Biomedicine & Pharmacotherapy 113 (2019) 108747

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# Novel naphthalene-enoates: Design and anticancer activity through regulation cell autophagy

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ARTICLE INFO

*Keywords:*
Synthesis
Naphthalene derivatives
Anticancer activity
Autophagy

ABSTRACT

Eleven dihydroxy-2-(1-hydroxy-4-methylpent-3-enyl)naphthalene derivatives as anticancer agents through regulating cell autophagy were designed and synthesized. The anticancer activity results indicated that most compounds manifested obvious un-toxic effect on GES-1 and L-02 with $IC_{50}$ from 0.58 to 1.41 mM. Among them, (*S,Z*)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(3,4-dihydroisoquinolin-2(1 H)-yl)-4-oxobut-2-enoate (compound **4i)** could induce cancer cells apoptosis. Further experiments showed that autophagy played an important role in the pro-apoptotic effect of this compound. Preliminary mechanism indicated that this compound could inhibit phosphoinositide 3-kinase/protein kinase B and the mammalian target of rapamycin (PI3K/AKT/mTOR) pathway by mediating apoptosis in an autophagy-dependent manner.

## 1. Introduction

Autophagy is a degradation process of the highly conserved protein or organelle found in eukaryotes [1,2]. It entails the degradation of cellular organelles and macromolecules in autophagic vesicles (autophagosomes), which fuse with lysosomes to form autophagolysosomes. Autophagy can be activated by starvation, oxidative stress, radiation or chemical insults and so on [3]. So far, autophagy has been found to be associated with cancer [4], neurodegenerative [5], inflammatory [6] and cardiovascular [7]. Autophagy is required for apoptosis to occur, maybe shows an essential impact on apoptosis and even more switching on cell apoptosis [8–10]. Among them, LC3-II is the key protein associated with autophagy, changes of its expression indicating an accumulation of cellular autophagosomes, which may representant increased production of autophagosomes or impaired autophagy [11]. Protein p62 is an autophagic substrates which can verify the lysosome degradation [12]. The phosphoinositide 3-kinase (PI3K) pathway has regulatory roles in cell survival, proliferation, differentiation, which plays an important role in oncology associated with autophagy [13,14].

(*R*)-dihydroxy-2-(1-hydroxy-4-methylpent-3-enyl)naphthalene derivatives, an active scaffold isolated from natural products [15] have extensive pharmacological activities [16,17], such as anti-inflammatory, anticancer [18,19], regulation of microtubule tubulin polymerization [20], so, its derivatives have been widely attracted [21–24]. However, due to its relatively side effects and toxicity, the potential utility as clinical anticancer agent is limited. In order to develop the molecules with enhances properties and low toxicity, number of its analogs have been designed and synthesized [25–27]. In order to discover new compounds with anticancer activity against PI3K, the crystal structure of phosphoinositide 3-kinase gamma (PDB:2CHW) was used as target for virtual screening in this study. We first defined the pocket through the ligand (PIK-39) in the crystal structure, this pocket contains some important residues, such as Met804, Trp812, Lys890, Thr887, Met953 and Ile831 *etc*. Among them, Met804 and Trp812 built a novel hydrophobic pocket, which is the entrance to the ATP binding site among these residues [28]. Focusing on this model, when introduced phenylamino/quinoline-butyrate moiety into the dihydroxy-naphthalene-1,4-dione skeleton, lower interaction energy was found (Table 1), furthermore, the docking results indicated that interactions between the groups of esterification and the key residues Trp812, Lys890 were formed. So, these compounds may be of great significance, and should be increase their activity, reduce toxicity (Fig. 1). The further study showed that apoptosis of A875 cells could be induced by its analogues [29] through inhibiting the PI3K/Akt signaling pathway [30]. Because PI3K/AKT/mTOR pathway negatively regulate autophagy [31–34], and then influence cell apoptosis. So, based on above finding, a series of dihydroxy-naphthalene-1,4-dione-cinnamamide analogues were synthesized, a new mechanism for title compound was

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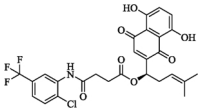
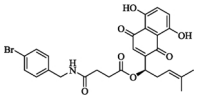
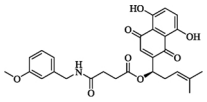
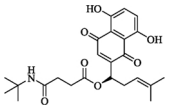
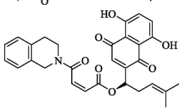
https://doi.org/10.1016/j.biopha.2019.108747
Received 17 February 2019; Accepted 27 February 2019

**Table 1**
Representative dihydroxy-naphthalene-1,4-dione derivatives through virtual screening.

| Compounds | -CDOCKER INTERACTION ENERGY (kcal/mol) |
|---|---|
| PIK-39 | 53.0399 |
|  | 59.9951 |
|  | 64.7394 |
|  | 61.0376 |
|  | 57.2896 |
|  | 54.1587 |
| Shikonin | 40.0391 |

then proposed in an effort to verify the biological activity [35].

## 2. Material and methods

### 2.1. Chemistry

All chemical procedures were screened by Thin-Layer Chromatography (TLC). Melting points recorded by XT4MP device without correction (Taike Corporation, Beijing). Spectra of nuclear magnetic resonance were detected through Brucker AM500. Reagents were commercial available.

### 2.2. General procedure for the synthesis of title compounds **4a~4k**

A solution of carboxylic acid, dicyclohexylcarbodiimide and 4-dimethylaminopyridine (DMAP) in dichloromethane (DCM) was stirred in ice bath for 15 min. Then was added shikonin, the solution was stirred in ice bath and stirred for 6 h slowly to room temperature. After TLC shows the reaction was completed, the solution was concentrated and cooled to 0 °C and filtered to removal all the dicyclohexylurea (DCU), the filtrate was evaporated, the resulting product was purified through prepared layer chromatography. Compound **4** is light red solid (Scheme 1).

**4a:** (R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(2-chloro-5-(trifluoromethyl)phenylamino)-4-oxobutanoate

Red solid. Yield: 75.2%. m.p.68.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.57 (s, 1 H), 12.39 (s, 1 H), 8.74 (s, 1 H), 7.88 (s, 1 H), 7.44 (d, *J* = 8.4, 1 H), 7.29-7.26 (m, 1 H), 7.18-7.13 (m, 2 H), 7.06 (d, *J* = 0.8, 1 H), 6.05 (dd, *J* = 6.7, 5.0, 1 H), 5.11 (t, *J* = 7.3, 1 H), 2.84 (t, *J* = 6.2, 2 H), 2.80-2.75 (m, 2 H), 2.66-2.60 (m, 1 H), 2.53-2.46 (m, 1 H), 1.66 (s, 3 H), 1.56 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 177.44, 175.98, 171.64, 169.57, 168.06, 167.53, 147.44, 136.26, 134.99, 133.09, 132.83, 131.57, 130.11, 129.36, 128.81, 124.33, 122.52, 121.03, 117.46, 111.79, 111.53, 70.20, 32.77, 29.14, 25.70, 25.58, 17.94.

**4b:** (R)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-oxo-4-(4-(trifluoromethyl)phenylamino)butanoate

Red solid. Yield: 74.8%. m.p.125.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.56 (s, 1 H), 12.40 (s, 1 H), 7.65 (br, 1 H), 7.59 (d, *J* = 8.3, 2 H), 7.52 (d, *J* = 8.5, 2 H), 7.18-7.13 (m, 2 H), 7.04 (s, 1 H), 6.04 (dd, *J* = 6.7, 5.1, 1 H), 5.10 (t, *J* = 7.2, 1 H), 2.86-2.82 (m, 2 H), 2.71-2.67 (m, 2 H), 2.64-2.58 (m, 1 H), 2.53-2.45 (m, 1 H), 1.65 (s, 3 H), 1.55 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 177.27, 175.74, 172.03, 169.57, 168.26, 167.73, 147.39, 140.69, 136.33, 133.18, 132.94, 131.49, 126.22, 126.19, 124.89, 123.09, 119.13, 117.39, 111.76, 111.53, 70.19, 32.78, 32.06, 29.33, 25.72, 17.94.

**4c:** (S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(4-bromobenzylamino)-4-oxobutanoate

Red solid. Yield: 69.4%. m.p.121.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.58 (s, 1 H), 12.40 (s, 1 H), 7.39 (d, *J* = 8.3, 2 H), 7.17 (s, 2 H), 7.10 (d, *J* = 8.3, 2 H), 7.00 (s, 1 H), 6.01 (dd, *J* = 6.4, 5.0, 1 H), 5.91 (br, 1 H), 5.10 (t, *J* = 7.3, 1 H), 4.37 (t, *J* = 6.2, 2 H), 2.79 (t, *J* = 6.7, 2 H), 2.63-2.57 (m, 1 H), 2.52 (t, *J* = 6.7, 2 H), 2.47-2.44 (m, 1 H), 1.67 (s, 3 H), 1.56 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 177.66, 176.16, 171.88, 170.95, 167.90, 167.36, 147.71, 137.14, 136.21, 133.05, 132.82, 131.69, 131.50, 129.30, 121.32, 117.51, 111.77, 111.52, 69.94, 42.97, 32.79, 30.83, 29.46, 25.76, 17.95.

**4d:** (S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(3-methoxybenzylamino)-4-oxobutanoate

Red solid. Yield: 57.3%. m.p.87.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.57 (s, 1 H), 12.40 (s, 1 H), 7.20 (t, *J* = 7.8, 1 H), 7.17 (s, 2 H), 7.00 (s, 1 H), 6.81 (d, *J* = 7.5, 1 H), 6.79-6.74 (m, 2 H), 6.01 (dd, *J* = 6.4, 5.2, 1 H), 5.86 (br, 1 H), 5.10 (t, *J* = 7.3, 1 H), 4.40 (d, *J* = 5.7, 2 H), 3.76 (s, 3 H), 2.81-2.77 (m, 2 H), 2.63-2.57 (m, 1 H), 2.52 (t, *J* = 6.7, 2 H), 2.50-2.41 (m, 1 H), 1.67 (s, 3 H), 1.56 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 177.90, 176.43, 171.85, 170.80, 167.64, 167.10, 159.83, 147.79, 139.60, 136.17, 132.93, 132.68, 131.57, 129.67, 119.83, 117.54, 113.09, 113.01, 111.80, 111.55, 69.88, 55.18, 43.62, 32.80, 30.87, 29.50, 25.75, 17.94.

**4e:** (S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(4-methoxybenzylamino)-4-oxobutanoate

Red solid. Yield: 42.1%. m.p.107.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.57 (s, 1 H), 12.41 (s, 1 H), 7.17 (s, 3 H), 7.15 (s, 1 H), 7.01 (s, 1 H), 6.82 (d, *J* = 8.6, 2 H), 6.01 (dd, *J* = 6.3, 5.3, 1 H), 5.79 (br, 1 H), 5.10 (t, *J* = 7.3, 1 H), 4.35 (d, *J* = 5.6, 2 H), 3.77 (s, 3 H), 2.80-2.76 (m, 2 H), 2.63-2.57 (m, 1 H), 2.51-2.45 (m, 3 H), 1.67 (s, 3 H), 1.56 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 177.96, 176.48, 171.83, 170.67, 167.60, 167.07, 158.97, 147.83, 136.17, 132.92, 132.68, 131.60, 130.10, 129.07, 117.54, 114.02, 111.79, 111.54, 69.84, 55.24, 43.18, 32.80, 30.86, 29.52, 25.76, 17.95.

**4f:** (S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(furan-2-ylmethylamino)-4-oxobutanoate

Red solid. Yield: 66.2%. m.p.115.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.57 (s, 1 H), 12.42 (s, 1 H), 7.32 (s, 1 H), 7.19-7.15 (m, 2 H), 7.00 (s, 1 H), 6.28 (dd, *J* = 2.9, 1.9, 1 H), 6.20 (d, *J* = 2.6, 1 H), 6.03-5.98 (m, 1 H), 5.89 (s, 1 H), 5.10 (t, *J* = 7.2, 1 H), 4.42 (d, *J* = 5.5, 2 H), 2.81-2.73 (m, 2 H), 2.63-2.57 (m, 1 H), 2.51-2.44 (m, 3 H), 1.67 (s, 3 H), 1.56 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 178.02, 176.52, 171.77, 170.68, 167.56, 167.03, 151.03, 147.84, 142.17, 136.17, 132.91, 132.68, 131.61, 117.53, 111.79, 111.56, 110.42, 107.46, 69.85, 36.60, 32.79, 30.71, 29.41, 25.75, 17.94.

**4 g:** (S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(isopropylamino)-4-oxobutanoate

Red solid. Yield: 67.4%. m.p.87.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.57 (s, 1 H), 12.42 (s, 1 H), 7.19-7.14 (m, 2 H), 7.00 (s, 1 H), 6.01 (dd, *J* = 6.4, 5.1, 1 H), 5.39 (d, *J* = 6.7, 1 H), 5.10 (t, *J* = 7.3, 1 H), 4.09-4.01 (m, 1 H), 2.78-2.69 (m, 2 H), 2.61-2.56 (m, 1 H), 2.50-2.46 (m, 1 H), 2.43 (t, *J* = 6.9, 2 H), 1.67 (s, 3 H), 1.56 (s, 3 H), 1.11 (dd, *J* = 13.8, 6.6, 6 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 178.05, 176.55, 171.88, 169.98, 167.53, 167.01, 147.91, 136.15, 132.90, 132.67, 131.58, 117.54, 111.79, 111.54, 69.77, 41.50, 32.80, 31.00, 29.55, 25.75, 22.72, 22.69, 17.94.

**4h:** (S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(tert-butylamino)-4-oxobutanoate

Red solid. Yield: 79.1%. m.p.76.8 °C. $^1$H NMR (600 MHz, $CDCl_3$)

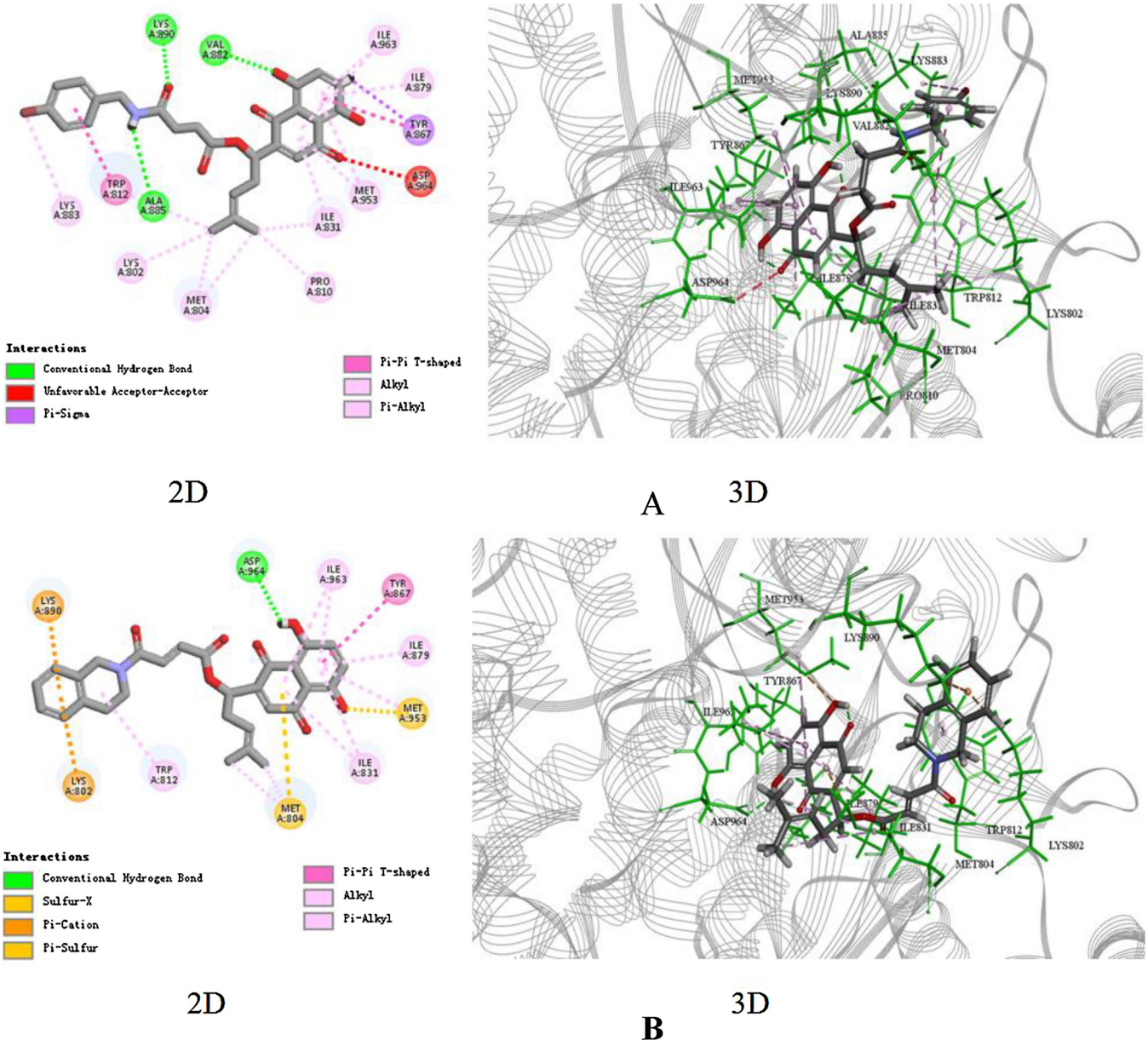
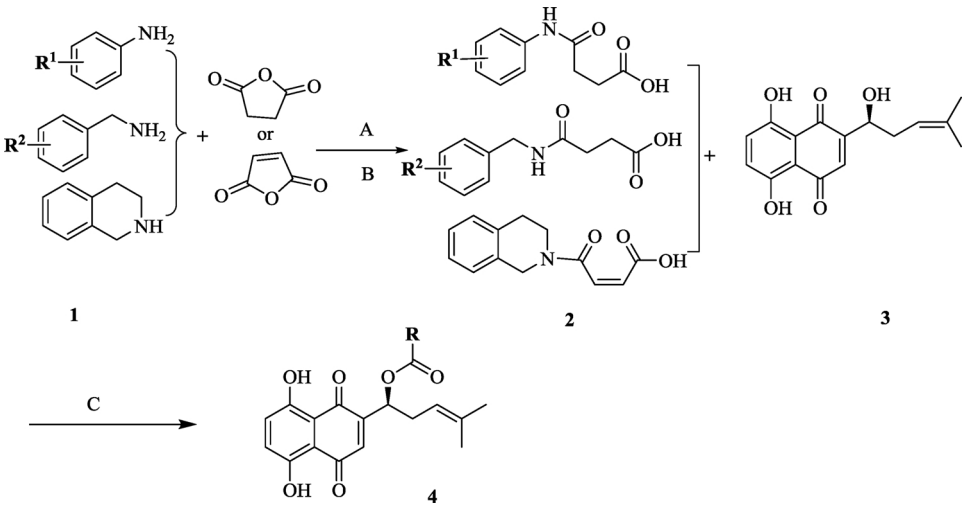



**Fig. 1.** Docking results.




**Scheme 1.** Synthesis of compounds **4a˜4k**.

δ = 12.57 (s, 1 H), 12.42 (s, 1 H), 7.19-7.15 (m, 2 H), 7.01 (s, 1 H), 6.00 (dd, *J* = 6.7, 4.9, 1 H), 5.37 (s, 1 H), 5.10 (t, *J* = 7.3, 1 H), 2.76-2.71 (m, 2 H), 2.63-2.56 (m, 1 H), 2.49-2.45 (m, 1 H), 2.39 (t, *J* = 6.8, 2 H), 1.67 (s, 3 H), 1.56 (s, 3 H), 1.31 (s, 9 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 178.24, 176.76, 171.95, 170.14, 167.35, 166.82, 147.97, 136.10, 132.82, 132.59, 131.66, 117.58, 111.79, 111.54, 69.75, 51.29, 32.79, 31.63, 30.72, 29.50, 28.74, 26.15, 25.74, 17.94.

**4i:** (S,Z)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-(3,4-dihydroisoquinolin-2(1 H)-yl)-4-oxobut-2-enoate

Red solid. Yield: 72.5%. m.p. 64.8 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.56 (s, 1 H), 12.43 (s, 1 H), 7.20-7.05 (m, 7 H), 6.01 (t, J = 5.8, 1 H), 5.12 (t, J = 7.1, 1 H), 4.71 (s, 1 H), 4.62 (s, 1 H), 4.13-4.07 (m, 1 H), 3.67 (t, J = 6.0, 1 H), 3.50-3.42 (m, 1 H), 2.89 (t, J = 5.9, 1 H), 2.78 (t, J = 6.0, 2 H), 2.64-2.59 (m, 1 H), 2.51-2.45 (m, 1 H), 1.67 (s, 3 H), 1.56 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ = 169.71, 168.25, 162.92, 157.88, 157.37, 147.73, 139.10, 135.61, 126.99, 123.64, 123.42, 122.87, 122.18, 119.53, 117.09, 115.67, 108.65, 102.79, 102.55, 60.68, 44.38, 40.11, 20.61, 17.76, 16.75, 16.58, 14.95, 8.94.

**4 j:** (S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-oxo-4-(pyrrolidin-1-yl)butanoate

Red solid. Yield: 47.4%. m.p.98.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.56 (s, 1 H), 12.42 (s, 1 H), 7.18-7.14 (m, 2 H), 7.04 (s, 1 H), 6.04-5.98 (m, 1 H), 5.15-5.09 (m, 1 H), 3.45 (t, *J* = 6.8, 2 H), 3.41 (t, *J* = 6.6, 2 H), 2.76 (t, *J* = 6.5, 2 H), 2.66-2.59 (m, 1 H), 2.57 (t, *J* = 6.4, 2 H), 2.52-2.44 (m, 1 H), 1.97-1.90 (m, 2 H), 1.87-1.81 (m, 2 H), 1.67 (s, 3 H), 1.56 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 178.77, 177.29, 172.15, 169.30, 166.84, 166.33, 148.15, 135.99, 132.64, 132.41, 131.86, 117.66, 111.79, 111.55, 69.64, 46.40, 45.74, 32.78, 29.24, 29.06, 26.02, 25.77, 24.35, 17.95.

**4k:** (S)-1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl 4-oxo-4-(thiazolidin-3-yl)butanoate

Red solid. Yield: 34.7%. m.p.87.0 °C. $^1$H NMR (600 MHz, $CDCl_3$) δ = 12.57 (s, 1 H), 12.43 (s, 1 H), 7.19-7.14 (m, 2 H), 7.04 (s, 1 H), 6.01 (dd, *J* = 6.4, 5.0, 1 H), 5.12 (t, *J* = 7.2, 1 H), 4.59 -4.53 (m, 1 H), 4.51-4.46 (m, 1 H), 3.85-3.81 (m, 1 H), 3.73 (t, *J* = 6.2, 1 H), 3.08 (t, *J* = 6.2, 1 H), 2.98 (td, *J* = 6.3, 1.4, 1 H), 2.77 (t, *J* = 6.5, 2 H), 2.67-2.59 (m, 3 H), 2.52-2.48 (m, 1 H), 1.68 (s, 3 H), 1.56 (s, 3 H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 178.40, 176.91, 171.88, 169.09, 167.22, 166.71, 147.95, 136.07, 132.78, 132.55, 131.79, 117.62, 111.81, 111.58, 69.83, 48.59, 48.07, 32.78, 31.23, 29.76, 29.01, 25.76, 17.95.

### 2.3. Cell culture

Human glioma cell (U-87), human hepatocellular carcinoma cell (SMMC-7721), human gastric cancer cell (SGC-7901), human gastric cancer cell (MGC-803) and human normal liver cell (L-02), gastric mucosa cells (GES-1) were maintained in dulbecco's modifed eagle medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) along with 1% penicilin and streptomycin. Cells were grown at 37 °C in a atmosphere with 5% $CO_2$.

### 2.4. Cell viability assay

U-87, SMMC-7721, SGC-7901, MGC-803 and GES-1, L-02 cells were seeded in 96-well plates and permitted to grow for 20 h. Then the concentrations of the compounds (0.16, 0.8, 4, 20, 100 μM) were added, MTT assay was used to test the cell viability after 48 h. MTT (20 μL, 5 mg/mL) was added into each well. After 4 h of incubation at 37 °C, 150 μL dimethyl sulfoxide (DMSO) were added to each well, Then shaking the plates for 15 min. Finally, $IC_{50}$ value was determined by the absorbance at 490 nm.

### 2.5. Cell apoptosis

SGC-7901 cells were treated with different concentrations of compound, and were harvested after 48 h, resuspended in 400 μL binding buffer. 5 μL Annexin V-Fluorescein isothiocyanate (FITC) was then added in each tube and incubated on ice in the dark for 15 min. The ratio of apoptosis was analyzed by flow cytometry after Propidium Iodide (10 μL respectively) being added. In addition, all samples ought to be detected by flow cytometry immedieately. At last, flowjo software was used to analyze the acquired results.

### 2.6. Acridine orange staining assay for autophagy detection

Cells ($1 \times 10^4$/ mL/well) seeded in coverslip Laser confocalwere exposed to various concentrations of compound for 48 h. 1 μg/mL acridine orange (AO) was added in the cells for 15 min, then the graphs were taken by confocal microscope.

### 2.7. Green fluorescence protein-protein light chain 3 (GFP-LC3) transfection

GFP-LC3 was applied to study autophagy. Cells ($1.5 \times 10^5$/mL/vessel) were grown in coverslip Laser confocal. The next day, SGC-7901 cells were transected with the plasmid GFP-LC3 gene (0.4 μg/vessel) and 1.5 μL of Lipofectamine 2000. The transected cells were treated with compound for 24 h. Then SGC-7901 cells were fixed in paraformaldehyde for 15 min. Finally, cells were photographed with a laser confocal microscope.

### 2.8. Western blot analysis

After treatment, cells were harvested by trypsinisation, and were resuspended with lysis buffer on ice for 30 min. After centrifugation at 12,000 r for 30 min at 4 °C, quantified by the Bradford method (Bio-Rad, Richmond CA). Equal amounts of protein extracts were separated by 12% sodium dodecyl sulfonate-Polyacrylamide (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were treated with specified antibodies (LC3, Beclin1 and P62 were from Abcam; Bax, Bcl-2, PARP, PI3kγ, p-PI3Kγ, Akt, p-Akt, mTOR, p-MTOR were from Cell Signaling Technology) followed by HRP-conjugated secondary antibody.

### 2.9. Statistical analysis

Data are reported as means ± SD. statistical analyses were calculated using Statistical Package for Social Sciences software (SPSS 17.0) and Graphpad Prism 5 software through an one way-ANOVA. All experiments are repeated at least three times. Differences between groups were statistically significant.

## 3. Results and discussion

### 3.1. Chemistry

To a solution of arylamine in toluene was added succinic anhydride. Then the solution was kept refluxing about 2.5 h. After TLC showed completion of reaction, the temperature was dropped to 25 °C. After filtration, the precipitate was rinsed with cooled toluene, and the solvent evaporated under reduced pressure to give white solid **2**. The product **2** was pure enough for next step [36]. The detailed synthesis process was attached to the Supporting information.

### 3.2. In vitro anticancer activity

The cytotoxicity *in vitro* of the compounds **4a˜4k** against U-87, SMMC-7721, SGC-7901, MGC-803 cells was evaluated using MTT, included the positive ADM (Adriamycin, a broad-spectrum anticancer drug). Cells were treated with compounds with different concentrations (0.16, 0.8, 4, 20, 100 μM) for 48 h [37]. It is obvious from the Table 2,

**Table 2**
In vitro anticancer activity of compounds **4a**˜**4k** against U-87, SMMC-7721, SGC-7901 and MGC-803 cell lines.

| Compd | IC50 μM [a] | | | |
|---|---|---|---|---|
| | U-87 | SMMC-7721 | SGC-7901 | MGCC-803 |
| **4a** | 6.42 ± 1.22 | –[c] | – | – |
| **4b** | 6.39 ± 0.31 | 11.45 ± 1.20 | 15.10 ± 1.87 | 12.79 ± 0.60 |
| **4c** | 3.44 ± 1.47 | 7.03 ± 3.95 | 3.97 ± 1.78 | 6.17 ± 3.32 |
| **4d** | 7.36 ± 2.22 | 20.38 ± 3.10 | 18.57 ± 1.30 | 7.84 ± 2.11 |
| **4e** | 5.15 ± 1.39 | 19.55 ± 3.55 | 13.24 ± 1.16 | 8.87 ± 2.78 |
| **4f** | 6.52 ± 2.56 | 42.82 ± 1.31 | 10.66 ± 0.79 | 4.71 ± 0.96 |
| **4 g** | – | – | – | – |
| **4h** | 15.81 ± 2.35 | 48.73 ± 3.58 | 20.37 ± 3.94 | 15.91 ± 0.30 |
| **4i** | 6.97 ± 1.91 | 8.32 ± 0.84 | 3.75 ± 0.79 | 7.86 ± 0.48 |
| **4 j** | 2.60 ± 1.97 | 7.67 ± 0.65 | 8.32 ± 1.45 | 2.38 ± 1.68 |
| **4k** | 5.64 ± 1.62 | 31.65 ± 1.35 | 9.18 ± 0.44 | 6.81 ± 1.23 |
| **AMD**[b] | 0.57 ± 0.32 | 0.46 ± 0.63 | 0.72 ± 0.12 | 0.48 ± 0.61 |

[a] The $IC_{50}$ value was defined as the concentration at which 50% survival of cells was observed. The standard deviation (SD) of three time independent experiments.
[b] AMD was used as a positive control.
[c] Not observed in the tested concentration range (> 100 μM).

**Table 3**
$IC_{50}$ values of some title compounds against human normal cells GES-1 and L-02 cells proliferation.[a]

| Compound | GES-1 ($IC_{50}$, mM) | L-02 ($IC_{50}$, mM) |
|---|---|---|
| **4a** | 1.01 ± 0.27 | 1.03 ± 0.31 |
| **4c** | 1.17 ± 0.11 | 1.25 ± 0.22 |
| **4e** | 1.33 ± 0.20 | 1.22 ± 0.19 |
| **4 g** | 0.85 ± 0.29 | 0.91 ± 0.33 |
| **4i** | 0.61 ± 0.15 | 0.58 ± 0.21 |
| **4k** | 1.16 ± 0.19 | 1.41 ± 0.22 |

[a] MTT assays were used for evaluation, and values were expressed as mean $IC_{50}$ of the triplicate experiment.

compounds **4i**, **4 j** showed certain activity against above four cell lines. Most compounds have a good inhibitory activity against U87 cells (compounds **4c** and **4 j**) with $IC_{50s}$ of 2.6˜3.5 μM, compared to that of the positive control ADM. Except compounds **4c**, **4i**, **4 j**, other compounds expressed poor activity against cell line. Among them, compounds **4c**, **4i**, **4 j**, **4k** reflected moderate activity against MGC-803 and SGC-7901.

Regular structure activity relationship was established, it is easy to see, when **R** is *N*-phenylamide, the substituents of the benzene ring have a great influence on the activity, and chlorine-substituent is disadvantageous to the activity (compound **4a**). When **R** is *N*-phenethyl amide, the target compounds generally have better activity (compound **4c**), among them, the substituent position of the benzene ring has little effect against the activity. When **R** is directly linked to lactam, different rings show poor effect on the activity (compounds **4i**, **4 j**, **4k**), but, when **R** amide is directly linked to alkyl, it is generally unfavorable to the activity (compound **4 g**).

### 3.3. Inhibition assay of human normal cells

In order to determine the selectivity of the potential cytotoxicity of some title compounds. We subsequently conducted a proliferative inhibition assay with human normal liver cell (L-02) and gastric mucosa cells (GES-1). As shown in Table 3, all compounds manifested obvious un-toxic effects on GES-1 and L-02 cells with $IC_{50s}$ from 0.58 to 1.41 mM. The data indicated that the compounds have good selectivity on somatic cells and tumor cells (Tables 2 and 3).

In addition, colony formation assays were also used to confirm the effect on cell vitality of the compound **4i**, the results showed that cell viability decreased in a concentration-dependent manner of this compound (2, 4, 8 μM) against SGC-7901 cells (Fig. 2). Compounds **4c** and **4i** had the same activity, compound **4c** showed better binding with target protein (Table 1 and Fig. 1), but compound **4i** was more soluble, so compound **4i** was chosen to explore the mechanism.

### 3.4. Induce apoptosis

To investigate whether the title compound **4i** induce the cell death due to apoptosis. We performed Annexin V-FITC/propidium iodide (PI) staining on SGC-7901 cells. Four quadrant images were usually analyzed by flow cytometric: the Q1 area represented damaged cells induced by mechanical forces or environmental stimulus, later period apoptotic cells and necrotic cells usually were located in the Q2 area, Q3 area denoted early apoptotic cells, the Q4 area often represented normal cells. As shown in Fig. 3A, the apoptosis percentage of SGC-7901 cells were respectively increased from 3.4% (1 μM) to 8.6% (2 μM) and 33.9% (4 μM). Correspondingly, the apoptosis ratios of control group was 2.2%. The results indicate title compound **4i** could promote SGC-7901 cells apoptosis dose-dependent. Generally, apoptosis occurs in two pathways: intrinsic pathway and the extrinsic pathway, which involve an ordered activation of a set of caspases. Finally, promote apoptosis [2]. The intrinsic pathway begins with the up-regulation of wild-type p53, followed by regulating Bcl-2 family proteins [3]. In order to confirmed that the apoptosis induced by title compound via the intrinsic pathway, we then assessed a series of key proteins related to the intrinsic pathway, the results showed that title compound could regulate Bax and cl-PARP, and then inhibit the expression of Bcl-2 (Fig. 3B).

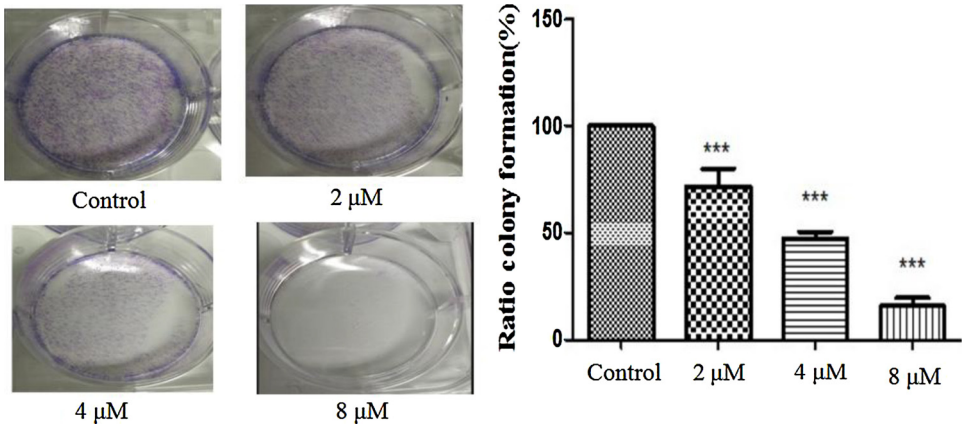



**Fig. 2.** Colony formation assays.

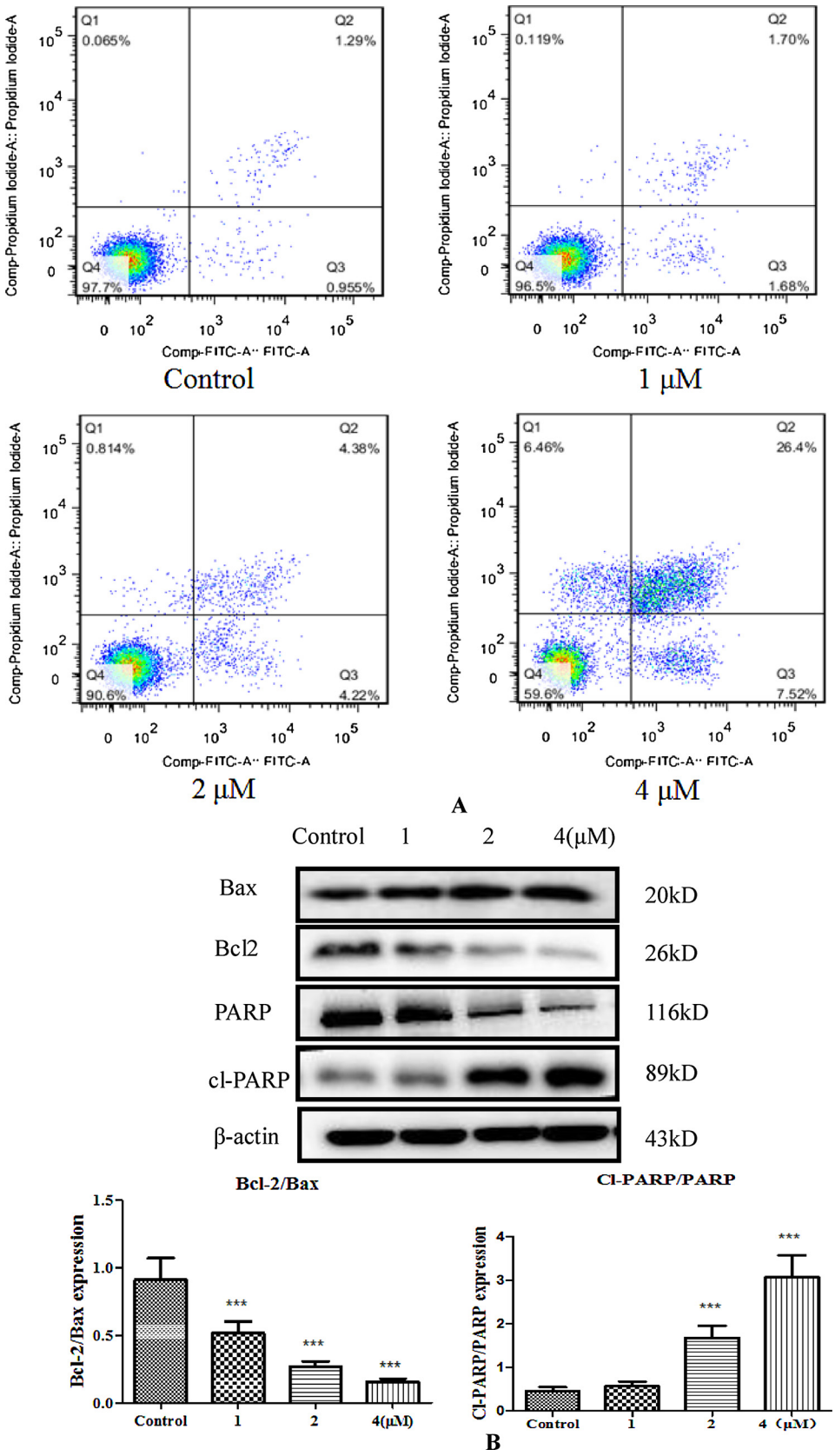



**Fig. 3.** Cell Apoptosis.

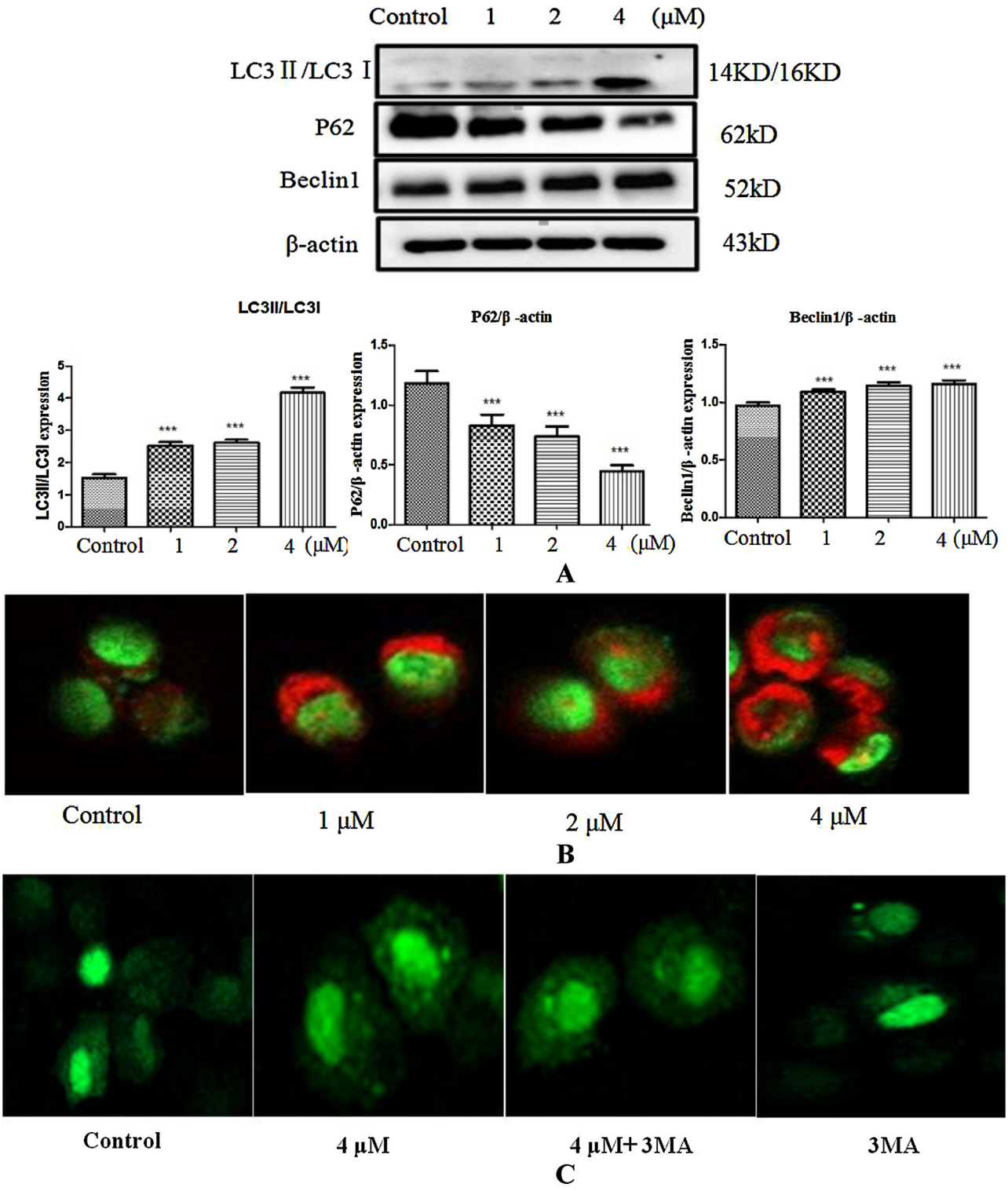



**Fig. 4.** Induces autophagy.

### 3.5. Induce autophagy

Autophagy is an adaptive mechanism by which cells defend against external and internal stresses [4,5]. Autophagy can be activated by starvation, oxidative stress, radiation or chemical insults and so on [6]. So far, autophagy has been found to be associated with cancer [7], neurodegenerative [11], inflammatory [12], and cardiovascular [38]. In this process, when expression of LC3-II increased, indicating an accumulation of cellular autophagosomes [39]. Protein p62 is also an autophagic substrates which can verify the lysosome degradation [40]. So, to explicate whether the title compound **4i** promote autophagy, we detected the expressions of LC3 II, p62 and Beclin1. Results indicated that title compound **4i** could increase the expressions of LC3 II and Beclin1, but decreased expression of p62 (Fig. 4A).

In addition, when occur autophagy, acidic autophagy lysosome increase. AO staining applied to observe visualize the acidic autophagy lysosome, the cytoplasm and the nucleus fluoresced dominant green when the concentrated dye in the vesicles showed bright red. According

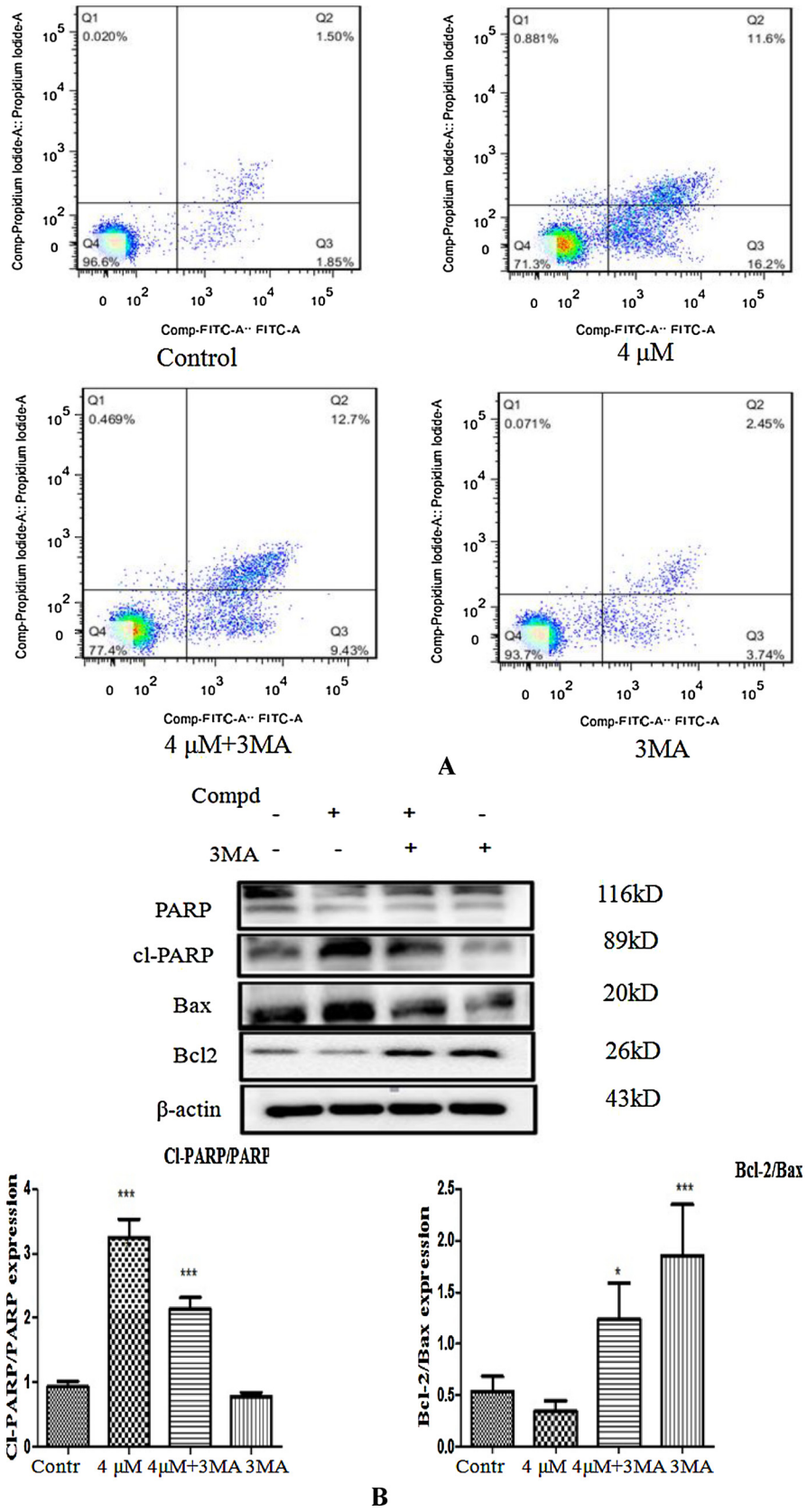



**Fig. 5.** Autophagy promote apoptosis.

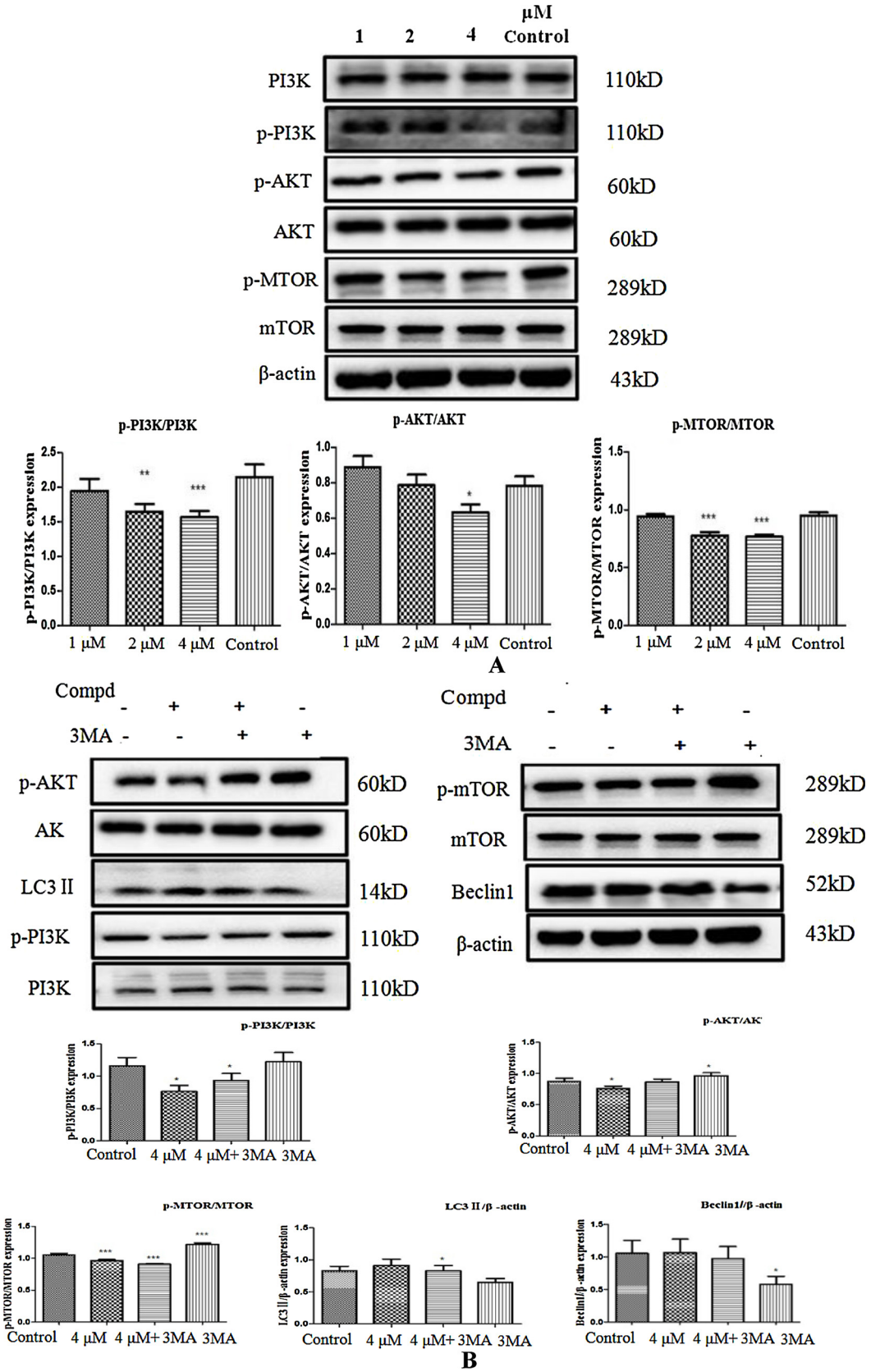



**Fig. 6.** Western blots observe the expressions.

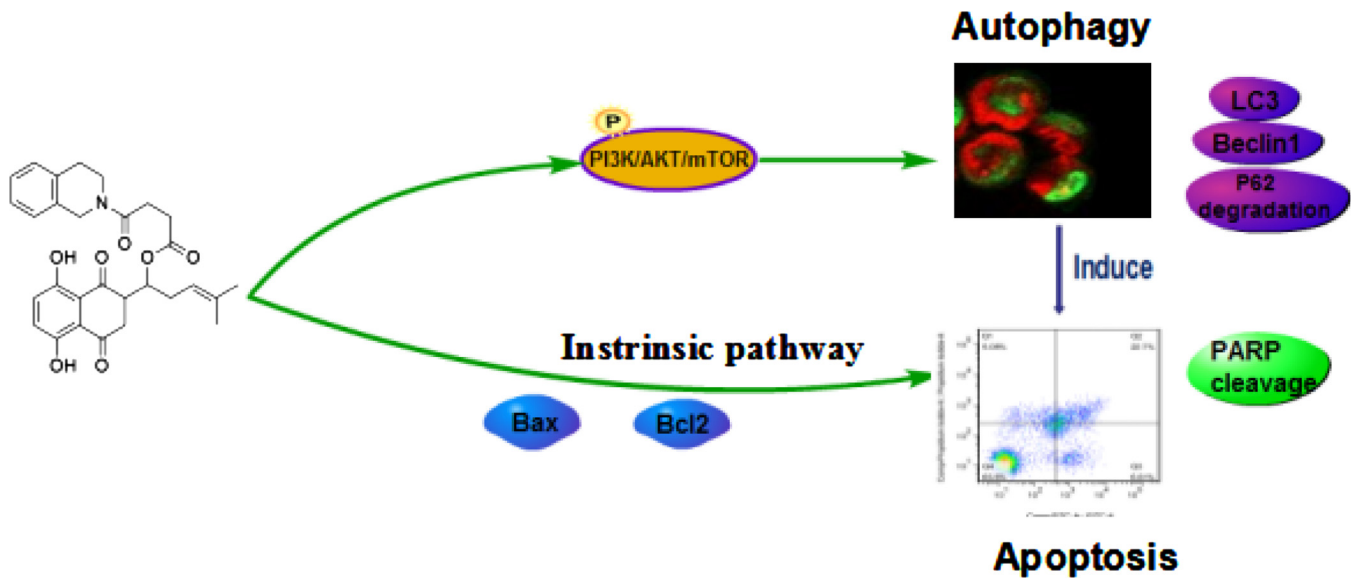



**Fig. 7.** Prelimilary mechanisms involved.

to the results (Fig. 4B, compared with the control, acidic autophagy lysosome was markedly increased after treatment with compound **4i** for 24 h at 1, 2, 4 μM.

A green fluorescent protein-conjugated form of LC3 (GFP-LC3) is another method for detecting autophagy. When autophagy occured, the GFP-LC3 fusion protein is translocated to the autophagosome membrane, and multiple bright green fluorescent spots are formed [41]. Form the Fig. 4C, obvious aggregation of LC3 puncta was observed after treat with compound **4i**, compared to non-treated cells.

### *3.6. The relationship between autophagy and apoptosis*

The functional relationship between autophagy and apoptosis is complex. It reported that autophagy facilitated apoptosis [42]. To explicate the function of autophagy in title compound **4i** induced apoptosis, 3-methyladenine (3-MA, an autophagic inhibitor) [43] was used. In Fig. 5A, apoptosis assay showed that the inhibition ratio of apoptosis had a decrease when autophagy was inhibited compared with the compound treatment alone. It suggested that autophagy induction may enhance the inhibitory effect of the title compound. Western blot was also used to further examine the conjecture (Fig. 5B). From the data, we found that the compound with 3-MA decreased the expressions of Bax, Bcl2 and cleaved PARP. In summary, our results from apoptosis assay and western blot analysis demonstrated that autophagy induced the SGC-7901 cells apoptosis [44,45].

### *3.7. Inhibition of the PI3K/AKT/mTOR pathway*

A growing body of research suggests that the PI3K/AKT/mTOR pathway inhibition contributes to the induction of autophagy. It can affect the survival of cell through autophagy and it was a significant target for cancer treatments [46–49]. To determine whether title compound **4i** influence the PI3K pathway, the expressions of representative proteins of PI3K/AKT/mTOR pathway were tested (Fig. 6A). In the present study, the expressions of PI3Kγ, AKT, mTOR made no difference with different concentration (compound **4i**) while p-PI3kγ, p-Akt, p-mTOR were decreased. Since autophagy inhibition decreased the percentage of apoptotic cells, the change in PI3K pathway was investigated in the presence of 3-MA (autophagy and PI3K inhibitor). As shown in Fig. 6B, Inhibition of p-PI3Kγ, p-AKT, p-mTOR were reversed when 3-MA (autophagy and PI3K inhibitor) blocked autophagy, suggesting that the PI3K pathway was negatively correlated with the autophagy induction by title compound (Fig. 7).

## 4. Conclusions

In summary, based on previous research and virtual screening, eleven dihydroxy-naphthalene-1,4-dione derivatives as potential anticancer agents were designed, synthesized, and investigated as autophagy regulators. The anticancer activity results indicated that one compound **4i** had selectivity on somatic cells over tumor cells and could promote apoptosis in gastric cancer cells. Further experiments using SGC-7901 cells showed autophagy played an important role in the pro-apoptotic effect of the compound **4i**. Furthermore, inhibition of PI3K/AKT/mTOR mediates the effect of the title compound **4i** on the induction of apoptosis in an autophagy-dependent manner. The results revealed the new anti-tumor mechanism for active naphthalene derivatives.

## Conflict of interest

No.

## Acknowledgments

The authors wish to thank the National Natural Science Foundation of China (No. 21572003, 21272008) and Scientific Research Fund of Anhui Medical University (No. 2017xkj055).

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.108747.

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