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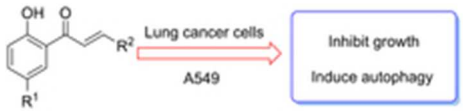

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ARTICLE

Discovery of 2'-hydroxychalcones as autophagy inducer in A549 lung cancer cells

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A series of 2'-hydroxychalcone derivatives was synthesized and the effects of all the compounds on growth of A549 lung cancer cell were investigated. The results showed that all compounds had inhibitory effects on the growth of A549 lung cancer cells and compound **2-7** possessed the highest growth inhibitory effect and induced autophagy of A549 lung cancer cells.

Introduction

Lung cancer is the leading cause of cancer-related death in the world at present. About 75% of all lung malignancies are non-small cell lung cancers (NSCLC). Despite advances in prevention and treatment, NSCLC is often diagnosed at an advanced stage and has a poor prognosis.

Current research suggests that standard chemotherapy and radiotherapy regimens have been optimized to maximal efficiency. Many studies have shown that small molecules can act as oncogenes or tumor suppressors and become a promising new treatment strategy.

Autophagy is an evolutionarily conserved pathway for degradation of cytoplasmic proteins and organelles via lysosome. Current research clearly shows that autophagy fulfills numerous functions in vital biological processes. Autophagy has become one of the most attractive topics in cancer research. Among the various biological functions of autophagy identified so far, the link between autophagy and cancer is often viewed as controversial, because of its apparently contradictory roles, such as survival and cell death.[1] Autophagy has dual roles in cancer, acting as both a tumor suppressor by preventing the accumulation of damaged proteins and organelles and as a mechanism of cell survival that can promote the growth of established tumors.[1-3] Autophagy can mediate cell death in a process known as autophagic cell death (ACD). ACD refers to cell death by autophagy (rather than cell death with autophagy) in which the final cell death process is mediated by autophagy rather than another cell death modality such as apoptosis or necroptosis.[4, 5] It is shown that some drugs, such as cisplatin, are able to induce autophagy in cancer cells and autophagy may be associated with drug resistance in tumors.[6] Furthermore, the effect of anticancer drugs on cancer cells may be increased by regulating the level of autophagy.[7-9] Current data suggest that depending on the context, either the induction or the inhibition of autophagy can provide therapeutic benefits to patients.[10]

Though the mounting knowledge about autophagy, the mechanisms through which the autophagic machinery regulates these diverse processes are not entirely understood.[11] Targeting autophagy in cancer provides new opportunities for drug development since more potent and specific inhibitors of autophagy are needed. The role of autophagy and its regulation in cancer cells continues to emerge and studies aim to define optimal strategies to modulate autophagy for therapeutic advantage.[12] Therefore, the design and synthesis of modulators of autophagy may provide novel therapeutic tools and may ultimately lead to new therapeutic strategies in cancer.

By so far, a number of anticancer compounds have been reported as autophagy inducers that target diverse signaling pathways in autophagy.[13-21] Autophagy is a highly complicated system sensing and responding to the status of different types of stress, which explains why there are multiple mechanisms of autophagy induction, and thus, abundant targets for drug discovery. The consequences of promoting autophagy in cancer cells are incompletely recognized and may largely depend on cellular context.[12] However, compared to other cancer types, autophagy inducers for treatment of lung cancer, esp. NSCLC, is relatively rare in reports.[22] And in most cases, those autophagy inducers in lung cancer produce a combination induction of apoptosis and autophagy.[23-27] Currently, aside from classic rapamycin and rapalogs,[28, 29] novel autophagy-specific compounds targeting for lung cancer therapy are in great need.

Chalcones, envisaged as open-chain flavonoids, show various biological activity, such as potent anti-inflammatory activity,[30, 31] anticancer activity,[32-39] lipid lowering agents that act by modulating lipoprotein lipase,[40] antiparasitic activity,[41] cathepsin B and L inhibitory activity,[42] cytoprotective activity[43] and anti-diabetic activity.[44] A few studies showed minor

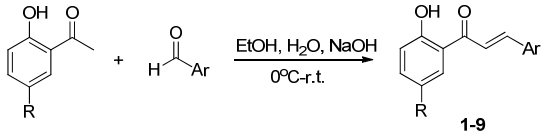
structural difference could lead to important difference in mechanistic effect for anticancer.[45]

In an ongoing study in our laboratory on discovery of modulators of apoptosis or autophagy as potential new anticancer agents,[46-55] herein, we have identified a series of chalcone derivatives with anticancer activity primarily by inducing autophagic cell death in A549 cells (an NSCLC cell line). Although a couple of studies have shown that chalcones could induce autophagy to some extent in different types of tumor,[24,25,45,56] the anticancer effects in NSCLC are rarely presented or not so significant as other cancer types.[25] Furthermore, the autophagy induced by these reported chalcones are largely dependent on the mechanisms overlapped and interrelated with apoptosis, accompanied by apoptosis or necroptosis as additional effects on tumor suppression. While our compounds **2-6**, specifically triggered autophagy without a concomitant remarkable apoptotic activity, indicating they might play a more prominent role in apoptosis-resistant cancers than those agents inducing both autophagy and apoptosis, since the anticancer effect only comes from ACD. Nevertheless, compound **7** inducing both autophagy and apoptosis in A549 cells, possessed the highest inhibitory effect. In this context, we speculated the robust autophagic induction by compound **7** was prior to and facilitated the consequent activation of apoptosis and thus caused much tumor cell death. Importantly, this series of chalcone compounds showed inhibitory effect on tumor cells equivalent or even greater than anticancer chalcones ever reported.

Results and discussion

Synthesis of compounds 1-9

The synthetic route of the proposed compound **1-9** is shown in Scheme 1. Starting materials 2-hydroxyacetophenone was prepared according to literatures.[57] The 2'-hydroxychalcone derivatives **1-9** were obtained by the reaction of 2-hydroxyacetophenone with substituted benzaldehyde under stirring at room temperature in 41.8-93.2% yields. The structures of compounds (**1-9**) were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS spectral data. In the ^{1}H NMR spectra of the compounds, the CH protons of the olefin resonated as a doublet at δ 7.40-7.67 ppm and 7.84-7.96 ppm, respectively. The hydroxy proton signal appeared at the range of δ = 12.63-12.93 ppm as a single peak.



1: R = Cl; Ar = Ph; **2**: R = Cl; Ar = 3,4-$(OCH_2O)C_6H_3$; **3**: R = Cl; Ar = 4-$MeOC_6H_4$;
4: R = Me; Ar = Ph; **5**: R = Me; Ar = 3,4-$(OCH_2O)C_6H_3$; **6**: R = Me; Ar = 4-$MeOC_6H_4$;
7: R = H; Ar = Ph; **8**: R = H; Ar = 3,4-$(OCH_2O)C_6H_3$; **9**: R = H; Ar = 4-$MeOC_6H_4$;

Scheme 1 The synthesis of compounds.

Inhibitory effects of the compounds on the proliferation of A549 cells

To define the biological effects of compounds **1-9** on A549 cells, we first observed the morphological changes of the cells treated with the compounds under a phase contrast microscope. Exposure of A549 cells to compounds **2-7** at 40 μM for 24 or 48 h resulted in a distinct decrease of cell viability as well as induced the changes of A549 cell morphology. Vacuolization and shrinkage of cells were observed, and the morphological changes were even more significant at 48 h for these cells (Fig. 1). In order to evaluate the inhibitory effects of these compounds on the proliferation of A549 cells we carried out the SRB assay with compounds **1-9** at 48 h. The data showed that a decrease of cell viability was observed with most compounds in a dose-dependent manner (Fig. 2). Among them, compound **7** showed a most potent inhibitory effect, according to the IC_{50}

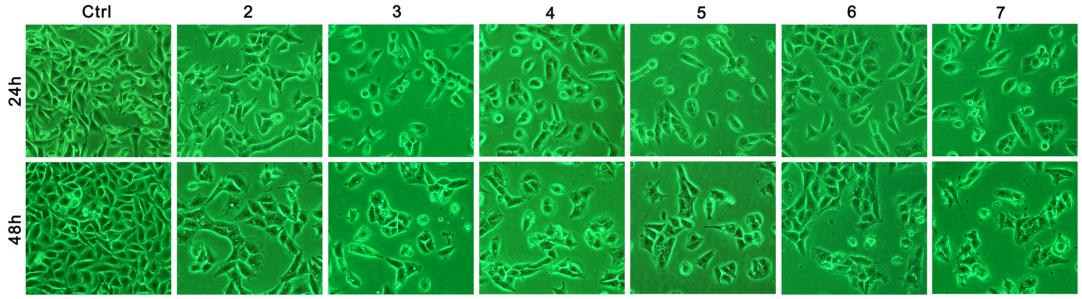


Fig 1. Effects of the compounds on A549 cell morphology at 24 or 48 h. A549 cells were treated with compounds **2-7** (40 μM) or DMSO 0.1% (v/v) as a vehicle control. Microscopic photographs (200×) were taken under an inverted phase contrast microscope (Nikon).

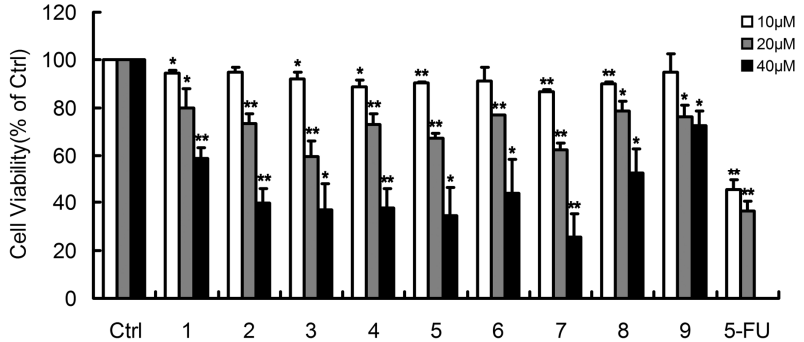


Fig 2. Effects of compounds **1-9** on A549 cell viability. A549 cells were treated with compounds **1-9** at the concentration of 10, 20 or 40 μM or treated with DMSO 0.1% (v/v) (Control) for 48 h. Cell viability was analyzed by SRB assay and illustrated in the labelled column. Results are presented as mean ± SE; (n =3, *p < 0.05 vs control; **p < 0.01 vs control).

values (Table 1). From IC_{50} data, we found that the cytotoxic potency was highly dependent on the substituents on the two aryl rings. Comparing compounds with same Ar and R = Cl, Me, H, respectively, the IC_{50} of compounds **1**, **4**, and **7** reduce in sequence; in contrast, the IC_{50} of compounds **3**, **6**, and **9** increase in sequence. However, for compounds **2**, **5** and **8**, the IC_{50} has no such sequence. Comparing compounds with same R and different Ar, the IC_{50} of compounds **1**, **2**, and **3** reduce in sequence; IC_{50} of compounds **4**, **5**, and **6** are almost no changes, but the IC_{50} of compounds **7**, **8** and **9** interestingly increase in sequence.

Table 1 IC_{50} of the compounds **1-9** and 5-FU for A549 cells at 48 h.

Compounds	1	2	3	4	5
IC_{50}(μM)	42.31±4.63	32.56±4.09	28.50±6.78	31.56±5.13	28.09±4.53
Compounds	6	7	8	9	5-FU
IC_{50}(μM)	35.93±6.80	24.58±4.47	44.03±5.06	58.12±6.01	7.27±4.08

Apoptosis and necrosis assays of compound treated A549 cells

Apoptosis (programmed cell death-type I) and necrosis are well-known mechanisms of cell death induced by anticancer therapies. The apoptotic process involves a sequence of cell shrinkage, increased cytoplasmic density, chromatin condensation, and segregation into sharply circumscribed masses abut the nuclear membrane which will produce apoptotic bodies. DNA staining with certain dyes, such as Hoechst 33258, allows the direct observation of the condensation of the cell nucleus. After exposed to compounds **2-7** at 20, 40 μM for 48 h, A549 cells were stained by Hoechst 33258 to detect condensation of nucleus that appeared as fluorescent blue. The results suggested that the compounds induce apoptosis slightly in A549 at both concentrations, except for compound **7**, indicating that apoptosis seems hardly to be a determinant factor for inhibiting growth of cells (Fig. 3). However, compound **7** significantly triggered apoptosis in A549 cells at 48 h, and the apoptosis rate reached 25%. To detect whether the compounds cause necrosis in A549 cells, we measured the LDH activity in cell culture medium. As shown in Fig. 4, there was no significant difference ($p>0.05$) in LDH release between the control group and the cells treated with compounds at the test range of concentration. To conclude, the inhibitory effects of cell viability were not due to apoptosis or necrosis in A549 cells.

Compounds induce autophagic cell death in A549 cells

Recently increasing evidence has demonstrated that autophagic cell death can be induced as an alternative cell-death mechanism when cells fail to undergo apoptosis. We therefore

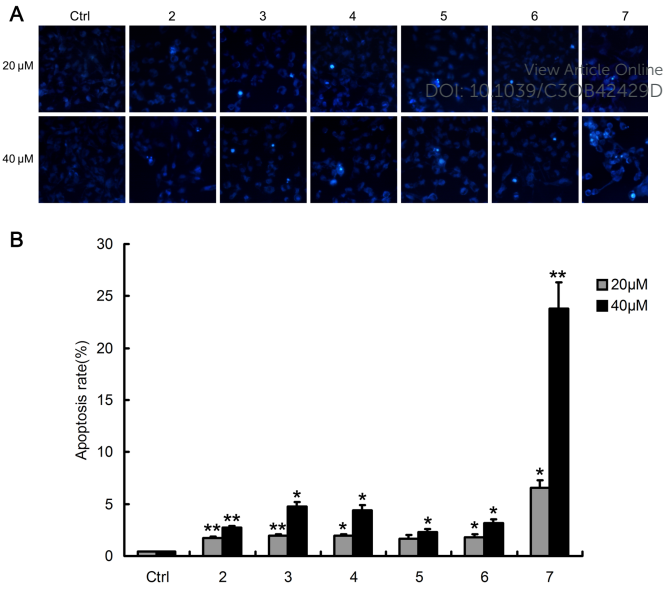


Fig 3. Apoptosis of A549 cells treated with compounds **2-7**. (**A**) fluorescent image of A549 cells stained by Hoechst 33258. (**B**) Fluorescence quantitative statistics for apoptotic cells. Fluorescent micrographs of Hoechst 33258 staining (200×) were taken under a fluorescent microscope (Nikon). (n =3, *p < 0.05 vs control; **p < 0.01 vs control).

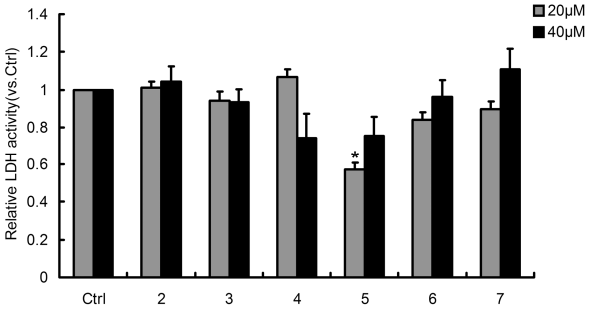


Fig 4. Effects of compounds **2-7** on LDH activities of A549 cells. The culture medium was collected as samples for LDH assay after 24 and 48 h treatment at the concentration of 20 or 40 μM. (n =3, *p < 0.05 vs control; **p < 0.01 vs control).

detected the autophagy activity in cells treated with compounds.

Vacuolization in morphological change has suggested a sign of autophagy activity. Since autophagy is a lysosomal degradation pathway for the breakdown of intracellular proteins and organelles, formation of acidic vesicles can be a viable marker for autophagy. We first performed aridine orange (AO) staining in A549 cells after treatment with compounds at 20 or 40 μM for 48 h. According to acidic compartments stained as fluorescent bright red, the cells showed elevated autophagic

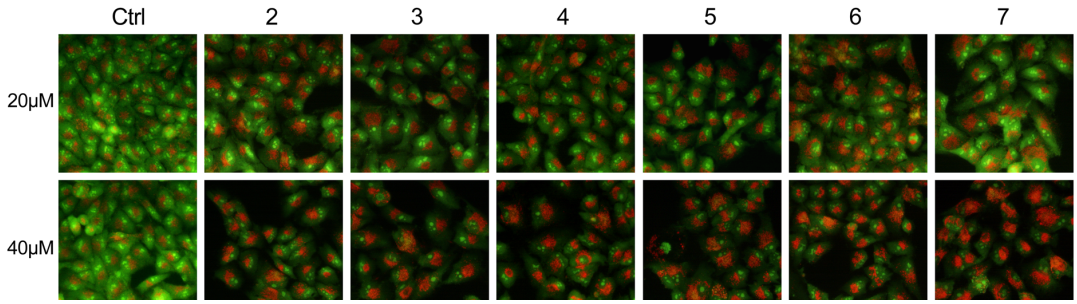


Fig 5. Compounds **2-7** increased the acidic vesicle level in the cells at 48 h. Microscopic photographs (400×) were taken under a fluorescent microscope (Nikon).

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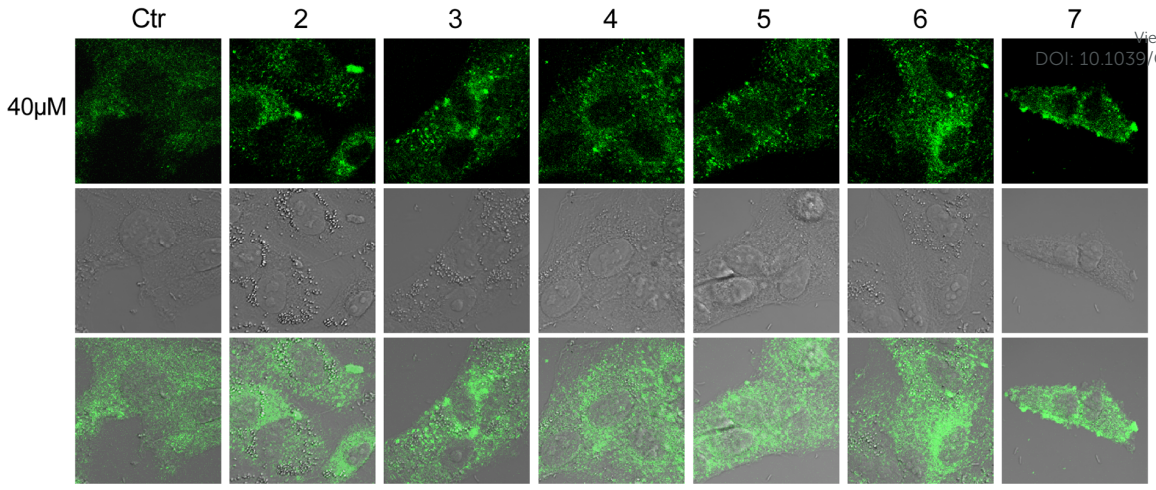


Fig 6. Compounds **2-7** increased punctate LC3-II in A549 cells at 48 h. Immunohistochemistry image was taken under a Confocal Laser scanning microscopy (Zeiss).

activity after the treatment compared with the control group (Fig. 5). Nevertheless, the examination of autophagy should be conducted by multiple methods because autophagosome accumulation may not represent fully functional autophagy. It has been widely accepted that LC3-II protein correlates with the induction of autophagy and both the ratio of LC3-II to LC3-I and the amount of LC3-II can be used to monitor autophagosome formation. Immunofluorescence of LC3-II is useful for *in vitro* and *in vivo* studies, and punctate LC3-II can be faithful evidence for autophagy. After treatment at 40 μM compounds for 48 h, we used anti-LC3-II antibodies for immunohistochemistry experiment and observed apparent increase in punctate LC3-II, comparing to control group (Fig. 6). We further examined the levels of LC3-II by immunoblot and the data showed both the enhancement of conversion of LC3-I to LC3-II and upregulation of LC3 levels (Fig. 7). All above data are consistent to demonstrate that the compounds can induce autophagic cell death in A549 cells and the inhibition of growth was mainly attributed to the autophagy induction.

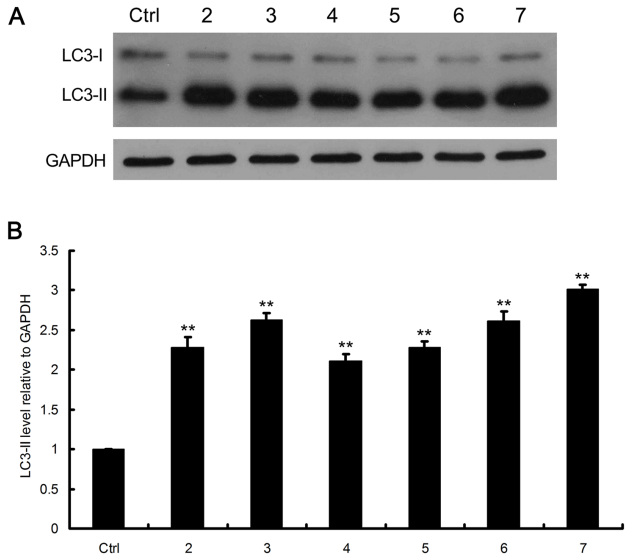


Fig 7. Western blot analysis of the protein level of LC3-II and GAPDH as a normalization control (**A**) and quantitative statistics (**B**). A549 cells were treated with compounds **2-7** at 40 μM for 48 h. (n =3, *p < 0.05 vs control; **p < 0.01 vs control).

Our results suggested that the compounds **2-7** induced excessive autophagy activity in A549 cells and led to autophagic cell death, which is another considerable mechanism used as a therapeutic strategy to treat cancer. Induction of autophagic cell death may be an ideal approach in those cancers that are resistant to apoptosis by anticancer therapies (e.g., chemotherapy, radiation). Moreover, those compounds also offered useful tools for studying the molecular mechanisms underlying the relation between autophagy and cancer or apoptosis. The cross-talking between autophagy and apoptosis is complex and confusing, since they are linked by effector proteins (e.g., Bcl-2, Bcl-XL, ATG5, p53) and share common pathways (e.g., PI3K/Akt/mTOR, NFκB, ERK). It's noteworthy that compound **7** performed the most significant inhibitory effects on A549 cells through combination of apoptosis and autophagy, even if other compounds had low level of activation for apoptosis. In this context, we speculated that the induction of apoptosis by compound **7** was probably triggered by autophagic activity, rather than the reverse. Even though many studies have suggested the concurrence of the two cell events under their overlapped mechanisms, there are also several examples in which the induction of autophagy facilitates the activation of apoptosis.[58, 59]

Experimental

General

Thin-layer chromatography (TLC) was conducted on silica gel 60 F_{254} plates (Merck KGaA). ^{1}H NMR spectra were recorded on a Bruker Avance 300 (300 MHz) spectrometer, and ^{13}C NMR spectra were recorded on a Bruker Avance 400 (100 MHz) spectrometer, using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. HRMS spectra were recorded on a Q-TOF6510 spectrograph (Agilent). Melting points were determined on an XD-4 digital micro melting point apparatus.

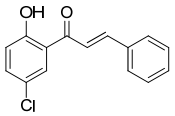
General procedure for the synthesis of compounds 1-9

To a stirred solution of substituted 2-hydroxyacetophenone (1.0 mmol) in ethanol (15 mL) was added 40% sodium hydroxide solution (2.0 mL) in an ice-salt bath. After stirred 30 min, 2-

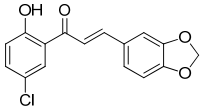
hydrazinylpyridine (1.2 mmol) was added and the reaction mixture was stirred for 12 h at room temperature as shown in Scheme 1. The progress of the reaction was monitored by TLC. After completion, the reaction mixture was neutralized by hydrochloric acid. The precipitates were filtered, washed with water, and consequently recrystallized from ethanol to afford **1-9.**

The spectroscopic data of compounds 1-9

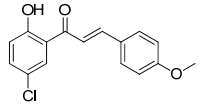
(E)-1-(5-chloro-2-hydroxyphenyl)-3-phenylprop-2-en-1-one (**1**) Yellow solid; yield: 87.9%; mp: 103-106 ◦C [lit. 110-112 ◦C][60]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.99 (d, 1H, J = 9.0 Hz, Ar-H), 7.43-7.49 (m, 4H, Ar-H), 7.63 (d, 1H, J = 15.6 Hz, -CH=CH-), 7.69-7.73 (m, 2H, Ar-H), 7.88 (d, 1H, J = 1.5 Hz, Ar-H), 7.96 (d, 1H, J = 15.6 Hz, -CH=CH-), 12.72 (s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 119.37, 120.26, 120.58, 123.54, 128.82, 128.88, 129.12, 131.30, 134.30, 136.20, 146.52, 162,08; HRMS: calcd for $[M+H]^+$ $C_{15}H_{12}ClO_2$: 259.0526; found: 259.0528.



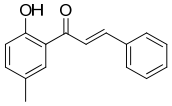
(E)-3-(benzo[d][1,3]dioxol-5-yl)-1-(5-chloro-2-hydroxyphenyl) prop-2-en-1-one (**2**). Yellow solid, yield 93.2%; mp 152-154 ◦C [lit. 145-146 ◦C][61]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.06 (s, 2H CH_2), 6.88 (d, 1H, J = 7.8 Hz, Ar-H), 6.98 (d, 1H, J = 8.7 Hz, Ar-H), 7.17-7.21 (m, 2H, Ar-H), 7.40 (d, 1H, J = 15.6 Hz, -CH=CH-), 7.41-7.45 (m, 1H, Ar-H) 7.85 (s, 1H Ar-H), 7.87 (d, 1H, J = 15.6 Hz, -CH=CH-), 12.80 (s, 1H, OH) ; ^{13}C NMR (100 MHz, $CDCl_3$): δ 101.88, 106.84, 108.82, 117.19, 120.21, 120.64, 123.46, 126.18, 128.69, 128.78, 136.01, 146.40, 148.58, 150.64, 162.02, 192.55; HRMS: calcd for $[M+H]^+$ $C_{16}H_{12}ClO_4$: 303.0424; found: 303.0419.



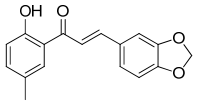
(E)-1-(5-chloro-2-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**3**). Yellow solid, yield 84.8%; mp 112-114 ◦C [lit. 118 ◦C][62]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.88 (s, 3H, OCH_3), δ 6.94-6.99 (m, 3H Ar-H), 7.43 (dd, 1H, J = 2.7, 8.7 Hz, Ar-H), 7.45 (d, 1H, J = 15.3 Hz, -CH=CH-), 7.65 (dd, 2H, J = 2.7, 11.4 Hz, Ar-H), 7.86 (d, 1H J = 2.4 Hz, Ar-H), 7.93 (d, 1H, J = 15.3 Hz, -CH=CH-), 12.85 (s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.48, 114.58, 116.75, 120.16, 120.70, 123.39, 127.04, 128.70, 130.83, 135.89, 146.40, 162.02, 162.31, 192.62; HRMS: calcd for $[M+H]^+$ $C_{16}H_{14}ClO_3$: 289.0631; found: 289.0628.



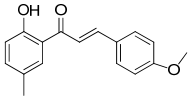
(E)-1-(2-hydroxy-5-methylphenyl)-3-phenylprop-2-en-1-one (**4**) Pale yellow solid, yield 41.8%; mp 117-119 ◦C [lit. 105-106 ◦C][63]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.36 (s, 3H CH_3), 6.95 (d, 1H, J = 8.7 Hz Ar-H), 6.88 (dd, 1H, J = 1.8, 8.4 Hz, Ar-H), 7.42-7.46 (m, 3H, Ar-H), 7.67 (d, 1H, J = 15.3 Hz, -CH=CH-), 7.66-7.68 (m, 3H, Ar-H), 7.92 (d, 1H, J = 15.3 Hz, -CH=CH-), 12.63 (s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.65, 118.36, 119.68, 120.16, 127.95, 128.71, 129.04, 129.40, 130.90, 134.65, 137.54, 145.23, 161.58, 193.59; HRMS: calcd for $[M+H]^+$ $C_{16}H_{15}O_2$: 239.1072; found: 239.1067.



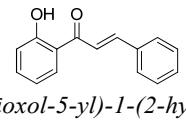
(E)-3-(benzo[d][1,3]dioxol-5-yl)-1-(2-hydroxy-5-methylphenyl) prop-2-en-1-one (**5**). Pale yellow solid, yield 86.2%; mp 115-118◦C [Lit. 111-112 ◦C][64]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.36 (s, 3H CH_3), 6.05 (s, 2H CH_2), 6.84 (d, 1H, J = 9.3 Hz, Ar-H), 6.93 (d, 1H, J = 8.4 Hz, Ar-H), 7.17 (dd, 1H, J = 1.5, 8.1 Hz, Ar-H), 7.21 (d, 1H, J = 1.5 Hz Ar-H), 7.31 (dd, 1H, J = 1.8, 8.4 Hz, Ar-H), 7.48 (d, 1H, J = 15.3 Hz, -CH=CH-), 7.69 (dd, 1H, J = 1.5, 9.9 Hz, Ar-H), 7.84 (d, 1H, J = 15.3 Hz, -CH=CH-), 12.70 (s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.60, 101.76, 106.77, 108.68, 118.04, 118.28, 119.72, 125.64, 127.79, 129.12, 129.22, 137.25, 145.01, 148.47, 150.22, 161.50, 193.34; HRMS: calcd for $[M+H]^+$ $C_{17}H_{15}O_4$: 283.0970; found: 283.0961.



(E)-1-(2-hydroxy-5-methylphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**6**). Pale yellow solid, yield 59.4%; mp 99-102◦C [lit. 94-96 ◦C][65]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.36 (s, 3H CH_3), 3.87 (s, 3H OCH_3), 6.92-6.97 (m, 3H, Ar-H), 7.30 (dd, 1H, J = 1.8, 8.4 Hz, Ar-H), 7.55 (d, 1H, J = 15.6 Hz, -CH=CH-), 7.63-7.69 (m, 3H, Ar-H), 7.89 (d, 1H, J = 15.6 Hz, -CH=CH-), 12.73 (s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.63, 55.40, 114.50, 117.66, 118.28, 119.78, 126.58, 127.71, 129.29, 130.56, 137.21, 145.10, 161.52, 161.99, 193.52; HRMS: calcd for $[M+H]^+$ $C_{17}H_{17}O_3$: 269.1178; found: 269.1178.

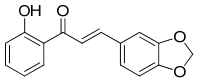


(E)-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one (**7**). Pale yellow solid, yield 55.7%; mp 94-96 ◦C [lit. 97.1-97.8 ◦C][66]; ^{1}H NMR (300 MHz, $CDCl_3$): 6.95 (t, 1H, J = 7.5 Hz, Ar-H), 6.98 (dd, 1H, J = 0.9, 8.4 Hz, Ar-H), 7.42-7.45 (m, 3H, Ar-H), 7.51 (t, 1H, J = 7.2 Hz, Ar-H), 7.67 (d, 1H, J = 15.6 Hz, -CH=CH-), 7.66-7.68 (m, 2H, Ar-H) 7.93 (dd, 1H J = 1.5, 8.1 Hz, Ar-H), 7.94 (d, 1H, J = 15.6 Hz, -CH=CH-), 12.81 (s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 118.65, 118.91, 120.03, 120.07, 128.72, 129.08, 129.72, 130.99, 134.58, 136.46, 145.49, 163.64, 193.74; HRMS: calcd for $[M+H]^+$ $C_{15}H_{13}O_2$: 225.0916; found: 225.0914.

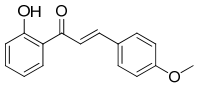


(E)-3-(benzo[d][1,3]dioxol-5-yl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**8**). Pale yellow solid, yield 64.9%; mp 143-145 ◦C [Lit. 137-138 ◦C][64]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.05 (s, 2H CH_2), 6.86 (d, 1H, J = 8.1 Hz, Ar-H), 6.94 (t, 1H, J = 7.5 Hz,

Ar-H), 7.02 (dd, 1H, *J* = 0.9, 8.4 Hz, Ar-H), 7.15-7.19 (m, 2H, Ar-H), 7.48 (d, 1H, *J* = 15.3 Hz, -CH=CH-), 7.49-7.50 (m, 1H, Ar-H), 7.85 (d, 1H, *J* = 15.3 Hz, -CH=CH-), 7.91 (dd, 1H, *J* = 1.5, 8.1 Hz,Ar-H), 12.89 (s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 101.80, 106.71, 108.74, 117.86, 118.58, 118.82, 120.04, 125.82, 129.01, 129.54, 136.24, 145.32, 148.49, 150.32, 163.56, 193.49; HRMS: calcd for $[M+H]^+$ $C_{16}H_{13}O_4$: 269.0814; found: 269.0818.



(E)-1-(2-hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**9**). Pale yellow solid, yield 77.0%; mp 93-95 ◦C [lit. 95-96◦C][67]; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.87 (s, 3H, OCH_3), 6.91-6.97 (m, 3H, Ar-H), 7.02 (dd, 1H, *J* = 0.9, 8.4 Hz, Ar-H), 7.49 (t, 1H, *J* = 7.8 Hz, Ar-H), 7.54 (d, 1H, *J* = 15.3 Hz, -CH=CH-), 7.64 (dd, 2H, *J* = 1.8, 6.9 Hz, Ar-H), 7.91-7.94 (m, 1H Ar-H), 7.91 (d, 1H, *J* = 15.3 Hz, -CH=CH-), 12.93 (s, 1H, OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.45, 114.53, 117.52, 118.58, 118.80, 120.12, 127.31, 129.58, 130.59, 136.17, 145.38, 162.05, 163.57, 193.66; HRMS: calcd for $[M+H]^+$ $C_{16}H_{15}O_3$: 255.1021; found: 255.1018.



Cell culture

A549 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% calf bovine serum (HyClone), at 37 °C in a humidified incubator with 5% CO_2. For the experimental study, cells were grown to 80−90% confluence, harvested with 0.05% trypsin (Sangon Biotech) in phosphate-buffered saline (PBS), plated at the desired cell concentration. All the cells were seeded at a density of 6250/cm^2 into plates or appropriate dishes 24 h before adding the compound.

Cell viability assay

Cells were seeded in 96-well plates. After 24 h, cells were treated with 0.1% DMSO (as control) or the compounds at indicated concentrations for specified time durations. Cell viability was evaluated by sulforhodamine B (SRB) assay. Briefly, pour off the medium and fix cells by adding 100 μL of cold 10% trichloroacetic acid (TCA) and incubate for 1 h at 4□. Discard the supernatant and then wash the plates five times with deionized water. Add 50μL of 0.4% (W/V) SRB solution in 1% acetic acid to each well and shake 5 min on titer plate shaker. Wash the plates five times with 1% acetic acid and subsequently add 100 μL of 10 mM unbuffered Tris base (pH 10.5) to dissolve the bound dye. Mix 5 min on a microtiter plate shaker and read optical densities at the wavelength of 540 nm using Infinite 200 pro multimode Plate reader (Tecan).

Hoechst 33258 staining

The living A549 cells were stained with 10 mg/mL of Hoechst 33258 in the medium for 15 min at 37□. Subsequently, the cells were gently washed twice with PBS, and were then observed under a fluorescence microscope (Nikon). The condensed DNA of apoptotic cells was identified by intense local staining in the nucleus, in contrast to diffused staining of DNA in normal cells. A minimum of 500 cells were counted, and each experiment was performed in triplicate.

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LDH assay

Cell culture medium was collected after 48 h treatment with 0.1% DMSO (as control) or the compound. LDH assay was performed using Lactate Dehydrogenase (LDH) kit (Nanjing Jiancheng Co., China) according to the manufacturer's instructions.

Acridine orange (AO) staining

Discard the medium after treatment and gently rinse the cells with PBS. The cells were stained with acridine orange dye (0.1 mg/mL) for 1 min. Subsequently, the cells were gently washed twice with PBS, and were then observed under a fluorescence microscope (Nikon). In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green, whereas acidic compartments fluoresce bright red.

Immunohistochemistry assay for LC3-II

After treatment, A549 cells were washed three times with 0.1 M PBS. Following fixation (15 min in 4% paraformaldehyde), cells were washed three times in 0.1 M PBS and blocked with sheep serum (1:30 dilution) in 0.1 M PBS for 20 min at room temperature. Then cells were incubated with rabbit anti-human LC3b antibody (1:100 dilution) in a humid chamber overnight at 4□. Then cells were washed three times with 0.1 M PBS and incubated for 30 min at 37□ with the secondary antibody, FITC-conjugated goat anti-rabbit IgG (1:200 dilution). Finally cells were washed three times with 0.1 M PBS, and were observed by a confocal laser scanning microscope (Zeiss, Germany).

Western blot analysis

Cells were washed twice with ice cold PBS and were then lysed in protein lysis buffer (0.5% SDS in 25 mM Tris–HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 μg/mL leupeptin and 10 μg/mL soybean trypsin inhibitor). The protein concentration of the cells was determined by the Bradford method. After adding loading buffer and boiling, equal amount of protein was loaded onto a 15% SDS polyacrylamide gel. After electrophoresis, the resolved protein was electrophoretically transferred to a Polyvinylidene Fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked with 5% non-fat milk in PBST (PBS containing 0.05% Tween 20) for 1 h at room temperature. Subsequently the membrane was probed with Rabbit anti-human LC3b antibody (1:1000), or anti-GAPDH mouse monoclonal antibody (1:1000) overnight at 4□, and was then washed twice with PBST, each time for 5 min. The membrane was subsequently incubated with HRP-conjugated goat anti-rabbit IgG (1:5000) or polyclonal goat anti-mouse immunoglobulins/HRP (1:5000) for 1 h at room temperature and then washed three times with PBST. Then the membrane was incubated with HRP substrate for 5 min and the fluorescence signal were detected with X-ray films. Intensity of the protein bands was quantified using Quantity-One software (Bio-Rad).

Statistical analysis

Data were presented as means ± SE from at least three independent experiments and analyzed by Student's t-test. Differences at $p<0.05$ were considered statistically significant.

Conclusions

A series of chalcone derivatives have been synthesized and the effects of all the compounds on growth of A549 lung cancer cell were investigated. All compounds had inhibitory effects on the growth of A549 lung cancer cells and compound **2-7** possessed the highest growth inhibitory effect and induced autophagy of A549 lung cancer cells. Moreover, compound **7** performed the most significant inhibitory effects on A549 cells through combination of apoptosis and autophagy.

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