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Synthesis of novel substituted pyrazole-5-carbohydrazide hydrazone derivatives and discovery of a potent apoptosis inducer in A549 lung cancer cells

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ABSTRACT

A series of novel 3-aryl-1-(4-*tert*-butylbenzyl)-1*H*-pyrazole-5-carbohydrazide hydrazone derivatives were synthesized and the effects of all the compounds on A549 cell growth were investigated. The results showed that all compounds had inhibitory effects on the growth of A549 lung cancer cells and compound (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl) ethylidene)-3-(4-chlorophenyl)-1*H*-pyrazole-5-carbohydrazide (**3e**) possessed the highest growth inhibitory effect and induced apoptosis of A549 lung cancer cells.

1. Introduction

Lung cancer is one of the leading causes of death worldwide.[1] Lung cancer cells might be resistant to current chemotherapy and eventually dominate the cell population and cause mortality. Thus, the development of novel, more effective anticancer drugs for lung cancer is urgently needed.

Apoptosis, a universal genetic program of cell death in higher eukaryotes, is a basic process involved in cellular development and differentiation. Apoptosis may be essential for the prevention of cancer cell proliferation, and its deregulation is widely believed to be involved in pathogenesis of many human diseases, including cancer,[2] and recently, many antitumor compounds have been found to induce the apoptotic process in tumor cells.[3–5] Therefore, identification of compounds that activate and promote apoptosis is an attractive strategy for the discovery and development of potential anticancer agents.

A number of hydrazide–hydrazone derivatives have been claimed to possess interesting bioactivity such as antibacterial–antifungal, anticonvulsant, antiinflammatory, antimalarial, analgesic, antiplatelets, antituberculosis and anticancer activities.[6–14] Aroylhydrazide-hydrazones containing hetero-ring such as pyridine, indole ring have attracted special attention.[15,16] A few of pyrazole carbohydrazide hydrazone derivatives have also been reported.[17,18] In our effort to discover and develop apoptosis inducers as potential new anticancer agents, we recently reported a novel synthesized 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carbohydrazide hydrazone and effects of them on A549 cell growth.[19] The study on structure–activity relationships showed that the hydrazone derived from salicylaldehyde (Chart 1, $R^1 = R^2 = R^3 = H$) had much more inhibitory effects. Furthermore, replacement of the benzyl group with a *t*-butylbenzyl moiety in 1 position of pyrazole resulted in a higher growth inhibitory effect. In view of this point and with the aim of exploring new anticancer compounds we synthesized a series of novel 3-aryl-1-(4-*tert*-butylbenzyl)-1*H*-pyrazole-5-carbohydrazide hydrazone derivatives and evaluated the effects of these compounds on

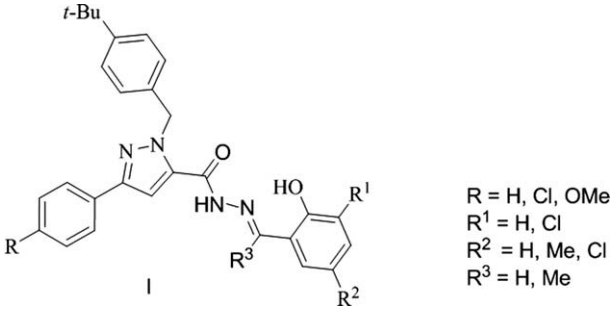

Chart 1. Structure of substituted 1*H*-pyrazole-5-carbohydrazide hydrazone.

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A549 cell growth. We found that (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene)-3-(4-chlorophenyl)-1*H*-pyrazole-5-carbohydrazide (**3e**) possessed the highest growth inhibitory effect and induced apoptosis of A549 cells.

2. Results and discussion

2.1. Synthesis of 3-aryl-1-(4-*tert*-butylbenzyl)-1*H*-pyrazole-5-carbohydrazide hydrazone

The synthesis of 3-aryl-1-(4-*tert*-butylbenzyl)-1*H*-pyrazole-5-carbohydrazide hydrazone derivatives has been accomplished as outlined in Scheme 1 starting from 3-aryl-1-(4-*tert*-butylbenzyl)-1*H*-pyrazole-5-carbohydrazide (**1**) that can be synthesized as described in our previous paper[20] and substituted 1-(2-hydroxyphenyl)ethanone (**2**). For example, (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide (**3h**) was synthesized in 87% yield by the reaction of 1-(4-*tert*-butylbenzyl)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide with 1-(4-chloro-2-hydroxyphenyl)ethanone in ethanol over a 4 h reflux period.

The structures of hydrazone derivatives were determined by IR, ^{1}H NMR and mass spectroscopy. Thus, for example **3h**, obtained as white crystal, gave a [M+H]-ion peak at *m/z* 531.3 in the ESI-MS, in accord with the molecular formula $C_{30}H_{32}ClN_4O_3$. In the IR spectra, the carbonyl group absorptions in hydrazide moiety and NH bands in CONH were observed in the 1694 cm^{-1} and 3000–2834 cm^{-1} region, respectively. The ^{1}H NMR spectra indicated the chemical shift of the NH proton at δ = 11.46 ppm in the form of singlet peak. Strong deshielding of this proton can be explained by hydrogen bond formation. The signal of hydroxy group substituted on benzene ring appears at 13.21 ppm, that is, in the range characteristic for intramolecular bonded. Two *ortho*-aromatic protons signals in 4-*tert*-butylbenzyl moiety appeared at the range of δ = 7.02 and 7.78 ppm as doublet peaks (*J* = 8.7 Hz). Two *ortho*-aromatic protons signals in 4-methoxybenzene moiety appeared at the range of δ = 7.18 and 7.34 ppm as doublet peaks (*J* = 8.3 Hz). Two *ortho*-aromatic protons signals in 5-chloro-2-hydroxyphenyl moiety appeared at the range of δ = 6.95 ppm as doublet peaks (*J* = 8.8 Hz) and δ = 7.36 ppm as double doublet peaks (*J* = 8.8 and 2.2 Hz). The doublet peaks at δ = 7.66 is C_6 aromatic proton signals in 5-chloro-2-hydroxyphenyl moiety. Two singlet signals appeared at δ = 5.72 and 7.52 ppm are consistent with methylene protons in 4-*tert*-butylbenzyl and pyrazole moiety, respectively. Three singlet signals appeared at δ = 1.23, 2.50 and 3.80 ppm are consistent with methyl protons in 4-*tert*-butylbenzyl, ethylidene and methoxy moiety, respectively. Furthermore, the structure of (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide **3h** was confirmed by the X-ray diffraction.

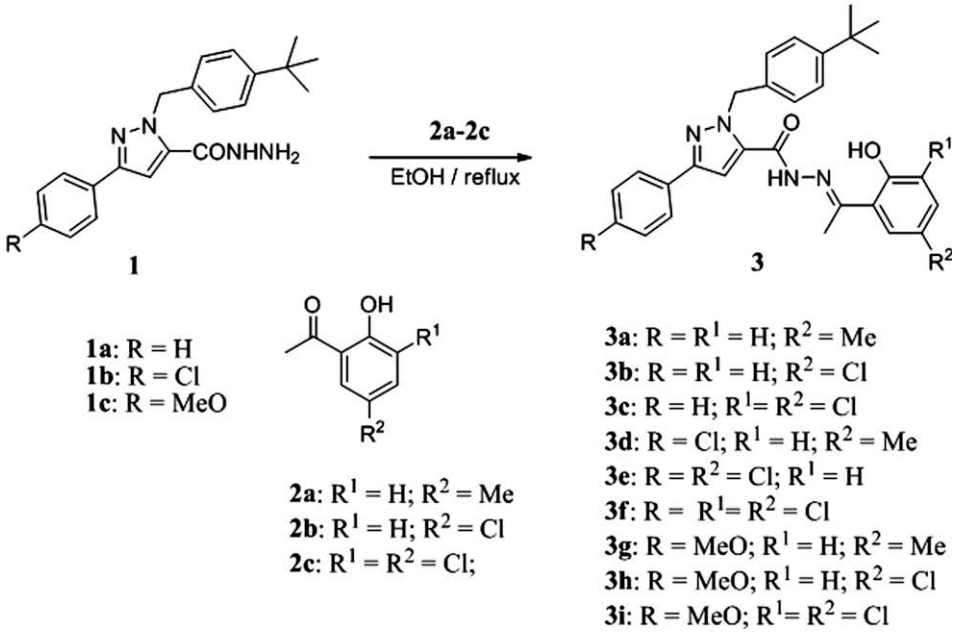
2.2. Single-crystal structural characterization of compound 3h by X-ray

The spatial structure of compound **3h** was determined by using X-ray diffraction analysis. The single crystals were grown from acetone at room temperature. The molecular view of **3h** is shown in Figure 1.

The X-ray analysis revealed unambiguous proof of the regiochemistry of 4-*tert*-butylbenzyl group and the geometry stereochemistry of substituent group on the N=C double bond in (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide and definitely assigned the structure of **3h** (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide.

The molecule of **3h** (Fig. 1) consists of four fragments, a planar pyrazole ring, aryl ring bonded to pyrazole, 4-*tert*-butylbenzyl and *N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene moiety. The pyrazole ring makes dihedral angles of 14.40(13)° 10.1 7(13)° and 81.63(14)° with the 4-methoxyphenyl ring, 5-chloro-2-hydroxyphenyl and 4-*tert*-butylbenzyl, respectively.

The carbohydrazide part of the molecules of **3h** adopts an extended conformation with torsion angles N3–C10–C9–O2 [−171.61(9)°], C10–C9–N2–N1 [−178.79(13)°] and C7–N1–N2–C9 [−177.67(10)°] close to 180°. The C9–O2 [1.216(3)Å], N1–C7 [1.295(3)Å] and N1–N2 [1.368(3)Å] bond lengths are consistent with the double and single bond characters. The crystal structures of **3h** are stabilized by strong intermolecular hydrogen bonds.



Scheme 1. Synthesis of 3-aryl-1-(4-*tert*-butylbenzyl)-1*H*-pyrazole-5-carbohydrazide hydrazone.

2.3. Inhibitory effects of compounds 3a–i on the proliferation of A549 lung cancer cells

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay has been widely accepted as a reliable way to measure the cell proliferation rate, and conversely when metabolic events lead to apoptosis or necrosis. The data obtained by MTT assay showed that compounds **3a–i** had inhibitory effects on the growth of A549 cells in dosage-dependent manners. Compounds **3b**, **3d** and **3e** could inhibit the cell growth obviously at 1 μM after 48 h of the treatment. At 5μ M after 48 h of the treatment (at 2.5 μM in the case of **3i**), all the compounds effectively inhibited the cell growth (Fig. 2). Taken altogether, compound **3e** was the most potent compound in this series, having an IC50 value of 0.28 μM in suppressing A549 cell growth. Compound **3e** was almost 9-fold more potent than **3d** and >7-fold more potent than **3i**. Furthermore, compound **3e** was almost 22, 11 and 17-fold more effective than other compounds **I** (Chart 1) reported in the previous paper[19] in which R group is H, Cl and OMe, respectively, when $R^1 = R^2 = R^3 = H$, indicating that the nature of the R^1, R^2 and R^3 groups is critical for activity.

The growth inhibitory properties (IC50) for the compounds **3a–3i** are listed in Table 1.

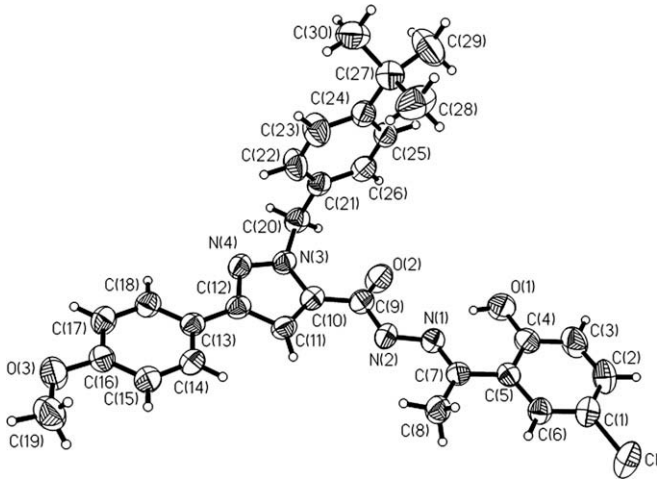


Figure 1. X-ray crystal structures of compound **3h**. Displacement ellipsoids are drawn at 50% probability level.

2.4. Compound 3e induced apoptosis in A549 cells

On the basis of the above results, compound **3e** was selected as a lead upon which further studies were conducted.

Apoptosis can be characterized by morphological and biochemical changes in the cell nucleus, including chromatin condensation and nuclear shrinking.[21] Morphological changes of cells treated with potent compound **3e** can be visually observed with light microscopy (Fig. 3). We found that the cells treated with compound **3e** at 1 μM for 48 h became shrinked. Such morphological changes were not apparent in the control cells. The findings indicate that apoptosis induction may be a mechanism by which compound **3e** kills the cancer cells.

To further demonstrate the cell death was due to apoptosis, we examined the DNA fragmentation of the treated cells by acridine orange assay and TUNEL assay. We found that compound **3e** induced A549 cell nuclear condensation and fragmentation, which are characteristics of apoptosis (Fig. 4). Therefore, our results demonstrated that compound **3e** promoted A549 cell apoptosis. We further assessed apoptosis using a TUNEL technique. We found that a significant number of cells in the treatment with compound **3e** were undergoing apoptosis, whereas almost no apoptotic cell was detected in the control group (Fig. 5).

To detect whether compound resulted in necrosis of A549 cells, LDH activity in cell culture medium was measured. As shown in Figure 6, there was no significant difference ($p > 0.05$) in LDH release between the cells of control group and the cells treated with the compound **3e** at 0.1, 0.5 and 1 μM for 24 and 48 h. The results

Table 1
Growth inhibitory properties IC_{50} (μM) for the compounds **3a**–**i** at 48 h

Compound	3a	3b	3c	3d	3e	3f	3g	3h	3i
IC_{50} (μM)	6.10	2.77	3.17	2.49	0.28	7.90	10.39	9.62	2.08

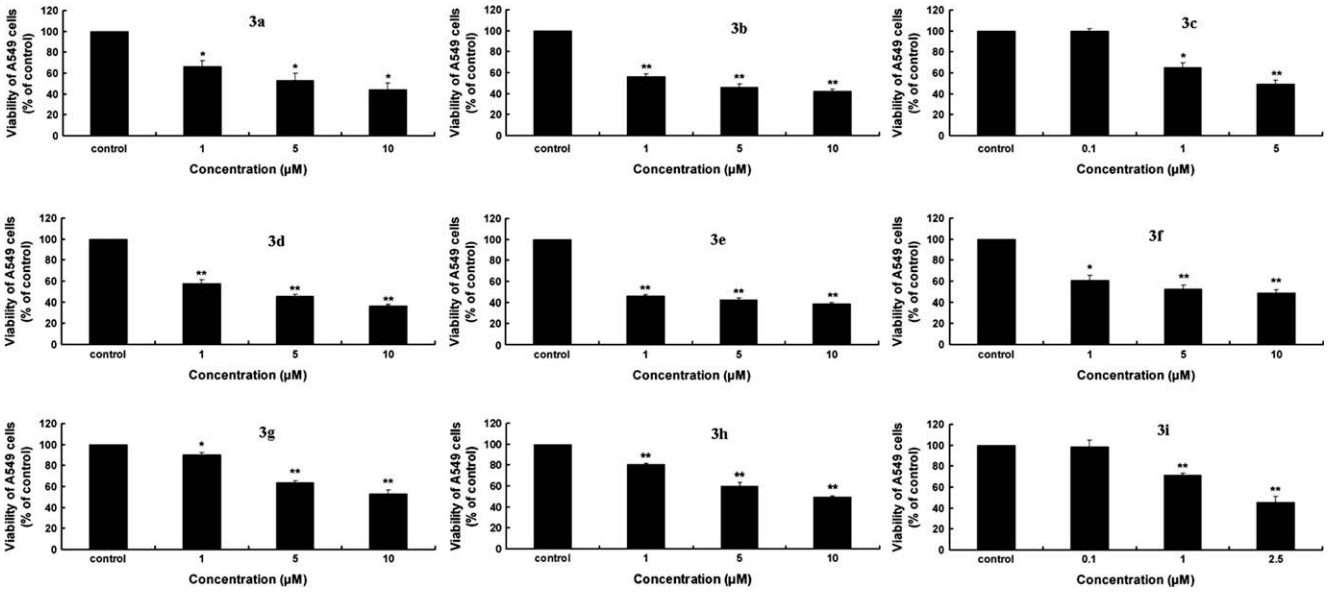


Figure 2. Viability of A549 cells treated with compounds **3a**–**3i**. Cells were seeded in 96-well plates at the density of 6250/cm^2. Cells were treated with the compounds at concentrations of 0, 1, 5, 10 μM (but 0.1, 1, 5 μM for **3c**; 0.1, 1, 2.5 for **3i**) for 48 h. The cell viability was determined by MTT assay. ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs control, $n = 3$.)

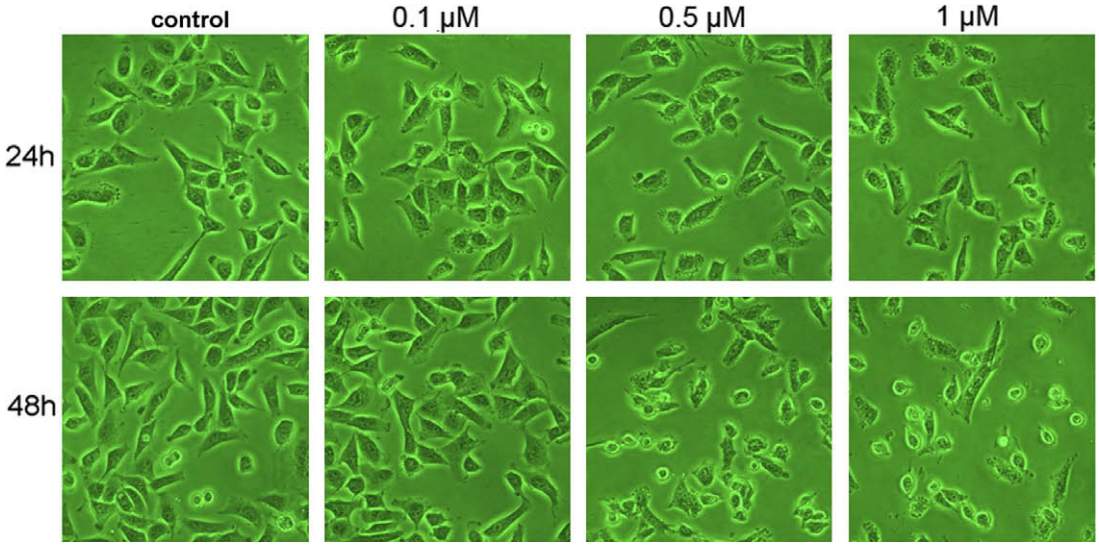


Figure 3. Morphology image of A549 treated with compound **3e** at 0.1, 0.5, and 1 μM for 24 and 48 h.

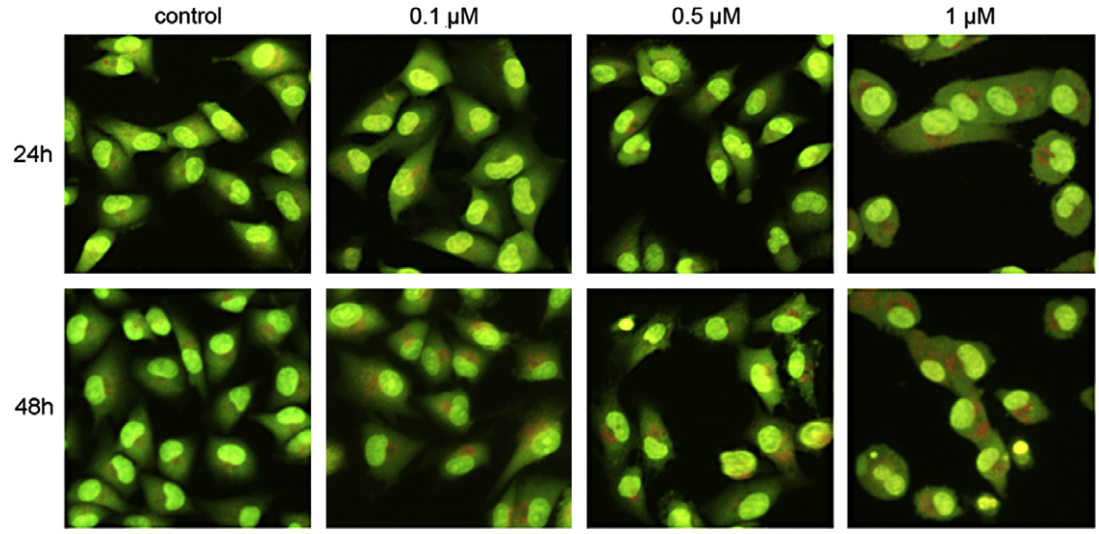


Figure 4. DNA fragmentation and apoptosis detections by acridine orange (AO) staining in A549 cells treated with 0.1, 0.5 and 1 μM of compound **3e** for 24 h and 48 h. ($n = 3$).

indicated that the compound **3e** at the test range of concentration did not cause necrosis in A549 cells (Fig. 6). Thus, our results suggested that the compound **3e** induced A549 cell apoptosis.

3. Conclusion

In summary, we have described a facile approach to prepare 3-aryl-1-(4-*tert*-butylbenzyl)-1*H*-pyrazole-5-carbohydrazide hydrazone derivatives **3a**–**3i** by the reaction of 3-aryl-1-(4-*tert*-butylbenzyl)-1*H*-pyrazole-5-carbohydrazide (**1**) and substituted 1-(2-hydroxyphenyl)ethanone (**2**). We found that compounds **3a**–**3i** could suppress A549 lung cancer cell growth. Compound **3e** was the most effective small molecule in inhibiting A549 cell growth and might perform its action through inducing apoptosis. The representative single-crystal structural characterization of the compound **3h** was studied by X-ray and it should be valuable for further investigation.

Studies on mechanisms by which compound **3e** induces apoptosis in A549 lung cancer cells are ongoing and will be reported in due course.

4. Experimental

4.1. Reagents and apparatus

Thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254} plates Merck KGaA). ^{1}H NMR spectra were recorded on a Bruker Avance 400 (400 MHz) spectrometer, using $CDCl_3$ or DMSO as solvents and tetramethylsilane (TMS) as internal standard. Melting points were determined on an XD-4 digital micro melting point apparatus and are uncorrected. IR spectra were recorded with an IR spectrophotometer Avtar 370 FT-IR (Termo Nicolet). MS spectra were recorded on a Trace DSQ mass spectrograph. X-ray diffraction data were recorded on a Bruker Smart CCD diffractometer.

Acridine orange (AO) was purchased from Shandong Chemical Industries (Jinan, China). RPMI 1640 was obtained from Gibco BRL (Grand Island, NY, USA) and Bovine calf serum was supplied by Beijing DingGuo Biotechnology Co., China. DMSO was bought from Shanghai Sangon Biological Engineering Technology and Services Company.

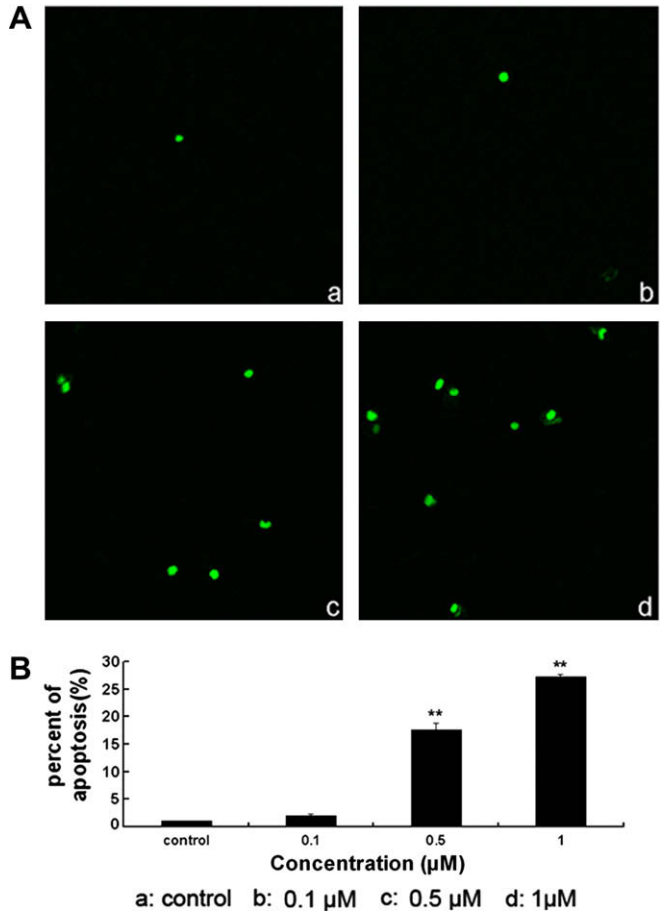


Figure 5. Quantification of apoptotic cells by TUNEL assay. (A) Fluorescent micrographs show the TUNEL-positive cells in A549 cells treated with compounds **3e** at 0.1, 0.5 and 1 μM for 48 h. (B) The quantity of apoptotic cells ($^{**}p < 0.01$ vs control group, $n = 3$).

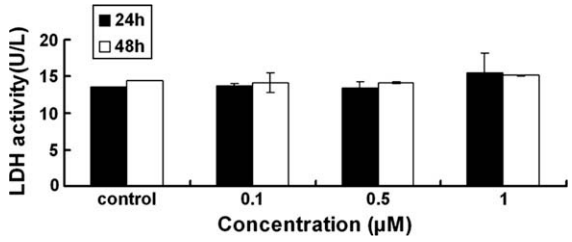


Figure 6. Effects of the compound **3e** on the release of LDH from A549 cells. The culture media from the cells treated with the compound at 0.1, 0.5 and 1 μM for 24 and 48 h, respectively. Light absorption was analyzed at 340 nm using a model Cintra 5 UV–vis spectrometer. There was no significant difference in LDH release among the four groups. ($P > 0.05$ vs control group, $n = 3$).

4.2. General procedure for the synthesis of 1-(4-*tert*-butylbenzyl)-3-aryl-1*H*-pyrazole- 5-carbohydrazide hydrazone derivatives (3a–3i)

To a stirred solution of 1 mmol of derivatives **1** in 10 ml of ethanol, there was added an equimolar amount of the appropriate keton derivatives **2**. The reaction mixture was maintained under reflux for 2–16 h, until TLC indicated the end of reaction. After this time, the reaction mixture stood over night and the solid formed was collected by filtration and washed with ethanol and recrystallized from ethanol to afford crystals. As a result of this process the compounds **3** were prepared in yield of 72–93%.

4.2.1. (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(2-hydroxy-5-methyl phenyl)ethylidene)-3-phenyl-1*H*-pyrazole-5-carbohydrazide (3a)

White solid, yield 84%; mp 203–206 °C; IR (KBr) *v*: 3476 (OH), 3054–2868 (NH), 1682 (C=O) cm^{-1}; ^1H NMR (DMSO, 400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.28 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 5.75 (s, 2H, CH_2), 6.82 (d, $J = 8.2$ Hz, 1H, ArH), 7.14 (d, $J = 8.2$ Hz, 1H, ArH), 7.20 (d, $J = 8.2$ Hz, 2H, ArH), 7.34 (d, $J = 8.2$ Hz, 2H, ArH), 7.38 (s, 1H, ArH), 7.46 (t, $J = 7.5$ Hz, 3H, ArH), 7.60 (s, 1H, 4-H), 7.86 (d, $J = 7.5$ Hz, 2H, ArH), 11.36 (s, 1H, NH), 12.87 (s, 1H, OH); ESI-MS: 481.4 $(M+H)^+$.

4.2.2. (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene)-3-phenyl-1*H*-pyrazole-5-carbohydrazide (3b)

White solid, yield 90%; mp 194–195 °C; IR (KBr) *v*: 3449 (OH), 3367–2869 (NH), 1686 (C=O) cm^{-1}; ^1H NMR (DMSO,400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.50 (s, 3H, CH_3), 5.75 (s, 2H, CH_2), 6.95 (d, $J = 8.8$ Hz, 1H, ArH), 7.19 (d, $J = 8.2$ Hz, 2H, ArH), 7.33–7.38 (m, 4H, ArH), 7.46 (t, $J = 7.6$ Hz, 2H, ArH), 7.61 (s, 1H, 4-H), 7.66 (s, 1H, ArH,), 7.85 (d, $J = 7.6$ Hz, 2H, ArH), 11.47 (s, 1H, NH), 13.20 (s, 1H, OH); ESI-MS: 501.3 $(M+H)^+$.

4.2.3. (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(3,5-dichloro-2-hydroxyphenyl)ethylidene)-3-phenyl-1*H*-pyrazole-5-carbohydrazide (3c)

Yellow solid, yield 76%; mp 205–206 °C; IR (KBr) *v*: 3486 (OH), 3121–2869 (NH), 1662 (C=O) cm^{-1}; ^1H NMR (DMSO, 400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.54 (s, 3H, CH_3), 5.75 (s, 2H, CH_2), 7.21 (d, $J = 8.2$ Hz, 2H, ArH), 7.34–7.38 (m, 3H, ArH), 7.47 (t, $J = 7.5$ Hz, 2H, ArH), 7.63 (s, 1H, 4-H), 7.65 (dd, $J = 2.3$, 19.0 Hz, 1H, ArH), 7.70 (dd, $J = 2.3$, 19.0 Hz, 1H, ArH), 7.86 (d, $J = 7.5$, 2H, ArH), 11.63 (s, 1H, NH), 14.25 (s, 1H, OH); ESI-MS: 535.3 $(M+H)^+$.

4.2.4. (*E*)-1-(4-*tert*-butylbenzyl)-3-(4-chlorophenyl)-*N*′-(1-(2-hydroxy-5-methylphenyl)ethylidene)-1*H*-pyrazole-5-carbohydrazide (3d)

Yellow solid, yield 76%; mp: 194–196 °C; IR (KBr) *v*: 3477 (OH), 3384–2867 (NH), 1678 (C=O) cm^{-1}; ^1H NMR (DMSO, 400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.28 (s, 3H, CH_3), 2.48 (s, 3H,CH_3), 5.74 (s, 2H, CH_2), 6.82 (d, $J = 7.8$ Hz, 1H, ArH), 7.13 (d, $J = 7.8$ Hz, 1H, ArH), 7.20 (d, $J = 8.2$ Hz, 2H, ArH), 7.34 (d, $J = 8.2$ Hz, 2H, ArH), 7.45 (s, 1H, ArH), 7.52 (d, $J = 8.6$ Hz, 2H, ArH), 7.60 (s, 1H, 4-H), 7.87 (d, $J = 8.6$ Hz, 2H, ArH), 11.77 (s, 1H, NH), 12.85 (s, 1H, OH); ESI-MS: 515.4 $(M+H)^+$.

4.2.5. (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene)-3-(4-chlorophenyl)-1*H*-pyrazole-5-carbohydrazide (3e)

Yellow solid, yield 72%; mp: 203–206 °C; IR (KBr) *v*: 3390 (OH), 2960–2867 (NH), 1691 (C=O) cm^{-1}; ^1H NMR (DMSO, 400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.50 (s, 3H, CH_3), 5.74 (s, 2H, CH_2), 6.95 (d, $J = 8.8$ Hz, 1H, ArH), 7.20 (d, $J = 8.2$ Hz, 2H, ArH), 7.33–7.35 (m, 3H, ArH), 7.52 (d, $J = 8.5$ Hz, 2H, ArH), 7.62 (s, 1H, 4-H), 7.66 (s, 1H, ArH), 7.87 (d, $J = 8.5$ Hz, 2H, ArH), 11.49 (s, 1H, NH), 13.18 (s, 1H, OH); ESI-MS: 535.2 $(M+H)^+$.

4.2.6. (*E*)-1-(4-*tert*-butylbenzyl)-3-(4-chlorophenyl)-*N*′-(1-(3,5-dichloro-2-hydroxyphenyl)ethylidene)-1*H*-pyrazole-5-carbohydrazide (3f)

Yellow solid, yield 93%; mp 246–248 °C; IR (KBr) *v*: 3478 (OH), 3123–2869 (NH), 1661 (C=O) cm^{-1}; ^1H NMR (DMSO, 400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.53 (s, 3H, CH_3), 5.74 (s, 2H, CH_2), 7.21 (d, $J = 8.3$ Hz, 2H, ArH), 7.35 (d, $J = 8.3$ Hz, 2H, ArH), 7.53 (d, $J = 8.7$ Hz, 2H, ArH), 7.65 (s, 1H, 4-H), 7.66 (s, 1H, ArH), 7.70 (s,

1H, ArH), 7.87 (d, J = 8.7 Hz, 2H, ArH), 11.66 (s, 1H, NH), 14.22 (s, 1H, OH); ESI-MS: 512.7 $(M-C_4H_9+H)^+$.

4.2.7. (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(2-hydroxy-5-methylphenyl)ethylidene)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide (3g)

White solid, yield 76%; mp 226–228 °C; IR (KBr) v: 3385 (OH), 2994–2833 (NH), 1691 (C=O) cm^{-1}; ^1H NMR (DMSO, 400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.28 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 5.72 (s, 2H, CH_2), 6.81 (d, J = 8.7 Hz, 1H, ArH), 7.02 (d, J = 8.7 Hz, 2H, ArH), 7.13 (d, J = 8.7 Hz, 1H, ArH), 7.19 (d, J = 8.3 Hz, 2H, ArH), 7.34 (d, J = 8.3 Hz, 2H, ArH), 7.45 (s, 1H, ArH), 7.50 (s, 1H, 4-H), 7.78 (d, J = 8.7 Hz, 2H, ArH), 11.33 (s, 1H, NH), 12.87 (s, 1H, OH); ESI-MS: 511.5 $(M+H)^+$.

4.2.8. (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(5-chloro-2-hydroxyphenyl)ethylidene)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide (3h)

White solid, yield 87%; mp 223–225 °C; IR (KBr) v: 3393 (OH), 3000–2834 (NH), 1694 (C=O) cm^{-1}; ^1H NMR (DMSO, 400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.50 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 5.72 (s, 2H, CH_2), 6.95 (d, J = 8.8 Hz, 1H, ArH), 7.02 (d, J = 8.7 Hz, 2H, ArH), 7.18 (d, J = 8.3 Hz, 2H, ArH), 7.33–7.37 (m, 3H, ArH), 7.52 (s, 1H, 4-H), 7.67 (s, 1H, ArH), 7.78 (d, J = 8.7 Hz, 2H, ArH), 11.46 (s, 1H, NH), 13.21 (s, 1H, OH); ESI-MS: 531.3 $(M+H)^+$.

4.2.9. (*E*)-1-(4-*tert*-butylbenzyl)-*N*′-(1-(3,5-dichloro-2-hydroxyphenyl)ethylidene)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide (3i)

Yellow solid, yield 91%; mp 248–250 °C; IR (KBr) v: 3486 (OH), 2966–2835 (NH), 1694 (C=O) cm^{-1}; ^1H NMR (DMSO, 400 MHz) δ: 1.23 (s, 9H, $3CH_3$), 2.53 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 5.73 (s, 2H, CH_2), 7.02 (d, J = 8.4 Hz, 2H, ArH), 7.20 (d, J = 8.4 Hz, 2H, ArH), 7.34 (d, J = 8.4 Hz, 2H, ArH), 7.54 (s, 1H, 4-H), 7.65 (dd, J = 2.0, 18.2 Hz, 1H, ArH), 7.69 (dd, J = 2.0, 18.2 Hz,1H, ArH), 7.78 (d, J = 8.4 Hz, 2H, ArH), 11.61 (s, 1H, NH), 14.25 (s. 1H, OH); ESI-MS: 600.6 $(M+2NH_4)^+$.

4.3. Cell culture

A549 lung cancer cells were cultured in RPMI 1640 medium at 37 °C with 5% CO_2, and 95% air, supplemented with 10% (v/v) bovine calf serum and 80 U/ml gentamicin. The cells were seeded onto 96-well plates or other appropriate dishes containing the medium at the density of 6250/cm^2.

4.4. Cell viability assay

As the previous report, cells were seeded onto 96-well plates and treated with compounds **3a–3i** at 1, 5, and 10 μM for 24 and 48 h, respectively. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay according to Price et al.[22] The light absorption was measured at 570 nm using Spectra MAX 190 microplate spectrophotometer (GMI Co., USA).

4.5. Acridine orange (AO) staining

The cells were incubated with compound **3e** at 0.1, 0.5, 1 μM for 24 and 48 h, and stained with 5 μg/ml of acridine orange (AO) at room temperature for 1 min. Then the cells were observed and photographed using a Nikon fluorescence microscope.

4.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

The TdT-mediated dUTP nick-end labeling technique was used to detect in situ nuclear DNA fragmentation and measure the apoptosis ratio.[23] In brief, after cells were treated in the presence or absence of compound **3e** at 0.1, 0.5 and 1 μM for 48 h, DNA fragmentation was detected by the DeadEndTM Fluorometric TUNEL System (Promega, USA) according to the manufacturer's protocol. Cells were evaluated by the laser scanning confocal microscope (TCS-SP2, Leica, Germany). The percent apoptosis rate was quantified according to the TUNEL-positive rate.

4.7. LDH assay

Lactate dehydrogenase (LDH) assay was performed on cells treated with compound **3e** at 0.1, 0.5 and 1 μM for 24 and 48 h using a LDH kit (Nanjing Jiancheng, China) according to the manufacturer's protocol. Light absorption was measured at 340 nm using a model Cintra 5 UV–vis spectrometer (GBC, Australia).

4.8. Statistical analyses

Data were presented as means ± SE and analyzed by SPSS software. Pictures were processed with Photoshop software. Mean values were derived from at least three independent experiments. Differences at $p < 0.05$ were considered statistically significant.

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