induced by EGF at 1.28 μM. Interestingly, 4-substituted phenylethylamino-5,6,7-trimethoxyquinazoline compounds also possessed strong antiphosphorylation activities, such as **6x**. MTT assay showed that some of the compounds had moderate to strong anticancer activities. The inhibition rate of **6x** against PC3, BGC823, and PC3 cells at 10 μM were 90.6% ± 9.1%, 88.2% ± 6.1%, and 90.4% ± 2.8%, respectively. Also, the IC_{50} values of **6x** were 6.2 ± 0.9, 3.2 ± 0.1, and 3.1 ± 0.1 μM against PC3, BGC823, and Bcap37 cells, respectively, which were much higher than those of PD153035 (8.3 ± 1.1, 14.4 ± 1.5, and 15.7 ± 1.2 μM, respectively). The apoptosis-inducing activity of **6x** in PC3 cells was investigated by AO/EB staining, Hoechst 33258 staining, DNA ladder, and flow cytometry analyses. This compound clearly demonstrated cell apoptosis-inducing effects, with the inducing ratio higher than those ratios observed for the positive control HCPT. Moreover, the apoptosis ratio of **6x** was 31.8% at 10 μM for 72 h, which was higher than that of HCPT (17.0% at 10 μM for 72 h). Further studies on the specific mechanisms of **6x** in PC3 cells are currently underway.

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 a 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. ^1H NMR and ^{13}C NMR spectra (solvent DMSO-d_6, 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. Microwave reactions were performed on a variable power Focused Microwave Synthesis, Discover™ LabMate equipped with a high-sensitivity IR sensor for temperature control and measurement.

4.2. Chemistry

*4.2.1. Preparation of 2,3,4-trimethoxy-6-nitrobenzoic acid (**1**) [31]*

2,3,4-Trimethoxybenzoic acid was added in portions to 45 mL of concentrated 70% nitric acid at 0 °C (5.0 g, 0.02 mol). After stirring

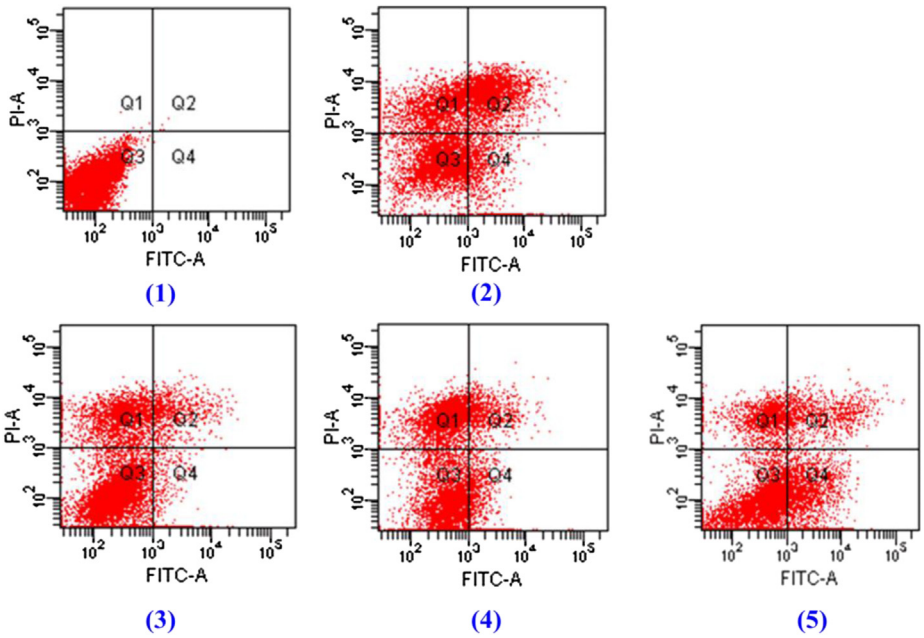


Fig. 4. Annexin V/PI apoptosis ratio detection assay. (1) Cells were treated with 0.1% DMSO for 72 h (2) Cells were treated with HCPT (10 μM) for 72 h (3–5) Cells were treated with **6x** at 1, 5, and 10 μM, respectively for 72 h.

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 (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.

*4.2.2. Preparation of methyl 2,3,4-trimethoxy-6-nitrobenzoate (**2**)*

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.

*4.2.3. Preparation of methyl 6-amino-2,3,4-trimethoxybenzoate (**3**)*

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.

*4.2.4. Preparation of 5,6,7-trimethoxyquinazoline-4-one (**4**)*

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°Cfor 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.

*4.2.5. Preparation of 4-chloro-5,6,7-trimethoxyquinazoline (**5**)*

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

Table 5
Apoptosis rate of compound **6x** and HCPT tested on PC3 cells.

Compd	24 h			48 h			72 h		
6x	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM
	5.1%	8.2%	10.9%	9.5%	11.1%	16.4%	11.6	19.8%	31.8%
HCPT	10 μM	20 μM	30 μM	10 μM	20 μM	30 μM	10 μM	20 μM	30 μM
	5.7%	7.4%	11.9%	8.3%	11.9%	12.8%	17.0%	19.8%	38.2%

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.

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

In the MW method, a mixture of 4-chloro-5,6,7-trimethoxy quinazoline (3.0 mmol) and aryl amine (3.0 mmol) in 2-propanol (10 mL) was stirred for 3 min and then reacted under MW at 100 W and 80 °C for 20 min. Upon reaction completion as monitored by thin-layer chromatography (TLC), the solvent was removed under reduced pressure. The residue was dissolved with methanol or chloroform and purified by TLC (petroleum ether/ethyl acetate, 1:2, *V*:*V*). The target compound was eluted from the silica gel with ethyl acetate to give the title compounds.

In the CH method, a solution of 4-chloro-5,6,7-trimethoxy quinazoline (4.0 mmol) and aryl amine (4.0 mmol) in 2-propanol (6 mL) was stirred under reflux for 2–12 h. Upon reaction completion as monitored by TLC, the solvent was removed under reduced pressure. The resulting solid was purified by silica gel or recrystallized with ethanol or methanol to give the title compounds.

*4.2.6.1. 5,6,7-Trimethoxy-N-(4-nitrophenyl)quinazoline-4-amine (**6a**).* Yield: 85.8%; pale yellow needles; mp: 170–173 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.00–4.36 (3s, 9H, $3OCH_3$), 7.90 (s, 1H), 7.99 (d, 2H, J = 9.2 Hz), 8.35 (d, 2H, J = 9.2 Hz), 8.70 (s, 1H), 10.94 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 57.6, 61.8, 63.3, 98.1, 102.5, 122.8, 125.2, 137.7, 141.9, 142.4, 145.2, 148.8, 148.9, 158.7, 161.7. IR (KBr) ν: 812.4, 1141.9, 1268.5, 1456.3, 1619.3, 2876.8, 2998.2, 3010.0, 3312.2, 3460.5 cm^{-1}. Anal. calcd. for $C_{17}H_{16}N_4O_5$ (%): C, 57.30; H, 4.53; N, 15.72. Found: C, 56.95; H, 4.71; N, 15.65.

*4.2.6.2. 4-(5,6,7-Trimethoxyquinazoline-4-yl-amino)phenol hydrochloride (**6b**).* Yield: 89.7%; pale brown needles; mp: 207–209 °C. ^{1}H NMR (CD_3OD, 500 MHz):δ 3.93–4.18 (3s, 9H, $3OCH_3$), 6.68 (dd, 2H, J = 6.9 Hz, 2.3 Hz), 6.79 (d, 1H, J = 9.2 Hz), 6.84 (dd, 2H, J = 6.9 Hz, 2.9 Hz), 6.99 (s, 1H), 7.47 (d, 1H, J = 8.6 Hz), 8.30 (s, 1H). ^{13}C NMR (CD_3OD, 500 MHz): δ 56.1, 60.6, 62.9, 95.5, 101.8, 115.2, 125.6, 127.9, 136.4, 141.5, 149.4, 150.4, 156.5, 158.6, 161.2. IR (KBr) ν: 805.2, 1151.5, 1290.4, 1494.8, 1629.8, 2867.4, 2990.3, 3009.7, 3288.6, 3423.3 cm^{-1}. Anal. calcd. for $C_{17}H_{18}ClN_3O_4$ (%): C, 56.13; H, 4.99; N, 11.55. Found: C, 56.37; H, 4.88; N, 11.51.

*4.2.6.3. 5,6,7-Trimethoxy-N-(2-nitrophenyl)quinazoline-4-amine (**6c**).* Yield: 86.1%; yellow solid; mp: 124–127 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.97–4.28 (3s, 9H, $3OCH_3$), 7.13 (s, 1H), 7.18 (dt, 1H, J = 8.0 Hz, 1.7 Hz), 7.68 (dt, 1H, 8.0 Hz, 1.7 Hz), 8.19 (dd, 1H, J = 8.0 Hz, 1.7 Hz), 8.65 (s, 1H), 8.97 (dd, 1H, J = 8.0 Hz, 1.7 Hz), 11.97 (s, 1H, NH). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 56.3, 61.6, 62.3, 104.8, 105.4, 122.8, 124.7, 125.8, 134.6, 135.1, 138.9, 140.9, 148.6, 149.2, 153.9, 156.7, 158.7. IR (KBr) ν: 807.5, 1149.6, 1290.4, 1498.7, 1606.7, 2833.4, 2941.4, 3007.6, 3288.6, 3400 cm^{-1}. Anal. calcd. for $C_{17}H_{16}N_4O_5$ (%): C, 57.30; H, 4.53; N, 15.72. Found: C, 57.06; H, 4.21; N, 15.98.

*4.2.6.4. 5,6,7-Trimethoxy-N-(4-methylphenyl)quinazoline-4-amine hydrochloride (**6d**).* Yield: 84.8%; light yellow needles; mp: 171–173 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.41 (s, 1H, CH_3), 3.97–4.28 (3s, 9H, $3OCH_3$), 7.28 (d, 2H, J = 8.0 Hz), 7.53 (d, 2H, J = 8.0 Hz), 7.79 (s, 1H), 8.54 (s, 1H), 10.54 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 21.2, 57.4, 61.6, 62.9, 98.0, 102.0, 123.4, 130.1, 133.3, 133.4, 137.3, 137.5, 141.8, 149.3, 158.7, 160.9. IR (KBr) ν: 810.0, 1153.4, 1290.4, 1494.8, 1631.7, 2889.4, 2945.3, 3007.6, 3281.1, 3443.3 cm^{-1}. Anal. calcd. for $C_{18}H_{20}ClN_3O_3$ (%): C, 59.75; H, 5.57; N, 11.61. Found: C, 59.50; H, 5.76; N, 11.48.

*4.2.6.5. N-(4-Fluorophenyl)-5,6,7-trimethoxyquinazoline-4-amine (**6e**).* Yield: 89.0%; light yellow solid; mp: 172–175 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.97–4.31 (3s, 9H, $3OCH_3$), 7.18 (t, 2H, J = 8.6 Hz), 7.63–7.66 (m, 2H), 7.80 (s, 1H), 8.54 (s, 1H), 10.56 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 57.4, 61.6, 62.9, 97.7, 101.8, 116.2, 116.4, 125.4, 125.5, 131.8, 137.1, 141.8, 149.2, 158.8, 160.0, 161.1, 162.0. IR (KBr) ν: 802.8, 1155.4, 1290.4, 1504.5, 1631.8, 2851.2, 2980.0, 3021.5, 3285.6, 3437.3 cm^{-1}. Anal. calcd. for $C_{17}H_{16}FN_3O_3$ (%): C, 62.00; H, 4.90; N, 12.76. Found: C, 61.89; H, 4.96; N, 12.52.

*4.2.6.6. N-(3-Fluorophenyl)-5,6,7-trimethoxyquinazoline-4-amine hydrochloride (**6f**).* Yield: 37.2% (only obtained under CH); pale yellow solid; mp: 176–179 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.76–4.18 (3s, 9H, $3OCH_3$), 6.72 (s, 1H), 7.08 (dt, 1H, J = 8.6 Hz, 1.7 Hz), 7.39 (d, 1H, J = 8.6 Hz), 7.47 (dd, 1H, J = 14.9 Hz, 8.6 Hz), 7.81 (d, 1H, J = 10.9 Hz), 8.64 (s, 1H), 12.75 (b, 1H). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 56.8, 60.6, 60.7, 90.5, 100.8, 110.5, 110.7, 112.2, 112.4, 119.5, 131.0, 131.1, 135.8, 150.9, 159.7, 160.5, 163.6. IR (KBr) ν: 808.7, 1153.4, 1294.5, 1492.9, 1614.4, 2829.6, 2935.7, 3032.6, 3312.5, 3400.2 cm^{-1}. Anal. calcd. for $C_{17}H_{17}FClN_3O_3$ (%): C, 55.82; H, 4.68; N, 11.49. Found: C, 55.67; H, 5.01; N, 11.60.

*4.2.6.7. N-(3-Chlorophenyl)-5,6,7-trimethoxyquinazoline-4-amine (**6g**).* Yield: 81.9% (only obtained under MW); white solid; mp: 122–124 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.96–4.17 (3s, 9H, $3OCH_3$), 7.08 (s, 1H), 7.09 (d, 1H, J = 8.0 Hz), 7.31 (t, 1H, J = 8.0 Hz), 7.62 (d, 1H, J = 8.0 Hz), 7.99 (s, 1H), 8.61 (s, 1H), 9.93 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 57.1, 158.0, 61.6, 63.0, 104.3, 104.6, 119.3, 121.3, 123.7, 129.9, 134.6, 140.2, 140.8, 148.4, 148.6, 154.7, 157.0. IR (KBr) ν: 805.7, 1149.2, 1280.8, 1491.5, 1633.6, 2926.4, 2982.3, 3005.7, 3286.7, 3425.3 cm^{-1}. Anal. calcd. for $C_{17}H_{16}ClN_3O_3$ (%): C, 59.05; H, 4.66; N, 12.15. Found: C, 59.26; H, 4.42; N, 11.85.

*4.2.6.8. 5,6,7-Trimethoxy-N-(3-nitrophenyl)quinazoline-4-amine (**6h**).* Yield: 85.0%; yellow solid; mp: 192–194 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.97–4.21 (3s, 9H, $3OCH_3$), 7.12 (s, 1H), 7.55 (t, 1H, J = 8.1 Hz), 7.96 (dd, 1H, J = 8.1 Hz, 1.8 Hz), 8.15 (dd, 1H, J = 8.1 Hz, 1.8 Hz), 8.66 (s, 1H), 8.80 (s, 1H), 10.14 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 76.8, 77.1, 77.4, 104.2, 104.7, 115.9, 118.1, 126.8, 129.7, 140.3, 141.0, 148.3, 148.7, 148.8, 154.5, 156.9, 158.3. IR (KBr) ν: 800.5, 1141.9, 1280.6, 1494.3, 1617.6, 2851.1, 2979.4, 3020.6, 3323.3, 3435.4 cm^{-1}. Anal. calcd. for $C_{17}H_{16}N_4O_5$ (%): C, 57.30; H, 4.53; N, 15.72. Found: C, 57.03; H, 4.57; N, 15.42.

*4.2.6.9. N-(2-Fluorophenyl)-5,6,7-trimethoxyquinazoline-4-amine (**6i**).* Yield: 88.9%; white solid; mp: 139–142 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.98–4.33 (3s, 9H, $3OCH_3$), 7.25–7.29 (m, 3H), 7.81 (s, 1H), 8.52–8.55 (m, 1H), 8.67 (s, 1H), 11.09 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 57.5, 61.7, 62.9, 97.8, 102.5, 115.3, 115.6, 124.3, 125.0, 127.2, 137.3, 142.0, 149.2, 153.2, 155.1, 158.5, 161.3. IR (KBr) ν: 792.7, 1145.6, 1296.2, 1498.7, 1635.6, 2883.6, 2951.1, 3020.5, 3281.4, 3487.2 cm^{-1}. Anal. calcd. for $C_{17}H_{16}FN_3O_3$ (%): C, 62.00; H, 4.90; N, 12.76. Found: C, 61.78; H, 5.17; N, 12.92.

*4.2.6.10. N-(2-Bromo-4,6-dimethylphenyl)-5,6,7-trimethoxyquinazoline-4-amine hydrochloride (**6j**).* Yield: 83.2%; white solid; mp: 245–

247 °C. ^{1}H NMR (CD_3OD, 500 MHz): δ 2.24, 2.34 (2s, 6H, $2CH_3$), 3.94–4.20 (3s, 9H, $3OCH_3$), 7.02 (s, 1H), 7.15 (s, 1H), 7.39 (s, 1H), 8.16 (s, 1H). ^{13}C NMR (CD_3OD, 500 MHz): δ 17.7, 19.5, 56.2, 60.6, 62.2, 91.6, 96.4, 101.7, 121.7, 122.1, 130.4, 130.6, 138.1, 139.9, 141.7, 150.1, 150.7, 159.9, 161.4. IR (KBr) ν: 812.0, 1149.6, 1298.1, 1490.9, 1633.7, 2916.4, 2982.0, 3170.7, 3269.6, 3408.3 cm^{-1}. Anal. calcd. for $C_{19}H_{21}BrClN_3O_3$ (%): C, 50.18; H, 4.65; N, 9.24. Found: C, 50.30; H, 4.45; N, 9.11.

*4.2.6.11. N-(2-Chloro-4-bromophenyl)-5,6,7-trimethoxyquinazoline-4-amine (**6k**).* Yield: 81.0%; white needles; mp: 195–196 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.88–4.16 (3s, 9H, $3OCH_3$), 7.13 (s, 1H), 7.62 (dd, 1H, J = 9.2 Hz, 2.3 Hz), 7.85 (d, 1H J = 2.3 Hz), 8.55 (s, 1H), 8.80 (d, 1H, J = 9.2 Hz), 10.45 (s, 1H). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 56.8, 61.5, 63.1, 104.2, 104.6, 115.2, 124.9, 125.0, 131.1, 131.8, 136.0, 140.9, 148.3, 148.7, 154.1, 156.7, 158.6. IR (KBr) ν: 808.7, 1153.4, 1294.2, 1469.8, 1629.5, 2939.5, 2977.8, 3010.9, 3309.3, 3420.5 cm^{-1}. Anal. calcd. for $C_{17}H_{15}BrClN_3O_3$ (%): C, 48.08; H, 3.56; N, 9.89. Found: C, 47.78; H, 3.83; N, 10.04.

*4.2.6.12. N-(2-Bromo-5-fluorophenyl)-5,6,7-trimethoxyquinazoline-4-amine (**6l**).* Yield: 80.1%; pale yellow solid; mp: 158–161 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.91–4.26 (3s, 9H, $3OCH_3$), 7.22 (dt, 1H, J = 8.6 Hz, 2.9 Hz), 7.42 (s, 1H), 7.84 (dt, 1H, J = 8.6 Hz, 2.9 Hz), 8.06 (dd, 1H, J = 10.3 Hz, 2.9 Hz), 8.87 (s, 1H), 10.90 (s, 1H). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 57.4, 61.8, 63.5, 97.4, 102.6, 114.9, 115.2, 115.7, 134.4, 137.6, 141.4, 149.9, 150.7, 159.2, 160.6, 161.0, 162.6. IR (KBr) ν: 821.7, 1147.7, 1294.2, 1496.7, 1626.0, 2941.2, 2947.2, 3008.9, 3240.3, 3410.5 cm^{-1}. Anal. calcd. for $C_{17}H_{15}BrFN_3O_3$ (%): C, 50.02; H, 3.70; N, 10.29. Found: C, 50.34; H, 3.43; N, 10.51.

*4.2.6.13. N-(3-Fluorophenyl)-5,6,7-trimethoxyquinazoline-4-amine (**6m**).* Yield: 81.9% (only obtained under MW); white solid; mp: 158–161 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.98–4.31 (3s, 9H, $3OCH_3$), 7.04 (t, 1H, J = 8.0 Hz), 7.36 (d, 1H, J = 8.0 Hz), 7.45 (dd, 1H, J = 14.9 Hz, 8.6 Hz), 7.68 (d, 1H, J = 10.0 Hz), 7.85 (s, 1H), 8.62 (s, 1H), 10.68 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 57.4, 61.6, 62.9, 97.9, 102.0, 110.8, 113.9, 118.5, 130.5, 137.4, 142.0, 148.9, 149.0, 158.6, 161.2, 161.9, 163.9. IR (KBr) ν: 801.7, 1159.2, 1292.3, 1487.1, 1631.8, 2856.8, 2983.9, 3012.5, 3271.3, 3451.2 cm^{-1}. Anal. calcd. for $C_{17}H_{16}FN_3O_3$ (%): C, 62.00; H, 4.90; N, 12.76. Found: C, 61.76; H, 5.01; N, 12.50.

*4.2.6.14. N-(4-Bromophenyl)-5,6,7-trimethoxyquinazoline-4-amine (**6n**).* Yield: 82.4%; light yellow needles; mp: 143–145 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.87–4.13 (3s, 9H, $3OCH_3$), 7.09 (s, 1H), 7.56 (d, 2H, J = 9.2 Hz), 7.87 (d, 2H, J = 8.6 Hz), 8.49 (s, 1H), 9.98 (s, 1H). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 57.3, 61.7, 62.7, 103.9, 104.6, 115.6, 124.1, 131.9, 138.8, 140.8, 148.4, 148.6, 154.3, 156.9, 158.4. IR (KBr) ν: 817.8, 1139.9, 1298.1, 1467.8, 1608.6, 2947.2, 2990.6, 3003.2, 3294.6, 3411.7 cm^{-1}. Anal. calcd. for $C_{17}H_{16}BrN_3O_3$ (%): C, 52.32; H, 4.13; N, 10.77. Found: C, 52.05; H, 4.15; N, 10.87.

*4.2.6.15. N-((2-Fluoro-5-trifluoromethyl)phenyl)-5,6,7-trimethoxy quinazoline-4-amine (**6o**).* Yield: 80.7%; white solid; mp: 173–176 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.90–4.22 (3s, 9H, $3OCH_3$), 7.39 (s, 1H), 7.67 (t, 1H, J = 9.7 Hz), 7.79–7.81 (m, 1H), 8.36–8.37 (m, 1H), 8.85 (s, 1H), 10.80 (s, 1H). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 57.3, 61.7, 63.1, 97.6, 102.6, 117.4, 117.6, 123.8, 125.1, 125.8, 126.9, 138.9, 141.3, 149.9, 151.0, 157.2, 159.4, 160.9. IR (KBr) ν: 810.1, 1161.0, 1292.3, 1489.0, 1627.9, 2941.4, 2970.4, 3030.2, 3277.2, 3430.9 cm^{-1}. Anal. calcd. for $C_{18}H_{15}F_4N_3O_3$ (%): C, 54.41; H, 3.81; N, 10.58. Found: C, 54.48; H, 4.10; N, 10.35.

*4.2.6.16. 5,6,7-Trimethoxy-N-(4-(trifluoromethyl)phenyl)quinazoline-4-amine (**6p**).* Yield: 81.1%; yellow solid; mp: 143–145 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.90–4.23 (3s, 9H, $3OCH_3$), 7.38 (s, 1H), 7.84 (d, 2H, J = 8.6 Hz), 7.95 (d, 2H, J = 8.6 Hz), 8.83 (s, 1H), 10.83 (s, 1H). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 56.7, 56.8, 60.7, 91.6, 101.1, 123.6, 125.8, 126.6, 126.7, 135.8, 137.6, 142.5, 143.2, 151.1, 153.3, 159.6. IR (KBr) ν: 810.6, 1149.6, 1280.3, 1475.2, 1625.3, 2935.6, 2981.9, 3162.9, 3270.3, 3418.6 cm^{-1}. Anal. calcd. for $C_{18}H_{16}F_3N_3O_3$ (%): C, 56.99; H, 4.25; N, 11.08. Found: C, 56.66; H, 4.09; N, 11.40.

*4.2.6.17. N-(3-Trifluoromethylphenyl)-5,6,7-trimethoxyquinazoline-4-amine (**6q**).* Yield: 85.1%; yellow solid; mp: 163–166 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.85–4.18 (3s, 9H, $3OCH_3$), 7.31 (s, 1H), 7.35 (d, 1H, J = 8.0 Hz), 7.47 (t, 1H, J = 8.1 Hz), 7.58 (d, 1H, J = 8.1 Hz), 7.83 (s, 1H), 8.78 (s, 1H), 10.69 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 57.4, 61.7, 63.2, 97.1, 102.6, 124.2, 125.2, 126.7, 130.9, 131.2, 133.4, 138.2, 138.8, 141.2, 150.1, 150.6, 159.2, 160.7. IR (KBr) ν: 809.5, 1157.1, 1295.4, 1496.2, 1635.7, 2889.3, 2956.8, 3009.6, 3254.4, 3420.2 cm^{-1}. Anal. calcd. for $C_{18}H_{16}F_3N_3O_3$ (%): C, 56.99; H, 4.25; N, 11.08. Found: C, 56.78; H, 4.45; N, 11.26.

*4.2.6.18. N-(3-Chlorophenyl)-5,6,7-trimethoxyquinazoline-4-amine hydrochloride (**6r**).* Yield: 45.8% (only obtained under CH); yellow solid; mp: 192–195 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.88–4.14 (3s, 9H, $3OCH_3$), 7.10 (s, 1H), 7.16 (dd, 1H, J = 8 Hz, 1.2 Hz), 7.40 (t, 1H, J = 8.0 Hz), 7.72 (dd, 1H, J = 8.0 Hz, 1.2 Hz), 8.21 (d, 1H, J = 1.2 Hz), 8.53 (s, 1H), 10.01 (s, 1H). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 56.3, 60.1, 62.3, 95.4, 101.7, 118.6, 121.0, 122.0, 124.1, 131.0, 133.7, 135.5, 141.5, 150.1, 152.9, 156.0, 158.7. IR (KBr) ν: 803.6, 1147.6, 1278.5, 1485.2, 1643.4, 2931.8, 2968.5, 3015.6, 3290.3, 3417.7 cm^{-1}. Anal. calcd. for $C_{17}H_{17}Cl_2N_3O_3$ (%): C, 53.42; H, 4.48; N, 10.99. Found: C, 53.30; H, 4.26; N, 10.70.

*4.2.6.19. N-(2-Bromo-5-trifluoromethylphenyl)-5,6,7-trimethoxyqui nazoline-4-amine (**6s**).* Yield: 83.6%; pale yellow solid; mp: 185–188 °C. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 3.88–4.12 (3s, 9H, $3OCH_3$), 7.13 (s, 1H), 7.40 (dd, 1H, J = 8.6 Hz, 2.3 Hz), 7.96 (d, 1H, J = 8.1 Hz), 8.57 (s, 1H), 9.21 (s, 1H), 10.50 (s, 1H). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 57.2, 61.3, 63.4, 92.5, 100.4, 123.5, 124.1, 124.6, 125.4, 134.6, 135.9, 137.7, 141.4, 149.8, 150.6, 160.1, 160.5, 161.1. IR (KBr) ν: 792.7, 1145.8, 1287.5, 1464.0, 1627.9, 2887.4, 2954.9, 3037.9, 3238.5, 3405.1 cm^{-1}. Anal. calcd. for $C_{18}H_{15}BrF_3N_3O_3$ (%): C, 47.18; H, 3.30; N, 9.17. Found: C, 47.36; H, 3.38; N, 9.07.

*4.2.6.20. N-(4-Chlorophenyl)-5,6,7-trimethoxyquinazoline-4-amine dihydrochloride (**6t**).* Yield: 90.0%; light yellow needles; mp: 161–163 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.97–4.30 (3s, 9H, $3OCH_3$); 7.44 (d, 2H, J = 8.6 Hz), 7.65 (d, 2H, J = 8.6 Hz), 7.80 (s, 1H), 8.59 (s, 1H), 10.59 (s, 1H). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 54.3, 62.9, 63.5, 98.8, 102.3, 124.3, 129.5, 132.0, 134.9, 138.8, 141.9, 149.1, 149.8, 158.5, 160.8. IR (KBr) ν: 809.8, 1155.4, 1294.2, 1490.9, 1616.6, 2893.2, 2987.7, 3012.8, 3271.1, 3416.4 cm^{-1}. Anal. calcd. for $C_{17}H_{18}Cl_3N_3O_3$ (%): C, 48.77; H, 4.33; N, 10.04. Found: C, 48.81; H, 4.60; N, 9.75.

*4.2.6.21. N-(2-Fluoro-3-(trifluoromethyl)phenyl)-5,6,7-trimethoxyqu inazolin-4-amine hydro-chloride (**6u**).* Yield: 81.2%, yellow solid; mp: 152–154 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.99–4.36 (3s, 9H, $3OCH_3$), 7.27 (s, 1H), 7.41 (t, 1H, J = 8.0 Hz), 7.52 (t, 1H, J = 7.5 Hz), 8.71 (s, 1H), 8.86 (t, 1H, J = 7.5 Hz), 11.19 (s, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz): 57.5, 61.7, 63.1, 98.2, 102.7, 121.1, 123.4, 124.8, 124.9, 126.5, 126.6, 127.8, 138.1, 142.2, 149.0, 149.3, 158.6, 161.5. Anal. calcd. for $C_{18}H_{16}ClF_4N_3O_3$: C, 49.84; H, 3.72; N, 9.69. Found C, 49.54; H, 4.01; N, 9.48.

*4.2.6.22. N-(2-Bromo-4,6-difluorophenyl)-5,6,7-trimethoxyquinazolin-4-amine (**6v**).* Yield, 84.8%; yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.97–4.20 (3s, 9H, $3OCH_3$), 6.97 (dt, 1H, J = 9.2 Hz, 2.9 Hz), 7.09 (s, 1H), 7.26–7.29 (m, 1H), 8.47 (s, 1H), 9.07 (s, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz):

56.4, 61.4, 62.4, 104.1, 104.3, 104.6, 115.8, 116.0, 122.4, 123.5, 140.6, 148.7, 148.8, 155.0, 157.9, 158.3, 159.9. Anal. calcd. for $C_{17}H_{14}BrF2N_3O_3$: C, 47.91; H, 3.31; N, 9.86. Found C, 47.95; H, 3.59; N, 9.58.

*4.2.6.23. 4-Iodo-2-(5,6,7-trimethoxyquinazolin-4-ylamino)benzoic acid hydrochloride (**6w**).* Yield, 80.5%; white solid; mp: 195–198 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.91–4.24 (3s, 9H, 3OCH_3), 7.39 (s, 1H), 8.01 (dd, 1H, J = 8.6 Hz, 1.7 Hz), 8.17 (d, 1H, J = 8.6 Hz), 8.28 (d, 1H, J = 1.7 Hz), 8.86 (s, 1H), 12.26 (s, 1H); ^{13}C NMR ($CDCl_3$/CD_3OD, 500 MHz): 57.2, 61.5, 62.5, 89.4, 96.3, 103.0, 123.0, 126.6, 137.2, 140.1, 141.3, 141.6, 148.6, 150.0, 158.2, 161.5, 167.4. Anal. calcd. for $C_{18}H_{17}ClIN_3O_5$: C, 41.76; H, 3.31; N, 8.12. Found C, 41.86; H, 3.19; N, 8.09.

*4.2.6.24. N-(2-Fluorophenylethyl)-5,6,7-trimethoxyquinazolin-4-amine (**6x**).* Yield: 83.9%; yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.08 (t, 2H, J = 6.9 Hz), 3.89 (q, 2H, J = 6.3 Hz), 3.80–3.96 (3s, 9H, 3OCH_3), 6.98 (s, 1H), 7.04–7.10 (m, 2H), 7.20–7.23 (m, 1H), 7.26–7.28 (m, 1H), 7.80 (t, 1H, J = 5.2 Hz), 8.46 (s, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz): 40.9, 56.1, 61.2, 61.8, 103.1, 104.1, 115.4, 124.2, 126.1, 128.3, 131.2, 131.3, 139.9, 147.9, 149.2, 152.8, 157.5, 159.3, 162.4. Anal. calcd. for $C_{19}H_{20}FN_3O_3$: C, 63.85; H, 5.64; N, 11.76. Found C, 63.61; H, 5.52; N 11.99.

*4.2.6.25. 4,5-Dimethoxy-2-(5,6,7-trimethoxyquinazolin-4-ylamino) benzoic acid hydrochloride (**6y**).* Yield, 85.4%; white solid; mp: 207–210 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.94–4.24 (3s, 9H, 3OCH_3), 4.47 (s, 6H, 2OCH_3), 7.48 (s, 1H), 7.62 (s, 1H), 8.33 (s, 1H), 8.56 (s, 1H); ^{13}C NMR ($CDCl_3$/CD_3OD, 500 MHz): 60.0, 60.0, 60.3, 65.1, 66.0, 105.2, 109.3, 111.0, 112.8, 117.0, 123.8, 125,3, 139.2, 144.7, 148.7, 153.5, 156.4, 161.2, 163.1, 172.8. Anal. calcd. for $C_{20}H_{22}ClN_3O_7$: C, 53.16; H, 4.91; N, 9.30. Found C, 53.45; H, 4.70; N, 9.49.

*4.2.6.26. N-(3-Fluorophenylethyl)-5,6,7-trimethoxyquinazolin-4-amine (**6z**).* Yield: 83.7%; yellow oil; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.03 (t, 2H, J = 6.9 Hz), 3.89 (q, 2H, J = 6.3 Hz), 3.77–3.96 (3s, 9H, 3OCH_3), 6.92–7.01 (m, 2H), 6.98 (s, 1H), 7.07 (d, 1H, J = 7.5 Hz), 7.27–7.32 (m, 1H), 7.77 (t, 1H, J = 5.2 Hz), 8.47 (s, 1H); ^{13}C NMR ($CDCl_3$, 500 MHz): 41.6, 56.2, 61.2, 61.5, 104.0, 104.1, 113.4, 115.7, 124.6, 130.2, 139.9, 141.9, 148.0, 149.2, 155.3, 157.6, 159.3, 162.1, 164.0. Anal. calcd. for $C_{19}H_{20}FN_3O_3$: C, 63.85; H, 5.64; N, 11.76. Found C, 64.09; H, 5.93; N, 11.44.

4.3. Anticancer activity bioassay

4.3.1. Cell culture

The human prostate cancer cell line PC3, breast cancer cell line Bcap-37, and gastric cancer cell line BGC823 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 formazan, 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. AO/EB staining

Cells were seeded at a concentration of 5×10^4 cell/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).

4.3.4. Western blot analysis

PC3 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-pErk1/23, Stanta Cruz Biotechnology; or anti-actin) overnight at 4 °C. The membrane was then washed for 3–5 min with PBS plus 0.2% 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) [32].

4.3.5. Flow cytometry

Prepared PC3 cells (1×10^6/mL) were washed twice with cold PBS and then re-suspended gently in 200 μL of binding buffer. Thereafter, cells were stained in 5 μL of Annexin V-FITC and shaken well. Finally, the cells were mixed with 5 μL of PI, incubated for

20 min in the dark and subsequently analyzed. The experiments were performed three times independently and in each sample, 10,000 events were acquired in gate and analyzed by flow cytometer (BD FACSAria) using BD Diva software.

Conflicts of interest

None.

Author's contributions

Yang Zhang synthesized the compounds and carried out part of the bioassay experiments. Linhong Jin did part of the bioassay experiments. Hongmei Xiang also did part of the bioassay experiments. Jian Wu and Deyu Hu took part in the compound structural elucidation and bioassay experiments. Wei Xue carried out some structure elucidation experiments. Prof. Song Yang is the corresponding author 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.2013.05.043.

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