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# Synthesis and biological evaluation of novel 6-chloro-quinazolin derivatives as potential antitumor agents

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ABSTRACT

Series of novel derivatives of 6-chloro-quinazolin, which this moiety was linked to a 1,5-diaryl-1,4-pentadien-3-one system, have been synthesized and tested for their antitumor activities in vitro against a panel of three human cancer cell lines (MGC-803, Bcap-37, and PC3 cells). Bioassay results indicated that most of the prepared compounds demonstrated good activities against various cancer cells. 6-chloro-quinazolin derivatives **5a** and **5f** were the most active members in this study, and experimental results of fluorescent staining and flow cytometry analysis revealed that they could induce apoptosis in MGC-803 and Bcap-37 cells, with apoptosis ratios of 31.7% and 21.9% at 24 h of treatment at 10 μM in MGC-803 cells. Those two quinazoline derivatives could be considered as useful templates for future development to obtain more potent antitumor agents.



## 1. Introduction

Cancer is widely prevalent and considered to be the second to cardiovascular diseases as the cause of death all over the world [1]. It is one of the major challenges of this century, which concerns the medical community all over the world [2–4]. Although major advances have been made in the chemotherapeutic management of some patients, the task of discovering new anticancer agents remains critically important [5–7].

Quinazoline is the most commonly encountered heterocyclic compound in medicinal chemistry, because of its wide spectrum of pharmacological activities [8,9]. And a large number of its derivatives are considered to be an important chemical synthon of various physiological significance and pharmaceutical utility [10,11]. They have been used as medicines and display anti-tobacco mosaic virus (anti-TMV) [12–14], anti-cucumber mosaic virus (anti-CMV) [15,16], anti-HIV [17,18], anti-cancer [19], antimicrobial [20], antifungal [21], anti-inflammatory [22], anti-hypertensive [23], and anti-convulsant [24] activities. The quinazoline derivatives represent an attractive scaffold for designing interesting anticancer drugs [25,26], and they have attracted more interest because of their diverse biological activity notably as kinase inhibitors [26]. Some quinazoline derivatives interact with tubulin and interfere with its polymerization, others act by modulating aurora kinase activity or have an effect in critical phases in the cell cycle or act as apoptosis inducers [27]. Natural compounds have been an important source of clinically useful agents, for instance, useful anticancer agents [28]. Curcumin is a kind of natural phenols [29]. It is the principal curcuminoid of the popular Indian spice turmeric, which is a member of the ginger family [30]. Curcumin and its derivatives (analogs) have extensive bioactivities such as bactericidal [31,32], anticancer [33], antioxidative [34], anti-inflammatory [31], and anti-HIV [35] properties. They are known for their ability to resist mutation and raise antitumor activity [36], and could induce apoptosis in cancer cells without cytotoxic effects on healthy cells [37]. In previous work, our group reported that most of the newly synthesized 1,5-diaryl-1,4-pentadien-3-one derivatives (curcumin analogs) exhibited significant antitumor

*Abbreviationlist:* ADM, adriamycin; AO/EB, acridine orange/ethidium bromide; $^{13}C$ NMR, $^{13}C$ nuclear magnetic resonance; DMSO, dimethyl sulfoxide; FCM, flow cytometry; HCPT, 10-hydroxyl camptothecin; $^{1}H$ NMR, proton nuclear magnetic resonance; IR, Infra-red; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

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activities against BGC-823, Bcap-37, and PC3 cells [38]. We developed (1*E*,4*E*)-1-aryl-5-(4-(quinazolin-4-yloxy)phenyl)-1,4-pentadien-3-one derivatives as well, and the results of bioassay revealed that many of the title compounds displayed enhanced antitumor activity through the induction of cell apoptosis [39]. El-Azab et al. synthesized new series of 6-chloro-quinazolin derivatives and found that most of derivatives showed effective and selective activity against MCF-7, HEPG2, and HELA human cancer cell lines [40]. Thus, in view of the previous rationale and in continuation of an ongoing program aiming at developing more potential anticancer drugs, in the present study series of new derivatives of 6-chloro-quinazolin, in which this moiety was linked to a 1,5-diaryl-1,4-pentadien-3-one system, have been synthesized and screened for antitumor activity against various human cancer cell lines using MTT method. Preliminary mechanism of antitumor action was also studied by AO/EB staining, Hoechst 33258 staining, TUNEL assay, and flow cytometry analysis.

## 2. Results and discussion

### 2.1. Chemistry

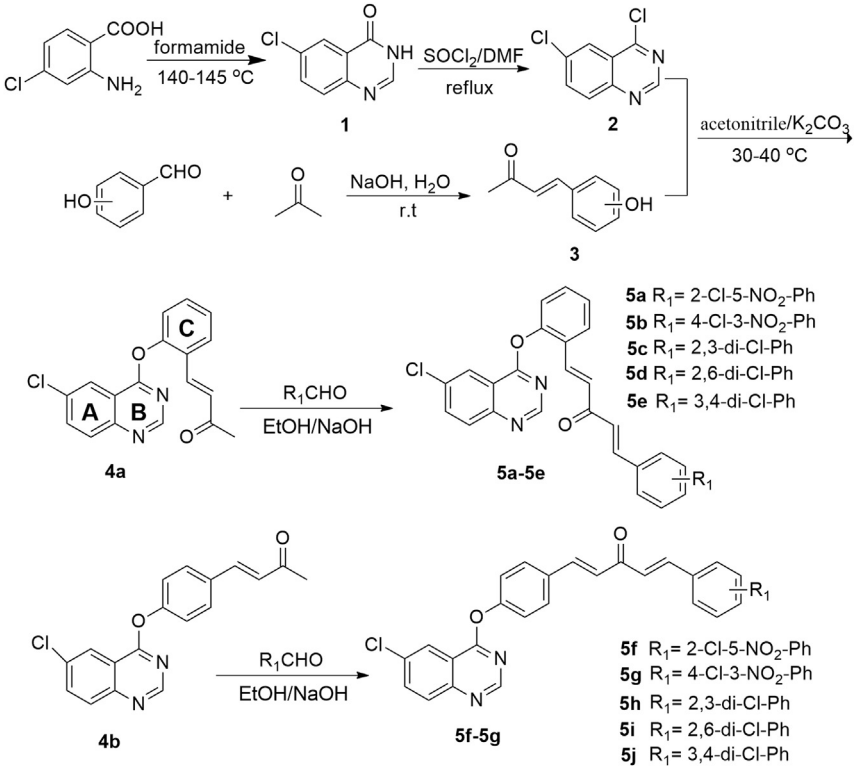
Scheme 1 outlines the synthetic pathways to obtain compounds **5a**–**5j**. Commercially available 2-amino-4-chlorobenzoic acid condensed with formamide at 140–145 °C for 4.5 h to get intermediate (**1**), which was further treated with $SOCl_2$ in the presence of DMF under reflux conditions to provide intermediate (**2**). Treatment of salicylaldehyde with acetone in the presence of sodium hydride at room temperature got intermediate 4-(4-hydroxyphenyl)-3-butylene-2-one (**3**). Intermediate (**2**) with 4-(4-hydroxyphenyl)-3-butylene-2-one (**3**) and $K_2CO_3$ in the presence of acetonitrile at 30–40 °C for 3.5 h afforded a mixture, which was recrystallized using anhydrous alcohol to yield intermediates **4a** and **4b**, respectively. And then, the target compounds **5a**–**5j** were synthesized by reacting the substituted aldehydes with **4a** or **4b** in the present of anhydrous alcohol in acetone at room temperature. Their structures were characterized by IR, $^1$H NMR, $^{13}$C NMR, and elemental analysis techniques.

### 2.2. Antiproliferative activity

To explore the antitumor potential of compounds **5a**–**5j**, a panel of cell lines representing a range of tumor types, including MGC-803, Bcap-37, and PC3, were treated with various concentrations of **5a**–**5j**, Adriamycin (ADM, positive control) or DMSO (0.1%) for 72 h. Cell viability was determined using the MTT method. Each experiment was repeated at least three times. The results are reported in terms of inhibition rates and $IC_{50}$ values, and summarized in Table 1.

As shown in Table 1, all the derivatives showed substantial cytotoxicity and displayed $IC_{50}$ values in the nanomolar range against MGC-803, Bcap-37, and PC3 cell lines. Namely, these compounds exhibited a broad spectrum of inhibition on human cancer cells, with $IC_{50}$ values ranging from 1 to 30 μM. The inhibitory ratios of compounds **5a** and **5f**, which are the most promising in this group compounds, were 91.6% and 86.7% on MGC-803 cells, 78.4% and 72.5% on Bcap-37 cells, and 86.9% and 76.8% on PC3 cells, respectively. The $IC_{50}$ values of compound **5a** were 1.96, 8.47, 2.51 μM, respectively, while for compound **5f**, the $IC_{50}$ values were 2.15, 9.84, 3.15 μM, respectively. The other compounds generally showed moderate activities. Interestingly, the MGC-803 cells were especially susceptible to all the derivatives, with a lower $IC_{50}$ values than that on others.

Based on these results, we deduced some preliminary structure–activity relationships. The biological results revealed that the derivatives showed different antitumor activities from moderate to




**Scheme 1.** Synthetic route for the preparation of compounds **5a**–**5j**.

**Table 1**
Effect of 6-chloro-quinazolin derivatives against cell viability of different cancer cell lines.

| Compd | Inhibition rates against different cells (%)[a] | | | $IC_{50}$ (μM)[b] | | |
|---|---|---|---|---|---|---|
| | MGC-803 | Bcap-37 | PC3 | MGC-803 | Bcap-37 | PC3 |
| **5a** | 91.6 ± 2.1 | 78.4 ± 7.8 | 86.9 ± 1.5 | 1.96 ± 0.42 | 8.47 ± 0.38 | 2.51 ± 0.29 |
| **5b** | 70.2 ± 11.2 | 52.5 ± 6.4 | 62.5 ± 6.9 | 5.32 ± 0.30 | 12.32 ± 0.46 | 9.76 ± 0.44 |
| **5c** | 76.5 ± 9.8 | 70.2 ± 8.5 | 73.8 ± 6.0 | 5.21 ± 0.43 | 11.53 ± 0.45 | 8.35 ± 0.27 |
| **5d** | 69.3 ± 14.2 | 67.1 ± 11.2 | 70.1 ± 14.2 | 5.17 ± 0.36 | 11.27 ± 0.39 | 7.98 ± 0.35 |
| **5e** | 56.7 ± 13.4 | 38.2 ± 12.6 | 42.2 ± 5.6 | 16.15 ± 0.22 | 21.15 ± 0.32 | 18.27 ± 0.39 |
| **5f** | 86.7 ± 11.5 | 72.5 ± 13.5 | 76.8 ± 8.5 | 2.15 ± 0.48 | 9.84 ± 0.27 | 3.15 ± 0.32 |
| **5g** | 66.2 ± 13.1 | 56.3 ± 3.8 | 56.3 ± 12.8 | 7.19 ± 0.51 | 16.19 ± 0.37 | 11.84 ± 0.27 |
| **5h** | 68.5 ± 11.5 | 61.7 ± 13.2 | 67.4 ± 11.0 | 7.30 ± 0.42 | 14.30 ± 0.48 | 10.19 ± 0.39 |
| **5i** | 61.7 ± 6.9 | 62.5 ± 9.7 | 67.5 ± 10.1 | 6.77 ± 0.35 | 13.77 ± 0.41 | 9.30 ± 0.48 |
| **5j** | 50.5 ± 8.7 | 34.5 ± 12.1 | 33.5 ± 3.2 | 16.90 ± 0.36 | 27.90 ± 0.52 | 18.77 ± 0.20 |
| ADM[c] | 95.8 ± 5.7 | 96.2 ± 1.9 | 93.5 ± 3.1 | 0.71 ± 0.24 | 0.94 ± 0.39 | 0.80 ± 0.27 |

[a] Inhibitory percentages of cells treated with 10 μM concentration of each compound for 72 h.
[b] Agent concentration (μM) that inhibited cell growth by 50% at 72 h after treatment.
[c] Adriamycin, positive control.

high inhibition rates against the same cell line at the same concentration. The variation among the different substitutes on $R_1$ may greatly affect the activities. When $R_1$ was 2-chloro-5-nitrophenyl, an apparent increase in antitumor activity was found. However, the compounds **5e** and **5j** showed the weakest activities, while $R_1$ was 3,4-dichlorophenyl. In addition, we also found that positions of substituents of C ring more or less affected the biological activities. For example, compounds **5a**–**5e** generally exhibited superior activity to compounds **5f**–**5g**.

### 2.3. Cell apoptosis induction

Apoptosis is a well identified biological response exhibited by cells after suffering DNA damage and is a useful marker for screening compounds for subsequent development as possible anticancer agents [41]. In our previous publication, we found that quinazoline derivatives and curcumin analogs could induce apoptosis in cancer cell lines [38,39]. In this work, to determine whether the title derivatives have similar effects on cancer cells, we selected compounds **5a** and **5f** to investigate their effect on MGC-803 and Bcap-37 cells by acridine orange (AO)/ethidium bromide (EB) and Hoechst 33258 staining under fluorescence microscopy.

Based on the differences in membrane integrity between necrotic and apoptosis, AO can pass through the cell membrane, but EB cannot. AO and EB are applied to investigate whether MGC-803 and Bcap-37 cells underwent cell death *via* apoptosis and/or necrosis. Under fluorescence microscope, live cells appear green (in the web version). Necrotic cells stain red (in the web version) but have a nuclear morphology resembling that of viable cells. Apoptosis cells appear green (in the web version), and morphological changes such as cell blebbing and formation of apoptotic bodies will be observed [42]. With HCPT used as a positive control at 10 μM, the cells treated with compounds **5a** and **5f** at different concentrations (1, 5, and 10 μM). After 12 h incubation, morphological changes were observed investigated using fluorescence microscopy. The results are illustrated in Fig. 1.

As shown in Fig. 1, green (in the web version) live MGC-803 and Bcap-37 cells with normal morphology were observed in the negative control group, whereas early apoptotic cells with yellow green (in the web version) dots or late apoptotic cells with orange dots (in the web version) could been found in positive group. In presence of the two compounds, the cells presented morphology consistent with apoptosis, including cell volume reduction, chromatin condensation and fragmentation of the nuclei condensation. These findings demonstrate that both of compounds **5a** and **5f** could induce apoptosis in cancer cells.

Blue-fluorescent Hoechst 33258 can cross the intact membrane of live cells. It stains the condensed chromatin of apoptotic cells more brightly than the looser chromatin of normal cells and enables the monitoring of nuclear changes associated with apoptosis. Thus, it shows apoptosis in all four types of cells, which are characterized by cytoplasmic and nuclear shrinkage, chromatin condensation and apoptosis body [43]. MGC-803 and Bcap-37 cells, treated with compounds **5a** and **5f** at concentrations of 1, 5, and 10 μM for 12 h, respectively, were stained with Hoechst 33258. HCPT was used as positive control at 10 μM or 12 h. The results are shown in Fig. 2.

Findings from the microscopic examination showed that the cells in the negative group displayed were normally blue (in the web version), while HCPT-treated cells appeared to be compact and condensed. The cells treated with compounds **5a** and **5f** exhibited strong blue (in the web version) fluorescence and revealed typical apoptotic morphology. The results once again indicated that the two compounds could induce apoptosis, consistent with the results for AO/EB double staining.

In addition, TUNEL assay is further carried out to confirm the cell apoptosis inducing activities of compounds **5a** and **5f**. This assay identifies nuclei containing fragmented DNA by enzymatically incorporating fluorescein-12-dUTP at the 3′ end of DNA fragments using a terminal deoxynucleotidyl transferase. Apoptotic cells can be stained brown (in the web version) with the assay and brown (in the web version) precipitates will be observed under light microscopy [44]. In this study, MGC-803 and Bcap-37 cells were treated with compounds **5a** and **5f** at 10 μM for 12 h, respectively. HCPT was used as a positive control at 10 μM for 12 h. The results are present in Fig. 3.

Fig. 3 shows that most nuclei were stained as a discernible brown (in the web version) in the treatment groups with HCPT, compounds **5a** and **5f**, compared with the negative control.

Annexin V-FITC/PI staining is performed to analyze the apoptosis of MGC-803 cells by flow cytometry. Early apoptotic cells are positive for annexin V and negative for PI, whereas late apoptotic cells are positive for both annexin V and PI. Therefore, fractions of cell populations in different quadrants are analyzed using quadrant statistics. The upper left quadrant (Q1) contains dead cells; the lower left quadrant contains (Q3) healthy cells; the upper and lower right quadrants (Q2 and Q4) contain viable apoptotic cells and non-viable apoptotic cells [45]. The results are given in Table 2. Table 2 shows that compounds **5a** and **5f** could induce apoptosis of MGC-803 cells, and the apoptosis of MGC-803 cells treated with the two compounds gradually increased in a dose-dependent and time-dependent manner. As shown in Fig. 4, the apoptosis ratios reached the peak (31.7% and 21.9%) after 24 h of

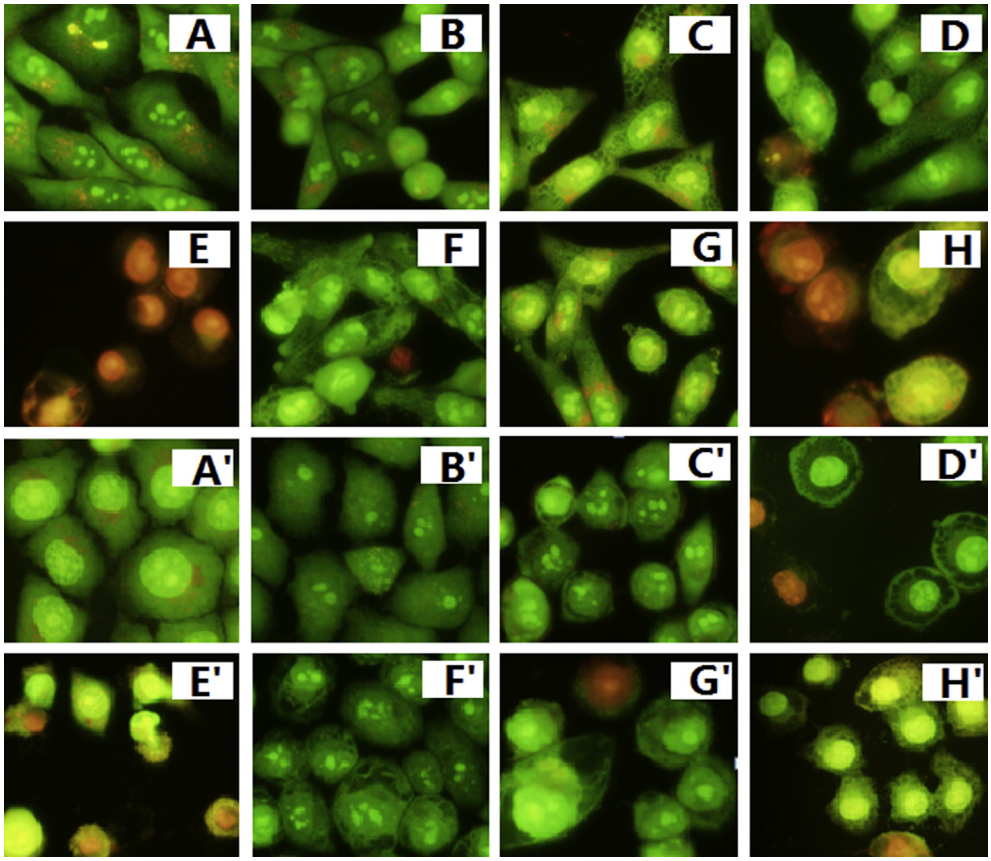



**Fig. 1.** MGC-803 and Bcap-37 cells were stained by AO/EB and observed under fluorescence microscopy. For MGC-803 cells group, A: without treatment; B: treated with HCPT (10 μM) for 12 h; C, D, and E: treated with compound **5a** at 1, 5, and 10 μM for 12 h; F, G, and H: treated with compound **5f** at 1, 5, and 10 μM for 12 h. For Bcap-37 cells group, A′: without treatment; B′: treated with HCPT (10 μM) for 12 h; C′, D′, and E′: treated with compound **5a** at 1, 5, and 10 μM for 12 h; F′, G′, and H′: treated with compound **5f** at 1, 5, and 10 μM for 12 h.

treatment at 10 μM, which were markedly higher than those of the positive control HCPT (18.6%).

## 3. Conclusion

The objective of the present study was to synthesize and investigated the antitumor activities of novel 6-chloro-quinazolin derivatives. The results of the antitumor screening revealed that most of the compounds were found to exhibit good antitumor activities against the MGC-803, Bcap-37, and PC3 cell lines, such as compounds **5a** and **5f**. The inhibitory ratios of compound **5a** and **5f** were 91.6% and 86.7% on MGC-803 cells, 78.4% and 72.5% on Bcap-37 cells, and 86.9% and 76.8% on PC3 cells, respectively. Also, the $IC_{50}$ values of compound **5a** were 1.96, 8.47, 2.51 μM, respectively, while for compound **5f**, the $IC_{50}$ values were 2.15, 9.84, 3.15 μM, respectively. In addition, further studies revealed that compounds **5a** and **5j** could induce apoptosis in MGC-803 and Bcap-37 cells, and the apoptosis ratios in MGC-803 cells reached the peak (31.7% and 21.9%) at 10 μM for 24 h, respectively, which were higher than that of HCPT (18.6%). Those two 6-chloro-quinazolin derivatives could be considered as useful templates for future development to obtain more potent antitumor agents.

## 4. Experimental

### 4.1. General

Melting points were determined using an XT-4 binocular microscope (Beijing Tech Instrument Co., China) and were not corrected. IR spectra were recorded as KBr discs using a Bruker VECTOR 22 spectrometer. $^1H$ and $^{13}C$ NMR spectra were recorded in a $CDCl_3$ solvent using a JEOL-ECX 500 NMR spectrometer operating at 500 and 125 MHz at room temperature and using TMS as an internal standard. Elemental analysis was performed on an Elementar Vario-III CHN analyzer. All reagents obtained from Yuda Chemistry Co., Ltd were of analytical reagent grade, and used without further purification unless otherwise noted. Analytical TLC was performed on silica gel $GF_{254}$. Column chromatographic purification was performed using silica gel (200–300 mesh) (Qingdao Marine Chemistry Co., Qingdao, China).

### 4.2. Synthetic procedures

6-chloroquinazolin-4(3*H*)-one (**1**) and 4,6-dichloroquiazoline (**2**) were prepared according to a previously described method [46]. Intermediate 4,6-dichloroquiazoline (**2**) was prepared following standard synthetic protocols [47]. Intermediate (*E*)-4-(2-hydroxyphenyl)-3-butylene-2-one or (*E*)-4-(4-hydroxyphenyl)-3-butylene-2-one (**3**) was prepared according to a previously reported [48].

### 4.3. General synthetic procedures for the preparation of compounds *5a–5j*

Compounds **5a–5j** were synthesized (Scheme 1). Intermediate (**2**) (10 mmol), intermediate (**3**) (10 mmol) and $K_2CO_3$ (70 mmol) were added to acetonitrile (20 mL); the mixture was then stirred at 30–40 °C for 3.5 h. After the reaction was completed, the mixture

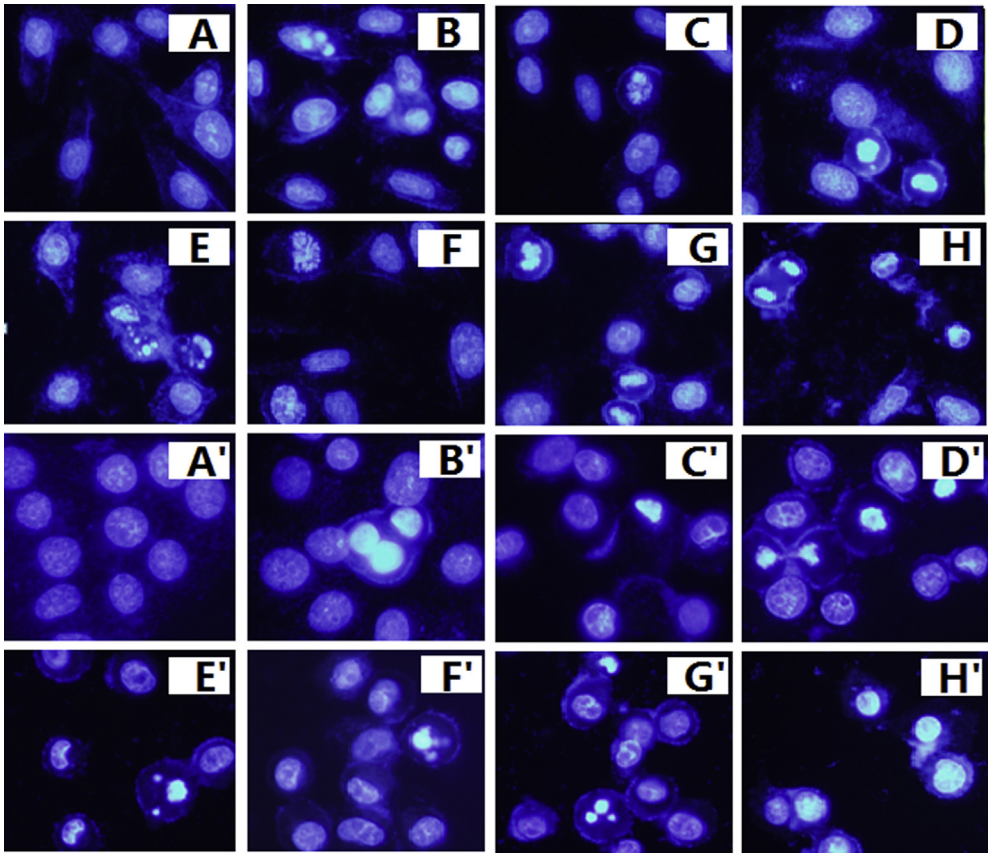



**Fig. 2.** MGC-803 and Bcap-37 cells were stained by Hoechst 33258 and observed under fluorescence microscopy. For MGC-803 cells group, A: without treatment; B: treated with HCPT (10 μM) for 12 h; C, D, and E: treated with compound **5a** at 1, 5, and 10 μM for 12 h; F, G, and H: treated with compound **5f** at 1, 5, and 10 μM for 12 h. For Bcap-37 cells group, A′: without treatment; B′: treated with HCPT (10 μM) for 12 h; C′, D′, and E′: treated with compound **5a** at 1, 5, and 10 μM for 12 h; F′, G′, and H′: treated with compound **5f** at 1, 5, and 10 μM for 12 h.

was filtered and then concentrated under reduced pressure. The obtained residue was recrystallized with anhydrous alcohol and then filtered to obtain intermediate **4a** or **4b**, respectively. Intermediate **4a** or **4b** (0.5 mmol), substituted aldehydes (0.5 mmol), and sodium hydroxide (1%) were added to 75 vol % ethanol/water solution (20 mL) and stirred at room temperature. After the reaction was completed, the mixture was concentrated under reduced pressure. The residue was suspended in water (20 mL), adjusted with 5% HCl to pH 7, and filtered. The filtration residue was recrystallized with anhydrous alcohol to give compounds **5a**–**5j**. Their structures were characterized by IR, $^{1}$H NMR, $^{13}$C NMR, and elemental analysis techniques.

*4.3.1. (4E)-1-(2-chloro-5-nitrophenyl)-5-(2-((6-chloroquinazolin-4-yl)oxy)phenyl)penta-1,4-dien-3-one (**5a**)*

Yield: 57.5%; yellow powder; mp: 187–189 °C; IR (KBr, cm$^{-1}$) $\nu$: 3444, 1678, 1620, 1595, 1475, 1359, 1215, 983; $^{1}$H NMR ($CDCl_3$, 500 MHz) $\delta$: 8.73 (s, 1H, Qu-2-H), 8.42 (d, $J$ = 2.9 Hz, 2H, Qu-7,8-H),

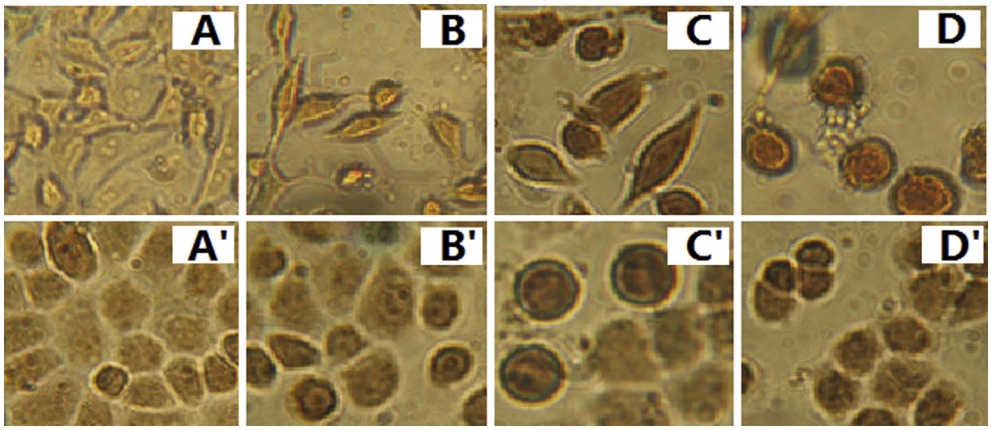



**Fig. 3.** MGC-803 and Bcap-37 cells were stained by TUNEL and observed under light microscopy. For MGC-803 cells group, A: without treatment; B, C, and D: treated with HCPT, compound **5a**, and **5f** at 10 μM for 12 h, respectively. For Bcap-37 cells group, A′: without treatment; B′, C′, and D′: treated with HCPT, compound **5a**, and **5f** at 10 μM for 12 h, respectively.

**Table 2**
Apoptosis ratios of compounds **5a**, **5f** and HCPT tested on MGC-803 cells.

| Compd | 6 h[a] | | | 12 h | | | 24 h | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 μM[b] | 5 μM | 10 μM | 1 μM | 5 μM | 10 μM | 1 μM | 5 μM | 10 μM |
| **5a** | 2.0% | 6.7% | 10.3% | 3.5% | 9.2% | 15.7% | 5.2% | 16.8% | 31.7% |
| **5f** | 1.2% | 1.7% | 4.2% | 1.8% | 5.6% | 8.9% | 4.3% | 9.8% | 21.9% |
| HCPT | 1.3% | 1.8% | 3.5% | 2.1% | 6.0% | 9.1% | 3.8% | 7.3% | 18.6% |

[a] Treating time.
[b] Agent concentration.

8.14 (d, $J$ = 6.3 Hz, 1H, Cl–Ar–CH═), 7.82–7.98 (m, 6H, Qu-5-H, Cl-Ar-3,4,6-H, Ar–CH═, Ar-3-H), 7.56 (d, $J$ = 16.5 Hz, 1H, Cl–Ar–C═CH), 7.42 (t, 1H, Ar-5-H), 7.26 (d, $J$ = 15.5 Hz, 1H, Ar-6-H), 7.14 (t, 1H, Ar-4-H) 6.97 (d, $J$ = 15.5 Hz, 1H, Ar–C═CH); $^{13}$C NMR ($CDCl_3$, 125 MHz) $\delta$: 187.6, 166.0, 154.3,153.5, 151.5, 149.5, 146.8, 141.5, 140.9, 137.7, 136.8, 135.5, 134.6, 132.1, 130.2, 129.9, 128.3, 126.9, 126.8, 125.1, 123.4, 122.5, 116.8; Anal. Calcd for $C_{25}H_{15}Cl_2N_3O_4$: C 60.99; H 3.07; N 8.54; Found: C 61.24; H 3.14; N 8.81.

### 4.4. Cell culture

Human gastric cancer cell line (MGC-803), human breast cancer cell line (Bcap-37), and human prostate cancer cell line (PC3) were obtained from the Institute of Biochemistry and Cell Biology, China Academy of Science. The cells were maintained as a monolayer in RPMI 1640 supplemented with 10% heat inactivated FBS. Cell culture was carried out in an environment of 5% $CO_2$ and 95% 'normal' air at 37 °C. All the cells were passed twice weekly and routine examination was also done for mycoplasma contamination. Cells in logarithmic growth phase were used for further experiments.

### 4.5. MTT assay

The MTT assay was used to evaluate the effect of drugs on the proliferation of MGG-803, Bcap-37, and PC3 human cancer cell lines. In brief, the cells were seeded in 96-well plates at $2 \times 10^3$ cells/well. After overnight incubation, the culture medium was replaced by fresh medium containing drugs at the final concentrations for 72 h. At the end of incubation, the medium was removed and, after two washes with PBS, 100 μL of MTT was added to the cells. Subsequently, the microplates were incubated for 4 h. The medium in each well was aspirated and the violet formazan crystals that formed as a result of MTT reduction within metabolically viable cells were dissolved with 100 μL of SDS per well for 12 h. The optical density (OD) of each well was determined at 595 nm by using a microplate reader (Bio-Rad Laboratories, Model 680). All the observations and assays were repeated in triplicate.

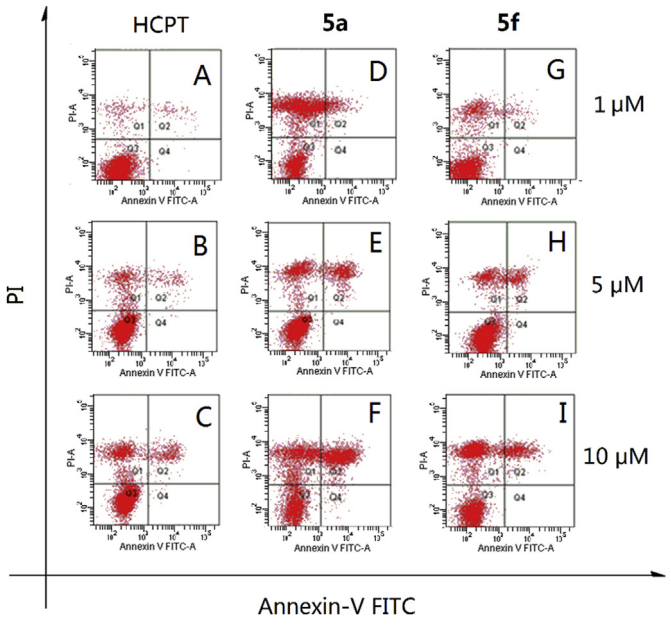



**Fig. 4.** Apoptosis of MGC-803 cells exposure to HCPT: 1 μM (A), 5 μM (B), 10 μM (C), compound **5a**: 1 μM (D), 5 μM (E), 10 μM (F) and compound **5f** 1 μM (G), 5 μM (H), 10 μM (I) for 24 h.

### 4.6. AO/EB staining

The MGC-803 or Bcap-37 cells were seeded in a 6-well plate. After overnight incubation, the cells were treated with drugs with different concentrations for 12 h at 37 °C. Cells were then washed with PBS and stained with AO/EB (Beyotime) at room temperature. And then the cells were observed and photographed by fluorescence microscope (OLYPUS Co., Japan).

### 4.7. Hoechst 33258 staining

The MGC-803 or Bcap-37 cells were cultured in 6-well plates. After overnight incubation, the cells were treated with drugs with different concentrations for 12 h at 37 °C. Cells were washed with PBS and stained with Hoechst 33258 (Beyotime) at room temperature for 5 min. The apoptosis was classified by morphology and color of the cells, and quantified. Finally, after washing twice with PBS, morphological changes were observed under a fluorescence microscope by using 350 nm excitation and 460 nm emission.

### 4.8. TUNEL assay

The MGC-803 or Bcap-37 cells were cultured in 6-well plates. After overnight incubation, the cells were treated with HCPT, compounds **5a** and **5f** at 10 μM for 12 h at 37 °C, respectively. Cells were washed with PBS and were performed as previously described. Finally, the cells were rewashed twice with PBS, and were consequently imaged under an XDS-1B inverted biological microscope.

### 4.9. Flow cytometry analysis

MGC-803 cells were treated with drugs at different concentrations (1 μM, 5 μM, and 10 μM) for 6, 12, and 24 h, respectively. The cells were washed with ice-cold PBS twice and then resuspended gently in 500 μL binding buffer. The cells were then stained with 5 μL of Annexin V-FITC and shaked well. 5 μL of PI was added to these cells and incubated in the dark for 20 min at 20–25 °C. Data acquisition was carried out using FACS.

### 4.10. Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were performed using SPSS 10.0, and analyzed using one-way ANOVA. These data were from experiments performed in triplicate. Statistical significance was set at $P < 0.05$.

## Conflicts of interest

None.

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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.2014.07.053.

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