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Synthesis of novel oxime-containing pyrazole derivatives and discovery of regulators for apoptosis and autophagy in A549 lung cancer cells

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ABSTRACT

A series of novel oxime-containing pyrazole derivatives were synthesized by the reaction of ethyl 3-phenyl-1*H*-pyrazole-5-carboxylate derivatives and 2-bromo-1-phenylethanone followed by the reaction with hydroxylamine hydrochloride. The structures were determined by IR, ^{1}H NMR, HRMS, and X-ray analysis. A dose- and time-dependent inhibition of proliferation was observed in A549 lung cancer cell after compound treatment. Inhibition of growth was mainly attributed to the autophagy induction.

Cancer is a major public health problem in the world and lung cancer is the leading cause of death from malignancies worldwide in both men and women. Chemotherapy is still one of the primary modalities for the treatment of cancer. However, the use of available chemotherapeutics is often limited mainly due to undesirable side effects and a limited choice of available anticancer drugs. This clearly underlies the urgent need of developing novel chemotherapeutic agents with more potent antitumor activities.

Apoptosis and autophagy have been proposed to be involved in diverse human diseases including cancer. The regulation of autophagy in cancer cells is complex since it can enhance tumor cell survival in response to certain stresses, yet it can also act to suppress the initiation of tumor growth. Several drugs that are active on autophagy signaling pathways are already used for the treatment of cancer or are under preclinical development. It should, however, be noted that in most cases it is still unclear whether the modulation of autophagy by these compounds has a positive or a negative effect for the treatment. Understanding the signaling pathways involved in the regulation of autophagy as well as the autophagy process itself represents new directions in the development of anticancer therapies.[1–3]

Many pyrazole derivatives are well acknowledged to possess a wide range of anticancer bioactivities.[4–6] Although the skeleton of pyrazole plays an important role in biological effects, the type of peripheral substituent is also crucial. Thus, pyrazole derivatives containing oxime have also attracted considerable attention.[7–12] Such modifications may be of interest in the development of new antitumor compounds with improved characteristics.

In our effort to discover and develop apoptosis or autophagy regulator as potential new anticancer agents, we synthesized a series of novel pyrazole derivatives and the evaluation of biological activity showed that these compounds can inhibit A549 lung cancer cell growth by inducing apoptosis or autophagy.[13–23] The findings encouraged further us to modify the structure of pyrazole derivatives and identify the interaction mechanism of small molecules with targets. Herein we would like to report the synthesis and single-crystal characterization of ethyl 3-aryl-1-(2-(hydroxyimino)-2-arylethyl)-1*H*-pyrazole-5-carboxylate derivatives as well as their biological activities in suppressing the growth of A549 lung cancer cells by inducing apoptosis or autophagy.

Chemistry: The synthesis of novel ethyl 1-(2-oxo-2-phenylethyl)-3-phenyl-1*H*-pyrazole-5-carboxylate derivatives (**3**) has been performed as shown in Scheme 1 from 2-bromo-1-phenylethanone (**2**) and ethyl 3-phenyl-1*H*-pyrazole-5-carboxylate derivatives (**1**) that can be synthesized as described in our previous paper.[23] For example, compound **3a** was synthesized by the reaction of ethyl

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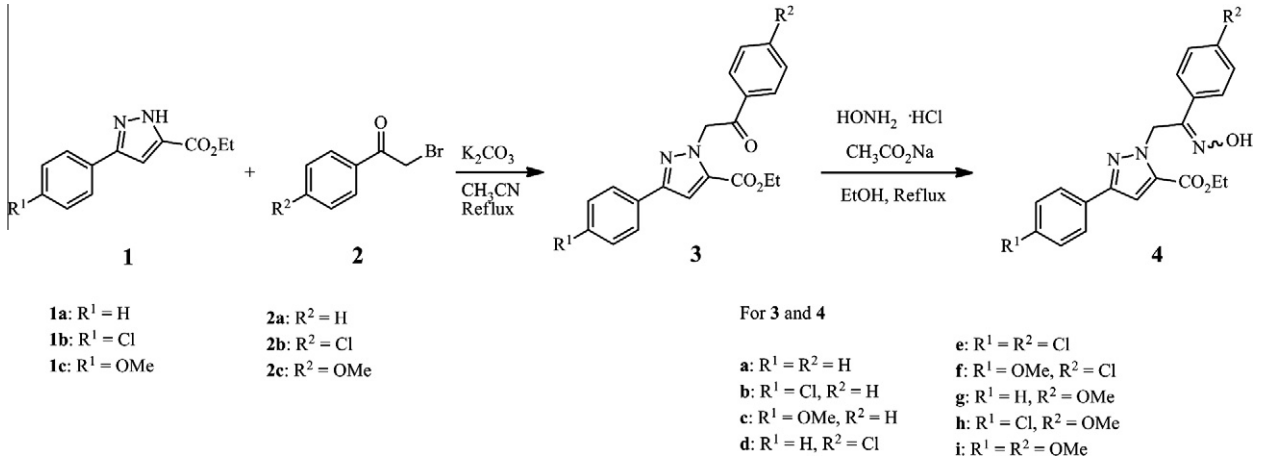

Scheme 1. Synthesis of ethyl 1-(2-oxo-2-phenylethyl)-3-phenyl-1*H*-pyrazole-5-carboxylate and ethyl 1-(2-(hydroxyimino)-2-phenylethyl)-3-phenyl-1*H*-pyrazole-5-carboxylate derivatives.

3-phenyl-1*H*-pyrazole-5-carboxylate (**1a**) with 2-bromo-1-phenylethanone (**2a**) in the presence of potassium carbonate in acetonitrile over a 2 h reflux period. After flash chromatography on silica gel, ethyl 1-(2-oxo-2-phenylethyl)-3-phenyl-1*H*-pyrazole-5-carboxylate (**3a**) was obtained in 71% yield. Compound **3** reacted with hydroxylamine hydrochloride in the mixture of ethanol and sodium acetate anhydrous over a 2–4 h reflux period to afford ethyl 3-aryl-1-(2-(hydroxyimino)-2-arylethyl)-1*H*-pyrazole-5-carboxylate derivatives (**4**) in 85–99% yields.

The structures of ethyl 1-(2-oxo-2-arylethyl)-3-aryl-1*H*-pyrazole-5-carboxylate derivatives (**3**) and ethyl 3-aryl-1-(2-(hydroxyimino)-2-arylethyl)-1*H*-pyrazole-5-carboxylate derivatives (**4**) were determined by IR, ^{1}H NMR, and HRMS spectroscopy. For example, the compound **3a** showed bands at 1711 and 1271 cm^{-1} arising from C=O stretching and asymmetric C–O–C stretching vibrations in IR spectrum, respectively. In ^{1}H NMR spectra, signal of two *ortho*-aromatic protons in 2-oxo-2-phenylethyl moiety appeared at the range of δ = 8.00 and 8.02 ppm (J = 7.4 Hz). While one triplet signal of the *para*-aromatic protons upfielded and resonated at the range of δ = 7.62 and 7.65 ppm (J = 7.4 Hz). Two protons of benzene bonded to pyrazole ring appeared as doublet peaks at rang of δ = 7.81 and 7.83 ppm (J = 7.4 Hz). In the same benzene moiety, signals of other two protons appeared at rang of δ = 7.39, 7.41, and 7.43 ppm (J = 7.4 Hz) as triplet peaks. Singlet signal of pyrazole proton appeared at δ = 7.25 ppm. In the HRMS, in accordance with the molecular structure of $C_{20}H_{18}N_2O_3$, compound **3a** gave a [M+H]-ion peak at m/z 335.1378. In the case of compound **4**, for example, in the HRMS, compound **4a** gave a [M+H]-ion peak at m/z 350.1516 (calcd 350.1505) in accordance with the molecular structure of $C_{20}H_{19}N_3O_3$. It is well known that *Z*/*E* isomerism is obvious while synthesizing the oximes from unsymmetrical ketones.[24–26] In the present study, indeed, the mixtures of *Z*/*E* isomers were achieved in all cases, and the ratios of *Z*/*E* were around 2.6:1–4.1:1 according to ^{1}H NMR spectroscopy. The signals of methylene bonded to pyrazole moiety in *Z* isomer were deshielded and the deshielding magnitude was about 0.29 ppm comparing with *E* isomer. The difference may be attributed to the interaction of N–O and C–H of methylene. More interestingly, a purified *Z* isomer determined by X-ray, such as compound **4d**, was converted to a mixture of *Z*/*E* isomers (*Z*/*E* = 13.2:1) in deuteriochloroform due to equilibrium of isomerization at room temperature.

Crystallography: Suitable crystals of **4c** and **4d** for X-ray diffraction were grown by slow crystallization from ethanol. Novel compounds of **4c** and **4d** crystallized in the same space group, $P2_1/c$. The two molecular structures with the atom-numbering scheme are shown in Figure 1, and important hydrogen bonds are given in Table 1. Both **4c** and **4d** consist of three ring systems and an oxime frame in the molecular structures. In the two compounds, the carbonyl groups occupy an almost planar position in relation

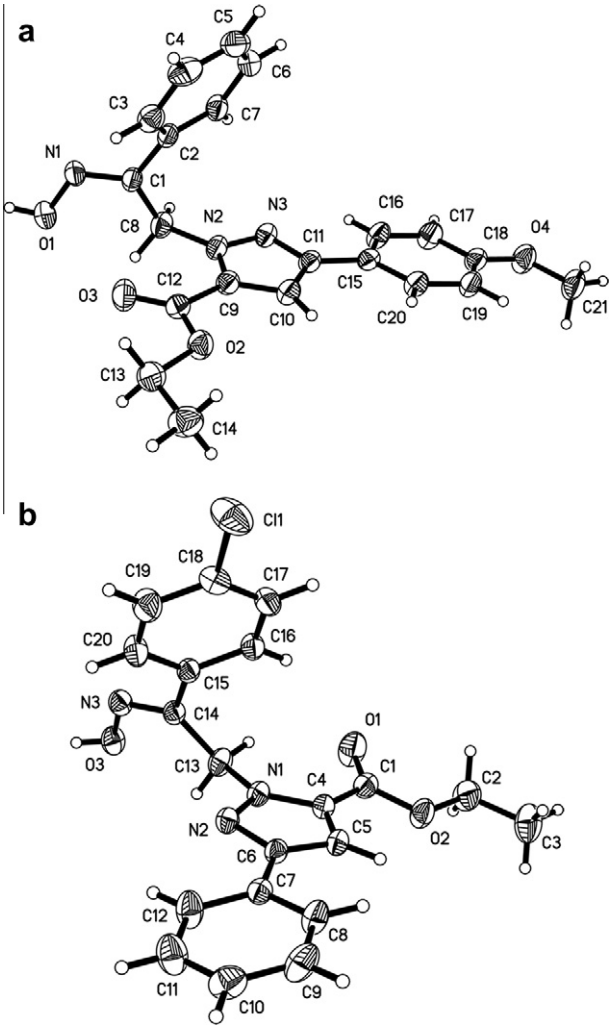

Figure 1. View of the molecules **4c** (a) and **4d** (b) with the atomic labeling. Displacement ellipsoids are drawn at the 30% probability level.

Table 1
Hydrogen-bond geometry for structures of **4c** and **4d** (Å, °)

Compound	D–H···A	D–H	H···A	D···A	∠D–H···A
4c	O1–H1···N1[a]	0.82	2.11	2.8059(18)	143
	C6–H6···O3[b]	0.93	2.55	3.407(2)	154
	C8–H8A···O1	0.97	2.28	2.665(2)	103
4d	O3–H3···N2[c]	0.82	2.04	2.8335(18)	164
	C13–H13A···O3	0.97	2.24	2.623(2)	102
	C13–H13B···O1	0.97	2.26	2.982(2)	130

[a] Symmetry codes: $1-x, 2-y, 2-z$.
[b] $x+1, y, z$.
[c] $1-x, 1-y, 2-z$.

to the mean plane of the pyrazole rings. Torsion angles C8–C1–N1–O1 [2.0(2)°] in **4c** and C13–C14–N3–O3 [−1.8(2)°] in **4d** revealed the two compounds adopt *Z* configuration about the C=N double bonds in the crystals.

*Inhibitory effects of compounds **4a–i** on the proliferation of A549 lung cancer cells*: All the compounds synthesized were evaluated for their cytotoxicity against A549 lung cancer cell line. After incubated with compounds at the concentration of 10, 20 or 40 μM for 48 h, respectively, the cells were treated with SRB to measure their growth/viability (% of the untreated control) using a spectrophotometer as described previously.[14,27,28] The reading of SRB staining is known to accurately reflect the levels of total cellular macromolecules.[27] Figure 2 indicates the cytotoxic effects of the compounds tested at different concentration on the viability of A549 lung cancer cells that were incubated for 48 h. The viability of A549 cells was significantly dose-dependently suppressed by these compounds. For example, exposure of cells to compound **4e**, at 10, 20 and 40 μM for 48 h, the cell viability reduced significantly from 100% to 58.6%, 51.9% and 10.3%, respectively. The IC_{50} concentration for each compound was calculated with reference to a standard curve (control cells), which represents the concentration that results in a 50% decrease in cell growth after 48 h of incubation. For each compound, the 50% growth inhibition (IC_{50}) was reported in Table 2.

From these data, it is possible to relate the structural characteristics of the compounds to their antitumor activity. When substituent R^2 in benzene bonded to oxime moiety is chlorine, the inhibition effect of growth is stronger. Taken together, compound **4e** was the most effective compound in suppressing A549 cell growth.

*Apoptosis assays of morphological changes and DNA fragmentation in compound (**4a–i**) treated A549 cells*: Apoptosis is an important

Table 2
In vitro cytotoxicity of synthesized compounds (**4a–i**) at 48 h

Compounds	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**	**4h**	**4i**
IC_{50} (μM)	29.8	31.4	42.7	26.3	14.5	16.3	29.1	17.8	36.5

determinant factor of the response of tumors to chemotherapeutic agents. The morphological features of apoptosis include chromatin condensation, cell shrinkage, and nuclear fragmentation.[29] To further investigate the induction of apoptosis, we examined the morphological changes induced by compounds **4a–i** in A549 cells using Hoechst 33258 staining. The A549 cells were exposed to 10, 20 and 40 μM for 48 h. As illustrated in Figure 3, compared with vehicle (0.1% DMSO), most of which contained intact genomic DNA, cells cultured with compounds **4a–f** had some cells with condensed chromatin. Granulation of the nucleus appeared as fluorescent blue in the detail of the Hoechst 33258 stained cells (those from which vehicle was absent). Since nucleus granulation is a feature of the morphological change in apoptosis, these results suggest that **4a–f** may induce partly apoptosis in A549 cells. However, the apoptosis magnitude of cells seems hardly to be decisive for inhibiting growth of cells. In addition, in the case of compounds **4g–i,** the morphological features of apoptosis were less found.

*Compounds **4b**, **4d–f**, **4h**, and **4i** induced autophagic cell death in A549 cells*: In order to determine if the growth inhibitory effects were due to necrosis that is believed to be an unwanted side effect of cancer-fighting agents, we performed the LDH assay and the results showed that 40 μM of compounds **4** at 48 h did not cause a significant ($P > 0.05$) increase in LDH release as shown in Figure 4.

Like apoptosis, autophagy is also believed to be a frequent cell death mechanism and a number of studies have reported that autophagy is activated in response to various anticancer therapies.[30,31] Growing evidence indicates that nonapoptotic programmed cell death is principally attributed to autophagy (type II programmed cell death).[32] Considering the majority of compounds **4** treated A549 cells do not primarily display typical features of apoptosis, we therefore assessed whether compounds **4** induces autophagy in lung cancer.

Autophagy is the process of sequestering cytoplasmic proteins into lytic components and is characterized by the formation and promotion of acidic vesicular organelles (AVO). The volume of the cellular acidic compartment, as a marker of autophagy, can be visualized by acridine orange (AO) staining.[33,34] To assess the occurrence of acidic vesicular organelles, we treated A549 cells

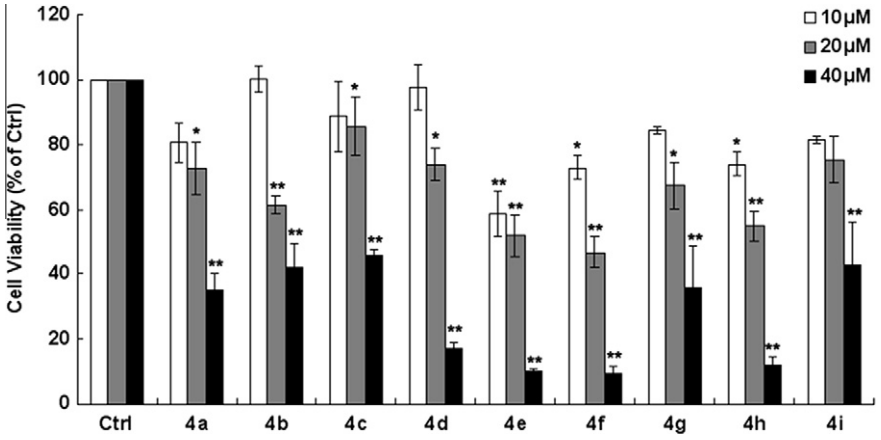

Figure 2. Effects of the compounds on A549 cell viability. A549 cells were treated with compounds **4a–i** at concentrations of 10, 20 or 40 μM or left untreated (control) for 48 h. Cell viability was analyzed by SRB assay and illustrated in column figures. Results are presented as mean ± SE; ($n = 3$. *, $P < 0.05$ vs control; **, $P < 0.01$ vs control).

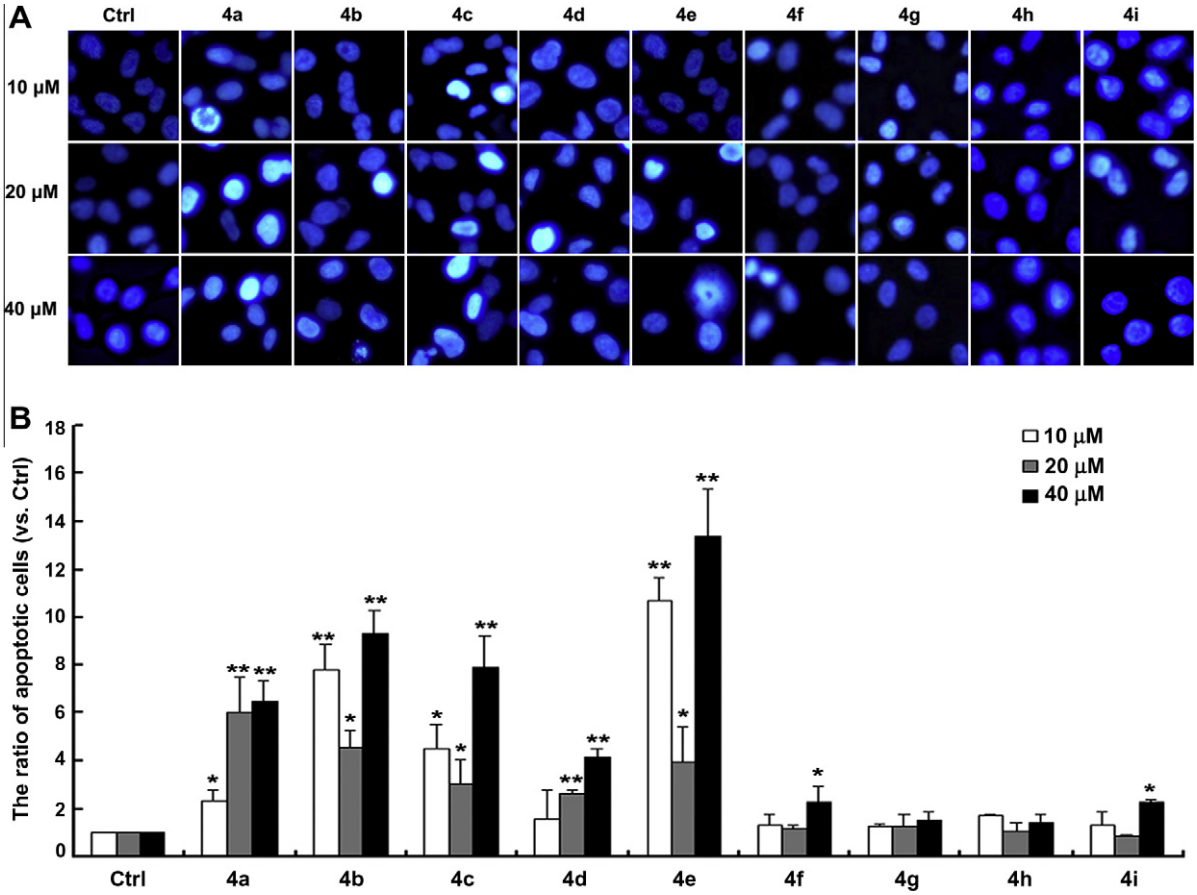

Figure 3. Compounds **4a–i** induces A549 cell apoptosis significantly. (A) Fluorescent micrographs of Hoechst 33258 staining (400×). (B) Ratio of apoptotic cells vs control. Results are presented as mean ± SE; (n = 3. *, P <0.05 vs control; **, P <0.01 vs control).

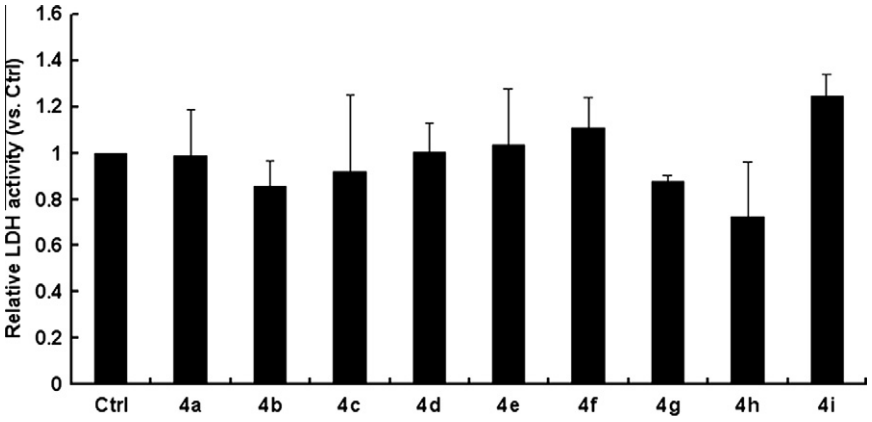

Figure 4. Effects of the compounds on LDH release in A549 cells. A549 cells were treated with compounds **4a–i** at the concentration of 40 μM or left untreated (control) for 48 h. LDH release was determined. Results are presented as mean ± SE; n = 3.

with compounds **4** for 48 h and then stained them with acridine orange. As shown in Figure 5, compounds **4b**, **4d–f**, and **4h–i** treatment resulted in the appearance of AVO when cells were stained with acridine orange after 48 h treatment. It is acceptable that LC3 is the credible marker of the autophagosome in mammalian cells. Enhancement of LC3-II level occurs when autophagy is induced. Thus, induction or suppression of autophagy can be easily monitored by examining the levels of LC3-II by immunoblot.[35] To further identify autophagosome, we determined the protein level of LC3-II in the A549 cells treated with compounds **4b** and **4d–i,** which were chosen based acridine orange (AO) staining, at 24 h by Western blot analysis.[36] It can be seen from Figure 6 that protein levels of LC3-II in the A549 cells treated with compounds at 24 h, except **4g**, were elevated (P <0.05).

In summary, a series of novel oxime-containing pyrazole derivatives were synthesized and characterized by X-ray analysis. A dose- and time-dependent inhibition of proliferation was observed in A549 lung cancer cell after compound treatment. The morphological feature of apoptosis was demonstrated by Hoechst 33258 staining and the induction of autophagy was determined by acridine orange (AO) staining and enhancement of LC3-II level. Inhibition of growth was mainly attributed to the autophagy induction.

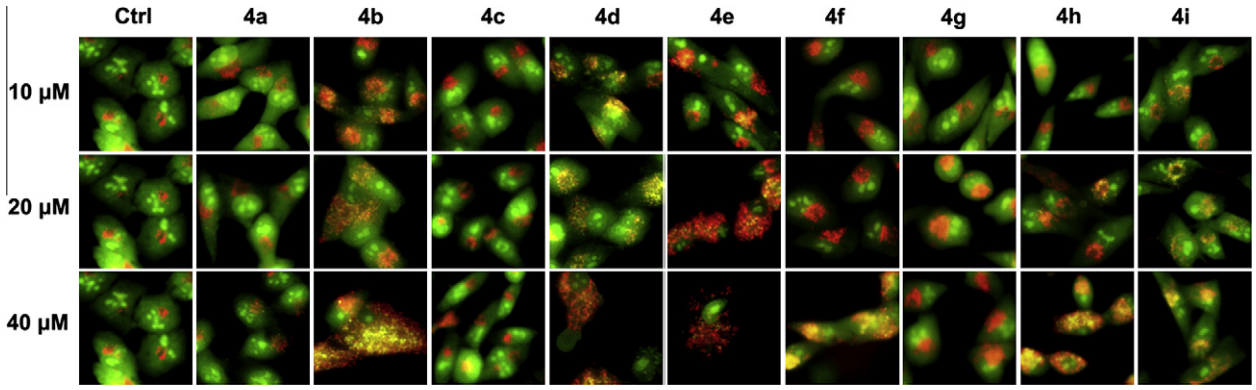

Figure 5. Compounds **4a**–**i** increased the acidic vesicle level in the cells at 48 h (one representative result of three independent experiments $n = 3$). Microscopic photographs (400×) were taken under a fluorescent microscope (Nikon).

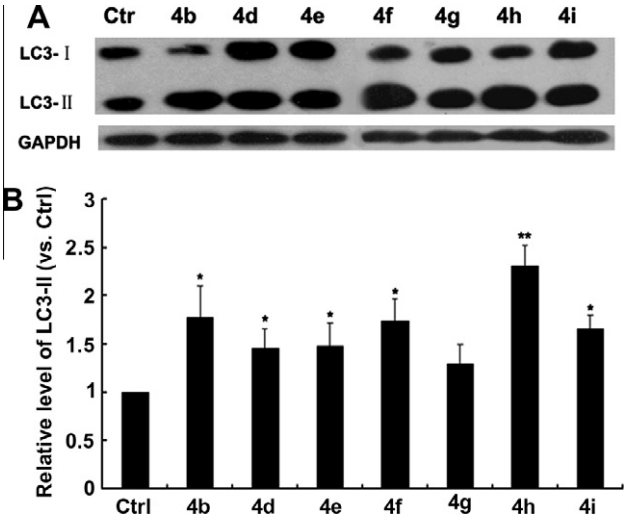

Figure 6. Compounds **4b**, **4d**–**i** induce autophagy in A549 cells. (A) Western blot analysis of the protein level of LC3-II and GAPDH as a normalization control. A549 cells were treated with 20 μM **4d**, **4e** and 40 μM **4b**, **4f**–**i**. (B) Quantification of LC3-II protein level. (*, $P < 0.05$ vs control, **, $P < 0.01$ vs control, $n = 3$).

The study of structure–activity relationships showed that the cytotoxic potency of the compounds was highly dependent on the substitution types and patterns on the aryl ring.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.121.

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