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Synthesis and structure–activity relationships of novel 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives as potential agents against A549 lung cancer cells

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Abstract—A series of novel 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives were synthesized, and the effects of all the compounds on A549 cell growth were investigated. The results showed that all the nine compounds had inhibitory effects on the growth of A549 cells and induced the cell apoptosis. The study on structure–activity relationships and prediction of lipophilicities of compounds showed that compounds with log *P* values in the range of 3.12–4.94 had more inhibitory effects on the growth of A549 cells.

1. Introduction

Lung cancer is one of the leading causes of death in the world. Since current treatment modalities are inadequate, novel therapies are needed to reduce the effects of the increasing incidence in pulmonary neoplasm.[1,2] An elaborate search of new anticancer agents has primarily been triggered by the unveiling of new molecular targets on which they intervene, followed by the discovery of novel classes of compounds that interact with such targets.[3,4] In our effort to discover and develop apoptosis inducers as potential new anticancer agents, we have identified several classes of molecules as novel apoptosis inducers, including safrole oxide, 1-alkoxy-3-(3′,4′-methylenedioxy)phenyl-2-propanol, morpholinone derivatives, 2,3-dihydro-3-hydroxymethyl-1,4-benzoxazine derivatives.[5–17]

Many pyrazole derivatives are known to exhibit a wide range of biological properties such as anti-hyperglycemic, analgesic, anti-inflammatory, anti-pyretic, anti-bacterial, hypoglycemic, sedative–hypnotic activity,[18,19] and anticoagulant activity.[20] Recently, some arylpyrazoles were reported to have non-nucleoside HIV-1 reverse transcriptase inhibitory activity.[21] Extensive studies have been devoted to arylpyrazole derivatives such as Celecoxib, a well-known cyclooxygenase-2 inhibitor.[22–24] In the previous paper, we investigated the effects of ethyl 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carboxylate derivatives on the growth of A549 lung cancer cell and found that these compounds could suppress A549 lung cancer cell growth.[25] Recently, we described the synthesis and preliminary biological evaluation of ethyl 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carboxylate derivatives **3**.[26] We found that the compounds **3a**–**3d** promoted human umbilical vein endothelial cell (HUVEC) apoptosis to a certain extent at the concentrations of 5–20 μM and the effect on the cell viability was dose-dependent. However, the compounds **3** have no obvious effects on the A549 cell growth in the test dose. Not surprisingly, the difference of compounds **3** from ethyl 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carboxylate derivatives in the structures caused the different bioactivities. The modification of pyrazole such as basic substituent moiety should provide potential bioactivities. A few of heterocyclic carbohydrazide derivatives have been reported.[27] However, there were no reports on the

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synthesis and biological evaluation of 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives. We thus tried to replace the ester group of compounds **3** with hydrazine group to give 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives **4**. We herein would like to report the synthesis of **4** and the findings of their biological activities in suppressing the growth of A549 lung cancer cells.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds **4** has been accomplished as outlined in Scheme 1 starting from compounds **3** that can be synthesized from **1** and the commercially available **2** as described in previous paper.[26] For example, compound **4a** was synthesized by the reaction of compound **3a** with hydrazine hydrate in the condition of refluxing (4 h) in methanol in yield 93%. The structures of **4a**–**4i** were determined by IR, ^{1}H NMR, mass spectroscopy, and elemental analyses. Thus, for example **4f**, obtained as yellow crystal, gave a [M+H]-ion peak at *m*/*z* 362.3 in the ESI-MS, in accord with the molecular formula $C_{16}H_{13}Cl_2N_5O$. The carbonyl group absorptions in hydrazide moiety were observed in the 1675 cm^{-1} in IR spectra and NH and NH_2 bands in $CONHNH_2$ were observed in the 3203 cm^{-1} and 3300–3260 cm^{-1} regions, respectively. The ^{1}H NMR spectra indicated the chemical shift of the NH_2 at $\delta = 4.11$ ppm in the form of singlet broad peak. Two *ortho*-aromatic proton signals in pyridine moiety appeared at the range of $\delta = 7.27$ and 7.70 ppm as doublet peaks ($J = 8.4$ Hz). Two *ortho*-aromatic proton signals in benzene moiety appeared at the range of $\delta = 7.39$ and 7.72 ppm as doublet peaks ($J = 8.7$ Hz). Four singlet signals appearing at $\delta = 5.78$, 6.78, 7.25, and 8.45 ppm are consistent with methylene protons, pyrazole proton, NH proton in carbohydrazide moiety and ArH in pyridine moiety, respectively. The elemental analyses shows satisfactory accord. Furthermore, the structure was confirmed by the X-ray diffraction as shown in Figure 1.

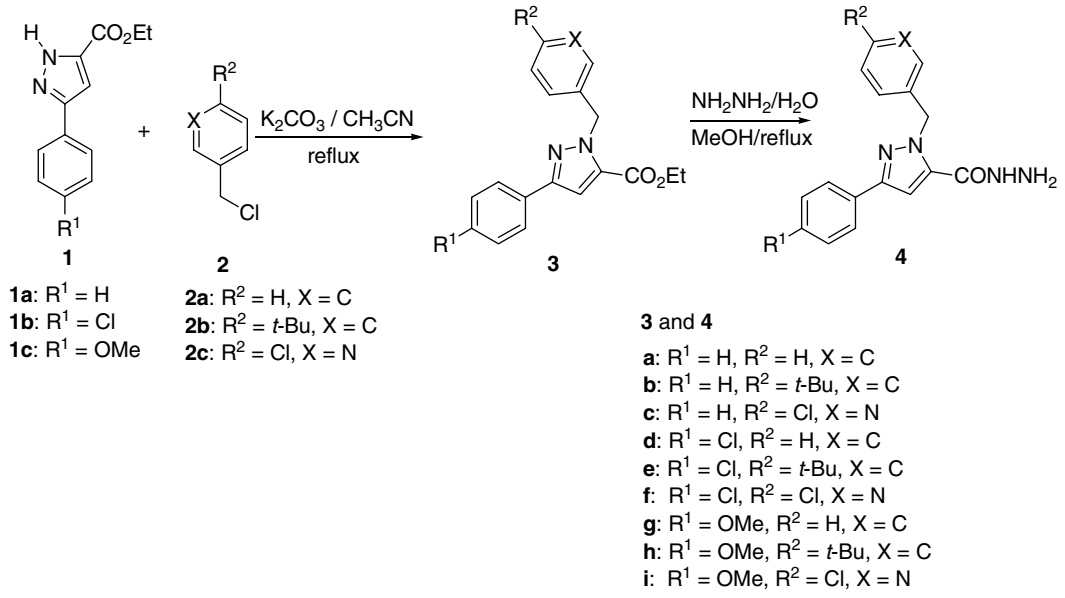
2.2. Evaluation of biological activity

2.2.1. Effects of the compounds on the viability of A549 lung cancer cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay is 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**–**3i** have no effects on the growth of A549 cells in dosage- and time-dependent manners in the test dosages (data not shown), however, all the nine compounds **4a**–**4i** had inhibitory effects on the growth of A549 cells in dosage- and time-dependent manners as indicated by the results in Figure 2. As typically shown in Figure 3, exposure of cells to **4b**, **4e**, and **4h** at 40 μM for 24 h resulted in cell viability decrease from 100% to 61%–52% ($p < 0.01$). When the exposure continued onto 48 h, compared with the control group, the cell viability reduced more significantly from 100% to 32%–26% ($p < 0.01$). Further, exposure of cells to **4b**, **4e**, and **4h** at 80 μM for 48 h, the cell viability reduced more significantly from 100% to 15%, 13%, and 6% ($p < 0.01$), respectively.

2.2.2. Structure–activity relationships and prediction of lipophilicity. Growth inhibitory properties (IC_{50}) for the compounds **4a**–**4i** are listed in Table 1. The data suggested that compounds **4b**–**4f** and **4h** could inhibit the cell growth obviously in the concentrations of 20–80 μM after 48 h of the treatment. From these results we could propose that the hydrazine group possessing basicity plays a more important role than the ester group in molecule. Furthermore, the compounds with *tert*-butyl group **4b**, **4e**, and **4h** were more effective to inhibit A549 cell growth, particularly compound **4e** was the most effective compound in suppressing A549 cell growth. By comparing the cytotoxic activities of the compounds **3a**–**3i** and **4a**–**4i**, it was shown that the cytotoxic potency was highly dependent, as expected, on the substitution types and patterns on the aryl ring. Replacement of the hydrogen at the 4-position of 3-aryl ring with electron-donating methoxy group and chlorine did not significantly alter the cytotoxicities against the cancer cells tested. However, replacing the hydrogen at the 4-position of 1-aryl ring with a bulkier *tert*-butyl group resulted in significant activity increasing (compounds **4b**, **4e**, and **4h**), which was probably caused by favorable steric interactions of the bulky hydrophobic alkyl group at 4-position of 1-aryl ring with the binding site. In another variation, we replaced the substituted benzene ring with 2-chloropyridine ring such as **4c** and **4f** and found that it significantly affected the activity compared with benzene and 4-chlorobenzene.

Lipophilicity is an important indicator of how easily molecules can cross cell membranes. It is generally accepted that the more lipophilic a molecule, the more favorably it will interact with the fatty acid tails of the lipid bilayer, thus allowing the molecule to traverse the membrane more easily. The partition coefficient log *P* is a parameter which describes the manner in which a drug partitions between polar and non-polar phases, and it has been demonstrated to be an indispensable tool in predicting the transport and activity of drugs.[28–31] Prediction of lipophilicity (log *P*) and aqueous solubility (log *S*) of compounds **4a**–**4i** was calculated using ALOGPS 2.1 software.[32,33] The compounds **4** showed lipophilicity with log *P* values in the range of 2.52–4.94 (Table 2) and aqueous solubility with log *S* values in the range of 1.50–41.20 mg/L. These studies indicate that compounds possessing the lipophilicity with log *P* values in the range of 3.12–4.94 have more inhibitory effects on the growth of A549 cells.

2.2.3. Effects of the compounds on the morphology of A549 cells. Morphological changes are associated with the physiological and pathological processes in A549 cells. The morphology of the cells treated with compounds **4b**, **4e**, and **4h** was studied to verify whether the cells underwent apoptosis. The obviously morphological changes of the cells treated with compounds **4b**,



Scheme 1. Synthesis of compounds **4a**–**4i**.

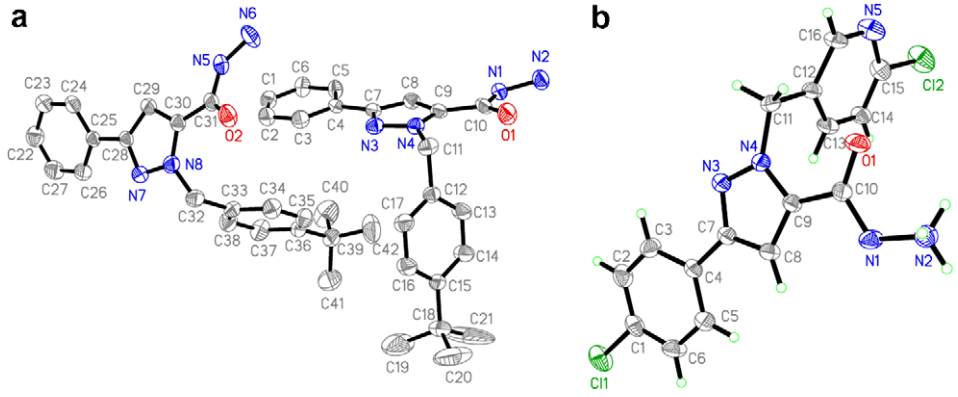


Figure 1. X-ray crystal structures of **4b** (a) and **4f** (b). Displacement ellipsoids are drawn at 50% probability level.

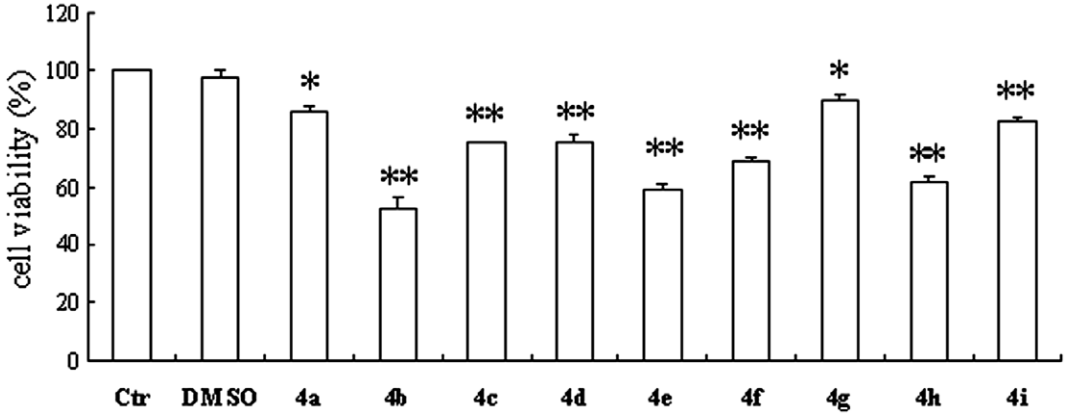


Figure 2. Effects of the compounds **4a**–**4i** on the viability of A549 lung cancer cells. Control (Ctr), the viability of the cells cultured in the medium without any compounds. DMSO, the viability of the cells cultured in the medium containing DMSO 0.1% (v/v) used as a vehicle control. Other bars show the viability of the cells treated with the compounds **4a**–**4i** at the concentration of 40 μM for 24 h.

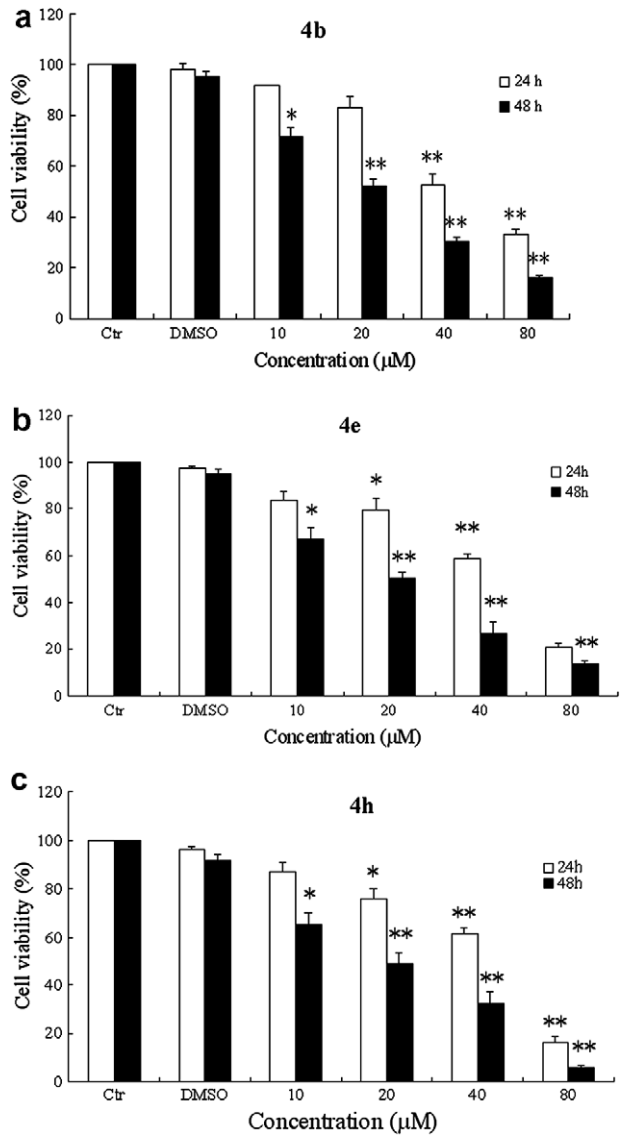


Figure 3. Effects of the compounds **4b**, **4e**, and **4h** on the viability of A549 lung cancer cells. Control (Ctr), the viability of the cells cultured in the medium without any compounds. DMSO, the viability of the cells cultured in the medium containing DMSO 0.1% (v/v) used as a vehicle control. Other bars show the viability of the cells treated with the compounds **4b**, **4e**, and **4h** at the concentrations of 10–80 μM for 24 and 48 h, respectively.

4e, and **4h** for 24 and 48 h were observed under a contrast phase microscope, as shown in Figure 4A and B. We found that the cells treated with compound **4e** at 40 μM for 24 h became shrinked, and the shrinkage is more remarkable in 48 h. Such morphological changes were not apparent in the control cells. The results told us the apoptosis should take place. It was confirmed by the analysis of DNA fragmentation and LDH activity assay.

2.2.4. Analysis of DNA fragmentation and LDH activity assay. DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing are the characteristics of apoptotic cells. The chromatin condensation and DNA fragmentation in the cells were observed by acridine orange (AO) staining under laser scanning confocal microscope. The results showed that obvious nuclear DNA fragmentation occurred in the cells treated with compounds **4b**, **4e**, and **4h**, respectively (Fig. 5). Such morphological changes were not apparent in the control cells. To confirm the mode of cell death induced by compounds **4b**, **4e**, and **4h** typically, LDH assay were performed on cells treated with or without **4b**, **4e** and **4h**. As shown in Figure 6, there were no obvious differences in LDH release between the cells in the control group (normal group) and the compound treatment groups. The results indicated that the compounds at the test range of concentrations did not cause necrosis in A549 cells. Thus, our results suggested that compounds **4b**, **4e**, and **4h** induced A549 cell apoptosis.

3. Conclusion

All of the compounds **4** could inhibit the growth of A549 cells in dosage- and time-dependent manners, typically compounds **4b**, **4e**, and **4h** induced A549 cells to apoptosis but did not cause necrosis in the cells. The study on structure–activity relationships and prediction of lipophilicities of compounds **4** showed that compounds with log *P* values in the range of 3.12–4.94 had more inhibitory effects on the growth of A549 cells.

4. Experimental

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, 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 re-

Table 1. Growth inhibitory properties IC_{50} (μM) for the compounds **4a**–**4i** at 24 and 48 h

Compound	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**	**4h**	**4i**
24 h	440.2	66.15	223.9	232.0	68.33	121.3	550.0	49.85	240.3
48 h	—	26.55	67.71	38.72	18.52	30.16	—	23.86	—

Table 2. Lipophilicity (log *P*) and aqueous solubility (log *S*) of compounds **4a**–**4i**

Compound	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**	**4h**	**4i**
log *P*	2.73	4.49	2.52	3.24	4.94	3.12	2.87	4.60	2.76
log *S*	41.20	3.06	30.53	11.66	1.50	15.60	26.80	3.07	28.78

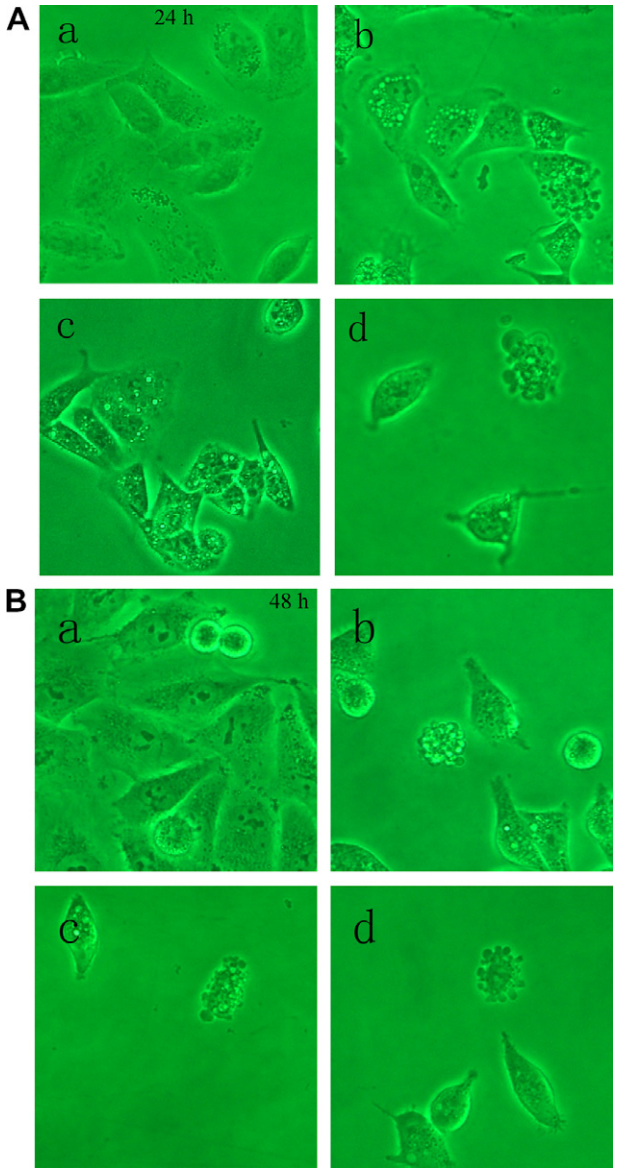


Figure 4. Effect of compounds **4b**, **4e**, and **4h** on A549 cell morphology for 24 h (A) and 48 h (B) at the concentration of 40 μM. The photographs were obtained by a phase contrast microscope (×800). (a) Control, (b) compound **4b**, (c) compound **4e**, (d) compound **4h**.

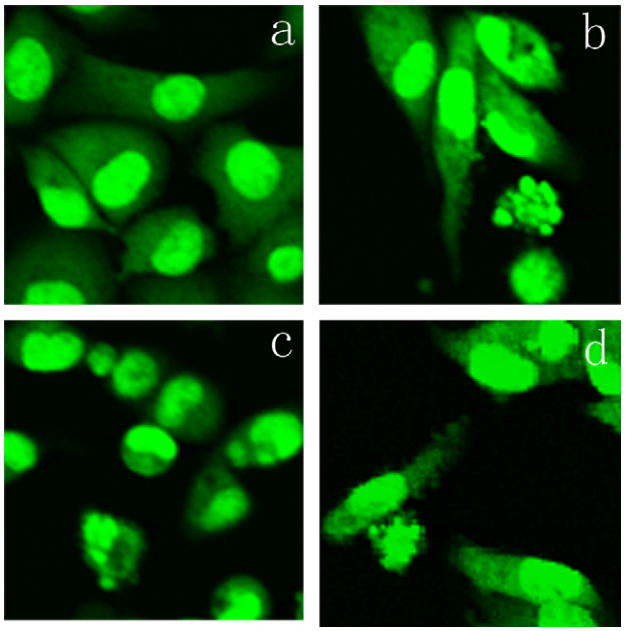


Figure 5. Effect of compounds **4b**, **4e**, and **4h** on A549 nuclear fragmentation. AO staining was performed after A549 cells were incubated with compounds **4b**, **4e**, and **4h** for 48 h. Then photographs were taken by a laser scanning confocal microscope (Leica, Germany). The pictures are representatives of three independent experiments. (a) Control, (b) compound **4b**, (c) compound **4e**, (d) compound **4h**.

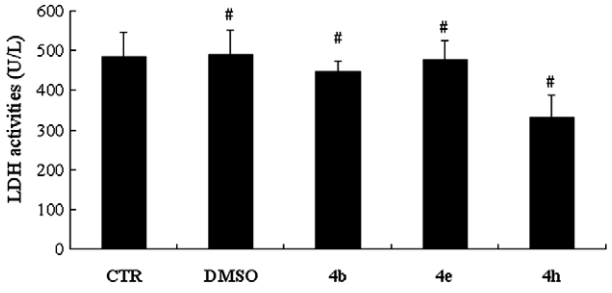


Figure 6. Effect of compounds **4b**, **4e**, and **4h** on LDH activities of A549 cells. The culture medium was collected as samples for LDH assay after 48 h treatment at the concentration of 40 μM. ($^{\#}p > 0.05$ vs control group, $n = 3$).

corded with an IR spectrophotometer Avtar 370 FT-IR (Termo Nicolet). Elemental analyses were performed on a Vario EL III (Elementar Analysensysteme GmbH) spectrometer. MS spectra were recorded on a Trace DSQ mass spectrograph. log *P* and log *S* were calculated using ALOGPS 2.1 software.

4.1. General procedure for the synthesis of 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives (4a–4i)

To a stirred solution of 1 mmol of **3a**–**3i** in methanol (5 ml), 1.2 ml of 80% hydrazine monohydrate was added. The reaction mixture was maintained under reflux for 1–5 h, until TLC indicated the end of reaction. After this time, the reaction mixture stood overnight and the solid formed was collected by filtration. Then the solid was recrystallized from ethanol. As a result of this process the following compounds were prepared in yields of 72–93% (**4a**–**4i**).

4.1.1. 1-Benzyl-3-phenyl-1*H*-pyrazole-5-carbohydrazide (4a). White solid, yield: 93%; mp: 132–133 °C; IR (KBr) *v*: 3302–3031 (NH, NH_2), 1681 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 4.03 (s, 2H, NH_2), 5.80 (s, 2H, CH_2), 6.80 (s, 1H, 4-H), 7.24–7.43 (m, 9H, ArH, NH), 7.79 (d, $J = 7.2$ Hz, 2H, ArH); ESI-MS: 293.5 $(M+H)^+$; Anal. Calcd for $C_{17}H_{16}N_4O$: C, 69.85; H, 5.52; N, 19.17. Found: C, 69.50; H, 5.25; N, 19.06.

4.1.2. 1-(4-*tert*-Butylbenzyl)-3-phenyl-1*H*-pyrazole-5-carbohydrazide (4b). White solid, yield: 76%; mp: 148–149 °C; IR (KBr) *v*: 3329–2865 (NH, NH_2), 1663

(C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.27 (s, 9H, $3CH_3$), 4.01 (s, 2H, NH_2), 5.77 (s, 2H, CH_2), 6.79 (s, 1H, 4-H), 7.24–7.43 (m, 8H, ArH, NH), 7.79 (d, $J = 7.8$ Hz, 2H, ArH); ESI-MS: 349.4 $(M+H)^+$; Anal. Calcd for $C_{21}H_{24}N_4O$: C, 72.39; H, 6.94; N, 16.08. Found: C, 72.16; H, 6.78; N, 15.96.

4.1.3. 1-((6-Chloropyridin-3-yl)methyl)-3-phenyl-1*H*-pyrazole-5-carbohydrazide (4c). White solid, yield: 86%; mp: 152–154 °C; IR (KBr) *v*: 3423–2944 (NH, NH_2), 1676 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 4.03 (s, 2H, NH_2), 5.79 (s, 2H, CH_2), 6.82 (s, 1H, 4-H), 7.26 (d, $J = 8.1$ Hz, 1H, PyH), 7.33–7.44 (m, 4H, ArH, NH), 7.68 (dd, $J = 2.1$, 8.1 Hz, 1H, PyH), 7.77 (d, $J = 7.2$ Hz, 2H, ArH), 8.45 (d, $J = 2.1$ Hz, 1H, PyH); ESI-MS: 328.5 $(M+H)^+$; Anal. Calcd for $C_{16}H_{14}ClN_5O$: C, 58.63; H, 4.31; N, 21.37. Found: C, 58.46; H, 4.39; N, 21.51.

4.1.4. 1-Benzyl-3-(4-chlorophenyl)-1*H*-pyrazole-5-carbohydrazide (4d). White solid, yield: 89%; mp: 180–182 °C; IR (KBr) *v*: 3266–2958 (NH, NH_2), 1630 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 3.98 (s, 2H, NH_2), 5.79 (s, 2H, CH_2), 6.77 (s, 1H, 4-H), 7.28–7.29 (m, 6H, ArH, NH), 7.37 (d, $J = 8.4$ Hz, 2H, ArH), 7.72 (d, $J = 8.4$ Hz, 2H, ArH); ESI-MS: 327.3 $(M+H)^+$; Anal. Calcd for $C_{17}H_{15}ClN_4O$: C, 62.48; H, 4.63; N, 17.15. Found: C, 62.52; H, 4.72; N, 17.06.

4.1.5. 1-(4-*tert*-Butylbenzyl)-3-(4-chlorophenyl)-1*H*-pyrazole-5-carbohydrazide (4e). White solid, yield: 79%; mp: 122–124 °C; IR (KBr) *v*: 3319–2860 (NH, NH_2), 1654 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.28 (s, 9H, $3CH_3$), 4.02 (s, 2H, NH_2), 5.75 (s, 2H, CH_2), 6.76 (s, 1H, 4-H), 7.24–7.31 (m, 3H, ArH, NH), 7.32 (d, $J = 8.4$, 2H, ArH), 7.37 (d, $J = 8.4$, 2H, ArH), 7.73 (d, $J = 8.4$ Hz, 2H, ArH); ESI-MS: 383.5 $(M+H)^+$; Anal. Calcd for $C_{21}H_{23}ClN_4O$: C, 65.87; H, 6.05; N, 14.63. Found: C, 65.66; H, 5.92; N, 14.32.

4.1.6. 3-(4-Chlorophenyl)-1-((6-chloropyridin-3-yl)methyl)-1*H*-pyrazole-5-carbohydrazide (4f). Yellow solid, yield: 84%; mp: 192–193 °C; IR (KBr) *v*: 3299-2936 (NH, NH_2), 1675 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 4.11 (s, 2 H, NH_2), 5.78 (s, 2H, CH_2), 6.78 (s, 1H, 4-H), 7.25 (s, 1H, NH), 7.27 (d, $J = 8.7$ Hz, 1H, PyH), 7.39 (d, $J = 8.7$ Hz, 2H, ArH), 7.70 (d, $J = 8.7$ Hz, 1H, PyH), 7.72 (d, $J = 8.7$ Hz, 2H, ArH), 8.45 (s, 1H, PyH); ESI-MS: 362.3 $(M+H)^+$; Anal. Calcd for $C_{16}H_{13}Cl_2N_5O$: C, 53.05; H, 3.62; N, 19.33. Found: C, 52.96; H, 3.72; N, 19.41.

4.1.7. 1-Benzyl-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide (4g). White solid, yield: 75%; mp: 142–144 °C; IR (KBr) *v*: 3306–2835 (NH, NH_2), 1673 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 3.84 (s, 3H, OCH_3), 4.01 (s, 2H, NH_2), 5.78 (s, 2H, CH_2), 6.72 (s, 1H, 4-H), 6.94 (d, $J = 8.4$ Hz, 2H, ArH), 7.23-7.28 (m, 6H, ArH, NH), 7.72 (d, $J = 8.4$ Hz, 2H, ArH); ESI-MS: 323.4 $(M+H)^+$; Anal. Calcd for $C_{18}H_{18}N_4O_2$: C, 67.07; H, 5.63; N, 17.38. Found: C, 66.86; H, 5.71; N, 17.26.

4.1.8. 1-(4-*tert*-Butylbenzyl)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide (4h). White solid, yield: 72%; mp: 142–144 °C; IR (KBr) *v*: 3325–2867 (NH, NH_2), 1656 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.27 (s, 9H, $3CH_3$), 3.84 (s, 3H, OCH_3), 4.01 (s, 2H, NH_2), 5.75 (s, 2H, CH_2), 6.71 (s, 1H, 4-H), 6.93 (d, $J = 8.4$ Hz, 2H, ArH), 7.26–7.32 (m, 5H, ArH, NH), 7.72 (d, $J = 8.4$ Hz, 2H, ArH); ESI-MS: 379.6 $(M+H)^+$; Anal. Calcd for $C_{22}H_{26}N_4O_2$: C, 69.82; H, 6.92; N, 14.80. Found: C, 69.29; H, 6.55; N, 14.71.

4.1.9. 1-((6-Chloropyridin-3-yl)methyl)-3-(4-methoxyphenyl)-1*H*-pyrazole-5-carbohydrazide (4i). Yellow solid, yield: 86%; mp: 157–159 °C; IR (KBr) *v*: 3410–2834 (NH, NH_2), 1669 (C=O) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 3.84 (s, 3H, OCH_3), 4.01 (s, 2H, NH_2), 5.76 (s, 2H, CH_2), 6.74 (s, 1H, 4-H), 6.94 (d, $J = 8.4$ Hz, 2H, ArH), 7.25 (d, $J = 7.8$ Hz, 1H, PyH), 7.42 (s, 1H, NH), 7.67 (d, $J = 7.8$ Hz, 1H, PyH), 7.70 (d, $J = 8.4$ Hz, 2H, ArH), 8.44 (s, 1H, PyH); ESI-MS: 358.5 $(M+H)^+$; Anal. Calcd for $C_{17}H_{16}ClN_5O_2$: C, 57.07; H, 4.51; N, 19.57. Found: C, 57.24; H, 4.62; N, 19.42.

4.2. Biological activity assay

4.2.1. Materials. RPMI 1640 was obtained from Gibco BRL Co. (Grand Island, USA) and bovine calf serum was from Beijing DingGuo Biotechnology Co. (China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco. Lactate dehydrogenase (LDH) assay kit was from ZhongSheng Co. (Beijing, China). Acridine orange (AO) from Fluka.

4.2.2. Cell culture. A549 lung cancer cells were cultured in RPMI 1640 medium, supplemented with 10% (v/v) newborn calf serum at 37 °C in 5% CO_2 and 95% air. The cells were routinely seeded at the density of 1000/cm^2 into 96-well plates or other appropriate dishes containing the medium.

4.2.3. MTT assay for cell viability. The compounds were dissolved in DMSO. The final concentration of DMSO was below 0.1% in the culture medium (v/v) (DMSO at these final concentrations did not affect the viability of the cells). Cells were seeded in 96-well plates and treated with compounds **4a**–**4i** 20–80 μM for 24 and 48, respectively. The cell viability was determined by the MTT assay following the procedure described by Price and McMillan.[34] The light absorptions were measured at 570 nm using SpectraMAX 190 microplate spectrophotometer (GMI Co., USA).

4.2.4. LDH assay for drug toxicity. The detection was performed as described previously.[35]

4.2.5. Nuclear fragmentation assay. Nuclear fragmentation was detected with AO staining and observed under a fluorescence microscope. Briefly, the cells were cultured in fresh medium with 0, 40 μM compounds **4b**, **4e**, and **4h**, respectively, for 48 h and stained with 5 μg/ml AO at room temperature. Then the cells were observed and photographed under an Olympus BH-2 fluorescence microscope.

4.2.6. Statistical analyses. Data were expressed as means ± SE, accompanied by the number of experiments performed independently, and analyzed by *t* test. Differences at $p < 0.05$ were considered statistically significant.

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