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Synthesis and preliminary biological evaluation of novel pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives as potential agents against A549 lung cancer cells

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ABSTRACT

A series of novel pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives were synthesized by the reaction of ethyl 3-aryl-1-(2-bromoethyl)-1*H*-pyrazole-5-carboxylate and amine in the general heating condition and microwave-assisted condition. The structures of the compounds were determined by IR, ^{1}H NMR and mass spectroscopy, in addition, representative single-crystal structures were characterized by using X-ray diffraction analysis. Preliminary biological evaluation showed that the compounds could inhibit the growth of A549 cells in dosage- and time-dependent manners. The study on structure-activity relationships showed that compounds with 4-chlorophenyl group at pyrazole moiety, such as 5-benzyl-2-(4-chlorophenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (**3o**) had much more inhibitory effects. Compound **3o** was the most effective small molecule in inhibiting A549 cell growth and might perform its action through modulating autophagy.

1. Introduction

Lung cancer is one of the leading causes of death worldwide.[1] Our understanding of the biology of cancer has undoubtedly improved in the last decade. One characteristic of cancer cells is their highly proliferative nature. Consequently, inhibition of proliferative pathways is considered an effective strategy to fight cancer and much attention has recently been paid to the discovery and development of new, more selective anticancer agents.[2–4]

Many pyrazole derivatives are known to exhibit a wide range of biological properties such as cannabinoid hCB1 and hCB2 receptor, anti-inflammatory, inhibitors of p38 Kinase, CB1 receptor antagonists, antimicrobial activity.[5–9] Extensive studies have been devoted to arylpyrazole derivatives such as Celecoxib, a well-known cyclooxygenase-2 inhibitor.[10–12] The incorporation of heterocyclic rings into prospective pharmaceutical candidates is a major strategy to obtain activity and safety advantages. As a consequence, much attention has been paid to the design and synthesis of fused-pyrazole derivatives.[13–17] However, a search of the literature revealed very few reports concerning pyrazolo-pyrazinones.[18–20] 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 and 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one.[21,22] The evaluation of biological activity showed that these compounds can inhibit A549 lung cancer cell growth. To extend the diversity of fused-pyrazole skeleton and screen anticancer agents, the modification of structure is needed.

Microwave-assisted chemistry has blossomed into a useful technique for a variety of applications in organic synthesis. There are some excellent reviews and reports on the broad use of microwave irradiation 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.[23–30] However, reports concerning microwave-assisted rapid synthesis of pyrazole-fused pyrazinone derivatives has not been reported.

Herein, we would like to report the microwave-assisted synthesis, structural characterization and preliminary biological evaluation of novel pyrazole-fused pyrazinone derivatives.

2. Results and discussion

2.1. Chemistry

The synthesis of pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives **3** has been accomplished as outlined in Scheme 1 starting from

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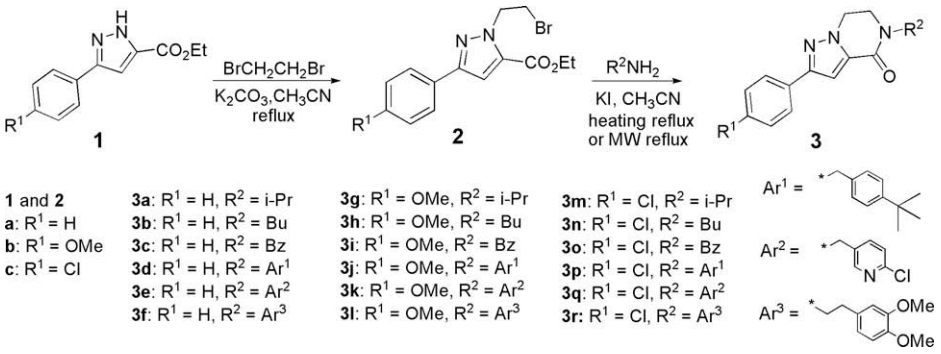

Scheme 1. Synthesis of pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives.

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 as the base in acetonitrile. After flash chromatography on silica gel, the ethyl 3-aryl-1-(2-bromoethyl)-1*H*-pyrazole-5-carboxylate **2** and the isomer, ethyl 5-aryl-1-(2-bromoethyl)-1*H*-pyrazole-3-carboxylate were obtained in 85 and 13% yields, respectively. The isomers can be easily distinguished by comparing the chemical shift in ^{1}H NMR spectra as described in our previous paper.[22] Thus, for example, 5-butyl-2-phenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (**3b**) was synthesized in 78.1% yield by the reaction of ethyl 1-(2-bromoethyl)-3-phenyl-1*H*-pyrazole-5-carboxylate (**2a)** with butylamine in acetonitrile over a 5 h reflux period. The structures of pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one **3** were determined by IR, ^{1}H NMR and mass spectroscopy. For example, 5-((6-chloropyridin-3-yl)-methyl)-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (**3k**), obtained in 67.3% yield as white crystal, gave a [M]-ion peak at m/z 368.8 in the ESI-MS, in accord with the molecular formula $C_{19}H_{17}ClN_4O_2$. In the IR spectra, the carbonyl group absorption was observed in the 1659 cm^{-1}. The ^{1}H NMR spectra indicated the chemical shift of the protons in methylene at δ = 4.75 (s, CH_2) and methyl at δ = 3.84 (s, CH_3). Two methylene protons in the pyrazine moiety appeared at 3.71 and 4.39 as triplet peaks (J = 6.2 Hz). A proton signal in pyrazole moiety appeared at 7.11 as singlet peak. Two *ortho*- aromatic protons signals in 4-methoxybenzene moiety appeared at the range of δ = 6.95 and 7.72 ppm as doublet peaks (J = 8.7 Hz), respectively. The signals of three protons in pyridine appeared at δ = 8.38 (d, J = 2.1 Hz), 7.71 (dd, J = 2.1, 8.2 Hz) and 7.34 (d, J = 8.2 Hz), respectively.

We focused our attention on the microwave-assisted synthesis technique after obtaining compounds **3** by classical heating method. 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 acetonitrile and added in a flask with a condenser, and irradiated under refluxing condition for 0.8–5.7 h. After work-up, desired compounds 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 reagent amine regardless of classical heating or microwave-assisted. From the Table 1 we can find that the more the steric hindrance of amine was, the lower the yield of compounds **3**. For example, in the case of **3a**, **3g** and **3m**, because the steric hindrance of isopropylamine is larger than *n*-butylamine, the yields of **3a**, **3g** and **3m** are 24.7, 22.8 and 36.6%, respectively, which are less than the yields of **3b**, **3h** and **3n** (78.1, 84.3 and 64.0**%**) in the classical heating condition. In the microwave-assisted condition, we did not obtained **3a**, **3g** and **3m** in good yields as other compounds.

Table 1
The yields of compounds **3** in the condition of classical heating and microwave-assisted.

Entry	Compounds	Classical heating		Microwave-assisted	
		Time (h)	Yields (%)	Time (h)	Yields (%)
1	**3a**	7	24.7	—	—
2	**3b**	5	78.1	2.5	75.8
3	**3c**	8	84.2	1.3	88.5
4	**3d**	9	64.0	3.2	54.3
5	**3e**	15	43.1	5.7	59.1
6	**3f**	10	74.3	0.8	94.4
7	**3g**	7	22.8	—	—
8	**3h**	7	84.3	2.7	80.9
9	**3i**	7	87.1	1.2	84.8
10	**3j**	8	68.7	3.2	67.6
11	**3k**	17	67.0	4	70.6
12	**3l**	4	68.2	1.2	71.3
13	**3m**	6	36.6	—	—
14	**3n**	5	64.0	2.4	66.7
15	**3o**	8	70.2	1.7	88.5
16	**3p**	9	54.8	3	83.1
17	**3q**	17	55.2	5	59.1
18	**3r**	3	56.6	1.2	86.4

In the case of the amine with electron-donating group, such as benzylamine (entry 3, 9 and 15) as well as 3,4-dimethoxyphenylethylamine (entry 6, 12 and 18), products **3** could be obtained in shorter time and satisfactory yields. On the other hand, in the case of amine with electron-withdrawing group, such as (6-chloropyridin-3-yl)methanamine (entry 5, 11 and 17), the reaction time were longer and the yields were lower in the both classical heating condition and microwave-assisted condition.

2.2. Single-crystal structural characterization by X-ray

The spatial structures of compounds **3k** and **3l** were determined by using X-ray diffraction analysis. The single crystals were grown from ethyl acetate at room temperature. The molecular views of **3k** and **3l** are shown in Figures 1 and 2. Crystal data and structure refinement for **3k** and **3l** are shown in Table 2.

The molecule of **3k** (Fig. 1) consists of four fragments, a planar pyrazole ring, aryl ring bonded to pyrazole, pyrazinone ring and arylalkyl group. An optimal electronic overlap of the π-system demands a coplanar arrangement. Indeed, the pyrazole ring and pyrazinone ring are approximately coplanar besides C7, which distance to the plane is 0.575 Å. The coplanar makes dihedral angles of 3.27(8)° and 73.65(8)° with the phenyl and pyridine rings, respectively, while that between the phenyl and pyridine rings is 76.91(9)°. In compound **3l** (Fig. 2), which is a close analogue of **3k**, the pyrazole ring and pyrazinone ring are approximately coplanar besides C12, which distance to the plane is 0.538 Å. The coplanar makes dihedral angles of 12.85(11)° and 88.23(10)° with

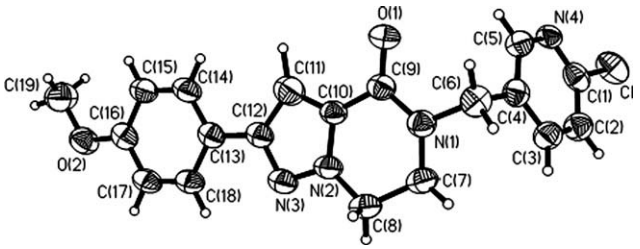

Figure 1. The molecular structure of compound **3k**, with displacement ellipsoids drawn at the 50% probability level.

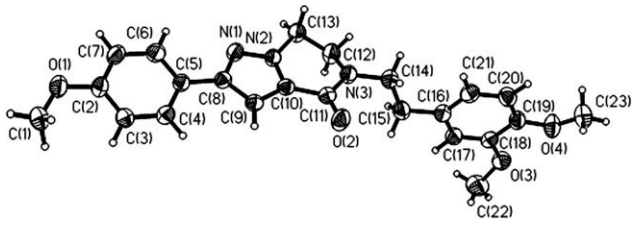

Figure 2. The molecular structure of compound **3l**, with displacement ellipsoids drawn at the 50% probability level.

the 4-methoxyphenyl and 3,4-dimethoxyphenyl rings, respectively, while that between the two phenyl rings is 75.84(12)°.

The single structure characterization should be valuable for further investigation.

2.3. 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 has been widely accepted as a reliable way to measure the cell proliferation rate, and conversely when metabolic events lead to apoptosis or necrosis. 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 of the 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 a arylalkyl group resulted in a significant activity increasing.[22] In the present study, we observed that the nature and the position of substituent on the molecule improve effectively biological functions. Growth inhibitory properties (IC_{50}) on the growth of A549 cells for the compounds **3a**–**3r** are listed in Table 3. The data suggested that compounds with 4-chlorophenyl group are more effective in dosage- and time-dependent manners (Figs. 3 and 4).

As typically shown in Figure 4, exposure of cells to compounds **3m** and **3o** at 32 μM for 12 h resulted in cell viability decrease from 100% to 81.4 and 78.7%, respectively. Continuing the exposure for 24 h resulted in cell viability decrease from 100% to 75.7 and 73.6%, respectively. When the exposure continued on to 48 h, compared with the control group, the cell viability reduced more significantly from 100% to 50 and 39.2% ($p < 0.01$). Further, exposure of cells to compounds **3m** and **3o** at 64 μM for 48 h, the cell viability reduced more significantly from 100% to 34.6 and 26.5%, respectively.

2.4. Analysis of DNA fragmentation and autophagy detection by acridine orange (AO) staining

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 not observed by acridine orange (AO) staining under a Nikon fluorescence microscope. The results showed that no obvious nuclear DNA fragmentation occurred in the cells treated with 16 μM or 32 μM of compound **3o** for 24 h and 48 h (Fig. 5). At the same time, the volume of the cellular acidic compartment, as a marker of autophagy, was visualized by acridine orange staining.[31–33] As shown in Figure 5, compound **3o** at 32 μM promoted autophagy dramatically. The data suggested that the compound might inhibit A549 cell growth through modulating autophagy.

Table 2
Crystal data and structure refinement for **3k** and **3l**.

	3k	**3l**
Empirical formula	$C_{19}H_{17}ClN_4O_2$	$C_{23}H_{25}N_3O_4$
Formula weight	368.82	407.46
Temperature	273(2) K	273(2) K
Wavelength	0.71073 Å	0.71073 Å
Crystal system	Monoclinic	Triclinic
space group	$P21/n$	$P\bar{1}$
Unit cell dimensions	a = 7.33070(10) Å, α = 90°	a = 7.4342(2) Å, α = 106.820(2)°
	b = 17.1568(2) Å, β = 92.8480(10)°	b = 12.0228(4) Å, β = 99.827(2)°
	c = 13.4779(2) Å, γ = 90°	c = 12.6883(4) Å, γ = 102.451(2)°
Volume	1693.04(4) A^3	1026.55(5) A^3
Z	4	2
Calculated density	1.447 Mg/m3	1.318 Mg/m^3
Absorption coefficient	0.248 mm^{-1}	0.091 mm^{-1}
$F(000)$	768	432
Crystal size	0.30 × 0.25 × 0.20 mm	0.40 × 0.10 × 0.10 mm
θ range for data collection	1.92–27.53°	1.73–27.52°
Limiting indices	$-9 \leqslant h \leqslant 9$, $-22 \leqslant k \leqslant 16$, $-17 \leqslant l \leqslant 17$	$-9 \leqslant h \leqslant 7$, $-15 \leqslant k \leqslant 15$, $-11 \leqslant l \leqslant 16$
Reflections collected/unique	12929/3914 [R(int) = 0.0223]	9746/4649 [R(int) = 0.0370]
Completeness to θ = 27.52°	99.9%	98.2%
Absorption correction	None	None
Max. and min. transmission	0.9520 and 0.9293	0.9909 and 0.9644
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data/restraints/parameters	3914/0/235	4649/0/347
Goodness-of-fit on F^2	0.935	1.000
Final R indices [$I > 2\sigma(I)$]	$R1$ = 0.0421, $wR2$ = 0.1148	$R1$ = 0.0583, $wR2$ = 0.1147
R indices (all data)	$R1$ = 0.0610, $wR2$ = 0.1294	$R1$ = 0.1288, $wR2$ = 0.1417
Largest diff. peak and hole	0.158 and −0.539 e $Å^{-3}$	0.193 and −0.219 e $Å^{-3}$

Table 3
Growth inhibitory properties for the compounds **3a**–**3r** at 48 h.

Compounds	3a	3b	3c	3d	3e	3f	3g	3h	3i	3j	3k	3l	3m	3n	3o	3p	3q	3r
IC_{50} (μM)	>64	>64	>64	>64	>64	31.5	>64	62.5	>64	50.2	>64	>64	31	29.8	22.3	>64	39.5	>64

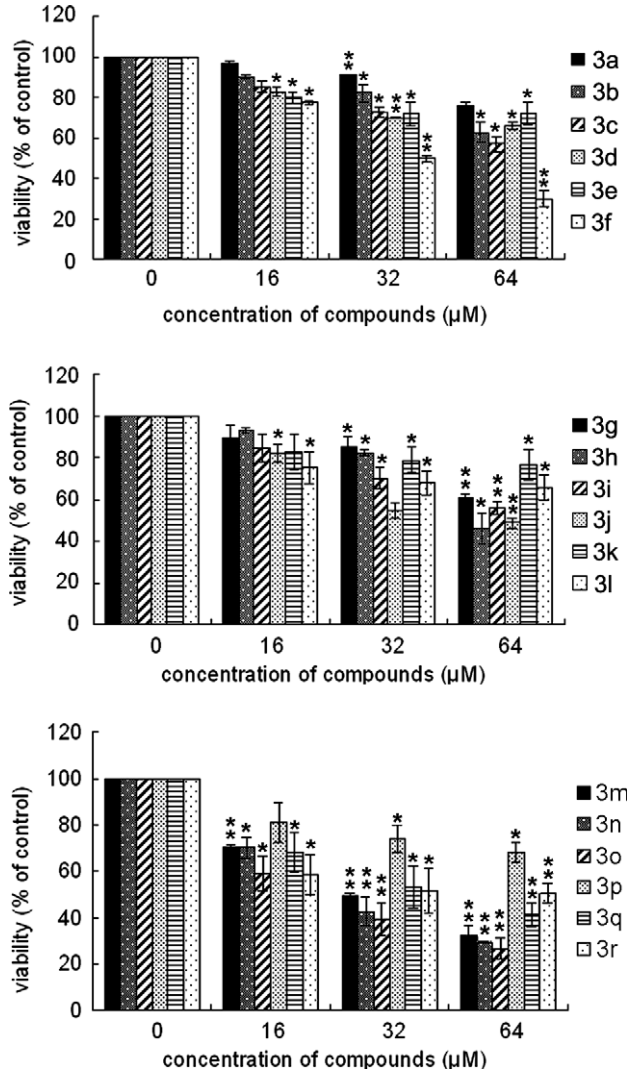

Figure 3. Viability of A549 cells treated with compounds **3a**–**3r**. Cells were seeded in 96-well plates at the density of 6250/cm^2. Cells were treated with the compounds at concentrations of 0, 16, 32, 64 μM, for 48 h. The cell viability was determined by MTT assay. ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs control, $n = 3$).

2.5. Necrosis detection by LDH activity assay

To further confirm the mode of cell death induced by compounds **3f**, **3j**, **3m**, **3n**, **3o** and **3q** typically, LDH assay were performed on cells treated with or without these compounds. As shown in Figure 6, there were no significant differences in LDH release between the cells in the control group (normal group) and the compounds treatment group. The results indicated that the compounds at the test range of concentration did not cause necrosis in the A549 cells. Thus, these compounds did not induce necrosis.

3. Conclusion

In summary, we have described a facile approach to prepare pyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives **3a**–**3l** by the reaction of ethyl 3-aryl-1-(2-bromoethyl)-1*H*-pyrazole-5-carboxylate with primary amine under the general heating condition and the microwave-assisted condition. We found that compounds **3f**, **3m**, **3n**, **3o**, **3q** could suppress A549 lung cancer cell growth. Compound **3o** was the most effective small molecule in inhibiting A549 cell growth and might perform its action through modulating autophagy. The representative single-crystal structural characterization of the compounds **3k** and **3l** were studied by X-ray and it should be valuable for further investigation.

4. Experimental

Thin-layer chromatography (TLC) was conducted on silica gel $60F_{254}$ plates (Merck KgaA). ^{1}H NMR spectra were recorded on a Bruker Avance 400 (400 MHz) spectrometer, using $CDCl_3$ as solvents 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.

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

The intermediates **2** were prepared according to the previous paper.[22]

A solution containing **2**, non-aromatic primary amines (10 equiv) and potassium iodide (0.2 equiv) in acetonitrile 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.

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

A solution containing **2**, non-aromatic primary amines (10 equiv) and potassium iodide (0.2 equiv) in acetonitrile was irradiated with high power region (700 W) to reflux under nitrogen for several hours (shown as Table 1). After work-up as the same with classical method, the products **3** were obtained.

4.3. Spectral data of compounds 3a–3r

4.3.1. 5-Isopropyl-2-phenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3a)

White solid, mp 179–181 °C; IR (KBr) *v*: 1651 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 1.24 (d, *J* = 7.1 Hz, 6H, CH_3), 3.70 (t, *J* = 6.1 Hz, 2H, CH_2NCO), 4.41 (t, *J* = 6.1 Hz, 2H, NCH_2), 4.99–5.06 (m, 1H, CH), 7.15 (s, 1H, 4-H), 7.34 (t, *J* = 7.3 Hz, 1H, ArH), 7.42 (t, *J* = 7.5 Hz, 2H, ArH), 7.81 (d, *J* = 7.3 Hz, 2H, ArH); ESI-MS: 255.8 $(M)^+$.

4.3.2. 5-Butyl-2-phenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3b)

White solid, mp 161–163 °C; IR (KBr) *v*: 1651 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 0.97 (t, *J* = 7.3 Hz, 3H, CH_3), 1.35–1.45

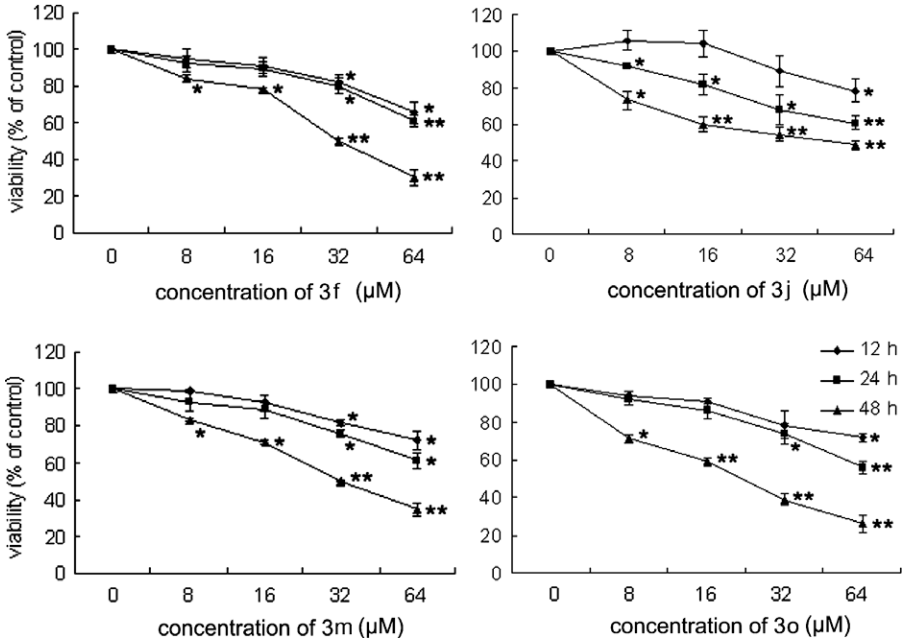

Figure 4. Viability of A549 cells treated with compounds **3f**, **3j**, **3m** and **3o**. Cells were treated with **3f**, **3j**, **3m** and **3o** at concentrations of 0, 16, 32, 64 μM, for 12, 24, and 48 h. Compounds **3f**, **3j**, **3m** and **3o** inhibit the cell growth in dosage- and time-dependent manners. ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs control, $n = 3$).

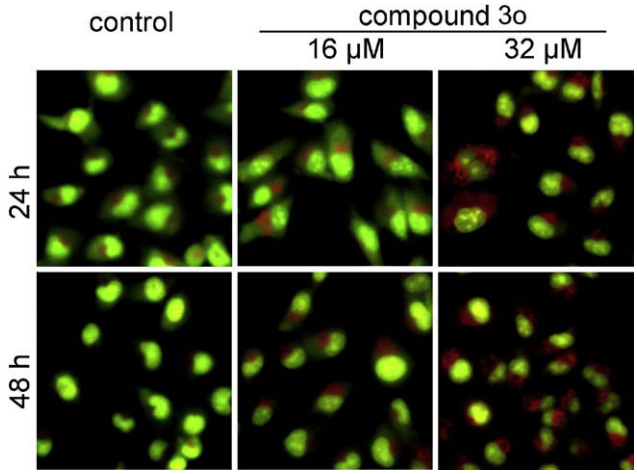

Figure 5. DNA fragmentation and autophagy detections by acridine orange (AO) staining in A549 cells treated with 16 μM or 32 μM of compound **3o** for 24 h and 48 h. ($n = 4$).

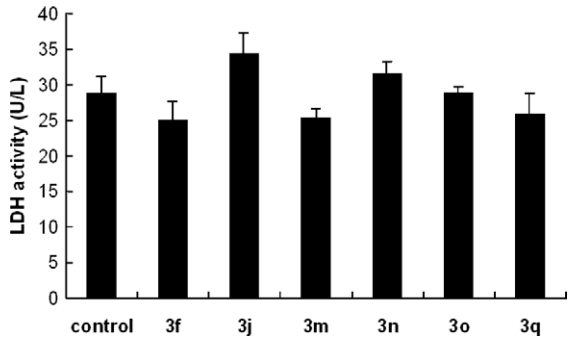

Figure 6. Effects of the compounds **3f**, **3j**, **3m**, **3n**, **3o** and **3q** on the release of LDH from A549 cells. The culture media from the cells treated with the compounds 64 μM for 48 h respectively. Light absorption was analyzed at 340 nm using a model Cintra 5 UV–vis spectrometer. There was no significant difference in LDH release among the four groups. ($p > 0.05$ vs control group, $n = 3$).

(m, 2H, CH_2), 1.59–1.67 (m, 2H, CH_2), 3.58 (t, $J = 7.4$ Hz, 2H, CH_2), 3.79 (t, $J = 6.2$ Hz, 2H, CH_2NCO), 4.44 (t, $J = 6.2$ Hz, 2H, NCH_2), 7.14 (s, 1H, 4-H), 7.32 (t, $J = 7.3$ Hz, 1H, ArH), 7.42 (t, $J = 7.5$ Hz, 2H, ArH), 7.81 (d, $J = 7.3$ Hz, 2H, ArH); ESI-MS: 270.6 $(M+H)^+$.

4.3.3. 5-Benzyl-2-phenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3c)

White solid, mp 174–175 °C; IR (KBr) ν: 1649 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 3.70 (t, $J = 6.2$ Hz, 2H, CH_2NCO), 4.40 (t, $J = 6.2$ Hz, 2H, NCH_2), 4.79 (s, 2H, CH_2Ph), 7.21 (s, 1H, 4-H), 7.32–7.37 (m, 6H, ArH), 7.43 (t, $J = 7.4$ Hz, 2H, ArH), 7.82 (d, $J = 7.8$ Hz, 2H, ArH); ESI-MS: 304.4 $(M+H)^+$.

4.3.4. 5-(4-*tert*-Butylbenzyl)-2-phenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3d)

White solid, mp 185–187 °C; IR (KBr) ν: 1644 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.31 (s, 9H, CH_3), 3.70 (t, $J = 6.2$ Hz, 2H, CH_2NCO), 4.38 (t, $J = 6.2$ Hz, 2H, NCH_2), 4.75 (s, 2H, CH_2Ph), 7.20 (s, 1H, 4-H), 7.26 (d, $J = 8.3$ Hz, 2H, ArH), 7.34–7.44 (m, 5H, ArH), 7.81 (d, $J = 7.1$ Hz, 2H, ArH); ESI-MS: 360.4 $(M+H)^+$.

4.3.5. 5-((6-Chloropyridin-3-yl)methyl)-2-phenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3e)

Yellow solid, mp 226–228 °C; IR (KBr) ν: 1649 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 3.73 (t, $J = 6.2$ Hz, 2H, CH_2NCO), 4.41 (t, $J = 6.2$ Hz, 2H, NCH_2), 4.76 (s, 2H, CH_2), 7.20 (s, 1H, 4-H), 7.32–7.36 (m, 2H, ArH, PyH), 7.42 (t, $J = 7.2$ Hz, 2H, ArH), 7.72 (dd, $J = 2.5$, 8.2 Hz, 1H, PyH), 7.80 (d, $J = 7.2$ Hz, 2H, ArH), 8.38 (d, $J = 2.3$ Hz, 1H, PyH); ESI-MS: 339.3 $(M+H)^+$.

4.3.6. 5-(3,4-Dimethoxyphenethyl)-2-phenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3f)

White solid, mp 152–154 °C; IR (KBr) ν: 1658 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 2.93 (t, $J = 7.1$ Hz, 2H, CH_2), 3.56 (t, $J = 6.0$ Hz, 2H, CH_2NCO), 3.80 (t, $J = 7.1$ Hz, 2H, CH_2), 3.86 (s, 6H, OCH_3), 4.27 (t, $J = 6.0$ Hz, 2H, NCH_2), 6.77–6.82 (m, 3H, ArH), 7.15 (s, 1H, 4-H), 7.33 (t, $J = 7.3$ Hz, 1H, ArH), 7.42 (t, $J = 7.5$ Hz, 2H, ArH), 7.80 (d, $J = 7.8$ Hz, 2H, ArH); ESI-MS: 378.6 $(M+H)^+$.

4.3.7. 5-Isopropyl-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3g)

White solid, mp 157–159 °C; IR (KBr) *v*: 1650 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.24 (d, *J* = 6.8 Hz, 6H, CH_3), 3.69 (t, *J* = 6.0 Hz, 2H, CH_2NCO), 3.85 (s, 3H, OCH_3), 4.42 (t, *J* = 6.0 Hz, 2H, NCH_2), 4.98–5.05 (m, 1H, CH), 6.95 (d, *J* = 8.6 Hz, 2H, ArH), 7.07 (s, 1H, 4-H), 7.74 (d, *J* = 8.6 Hz, 2H, ArH); ESI-MS: 286.0 $(M+H)^+$.

4.3.8. 5-Butyl-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3h)

White solid, mp 160–162 °C; IR (KBr) *v*: 1652 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 0.97 (t, *J* = 7.3 Hz, 3H, CH_3), 1.35–1.44 (m, 2H, CH_2), 1.59–1.66 (m, 2H, CH_2), 3.58 (t, *J* = 7.4 Hz, 2H, CH_2), 3.79 (t, *J* = 6.1 Hz, 2H, CH_2NCO), 3.85 (s, 3H, OCH_3), 4.44 (t, *J* = 6.1 Hz, 2H, NCH_2), 6.95 (d, *J* = 8.7 Hz, 2H, ArH), 7.07 (s, 1H, 4-H), 7.74 (d, *J* = 8.7 Hz, 2H, ArH); ESI-MS: 300.5 $(M+H)^+$.

4.3.9. 5-Benzyl-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3i)

White solid, mp 163–164 °C; IR (KBr) *v*: 1657 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 3.68 (t, *J* = 6.2 Hz, 2H, CH_2NCO), 3.84 (s, 3H, OCH_3), 4.35 (t, *J* = 6.2 Hz, 2H, NCH_2), 4.78 (s, 2H, CH_2Ph), 6.95 (d, *J* = 8.8 Hz, 2H, ArH), 7.13 (s, 1H, 4-H), 7.31–7.39 (m, 5H, ArH), 7.73 (d, *J* = 8.8 Hz, 2H, ArH); ESI-MS: 334.2 $(M+H)^+$.

4.3.10. 5-(4-*tert*-Butylbenzyl)-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3j)

White solid, mp 147–149 °C; IR (KBr) *v*: 1659 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.31 (s, 9H, CH_3), 3.69 (t, *J* = 6.2 Hz, 2H, CH_2NCO), 3.84 (s, 3H, OCH_3), 4.35 (t, 2H, *J* = 6.2 Hz, NCH_2), 4.74 (s, 2H, CH_2Ph), 6.95 (d, *J* = 8.8 Hz, 2H, ArH), 7.12 (s, 1H, 4-H), 7.26 (d, *J* = 8.2 Hz, 2H, ArH), 7.37 (d, *J* = 8.2 Hz, 2H, ArH), 7.73 (d, *J* = 8.8 Hz, 2H, ArH); ESI-MS: 390.0 $(M+H)^+$.

4.3.11. 5-((6-Chloropyridin-3-yl)methyl)-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3k)

White solid, mp 197–199 °C; IR (KBr) *v*: 1659 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 3.71 (t, *J* = 6.2 Hz, 2H, CH_2NCO), 3.84 (s, 3H, OCH_3), 4.39 (t, *J* = 6.2 Hz, 2H, NCH_2), 4.75 (s, 2H, CH_2), 6.95 (d, *J* = 8.7 Hz, 2H, ArH), 7.11 (s, 1H, 4-H), 7.34 (d, *J* = 8.2 Hz, 1H, PyH), 7.70 (d, *J* = 2.4 Hz, 1H, PyH), 7.72 (d, *J* = 7.6 Hz, 2H, ArH), 8.38 (d, *J* = 2.1 Hz, 1H, PyH); ESI-MS: 368.8 $(M)^+$.

4.3.12. 5-(3,4-Dimethoxyphenethyl)-2-(4-methoxyphenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3l)

White solid, mp 171–172 °C; IR (KBr) *v*: 1656 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 2.93 (t, *J* = 6.9 Hz, 2H, CH_2), 3.56 (t, *J* = 5.7 Hz, 2H, CH_2NCO), 3.79 (t, *J* = 6.9 Hz, 2H, CH_2), 4.24 (t, *J* = 5.7 Hz, 2H, NCH_2), 3.85 (s, 9H, OCH_3), 6.78–6.80 (m, 3H, ArH), 6.94 (d, *J* = 8.6 Hz, 2H, ArH), 7.06 (s, 1H, 4-H), 7.72 (d, *J* = 8.6 Hz, 2H, ArH); ESI-MS: 408.6 $(M+H)^+$.

4.3.13. 2-(4-Chlorophenyl)-5-isopropyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3m)

White solid, mp 188–190 °C; IR (KBr) *v*: 1654 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.24 (d, *J* = 6.8 Hz, 6H, CH_3), 3.69 (t, *J* = 6.1 Hz, 2H, CH_2NCO), 4.39 (t, *J* = 6.1 Hz, 2H, NCH_2), 4.98–5.04 (m, 1H, CH), 7.10 (s, 1H, 4-H), 7.38 (d, *J* = 8.6 Hz, 2H, ArH), 7.73 (d, *J* = 8.6 Hz, 2H, ArH); ESI-MS: ($C_{15}H_{16}ClN_3O$), 289.9 $(M)^+$.

4.3.14. 5-Butyl-2-(4-chlorophenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3n)

White solid, mp 173–175 °C; IR (KBr) *v*: 1651 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 0.97 (t, *J* = 7.3 Hz, 3H, CH_3), 1.37–1.43 (m, 2H, CH_2), 1.61–1.65 (m, 2H, CH_2), 3.58 (t, *J* = 7.4 Hz, 2H, CH_2), 3.78 (t, *J* = 6.1 Hz, 2H, CH_2NCO), 4.42 (t, *J* = 6.1 Hz, 2H, NCH_2), 7.10 (s, 1H, 4-H), 7.38 (d, *J* = 8.3 Hz, 2H, ArH), 7.73 (d, *J* = 8.3 Hz, 2H, ArH); ESI-MS: ($C_{16}H_{18}ClN_3O$), 303.9 $(M)^+$.

4.3.15. 5-Benzyl-2-(4-chlorophenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3o)

White solid, mp 142–145 °C; IR (KBr) *v*: 1647 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 3.69 (t, *J* = 6.1 Hz, 2H, CH_2NCO), 4.36 (t, *J* = 6.1 Hz, 2H, NCH_2), 4.78 (s, 2H, CH_2Ph), 7.17 (s, 1H, 4-H), 7.32–7.39 (m, 7H, ArH), 7.73 (d, *J* = 8.6 Hz, 2H, ArH); ESI-MS: ($C_{19}H_{17}ClN_3O$), 338.1 $(M+H)^+$.

4.3.16. 5-(4-*tert*-Butylbenzyl)-2-(4-chlorophenyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3p)

White solid, mp 212–214 °C; IR (KBr) *v*: 1649 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 1.13 (s, 9H, CH_3), 3.69 (t, *J* = 6.2 Hz, 2H, CH_2NCO), 4.36 (t, *J* = 6.2 Hz, 2H, NCH_2), 4.75 (s, 2H, CH_2Ph), 7.16 (s, 1H, 4-H), 7.26 (d, *J* = 8.2 Hz, 2H, ArH), 7.38 (dd, *J* = 3.1, 8.4 Hz, 4H, ArH), 7.73 (d, *J* = 8.6 Hz, 2H, ArH); ESI-MS: ($C_{23}H_{25}ClN_3O$), 394.3 $(M+H)^+$.

4.3.17. 2-(4-Chlorophenyl)-5-((6-chloropyridin-3-yl)methyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3q)

White solid, mp 192–195 °C; IR (KBr) *v*: 1652 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 3.73 (t, *J* = 6.2 Hz, 2H, CH_2NCO), 4.40 (t, *J* = 6.2 Hz, 2H, NCH_2), 4.76 (s, 2H, CH_2), 7.16 (s, 1H, 4-H), 7.35 (d, *J* = 8.2 Hz, 1H, PyH), 7.38 (d, *J* = 7.6 Hz, 2H, ArH), 7.70 (d, *J* = 2.4 Hz, 1H, PyH), 7.73 (d, *J* = 7.6 Hz, 2H, ArH), 8.38 (d, *J* = 2.3 Hz, 1H, PyH); ESI-MS: ($C_{18}H_{15}Cl_2N_4O$), 373.1 $(M+H)^+$.

4.3.18. 2-(4-Chlorophenyl)-5-(3,4-dimethoxyphenethyl)-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one (3r)

Yellow solid, mp 175–178 °C; IR (KBr) *v*: 1656 (C=O) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ: 2.93 (t, *J* = 7.1 Hz, 2H, CH_2), 3.56 (t, *J* = 6.1 Hz, 2H, CH_2NCO), 3.80 (t, *J* = 7.1 Hz, 2H, CH_2), 3.86 (s, 6H, OCH_3), 4.24 (t, *J* = 6.1 Hz, 2H, NCH_2), 6.78–6.82 (m, 3H, ArH), 7.10 (s, 1H, 4-H), 7.38 (d, *J* = 8.5 Hz, 2H, ArH), 7.72 (d, *J* = 8.5 Hz, 2H, ArH); ESI-MS: ($C_{22}H_{23}ClN_3O_3$), 412.3 $(M+H)^+$.

4.4. 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.

4.5. Cell viability assay

As the previous report, cells were seeded onto 96-well plates and treated with compounds **3a–3r** at 8, 16, 32 and 64 μM for 12, 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.[34] The light absorption was measured at 570 nm using Spectra MAX 190 microplate spectrophotometer (GMI Co., USA).

4.6. Acridine orange (AO) staining

The cells were incubated with compounds **3o** (16, 32 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.

4.7. LDH assay

Lactate dehydrogenase (LDH) assay was performed on cells treated with 64 μM compounds **3f**, **3j**, **3m**, **3n**, **3o** and **3q** for 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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