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Original article

Microwave-assisted synthesis, crystal structure of pyrazolo[1,5-*a*]pyrazin-4(5*H*)-ones and their selective effects on lung cancer cells

Ning Liu [a,1], Jin-Hua Zhang [b,1], Bao-Xiang Zhao [b,*], Jing Zhao [a,c], Le Su [a,c], Wen-Liang Dong [d], Shang-Li Zhang [a,c], Jun-Ying Miao [a,c,**]

[a] *Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Science, Shandong University, Jinan 250100, PR China*
[b] *Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, PR China*
[c] *The Key Laboratory of Experimental Teratology, Ministry of Education, Jinan 250012, PR China*
[d] *Shandong University of Traditional Chinese Medicine, Jinan 250355, PR China*

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ABSTRACT

A series of novel pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives with hydrophilic group was synthesized under general heating condition and microwave-assisted condition. The structures of compounds were determined by IR, ^{1}H NMR and HRMS, moreover, representative crystal structures were characterized by using X-ray diffraction analysis. Preliminary biological evaluation showed that some compounds could inhibit the growth of A549, H322 and H1299 cells in dosage dependent manners. The compounds could inhibit growth of A549, H322 and H1299 cells in different mechanism. Compounds **3e**–**h** inhibited growth of A549 cells by inducing a strong G1-phase arrest. Whereas these compounds inhibited growth of H1299 and H322 cells by inducing apoptosis.

1. Introduction

Lung cancer has been recognized to be one of the leading causes of death worldwide. Highly proliferative nature of cancer cells has undoubtedly been realized and consequently, inhibition of proliferative pathways is considered to be an effective strategy to fight cancer. Much attention has recently been paid to the discovery and development of new, more selective anticancer agents [1–3].

Apoptotic cell death regulators are currently considered to have significant potential as targets for cancer therapeutics although cell death can occur by mechanisms including necrosis, mitotic catastrophe and autophagy [4]. It is now recognized that one hallmark of cancer cells is their compromised ability to undergo apoptosis. Targeting critical apoptosis regulators is an attractive strategy for the development of new classes of therapies for the treatment of cancer and other human diseases.

Many pyrazole derivatives are known to exhibit a wide range of biological properties and fused-pyrazole derivatives have attracted considerable attention in the design and synthesis of potential anticancer agents [5–12]. However, a search of the literature revealed very few reports concerning pyrazolo-pyrazinones [13–15]. In our previous papers, we synthesized a series of novel fused-pyrazole derivatives including 6-(aroxymethyl)-2-aryl-6,7-dihydropyrazolo [5,1-*c*][1,4] oxazin-4-one derivatives, 5-alkyl-2-aryl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one and 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one [16–19]. The evaluation of biological activity showed that these compounds could inhibit A549 lung cancer cell growth. However, because the substituent in pyrazinone moiety was generally more hydrophobic, the modification of structure is needed to extend the diversity of fused-pyrazole skeleton and screen anticancer agents.

Microwave-assisted chemistry has blossomed into a useful technique for a variety of applications in organic synthesis. It has been demonstrated that the use of microwave heating can dramatically cut down reaction time, increase product purity and yields, and allow precise control of reaction conditions, all of which make it suited to meet the increased demands of high throughput chemistry [20–27]. In the previous paper, for example, we reported the microwave-assisted synthesis of pyrazole-fused pyrazinone

* Corresponding author. Tel.: +86 531 88366425; fax: +86 531 88564464.
** Corresponding author. Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, School of Life Science, Shandong University, Jinan 250100, PR China.
E-mail addresses: bxzhao@sdu.edu.cn, sduzhao@hotmail.com (B.-X. Zhao), miaojy@sdu.edu.cn (J.-Y. Miao).
[1] Equal contribution.

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derivatives, in which it is fast 3–10 fold than general heating method although it took 1–6 h to finish reaction [18].

Herein, we would like to report the microwave-assisted synthesis, structural characterization and preliminary biological evaluation of novel pyrazole-fused pyrazinone derivatives modified with hydrophilic group, such as hydroxyl group.

2. Chemistry

2.1. Synthesis

The synthesis of pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives (**3**) has been accomplished as outlined in Scheme 1 starting from ethyl 3-aryl-1-(2-bromoethyl)-1*H*-pyrazole-5-carboxylate (**2**) and amine. Firstly, the *N*-alkylation reaction of ethyl 3-aryl-1*H*-pyrazole-5-carboxylate (**1**) with excess 1,2-dibromoethane was achieved in the presence of potassium carbonate in acetonitrile according to our previous report [17]. After flash chromatography on silica gel, the ethyl 3-aryl-1-(2-bromoethyl)-1*H*-pyrazole-5-carboxylate (**2**) was obtained. Thus, for example, 5-(2-hydroxyethyl)-2-phenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (**3a**) was synthesized in 99% yield by the reaction of ethyl 1-(2-bromoethyl)-3-phenyl-1*H*-pyrazole-5-carboxylate (**2a**) with ethanolamine in acetonitrile over a 3 h reflux period. The structures of pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (**3**) were determined by IR, ^{1}H NMR and HRMS. For example, 2-(4-chlorophenyl)-5-(2-morpholinoethyl)-6,7-dihydropyrazolo [1,5-*a*]pyrazin-4(5H)-one (**3h**), obtained in 58% yield as white crystal, gave a [M + H]-ion peak at *m*/*z* 361.1435 in the HRESI-MS, in accord with the molecular formula $C_{18}H_{22}ClN_4O_2$. In the IR spectra, the carbonyl group absorption was observed in the 1653 cm^{-1}. The ^{1}H NMR spectra indicated two *ortho*- aromatic protons signals in 4-chlorobenzene moiety appeared at the range of $\delta = 7.74$ and 7.39 ppm, respectively, as doublet peaks with coupling constant 8.4 Hz. A proton signal in pyrazole moiety appeared at 7.11 as singlet peak. The chemical shift of the protons in methylenes (7-CH_2 and 6-CH_2) appeared at $\delta = 4.43$ and 3.89 as triplet peaks ($J = 6.2$ Hz), respectively. Other signals are consist with the structure although it is difficulty to attribute to assigned protons.

We focused our attention on the microwave-assisted synthesis technique after obtaining compounds **3** by classical heating method in order to shorten reaction time and free solvent. These reactions are performed in a modified domestic microwave oven due to its low cost and ready availability. In a typical experiment, ethyl 3-aryl-1-(2-bromoethyl)-1*H*-pyrazole-5-carboxylate (**2**) and amine were mixed in a flask, and irradiated for 1–2 min. After work-up, desired compounds **3** were obtained. Comparing two methods, microwave-assisted synthesis technique dramatically cut down reaction time, increase product yields as shown in Table 1. The yields of compounds **3** were depended on the structure of amine regardless of classical heating or microwave-assisted. Microwave-assisted reaction is fast 45–240 fold than general heating method.

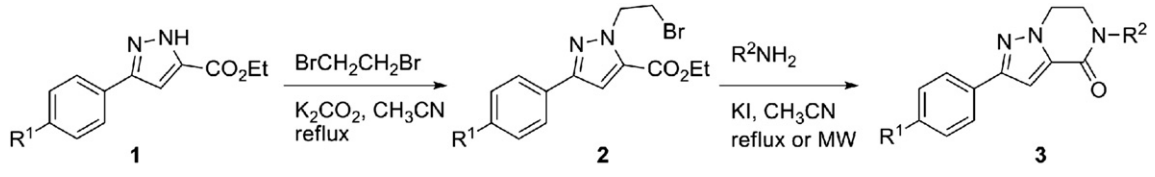
2.2. Single-crystal structural characterization by X-ray

The spatial structures of compounds **3h** and **3j** were determined by using X-ray diffraction analysis. The single crystals were grown from ethyl acetate solution at room temperature. The molecular views of **3h** and **3j** are shown in Figs. 1 and 2.

The molecule of **3h** consists of four fragments, a planar pyrazole ring, aryl ring bonded to pyrazole, pyrazinone ring and 2-morpholinoethyl group. An optimal electronic overlap of the π-system demands a coplanar arrangement. Indeed, the pyrazole ring and pyrazinone ring are approximately coplanar besides C12 with 0.326 Å deviation. The plane makes dihedral angle of 5.79(11)° with the 4-chlorophenyl ring. The morpholine ring is in a chair conformation. In compound **3j**, which is a close analogue of **3h**, the pyrazole ring and pyrazinone ring are approximately coplanar besides C7 with 0.385 Å deviation. The plane makes dihedral angles of 13.37(14)° with methoxyphenyl ring.

3. Pharmacology

Screening of synthesized substances was carried out using lung cancer A549, H322 and H1299 cell lines, which are p53 wild-type, p53-mutant and p53-null cells, respectively. Proliferation percentage was determined by the SRB assay [28,29]. Cells were incubated with substances at 8, 16, 32 and 64 μM for 48 h and the cell proliferation/viability determination using the survival percentage obtained with the cell treated only with the solvent (DMSO at 0.1%) as reference. The results are expressed as the average of triplicate assays. Effect factors related to antiproliferation such as apoptosis and cell cycle distribution were determined.



For **1** and **2**
a: R^1 = H
b: R^1 = Cl
c: R^1 = OMe

For R^2NH_2
a: R^2 = $HOCH_2CH_2$
b: R^2 = $HOCH_2CH_2CH_2$
c: R^2 = $HOCH_2CH_2OCH_2CH_2$
d: R^2 = $O(CH_2CH_2)_2NCH_2CH_2$

3a: R^1 = H, R^2 = $HOCH_2CH_2$
3b: R^1 = H, R^2 = $HOCH_2CH_2CH_2$
3c: R^1 = H, R^2 = $HOCH_2CH_2OCH_2CH_2$
3d: R^1 = H, R^2 = $O(CH_2CH_2)_2NCH_2CH_2$
3e: R^1 = Cl, R^2 = $HOCH_2CH_2$
3f: R^1 = Cl, R^2 = $HOCH_2CH_2CH_2$
3g: R^1 = Cl, R^2 = $HOCH_2CH_2OCH_2CH_2$
3h: R^1 = Cl, R^2 = $O(CH_2CH_2)_2NCH_2CH_2$
3i: R^1 = OMe, R^2 = $HOCH_2CH_2$
3j: R^1 = OMe, R^2 = $HOCH_2CH_2CH_2$
3k: R^1 = OMe, R^2 = $HOCH_2CH_2OCH_2CH_2$
3l: R^1 = OMe, R^2 = $O(CH_2CH_2)_2NCH_2CH_2$

Scheme 1. Synthesis of pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives.

Table 1
Yields of compounds **3a**–**l** in the condition of classical heating and microwave-assisted.

Compound **3**	General heating		Microwave	
	Time (min)	Yield (%)	Time (min)	Yield (%)
a	180	98	1	94
b	210	98	1	90
c	180	85	1	91
d	120	88	1	99
e	240	88	1	86
f	180	86	1	99
g	180	78	1	89
h	120	58	1	99
i	240	96	1	99
j	150	87	1	97
k	180	90	1	99
l	90	56	2	80

4. Results and discussion

4.1. Effects of the compounds on the viability of A549, H322, H1299 cells and normal human embryo lung cells

Firstly, cell viability of A549 cells treated with compounds **3a**–**l** was analyzed by SRB assay and the results showed that compounds **3e**–**h** exhibited an inhibitory effect on the proliferation of A549 cells in dose-dependent manner at 48 h (Fig. 3). However, other compounds did not exhibit effect on the cell viability. Thus, compounds **3e**–**h** were chosen to examine their effects on H322, H1299 cells and normal human embryo lung cells. The results indicated that these compounds had similar effects on the proliferation of H322 and H1299 cells in dose-dependent manner at 48 h as shown in Figs. 4 and 5, but had no effect on growth of human embryo lung cells (Fig. 6). In our previous paper, we reported that 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4 (5*H*)-one derivatives could inhibit A549 cell growth obviously in the concentrations of 5–40 μM after 48 h treatment and the cytotoxic potency was highly dependent on the substitution types and patterns on the pyrazinone ring, for example, replacing the alkyl at the *N*-position of pyrazinone ring with an arylalkyl group resulted in a significant activity increasing [17,18]. In the present study, we observed that the nature and the position of substituent on the molecule improved effectively biological functions. From Fig. 3 it can be found that compounds **3e**–**h** with 4-chlorophenyl group were more effective in dosage dependent manners, whereas, compounds **3a**–**d** with phenyl and compounds **3i-l** with methoxylphenyl group had no effects on the proliferation of A549 cells.

4.2. Compounds *3e*–*h* induced apoptosis in H322 and H1299 cells but did not in A549 lung cancer cells

To detect whether compounds **3e**–**h** resulted in apoptosis of the cells, we performed Hoechst 33258 staining assay. It is well known that 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 determined by Hoechst 33258 staining under a fluorescence microscope. It was interestingly found that compounds **3e**–**h** could induce apoptosis in H322 and H1299 cells, which are p53-mutant and p53-null cells, respectively, as shown in Figs. 7 and 8. However, from Fig. 9 it can be found that compounds **3e**–**h** did not induce apoptosis in A549 cells which are p53 wild-type cells. Thus, mechanism of compounds **3e**–**h** induced apoptosis of H322 and H1299 cell lines remains unclear. Pathway of apoptosis should be worthy to be investigated in the future.

4.3. Flow cytometry analysis of cell cycle distribution

In order to analyze effects of compounds **3e**–**h** on proliferation of A549 cells, the cell cycle progression was next investigated. It is well known that cell cycle dysregulation is a hallmark of tumor cells. The eukaryotic cell cycle consists of alternating rounds of DNA replication (S phase) and cell division (M phase) separated by the gap phases G0, G1 and G2. Regulation of proteins that mediate critical events of the cell cycle may be a useful anti-tumor target [30,31]. In order to get a more precise insight of growth inhibition, flow cytometry analysis was taken to detect whether compounds **3e**–**h** induce cell cycle arrest. The results showed that compounds **3f** and **3h** treatment with 64 μM at 48 h induced G1-phase arrest effectively (Fig. 10). After 48 h treatment with 64 μM, **3f** or **3h**, the G1 population was noticeably enhanced by 32% and 27%, respectively, compared with control. In the case of compounds **3e** and **3g**, the G1 population was enhanced by 18% and 13%, respectively. The increase in the G1-phase cell population was accompanied by a decrease in the S and G2-phase cell populations. The fact that compounds **3e**–**h** induced a strong G1-phase arrest is concomitant with the growth inhibitory effect.

4.4. Necrosis detection by LDH activity assay

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, LDH assay were performed on cells treated with compounds **3e**–**h** or 0.1% DMSO (as control). As shown in Fig. 11 for A549 cells, there were no significant differences in LDH release between the cells in the control group and the compounds treatment group. The results indicated that the compounds at the test range of concentration did not cause necrosis in the cells.

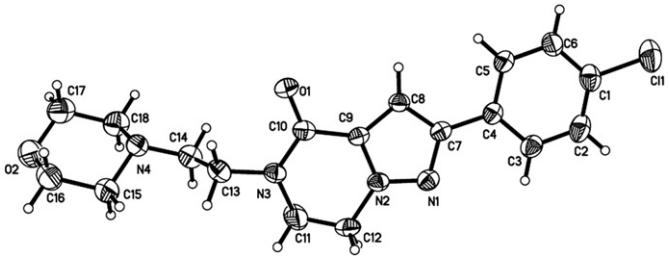


Fig. 1. The molecular structure of compound **3h**, with displacement ellipsoids drawn at the 50% probability level.

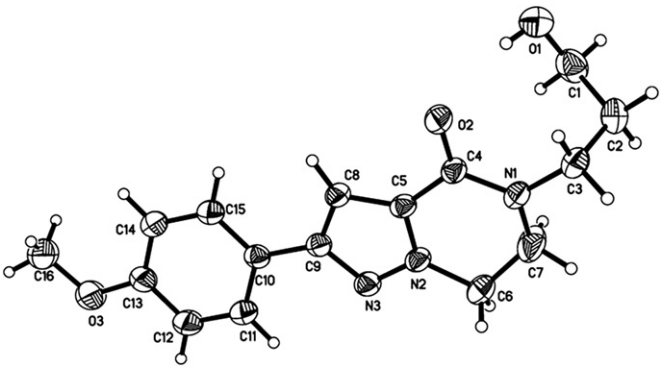


Fig. 2. The molecular structure of compound **3j**, with displacement ellipsoids drawn at the 50% probability level.

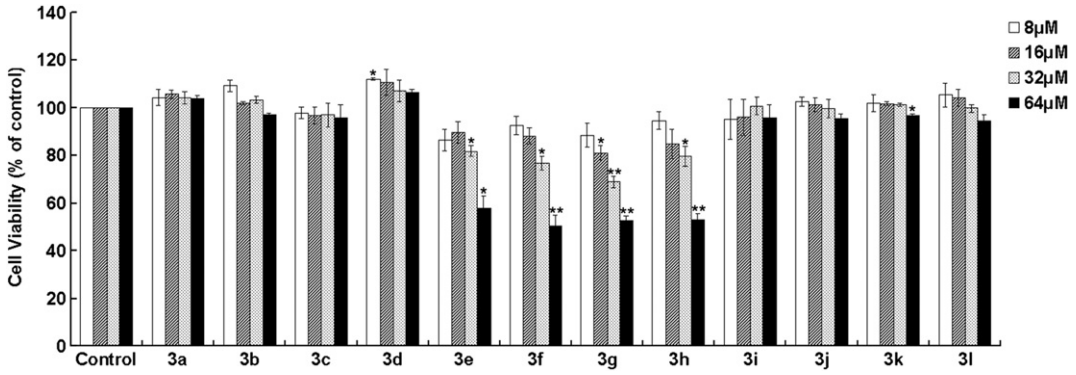


Fig. 3. Effects of the compounds on A549 cell viability. A549 cells were treated with compounds **3a**–**l** or 0.1% DMSO (control) for 48 h at the concentration of 8, 16, 32 or 64 μM. Cell viability was analyzed by SRB assay. Data are means ± SE from three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control).

5. Conclusion

In summary, we have described a facile approach to prepare pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives **3a**–**l** by the reaction of ethyl 3-aryl-1-(2-bromoethyl)-1*H*-pyrazole-5-carboxylate with amine under the general heating condition and the microwave-assisted condition. Microwave-assisted reaction is fast 45–240 fold than general heating method. We also studied the representative single-crystal structural characterization of the compounds **3h** and **3j** by X-ray that should be valuable for further investigation. We found that compounds **3e**–**h** with chlorophenyl group in pyrazole moiety could suppress A549, H322 and H1299 cells growth. The compounds might perform their action through inducing a strong G1-phase arrest rather than apoptosis for A549 cell, whereas by inducing apoptosis for H322 and H1299 cells. Mechanism of compounds **3e**–**h** induced apoptosis of H322 and H1299 cell lines should be worthy to be investigated in the future.

6. Experimental

6.1. 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 400 (400 MHz) spectrometer, using $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Melting points were determined on an XD-4 digital micro melting point apparatus and 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 Apex2CCD diffractometer.

6.2. General procedure for the synthesis of pyrazolo[1,5-a]pyrazin-4 (5H)-one derivatives (*3a*–*l*) by classical heating technique

The intermediates **2** were prepared according to the previous paper [17,18]. A mixture of **2** (1 mmol), amine (10 mmol) and potassium iodide (0.2 mmol) in acetonitrile (30 ml) was refluxed under nitrogen for several hours (shown as Table 1). Then the mixture was cooled, filtered, and the solvent was removed by evaporating under reduced pressure. The products **3** were obtained by column chromatography on silica gel using ethyl acetate as eluent.

6.3. General procedure for the synthesis of pyrazolo[1,5-a]pyrazin-4 (5H)-one derivatives (*3a*–*l*) by microwave-assisted technique

A mixture of **2** (1 mmol), amine (10 mmol) and potassium iodide (0.2 mmol) was irradiated with high power region (700 W) in free solvent for 1–2 min (shown as Table 1). After work-up as the same with classical method, the products **3** were obtained.

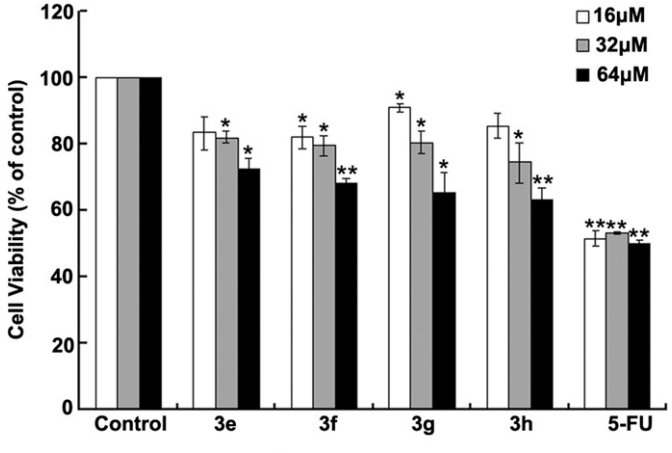


Fig. 4. Effects of the compounds on H322 cell viability. H322 cells were treated with compounds **3e**–**h** or 0.1% DMSO (control) for 48 h at the concentration of 16, 32 and 64 μM. Cell viability was analyzed by SRB assay. Data are means ± SE from three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control).

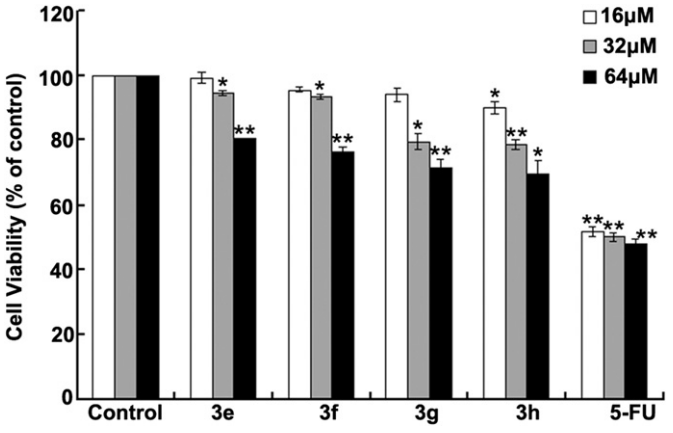


Fig. 5. Effects of the compounds on H1299 cell viability. H1299 cells were treated with compounds **3e**–**h** or 0.1% DMSO (control) for 48 h at the concentration of 16, 32 or 64 μM. Cell viability was analyzed by SRB assay. Data are means ± SE from three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control).

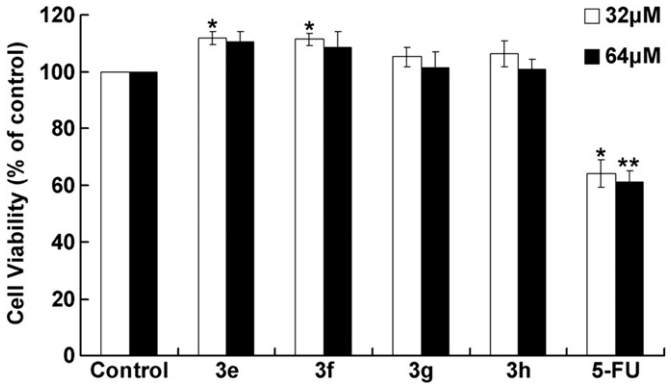


Fig. 6. Effects of the compounds on human embryo lung cells viability. Cells were treated with compounds **3e**–**h**, 5-FU (positive control) or 0.1% DMSO (vehicle control) for 48 h at the concentration of 32 and 64 μM. Cell viability was analyzed by SRB assay. Data are means ± SE from three independent experiments ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control).

6.4. Spectral data of compounds ***3a***–***l***

6.4.1. 5-(2-Hydroxyethyl)-2-phenyl-6,7-dihydropyrazolo[1,5-a] pyrazin-4(5H)-one (***3a***)

White solid, mp: 109–112 °C; IR (KBr) ν: 3380 (OH), 1638 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.80 (d, $J = 8.0$ Hz, 2H, ArH), 7.41 (t, $J = 7.5$ Hz, 2H, ArH), 7.33 (t, $J = 7.4$ Hz, 1H, ArH), 7.14 (s, 1H, 3-H), 4.45 (t, $J = 6.2$ Hz, 2H, CH_2), 3.89–3.95 (m, 4H, CH_2), 3.75 (t, $J = 5.1$ Hz, 2H, CH_2); HRMS *m/z* calcd for $C_{14}H_{16}N_3O_2$ $[M+H]^+$ 258.1243; Found: 258.1239. Calcd for $C_{14}H_{15}N_3O_2Na$ $[M+Na]^+$: 280.1062, Found: 280.1055.

6.4.2. 5-(3-Hydroxypropyl)-2-phenyl-6,7-dihydropyrazolo[1,5-a] pyrazin-4(5H)-one (***3b***)

White solid, mp: 179–181 °C; IR (KBr) ν: 3401 (OH), 1635 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.79 (d, $J = 7.7$ Hz, 2H, ArH), 7.40 (t, $J = 7.4$ Hz, 2H, ArH), 7.33 (t, $J = 7.3$ Hz, 1H, ArH), 7.19 (s, 1H, 3-H), 4.46 (t, $J = 6.2$ Hz, 2H, CH_2), 3.81 (t, $J = 6.2$ Hz, 2H, CH_2), 3.76 (t, $J = 6.1$ Hz, 2H, CH_2), 3.65 (t, $J = 5.4$ Hz, 2H, CH_2), 1.83–1.89 (m, 2H, CH_2); HRMS *m/z* calcd for $C_{15}H_{18}N_3O_2$ $[M+H]^+$ 272.1399; Found: 272.1397. Calcd for $C_{15}H_{17}N_3O_2Na$ $[M+Na]^+$: 294.1219, Found: 294.1220.

6.4.3. 5-(2-(2-Hydroxyethoxy)ethyl)-2-phenyl-6,7-dihydropyrazolo [1,5-a]pyrazin-4(5H)-one (***3c***)

White solid, mp: 97–99 °C; IR (KBr) ν: 3429 (OH), 1637 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.79 (d, $J = 7.7$ Hz, 2H, ArH), 7.40 (t, $J = 7.1$ Hz, 2H, ArH), 7.33 (t, $J = 7.5$ Hz, 1H, ArH), 7.13 (s, 1H, 3-H), 4.42 (t, $J = 6.2$ Hz, 2H, CH_2), 3.93 (t, $J = 6.2$ Hz, 2H, CH_2), 3.75–3.81 (m, 6H, CH_2), 3.65 (t, $J = 4.4$ Hz, 2H, CH_2); HRMS *m/z* calcd for $C_{16}H_{20}N_3O_3$ $[M+H]^+$ 302.1505; Found: 302.1500. Calcd for $C_{16}H_{19}N_3O_3Na$ $[M+Na]^+$: 324.1324, Found: 324.1325.

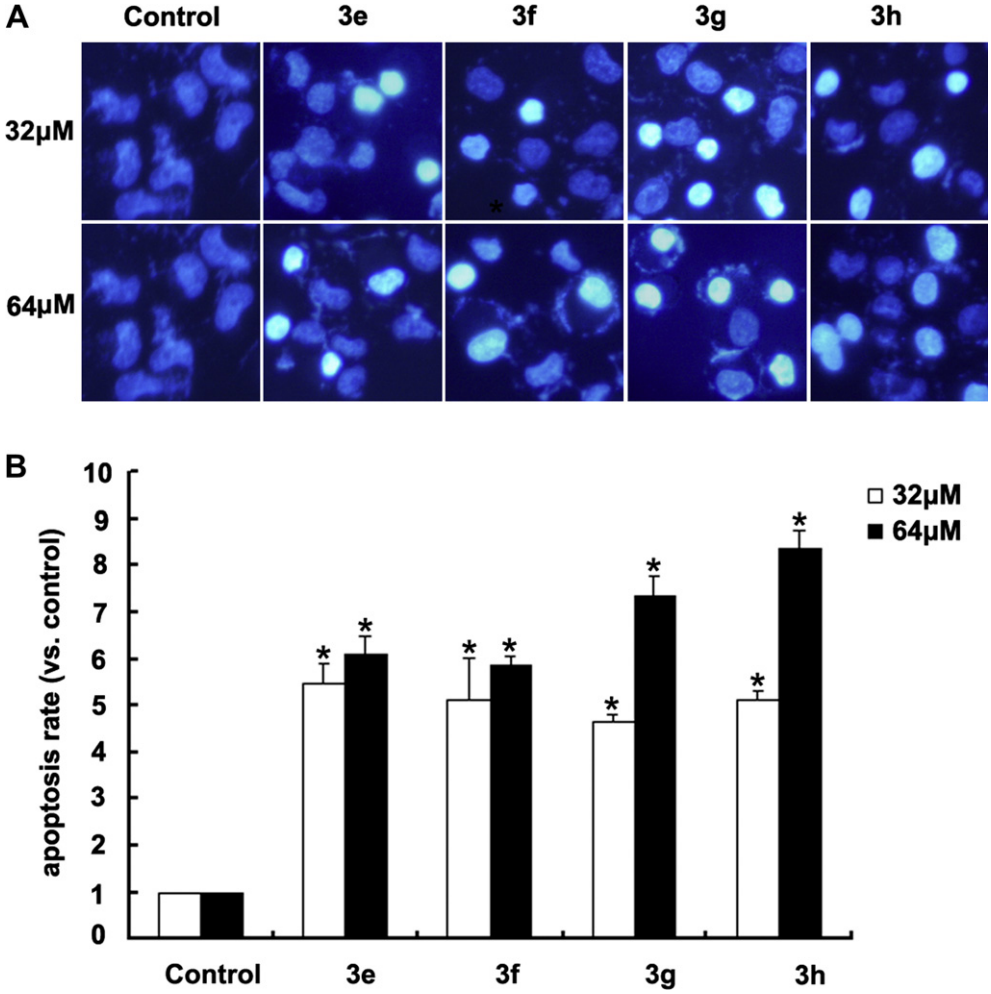


Fig. 7. Compounds **3e**–**h** induced apoptosis in H322 cell. Cells were treated with the compounds or 0.1% DMSO (control) for 48 h at the concentration of 32 or 64 μM, and then were stained with Hoechst 33258. The apoptosis rate calculated based on Hoechst 33258 staining, at least 500 cells were counted ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control).

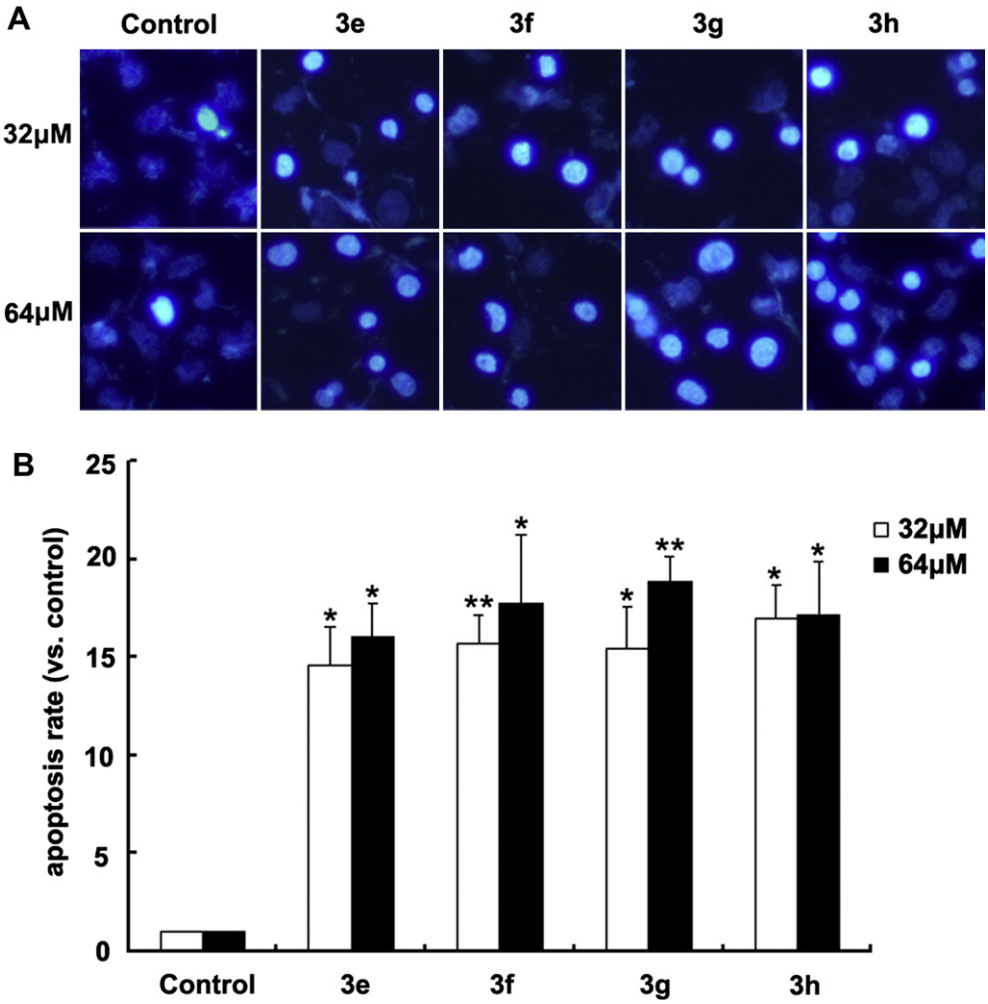


Fig. 8. Compounds **3e**–**h** induced apoptosis in H1299 cell. Cells were treated with the compounds or 0.1% DMSO (control) for 48 h at the concentration of 32 or 64 μM, and then were stained with Hoechst 33258. The apoptosis rate calculated based on Hoechst 33258 staining, at least 500 cells were counted ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control).

*6.4.4. 5-(2-Morpholinoethyl)-2-phenyl-6,7-dihydropyrazolo[1,5-a] pyrazin-4(5H)-one (**3d**)*

White solid, mp: 189–192 °C; IR (KBr) ν: 1654 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.80 (d, $J = 7.5$ Hz, 2H, ArH), 7.42 (t, $J = 7.1$ Hz, 2H, ArH), 7.34 (t, $J = 7.3$ Hz, 1H, ArH), 7.14 (s, 1H, 3-H), 4.44 (t, $J = 6.1$ Hz, 2H, CH_2), 3.89 (t, $J = 6.1$ Hz, 2H, CH_2), 3.65–3.75 (m, 6H, CH_2), 2.60–2.70 (m, 2H, CH_2), 2.50–2.60 (m, 4H, CH_2); HRMS *m/z* calcd for $C_{18}H_{23}N_4O_2$ $[M+H]^+$: 327.1821, Found: 327.1821.

*6.4.5. 2-(4-Chlorophenyl)-5-(2-hydroxyethyl)-6,7-dihydropyrazolo [1,5-a]pyrazin-4(5H)-one (**3e**)*

White solid, mp: 131–132 °C; IR (KBr) ν: 3394 (OH), 1636 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.73 (d, $J = 7.7$ Hz, 2H, ArH), 7.38 (d, $J = 7.7$ Hz, 2H, ArH), 7.11 (s, 1H, 3-H), 4.45 (t, $J = 6.1$ Hz, 2H, CH_2), 3.91–3.96 (m, 4H, CH_2), 3.76 (t, $J = 5.0$ Hz, 2H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 46.19, 47.33, 49.73, 61.59, 104.63, 126.89 (2C), 128.98 (2C), 131.02, 134.05, 135.65, 150.94, 158.70; HRMS *m/z* calcd for $C_{14}H_{15}ClN_3O_2$ $[M+H]^+$: 292.0853, Found: 292.0848.

*6.4.6. 2-(4-Chlorophenyl)-5-(3-hydroxypropyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one (**3f**)*

White solid, mp: 123–126 °C; IR (KBr) ν: 3381 (OH), 1646 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.73 (d, $J = 8.4$ Hz, 2H, ArH), 7.39 (d, $J = 8.4$ Hz, 2H, ArH), 7.12 (s, 1H, 3-H), 4.45 (t, $J = 6.2$ Hz, 2H, CH_2), 3.82 (t, $J = 6.2$ Hz, 2H, CH_2), 3.75 (t, $J = 6.2$ Hz, 2H, CH_2), 3.62 (t, $J = 5.6$ Hz, 2H, CH_2), 1.81–1.87 (m, 2H, CH_2); HRMS *m/z* calcd for $C_{15}H_{17}ClN_3O_2$ $[M+H]^+$: 306.1009, Found: 306.1005.

*6.4.7. 2-(4-Chlorophenyl)-5-(2-(2-hydroxyethoxy)ethyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one (**3g**)*

White solid, mp: 90–93 °C; IR (KBr) ν: 3385 (OH), 1668 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.73 (d, $J = 6.7$ Hz, 2H, ArH), 7.38 (d, $J = 6.7$ Hz, 2H, ArH), 7.11 (s, 1H, 3-H), 4.42 (t, $J = 6.2$ Hz, 2H, CH_2), 3.94 (t, $J = 6.2$ Hz, 2H, CH_2), 3.73–3.80 (m, 6H, CH_2), 3.61 (t, $J = 4.4$ Hz, 2H, CH_2); HRMS *m/z* calcd for $C_{16}H_{19}ClN_3O_3$ $[M+H]^+$: 336.1115; Found: 336.1103. Calcd for $C_{16}H_{18}ClN_3O_3Na$ $[M+Na]^+$: 358.0935, Found: 358.0918.

*6.4.8. 2-(4-Chlorophenyl)-5-(2-morpholinoethyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one (**3h**)*

White solid, mp: 191–193 °C; IR (KBr) ν: 1653 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.74 (d, $J = 8.4$ Hz, 2H, ArH), 7.39 (d, $J = 8.4$ Hz, 2H, ArH), 7.11 (s, 1H, 3-H), 4.43 (t, $J = 6.2$ Hz, 2H, CH_2), 3.89 (t, $J = 6.2$ Hz, 2H, CH_2), 3.68–3.73 (m, 6H, CH_2), 2.63 (t, $J = 6.2$ Hz, 2H, CH_2), 2.51–2.57 (m, 4H, CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 43.20, 46.19, 46.31, 53.12, 53.72, 56.35, 66.73, 66.84, 104.55, 126.88 (2C), 128.98 (2C), 131.11, 134.01, 135.82, 150.92, 157.75; HRMS *m/z* calcd for $C_{18}H_{22}ClN_4O_2$ $[M+H]^+$: 361.1431, Found: 361.1435.

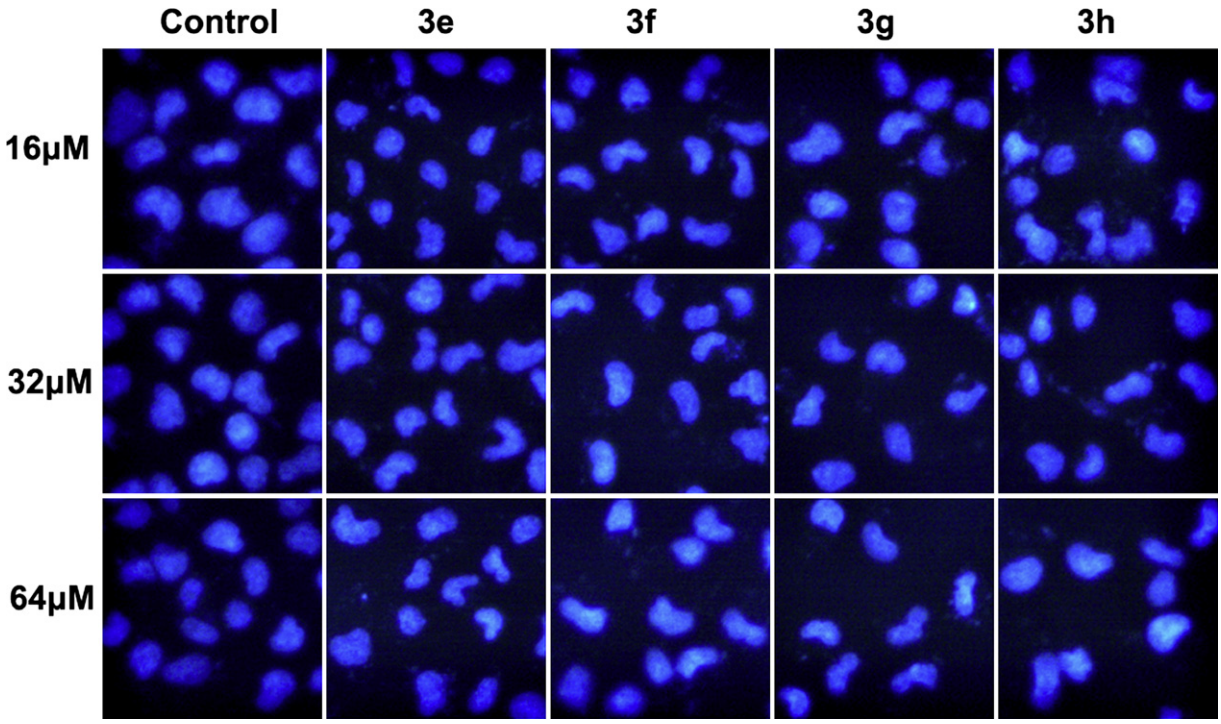


Fig. 9. Compounds **3e**–**h** did not induce apoptosis in A549 cell at 48 h significantly. Cells were treated with the compounds or 0.1% DMSO (control) for 48 h at the concentration of 16, 32 or 64 μM, and then were stained with Hoechst 33258. Fluorescent micrographs of Hoechst 33258 staining (200×) were taken under a fluorescent microscope (Nikon).

*6.4.9. 5-(2-Hydroxyethyl)-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one (**3i**)*

White solid, mp: 145–147 °C; IR (KBr) ν: 3400 (OH), 1640 (C═O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.72 (d, $J = 6.8$ Hz, 2H, ArH), 7.08 (s, 1H, 3-H), 6.94 (d, $J = 6.8$ Hz, 2H, ArH), 4.44 (t, $J = 6.2$ Hz, 2H, CH_2), 3.90–3.94 (m, 4H, CH_2), 3.84 (s, 3H, OCH_3), 3.76 (t, $J = 5.0$ Hz, 2H, CH_2); HRMS *m/z* calcd for $C_{15}H_{18}N_3O_3$ $[M + H]^+$: 288.1348. Found: 288.1342.

*6.4.10. 5-(3-Hydroxypropyl)-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one (**3j**)*

White solid, mp: 113–116 °C; IR (KBr) ν: 3409 (OH), 1632 (C═O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.73 (d, $J = 7.8$ Hz, 2H, ArH), 7.08 (s, 1H, 3-H), 6.95 (d, $J = 6.8$ Hz, 2H, ArH), 4.44 (t, $J = 6.2$ Hz, 2H, CH_2), 3.85 (s, 3H, OCH_3), 3.81 (t, $J = 6.2$ Hz, 2H, CH_2), 3.75 (t, $J = 6.1$ Hz, 2H, CH_2), 3.59–3.64 (m, 2H, CH_2), 3.41 (s, 1H, OH), 1.81–1.86 (m, 2H, CH_2); HRMS *m/z* calcd for $C_{16}H_{20}N_3O_3$ $[M + H]^+$: 302.1505; Found: 302.1502; Calcd for $C_{16}H_{19}N_3O_3Na$ $[M + Na]^+$: 324.1324, Found: 324.1323.

*6.4.11. 5-(2-(2-Hydroxyethoxy)ethyl)-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one (**3k**)*

White solid, mp: 103–106 °C; IR (KBr) ν: 3361 (OH), 1634 (C═O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.68 (d, $J = 8.4$ Hz, 2H, ArH), 7.00 (s, 1H, 3-H), 6.92 (d, $J = 8.4$ Hz, 2H, ArH), 4.34 (t, $J = 5.96$ Hz, 2H, CH_2), 3.82–3.92 (m, 8H, CH_2), 3.81 (s, 3H, OCH_3), 3.70–3.75 (m, 2H, CH_2); HRMS *m/z* calcd for $C_{17}H_{22}N_3O_4$ $[M + H]^+$: 332.1610; Found: 332.1609; Calcd for $C_{17}H_{21}N_3O_4Na$ $[M + Na]^+$: 354.1430, Found: 354.1428.

*6.4.12. 2-(4-Methoxyphenyl)-5-(2-morpholinoethyl)-6,7-dihydropyrazolo[1,5-a]pyrazin-4(5H)-one (**3l**)*

White solid, mp: 195–196 °C; IR (KBr) ν: 1653 (C═O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.73 (d, $J = 8.7$ Hz, 2H, ArH), 7.06 (s, 1H, 3-H), 6.95 (d, $J = 8.7$ Hz, 2H, ArH), 4.41 (t, $J = 6.1$ Hz, 2H, CH_2), 3.86 (t, $J = 6.3$ Hz, 2H, CH_2), 3.84 (s, 3H, OCH_3), 3.68–3.72 (m, 6H, CH_2), 2.62 (t, $J = 6.3$ Hz, 2H, CH_2), 2.53 (t, $J = 4.1$ Hz, 4H, CH_2); HRMS *m/z* calcd for $C_{19}H_{25}N_4O_3$ $[M + H]^+$: 357.1926, Found: 357.1929.

6.5. Cell culture

A549, H322 and H1299 lung cancer cells and human embryo lung 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.

6.6. Cell viability assay

Cells were seeded onto 96-well plates and treated with compounds **3a**–**l** at 8, 16, 32 and 64 μM for 48 h, respectively. The cell proliferation/viability determination using the survival percentage obtained with the cell treated only with the solvent (DMSO at 0.1%) as reference. The results are expressed as the average of triplicate assays.

6.7. LDH assay

Lactate dehydrogenase (LDH) assay was performed on cells treated with 64 μM compounds **3e**–**h**, for 48 h using a LDH kit (Nanjing Jiancheng, China) according to the manufacturer's protocol. Light absorption was measured at 440 nm using a model Cintra 5 UV–vis spectrometer (GBC, Australia).

6.8. Hoechst 33258 staining to detect apoptosis

For A549, the living cells were stained with 10 μg/mL of Hoechst 33258 in the medium for 10 min at 37 °C. For H322 and H1299, cell fixation was done with 4% formaldehyde in PBS for 10 min before

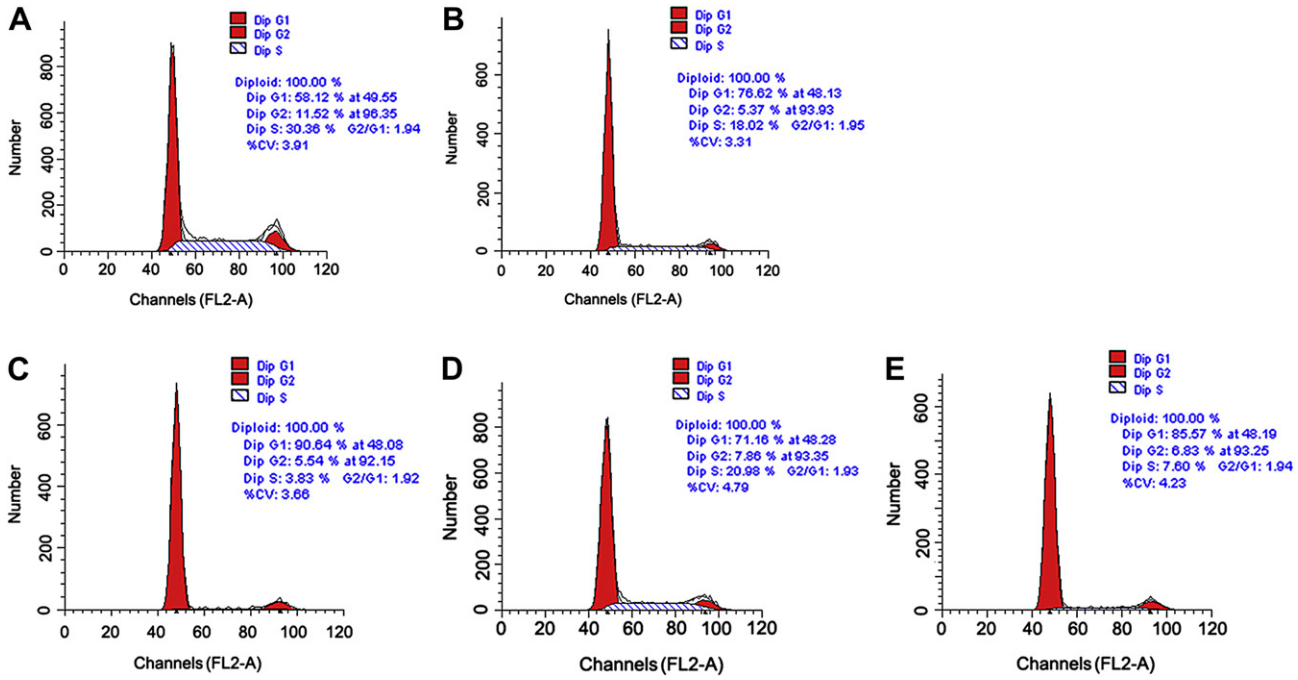


Fig. 10. Effect of compounds **3e**–**h** on cell cycle distribution of A549 cells. Cells were exposed to the compounds **3e**–**h** at 64 μM and incubated for 48 h. Values are expressed as percentage of the cell population in the G1, S, and G2 phase of cell cycle. (A) Control; (B) **3e**; (C) **3f**; (D) **3g**; and (E) **3h**. The result shown here is one representative experiment from three independent experiments.

staining with 2 μg/mL of Hoechst 33258 at 37 °C for 30 min. Subsequently the cells were gently washed once 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 was counted, and each experiment was performed in triplicate.

6.9. Flow cytometry analysis of cell cycle distribution

Cells were harvested and then fixed with 70% cold ethanol, stained with 50 μg/mL propidium iodide (PI) containing 10 μg/mL Rnase A at 4 °C for 1 h. The stained cells were analyzed using FACSCalibur flow cytometer (BD Bioscience, USA). Cell cycle distribution was analyzed by ModiFit software (BD Bioscience, USA).

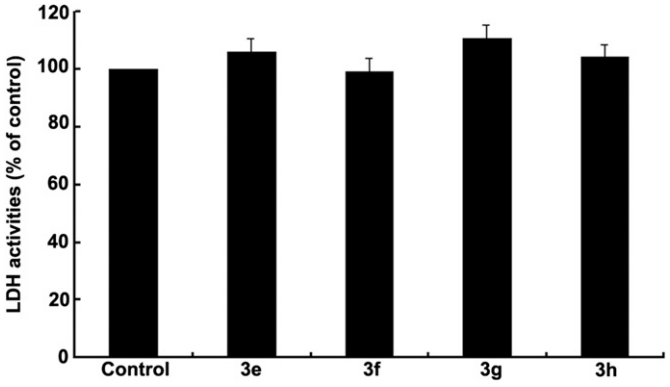


Fig. 11. Effects of compounds **3e**–**h** on the release of LDH from A549 cells. Cells were treated with compounds **3e**–**h** at the concentration of 64 μM for 48 h, and then the culture medium were collected for LDH assay. Results were presented as mean ± SE; $n = 3$.

6.10. 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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