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Synthesis of pyrazole peptidomimetics and their inhibition against A549 lung cancer cells

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ABSTRACT

A series of novel pyrazole peptidomimetics was synthesized from 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylic acid and amino acid ester. Structures of the compounds were characterized by means of IR, ^{1}H NMR and mass spectroscopy. Compounds **5e** and **5k** suppress effectively the growth of A549 lung cancer cells. Preliminary research on the mechanism of action showed that the inhibition might perform through combination of apoptosis, autophagy and cell cycle arrest.

Cancer is one of the leading causes of death. Lung cancer continues to be the major cause of cancer-related mortality in the world. Chemotherapy is still one of the primary modalities for the treatment of cancer. Many of the available anticancer agents exhibit undesirable side effects such as reduced bioavailability, toxicity and drug-resistance. Therefore, the search for novel and selective anticancer agents is urgently required.

Pyrazoles, with several sites for modification, offer the flexibility to design and construct structural analogs of biomedical interest. General and classical syntheses of substituted pyrazoles involve strategies based on the condensation of 1,3-dicarbonyl compounds.[1,2] The pyrazole scaffold represents a common motif in many pharmaceutical active and remarkable compounds demonstrating a wide range of pharmacological activities such as anticancer activity,[3–5] cyclooxygenase-2 inhibitor,[6–11] PDE4 inhibitors,[12] anti-inflammatory,[13,14] anti-angiogenic,[15,16] anti-bacterial[17] and anti-microbial.[18] Pyrazoles can also be used as chelating ligands to form metal complexes which possess bioactivities.[19–24] Pyrazole-fused heterocycle has been received much attention.[25–28]

Peptides have received much attention due to their antitumor activity.[29–35] Mimetic peptides are also of interest in drug discovery.[36–38] Drug–peptide conjugates have been explored to improve drug efficacy and lower side effects by directing the drug to a specific cell type.[39,40]

In our effort to discover and develop potential new anticancer agents, we reported a series of novel pyrazole derivatives with anticancer activity.[41–48] As an extension of our work on the synthesis and biological evaluation for pyrazole derivatives with structure diversity, we struggled to modify the structures of pyrazole derivatives with amino acid to form peptidomimetics.

Chemistry: The synthesis of 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylic peptidomimetics (**5**) has been accomplished as outlined in Scheme 1 from 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylic acid (**4**). The reaction of starting material pyrazole (**1**) and substituent benzyl chloride (**2**) afforded ethyl 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylate (**3**) according to our previous report.[49] The compounds **3** converted to 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylic acid (**4**) by the alkali hydrolysis. The condensation of 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylic acid (**4**) with amino acid ester under the presence of EDCI afforded 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylic peptidomimetics (**5**) in 18–73% yield. When ethyl 2-amino-3-mercaptopropanoate was used, the yield of desired compound is low, which attributed to competition reactions as shown in Scheme 2. 1-Benzyl-3-phenyl-1*H*-pyrazole-5-carboxylic acid reacts with ethyl 2-amino-3-mercaptopropanoate to form desired peptidomimetics **5c**. However, thiol in **5c** reacts with another 1-benzyl-3-phenyl-1*H*-pyrazole-5-carboxylic acid to afford carbothioate **6**. In addition, thiol in **5c** can oxidized to yield disulfide **7**. The structures of compounds **5a–l**, **6** and **7** were determined by IR, ^{1}H NMR and HRMS spectroscopy.

Inhibition of the compounds on the growth of A549 lung cancer cells: To define the biological activity, in terms of cell proliferation inhibition, compounds **5a–l** were tested in A549 cells at 40 μM

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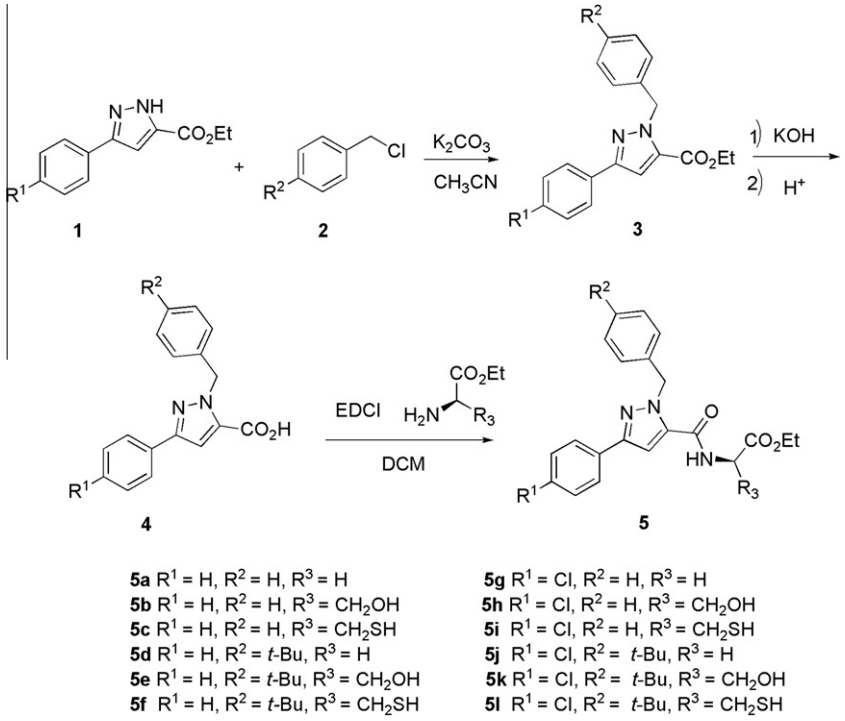
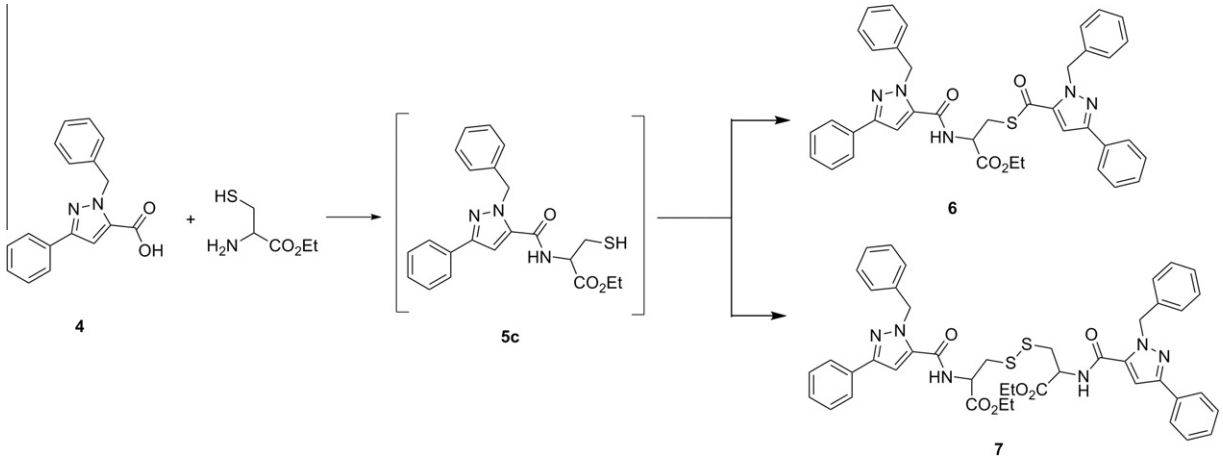


Scheme 1. Synthesis of 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylic peptidomimetics.



Scheme 2. The proposed route to form byproducts.

using Sulforhodamie B (SRB) colorimetric assay. Evaluating the number of viable cells at 48 h after treatment, a decrease of total cell number was observed with most compounds, in particular the effect on cell proliferation results more evident for **5e** and **5k**, comparing the number of viable cells to the control cells (Fig. 1).

The compounds **5b**, **5e**, **5g–k** possessed considerable activity and they were selected for an advanced assay against A549 cells at 40, 60 and 80 concentrations. It can be found that inhibitory is obviously dose-dependent for compounds **5b**, **5e**, **5h**, **5j** and **5k** (Fig. 2). Table 1 presents IC_{50} of compounds **5b**, **5e**, **5h**, **5j** and **5k**, which corresponds to concentration of the compounds causing 50% decrease in net cell growth. The antitumor activities of these compounds appear to relate to the pyrazole substituents and amino acid ester. The presence of *tert*-butyl group in benzyl moiety results in strong inhibition for cell growth. The compounds **5b**, **5e**, **5h** and **5k**, derived from serine ester, have more effective inhibitory

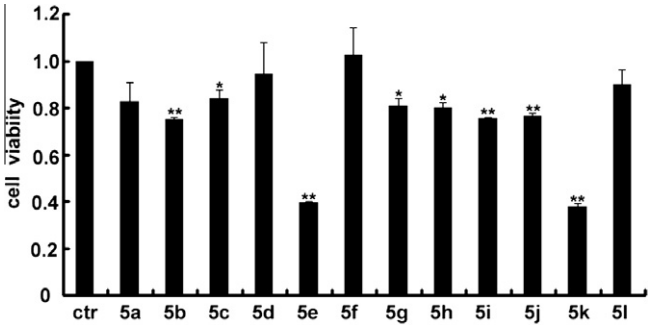


Figure 1. Effects of the compounds **5a–l** at 40 μM on A549 cell viability at 48 h.

effects comparing with compounds derived from glycine and cysteine ester. In addition, the chlorine group in benzene moiety

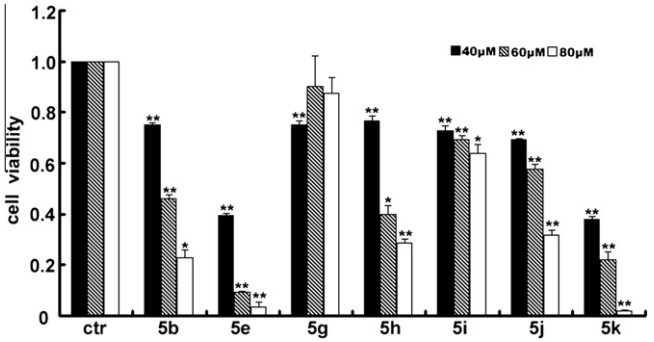


Figure 2. Effects of the selected compounds on A549 cell viability. A549 cells were treated with compounds **5b**, **5e**, **5g**–**k** at the concentration of 40, 60 or 80 μM or left untreated (control) for 48 h. Cell viability was analyzed by SRB assay and illustrated in the labeled column. Results are presented as mean ± SE; ($n = 3$, $^{*}p < 0.05$ vs control; $^{**}p < 0.01$ vs control).

Table 1
IC_{50} of the selected compounds and 5-FU for A549 cells at 48 h

Compounds	5-FU	**5b**	**5e**	**5h**	**5j**	**5k**
IC50 (μM)	4.21	56.07	36.12	56.81	63.41	38.77

affects also the inhibition of compounds for cell growth. Taken together, compounds **5e** and **5k** with both *tert*-butyl in benzyl moiety and serine ester moiety have most effective growth inhibition.

*Modulation of the compounds **5b**, **5e**, **5g–k** for apoptosis and autophagy in A549 lung cancer cells:* The mode of cell killing induced by most anticancer agents is apoptotic cell death. To shed more light on the mechanisms responsible for the cytotoxic effect of selected compounds, we analyzed first the cellular effects of the compounds on nuclear condensation by Hoechst 33258 staining. The morphology of apoptotic cells, including chromatin condensation and the formation of apoptotic bodies, can be determined after staining with Hoechst 33258, which binds to specific sites on double-stranded DNA. Hoechst 33258-stained A549 cells that were subsequently treated with selected compounds **5b**, **5e**, **5g–k** (80 μM) for 48 h showed intense fluorescence in the nuclei (Fig. 3), thereby indicating chromatin condensation. Apoptosis cells in groups treated with selected compounds significantly increased comparing with the control group. Among them, compounds **5e**, **5i** and **5k** caused a significant induction of apoptotic cells after 48 h.

Autophagy exerts dual functions in cancer, acting as both a tumor suppressor, for example, by preventing the accumulation of damaged proteins and organelles, and as a tumor promoter that supports tumor growth. Recent studies have suggested that, like apoptosis, autophagy is important in the regulation of cancer development and progression and in determining the response of tumor cells to anticancer therapy.[50,51] We next evaluated the effect of selected compounds on the induction of autophagy. LC3 is believed to be a credible marker of the autophagosome in mammalian cells. Enhancement of the conversion of LC3-I to LC3-II and upregulation of LC3 expression occurs when autophagy is induced. Thus, induction or suppression of autophagy can be easily monitored by examining the levels of LC3-II by immunoblot. The results showed an elevated protein level of LC3-II in the A549 cells treated with these compounds at 48 h ($p < 0.05$) (Fig. 4).

Taken together, compounds **5e**, **5i** and **5k** can induce both apoptosis and autophagy. However, **5e** and **5k** can lead to cell autoph-

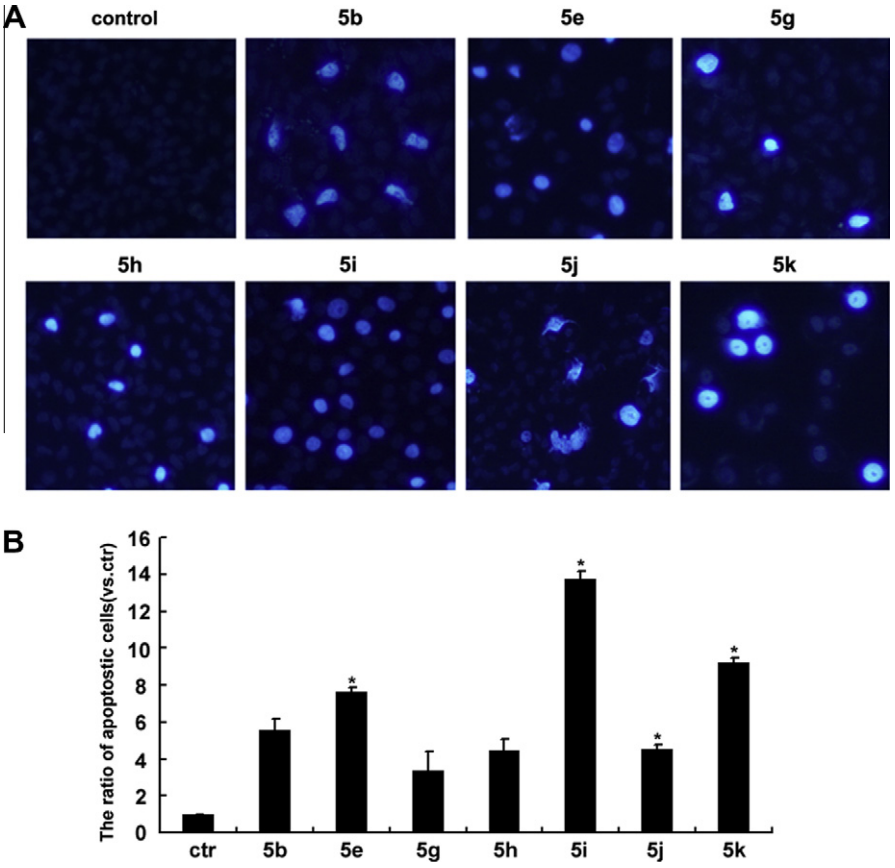


Figure 3. The compounds **5b**, **5e**, **5g–k** induced apoptosis in A549 cells at 80 μM for 48 h ($p < 0.01$ vs ctr, $n = 3$). Microscopic photographs (200×) were taken under a fluorescent microscope (Nikon).

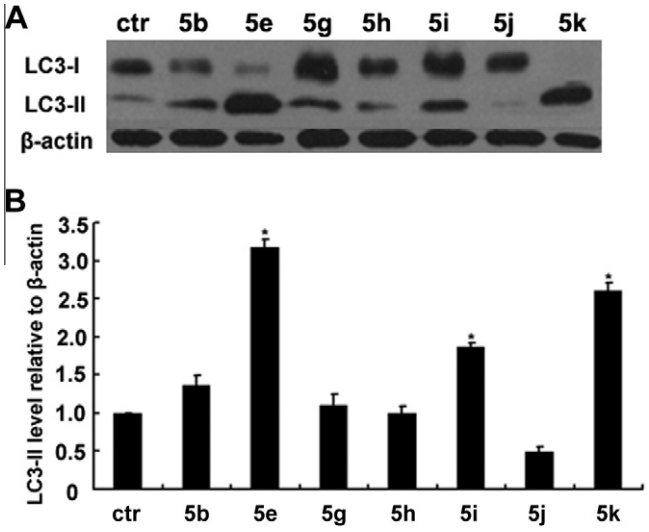


Figure 4. Western blot to test the protein level of LC3-II upon compounds treatment. A549 cells were treated with 80 μM **5b**, **5e**, **5g–k** for 48 h. The protein levels of LC3-II were detected by Western blot analysis (A). The relative levels of LC3-II were normalized by the level of β-actin, and represented as percent of control (B). ($^{*}p < 0.05$ vs control, $n = 3$).

Table 2
Effects of treatment with molecules at 80 μM after 48 h on cell cycle distribution

%	ctr	**5b**	**5e**	**5g**	**5h**	**5i**	**5j**	**5k**
G1	55.94	57.48	72.31	57.39	59.23	60.20	76.06	79.54
G2	9.39	8.08	6.06	8.57	0.69	7.25	4.04	0.92
S	34.67	34.44	21.63	34.04	40.08	32.55	19.91	19.53

agy in a high level which causes cell autophagic death, but **5i** induces cell apoptosis in a high level. A growing number of publications on the cross-regulation between apoptosis and autophagy show the interplay between these two important cellular events. However, the mechanisms mediating the complex regulation of apoptosis and autophagy are not yet fully understood. Therefore, continued investigation of crosstalk between apoptosis and autophagy is necessary.[52] In this study, we found compounds **5e** and **5k** suppress effectively the growth of A549 lung cancer cells through combination of apoptosis and autophagy, suggesting that these small molecules will be potential tools for investigating the mechanisms of the cross-regulation between apoptosis and autophagy.

Flow cytometry analysis of cell cycle distribution: Cell-cycle arrest in cancer cells has become a major indicator of anticancer effects.

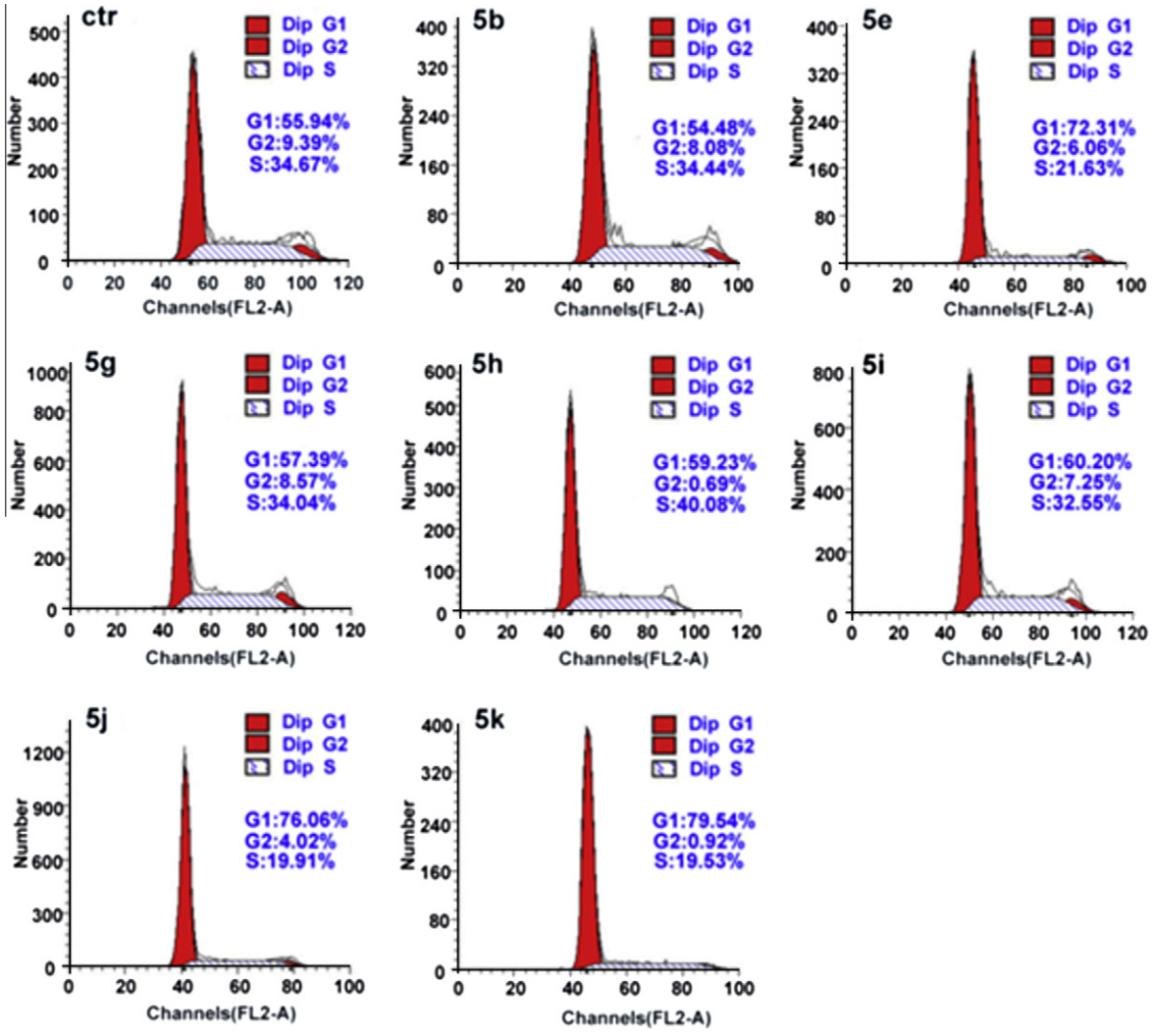


Figure 5. Effects of the selected compounds **5b**, **5e**, **5g–k** on cell cycle distribution of A549 lung cancer cells. Cells were exposed to DMSO, **5b**, **5e**, **5g–k** at 80 μM and incubated for 48 h.

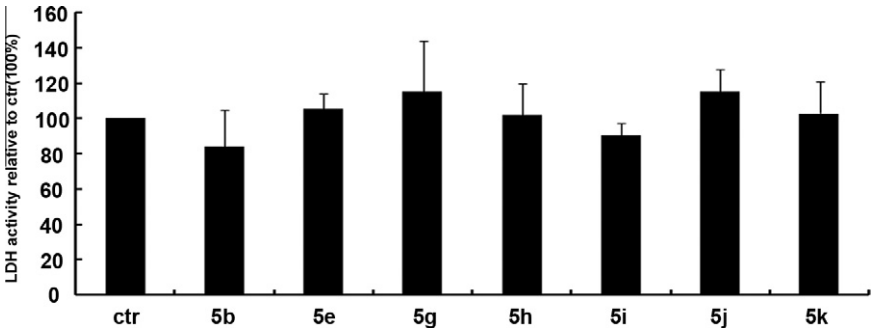


Figure 6. Effects of the compounds on LDH release in A549 cells. A549 cells were treated with compounds **5b**, **5e**, **5g–k** at the concentration of 80 μM or left untreated (control) for 48 h. LDH release was determined. Results are presented as mean ± SE; $n = 3$.

Anticancer agents may alter the regulation of the cell-cycle resulting in an arrest of cells in various phases of the cell cycle, thereby reducing the growth and proliferation of cancerous cells. Accordingly, we also inspected the cell cycle profiles of untreated A549 cells or cells subjected to 48 h treatment with the selected compounds. Compounds **5e**, **5j** and **5k** highly increased the G1 population. However, **5b**, **5g**, **5h** and **5i** increased slightly the G1 population (Fig. 5 and Table 2). Therefore, cell death induced by the selected compounds **5e**, **5j** and **5k** was associated with significant changes in the cell cycle distribution.

*Compounds **5b**, **5e**, **5g–k** do not cause necrosis in A549 lung cancer cells:* In order to determine if the growth inhibitory effects were due to necrosis that is believed to be an unwanted side effect of cancer-fighting agents, LDH assay were carried out on cells treated with compounds **5b**, **5e**, **5g–k** or 0.1% DMSO (as control). The results demonstrated that the compounds at the test range of concentration did not cause necrosis in the cells (Fig. 6).

In summary, we synthesized a series of novel pyrazole peptidomimics by the condensation of 3-aryl-1-arylmethyl-1*H*-pyrazole-5-carboxylic acid and amino acid ester. Preliminary biological evaluation indicates that some compounds suppress the growth of A549 lung cancer cells. Structure–activity relationship analysis shows that compounds **5e** and **5k** with both *tert*-butyl in benzyl moiety and serine ester moiety have most effective growth inhibition. Moreover, the inhibition is in association with apoptosis, autophagy and cell-cycle arrest in the G1 phase.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.09.032.

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