Bioorganic & Medicinal Chemistry xxx (2008) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

5-Alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives inhibit growth of lung cancer A549 cell by inducing apoptosis

Xiao-Hong Pan [a,d,†], Xia Liu [a,†], Bao-Xiang Zhao [b,*], Yong-Sheng Xie [b], Dong-Soo Shin [c], Shang-Li Zhang [a], Jing Zhao [a], Jun-Ying Miao [a,*]

[a] *Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, China*
[b] *Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, China*
[c] *Department of Chemistry, Changwon National University, Changwon 641-773, South Korea*
[d] *School of Pharmaceutical Sciences of Binzhou Medical University, Binzhou 264003, China*

ARTICLE INFO

Article history:
Received 1 June 2008
Revised 14 September 2008
Accepted 16 September 2008
Available online xxxx

Keywords:
Ferrocene
Pyrazole-fused pyrazinone
A549 cell
Apoptosis
Integrin β4
ROS

ABSTRACT

we found that 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives **8d**, **8e** and **8f** could effectively induce apoptosis in A549 lung cancer cells and elevate the levels of integrin β4 and ROS. The data suggested that these compounds might be promising agents for the cancer therapy, and these compounds would be useful tools for further investigate the functions of integrin β4 in regulation of the cancer cell apoptosis.

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] In the course of our search for novel anti-lung cancer drug candidates, we synthesized a series of novel pyrazole derivatives including ethyl 1-(2′-hydroxy-3′-aroxypropyl)-3-aryl-1*H*-pyrazole-5-carboxylate derivatives, 6-(aroxymethyl)-2-aryl-6,7-dihydropyrazolo[5,1-*c*][1,4]oxazin-4-one derivatives, ethyl 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carboxylate derivatives, 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carbohydrazide derivatives, and 1-arylmethyl-3-aryl-1*H*-pyrazole-5-carbohydrazide hydrazone derivatives.[10–14] The evaluation of biological activity showed that these compounds can inhibit A549 lung cancer cell growth.

It is well known that the incorporation of heterocyclic rings into prospective pharmaceutical candidates is a major tactic to gain activity and safety advantages. Ferrocene-containing compounds have attracted much more attention in medicinal chemistry.[15] Incorporation of a ferrocene fragment into a molecule of an organic compound often obtained unexpected biological activity, which is rationalized as being due to their different membrane permeation properties and anomalous metabolism. Many ferrocenyl compounds display interesting cytotoxic, antitumor, antimalarial, antifungal and DNA-cleaving activity.[16] Furthermore, the stability and non-toxicity of the ferrocenyl moiety is of particular interest rendering such drugs compatible with other treatment.[17] Therefore, the integration of one or more ferrocene units into a heterocyclic ring molecule has been recognized as an attractive way to endow a novel molecule functionally.[18–22]

In our previous paper, we have described the synthesis and evaluation of an unknown ferrocene-containing pyrazole-fused pyrazinone compounds, 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one.[23] The results showed that all

* Corresponding authors. Tel.: +86 531 88364929; fax: +86 531 88565610.
E-mail addresses: bxzhao@sdu.edu.cn (B.-X. Zhao), miaojy@sdu.edu.cn (J.-Y. Miao).
† Equal contribution authors.

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.09.046

compounds had almost inhibitory effects on the growth of A549 cells. As a continuation of this project, we are interested in the mechanism by which the 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives performed their functions.

Apoptosis, a universal genetic program of cell death in higher eukaryotes, is a basic process involved in cellular development and differentiation. Apoptosis may be essential for the prevention of cancer cell proliferation, and its deregulation is widely believed to be involved in pathogenesis of many human diseases, including cancer.[24] Further studies are needed to identify the signal(s) that mediate apoptosis program.

The integrins play key roles in the signaling networks that drive pathological angiogenesis and tumor progression. Integrin β4 as a target for cancer and anti-angiogenic therapy have attracted considerable attention.[25]

The aim of this study was to investigate whether the 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives (Chart 1) can induce apoptosis and modulate the level of integrin β4 in A549 lung cancer cells.

2. Results and discussion

2.1. Inhibitory effects of 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives on the proliferation of A549 lung cancer cells

As the previous report,[23] the proliferative inhibitory effects of 5-alkyl-2-ferrocenyl-6,7-dihydropyrazolo[1,5-*a*]pyrazin-4(5*H*)-one derivatives were in a dose-dependent manner. Among of them, **8d**, **8e** and **8f** have more effects on the growth of A549 cells. Thus, we selected 20 μM of the compounds **8d**, **8e** and **8f** as the most appropriate concentration for the following study.

2.2. Compounds 8d, 8e and 8f induce apoptosis in A549 cells

First we observed the morphological changes of the cells treated with compounds **8d**, **8e** and **8f** under a phase contrast microscope. The results showed that the morphological changes associated with apoptosis occurred. Many apoptotic cells were found in the treated cells, especially in the cells treated with compound **8d**

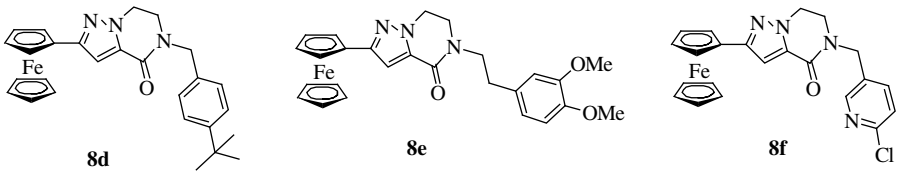

Chart 1. Structures of the compounds.

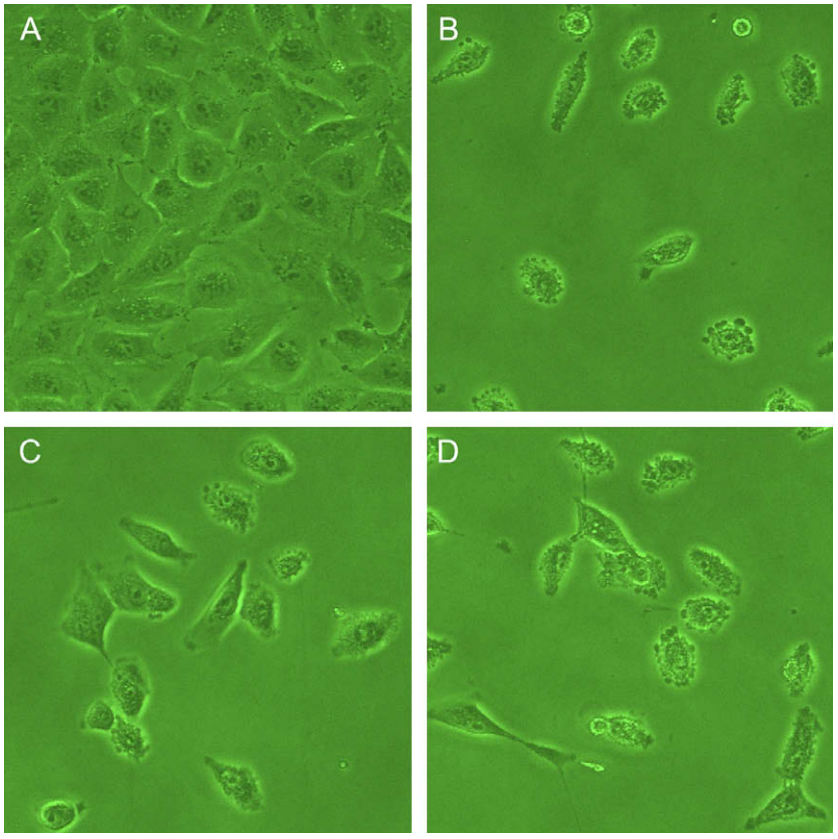

Figure 1. Morphology image of A549 cells treated with compounds **8d**, **8e** and **8f** (20 μM) for 48 h. The figures were obtained by using a phase contrast microscope (400×). (A) DMSO; (B) **8d**; (C) **8e**; (D) **8f**.

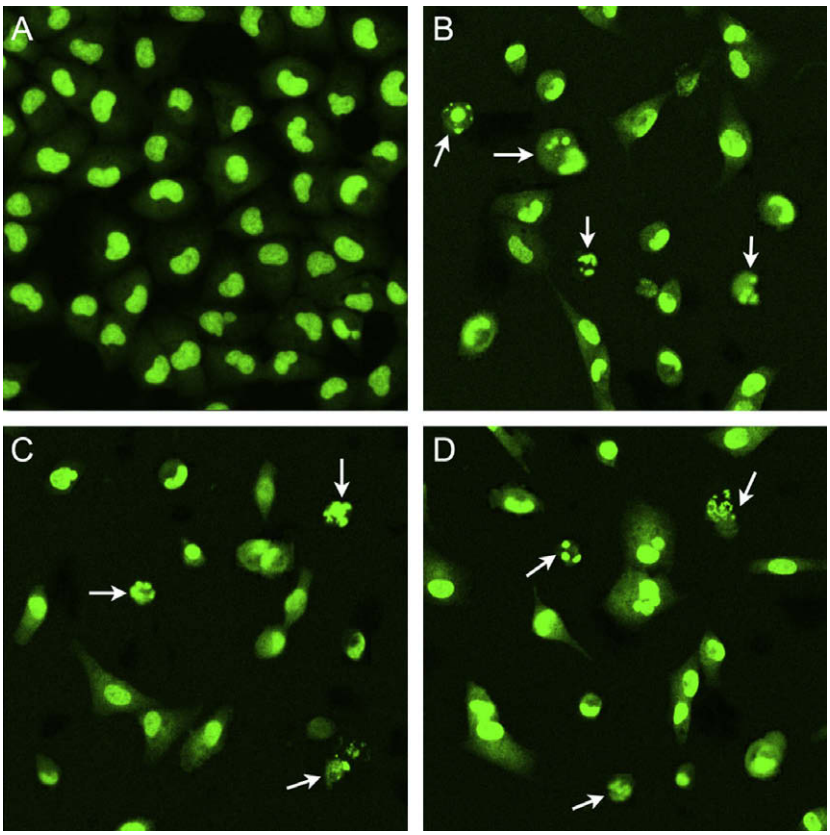

Figure 2. Nuclear DNA fragmentation image of A549 cells treated with compounds **8d**, **8e** and **8f** (20 μM) for 48 h. (A) DMSO; (B) **8d**; (C) **8e**; (D) **8f**. The arrowheads show the DNA fragmentation of nuclei.

(Fig. 1). To further demonstrate the cell death was due to apoptosis, we examined the DNA fragmentation of the treated cells by acridine orange assay and TUNEL assay. Treated with **8d**, **8e** and **8f** at 20 μM for 48 h, the cells presented chromatin condensation and DNA fragmentation (Fig. 2). We found compound **8d** was the most effective apoptosis inducer (Fig. 3). The results showed that the compounds **8d**, **8e** and **8f** inhibited A549 cells' proliferation by inducing apoptosis. Results from LDH assays showed that there was no significant difference in LDH release between the control group and the treatment groups with compounds **8d**, **8e** and **8f** at 20 μM for 24, 48 and 72 h (Fig. 4), indicating that **8d**, **8e** and **8f** did not induce necrosis in A549 cells.

2.3. Effects of compounds 8d, 8e and 8f on the level of integrin β4

To understand the mechanism by which these compounds induce apoptosis in A549 cells, we first examined the changes of integrin β4 level after treatments with the three compounds, respectively, for 48 h, because integrin β4 as a target for cancer and anti-angiogenic therapy have attracted considerable attention.[25] Surprisingly, the results showed that these compounds elevated the level of integrin β4 in A549 cells significantly (Fig. 5). Integrin β4 is a cell type specific protein, in breast cancer cells and normal mouse neurons, knockdown of this protein induces apoptosis,[26,27] however, in human umbilical vein endothelial cells, the level of integrin β4 is increased when apoptosis is induced by deprivation of serum and FGF-2.[28,29] Here, our data first suggested that elevating integrin β4 level might be induce apoptosis in A549 lung cancer cells.

2.4. Effects of compounds 8d, 8e and 8f on the levels of intracellular ROS

It is known that regulated changes in intracellular ROS levels can induce biochemical signaling processes that control basic cellular functions, such as proliferation and apoptosis which are prevalent in the development of cancer. Thus, to understand the possible mechanisms by which compounds **8d**, **8e** and **8f** induced apoptosis in A549 lung cancer cells, we further measured the level of intracellular ROS that has been reported to be affected by integrin β4.[30] As shown in Figures 6 and 8d–f significantly increased the level of ROS.

2.5. Effects of compounds 8d, 8e and 8f on the levels of intracellular ROS and apoptosis when integrin β4 was knockdown

To understand whether the cell death was induced by elevated expression of integrin β4, before the cells were incubated with the derivatives, we pretreated the cells with 40 nM integrin β4 siRNA for 24 h, which has been demonstrated to downregulate integrin β4 effectively in our lab (data not shown). As shown in Figures 7 and 8, when integrin β4 was knockdown compounds **8d** and **8e** could not elevate the level of intracellular ROS, at the same time, the abilities of compounds **8d** and **8e** to induce apoptosis were attenuated obviously. These data suggested that compounds **8d** and **8e** elevated ROS level and induced apoptosis partially through integrin β4 in A549 cells. Interestingly, we found that, when integrin β4 was knockdown, compound **8f** depressed ROS level dramatically and promoted apoptosis instead of inhibiting apoptosis.

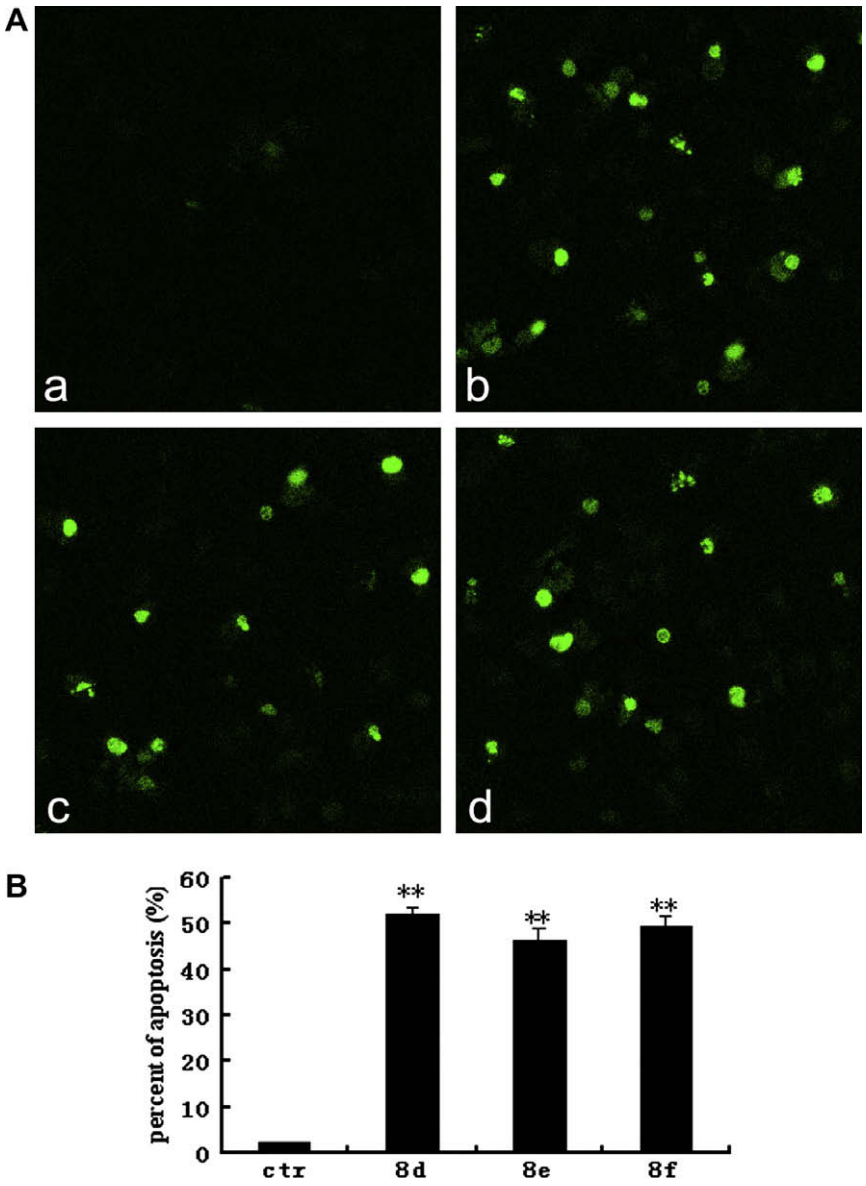

Figure 3. Quantification of apoptotic cells by TUNEL assay. (A) Fluorescent micrographs show the TUNEL-positive cells in A549 cells treated with compounds **8d**, **8e** and **8f** (20 μM), respectively, for 48 h. (a) DMSO; (b) **8d**; (c) **8e**; (d) **8f**. (B) The quantity of apoptotic cells ($^{**}p < 0.01$ vs control group, $n = 3$).

These data support the notion that very low level of ROS may have a proapoptotic effect as very high level of ROS,[28] although the detailed mechanism of compound **8f** action needs to be investigated.

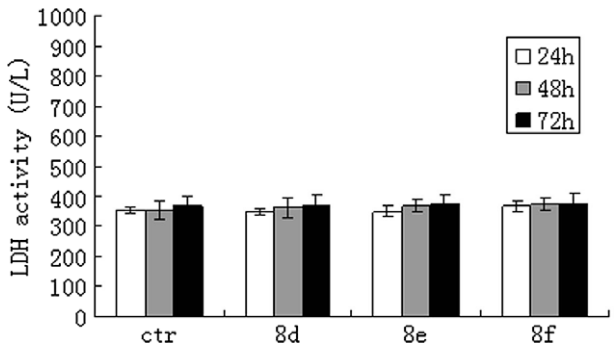

Figure 4. Effects of the compounds **8d**, **8e** and **8f** on the release of LDH from A549 cells. The culture media from the cells treated with the compounds 20 μM for 48 h, respectively. Light absorption was analyzed at 340 nm using a model Cintra 5 UV–vis spectrometer. There was no significant difference in LDH release among the four groups ($p > 0.05$ vs control group, $n = 5$).

3. Conclusion

The results showed that compounds **8d**, **8e** and **8f** could effectively induce apoptosis in A549 lung cancer cells. The data suggested that these compounds might be promising agents for the cancer therapy. Moreover, the results showed that compounds **8d**, **8e** and **8f** elevated the levels of integrin β4 and ROS when they induced apoptosis in A549 cells, suggesting that these compounds were useful tools for further investigate the functions of integrin β4 in regulation of the cancer cell apoptosis.

4. Experimental

4.1. Reagents and chemicals

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

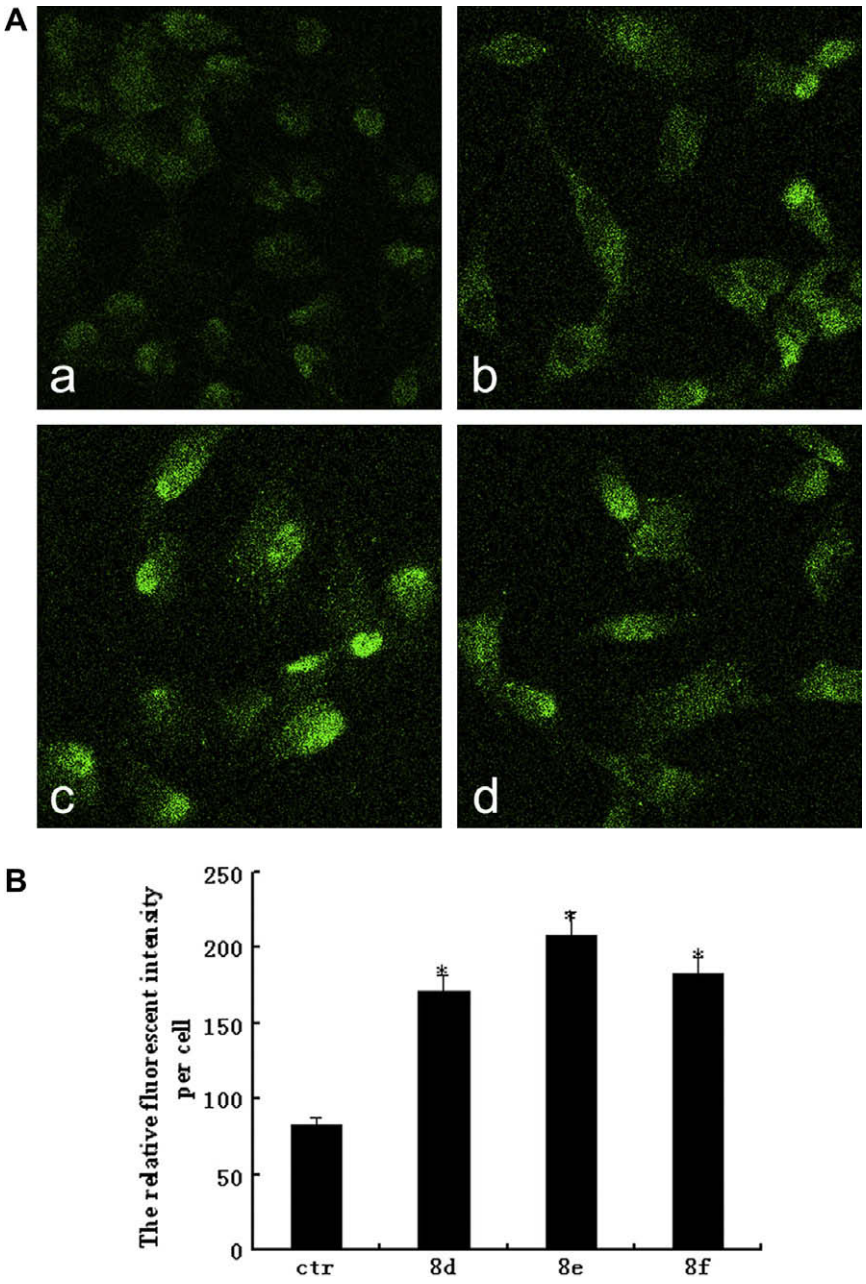

Figure 5. Effects of the compounds **8d**, **8e** and **8f** on the level of integrin β4 in A549 cells. (A) Fluorescent micrographs show the relative intensity of integrin β4 in A549 cells treated with compounds **8d**, **8e** and **8f** (20 μM), respectively, for 48 h. (a) DMSO; (b) **8d**; (c) **8e**; (d) **8f**. (B) The relative quantity of integrin β4 ($^{*}p < 0.05$ vs control group, $n = 3$).

from Shanghai Sangon Biological Engineering Technology and Services Company.

4.2. 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 penicillin/streptomycin. The cells were seeded onto 96-well plates or other appropriate dishes containing the medium at the density of 6250/cm^2.

4.3. Cell viability assay

As the previous report, cells were seeded onto 96-well plates and treated with compounds **8a–8f** at 2.5, 5.0, 10, 20 and 40 μM for 24 and 48 h, respectively. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay according to Price and McMillan[31] The light absorption was measured at 570 nm using Spectra MAX 190 microplate spectrophotometer (GMI Co., USA).

4.4. DNA fragmentation assay

The cells were incubated with compounds **8d**, **8e** and **8f** (20 μM) for 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 the laser scanning confocal microscope (TCS-SP2, Leica, Germany).

4.5. LDH assay

Lactate dehydrogenase (LDH) assay was performed on cells treated with 20 μM compounds **8d**, **8e** and **8f** for 48 h using a

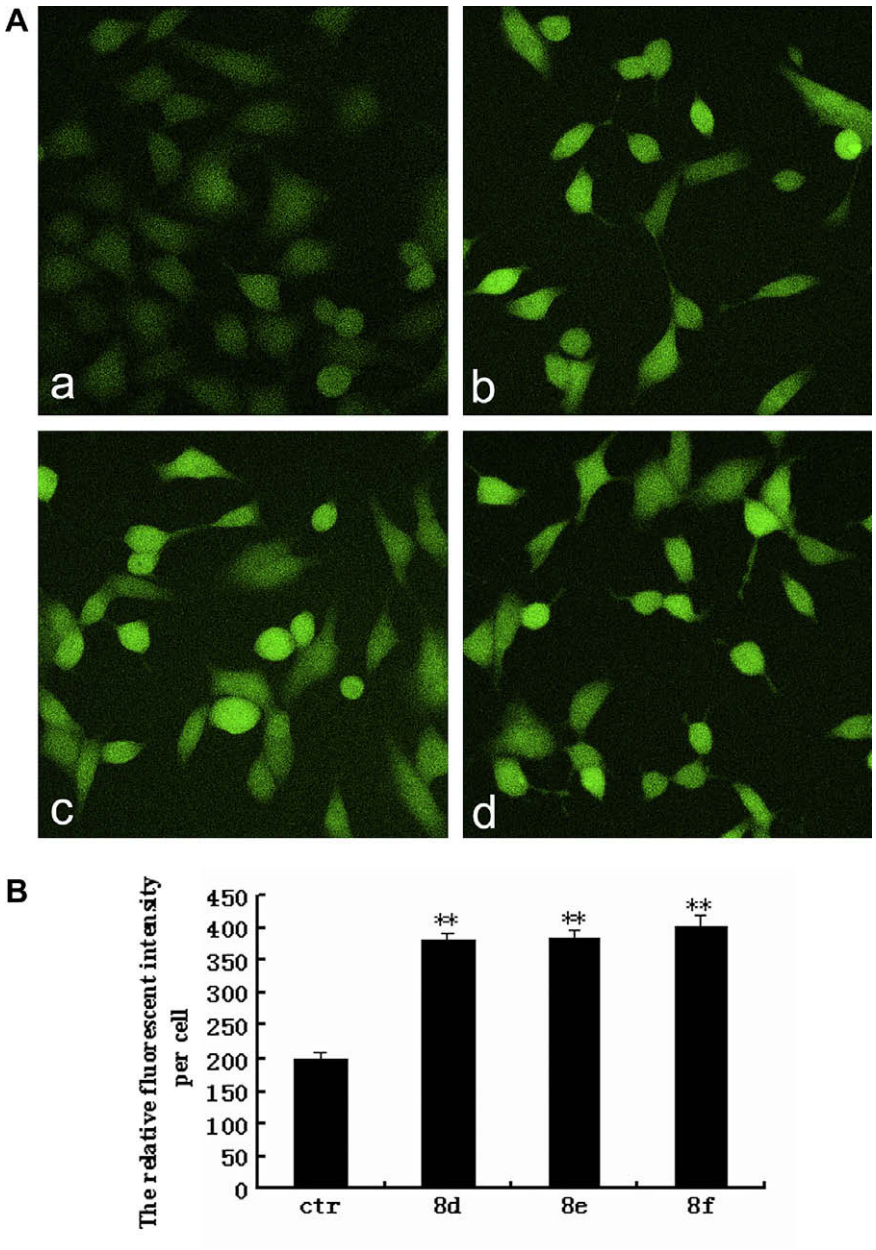

Figure 6. Effects of the compounds **8d**, **8e** and **8f** on the level of ROS in A549 cells. (A) Fluorescent micrographs show the relative intensity of ROS in A549 cells treated with compounds **8d**, **8e** and **8f** (20 μM), respectively, for 48 h. (a) DMSO; (b) **8d**; (c) **8e**; (d) **8f**. (B) The relative quantity of ROS ($^{**}p < 0.01$ vs control group, $n = 3$).

LDH kit (ZhongSheng, China) according to the manufacturer's protocol. Light absorption was measured at 340 nm using a model Cintra 5 UV–vis spectrometer (GBC, Australia).

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

The TdT-mediated dUTP nick-end labeling technique was used to detect in situ nuclear DNA fragmentation and measure the apoptosis ratio.[32] In brief, after cells were treated in the presence or absence of 20 μM compounds **8d**, **8e** and **8f** for 48 h, DNA fragmentation was detected by the DeadEndTM Fluorometric TUNEL System (Promega, USA) according to the manufacturer's protocol. Cells were evaluated by the laser scanning confocal microscope (TCS-SP2, Leica, Germany). The percent apoptosis rate was quantified according to the TUNEL-positive rate.

4.7. Immunofluorescence assay

The level of integrin β4 was examined using an immunofluorescence assay. After treatments, the cells were washed three times with 0.1 M PBS. Following fixation (15 min in 4% paraformaldehyde), the cells were washed three times in 0.1 M PBS and blocked with sheep serum (1:50 dilution) in 0.1 M PBS for 20 min at room temperature. Then cells were stained with rabbit anti-mouse integrin β4 IgG (Santa Cruz, USA, 1:100 dilution) in a humid chamber overnight at 4 °C. The cells were washed three times with 0.1 M PBS and incubated for 30 min at 37 °C with the secondary antibodies, FITC-conjugated goat anti-rabbit (Santa Cruz, USA, 1:200 dilution). Finally the cells were washed three times with 0.1 M PBS. Then the samples were evaluated by a laser scanning confocal microscope (Leica, Germany). We randomly selected the region of interest (ROI), and then zoomed in the same frames. The value of relative fluorescent intensity per cell equals the total value of

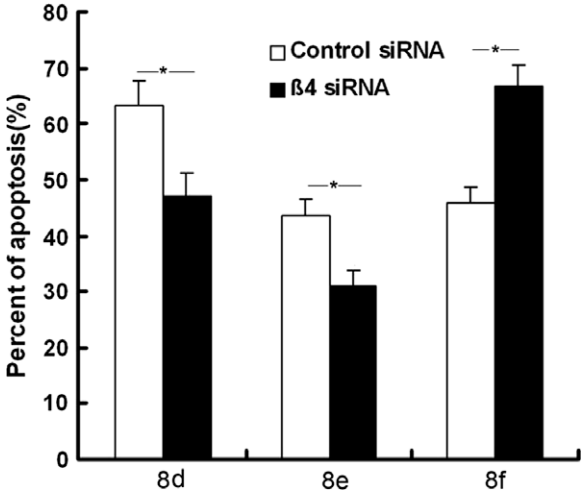

Figure 7. Effects of compounds **8d**, **8e** and **8f** on apoptosis when integrin β4 was knockdown in A549 cells. The cells were pretreated with 40 nM integrin β4 specific siRNA (β4 siRNA) or scramble siRNA (control siRNA) for 24 h, then treated with compounds **8d**, **8e** or **8f** (20 μM) for another 24 h, respectively. Quantification of apoptotic cells was examined by TUNEL assay. **8d**, compound **8d**; **8e**, compound **8e**; **8f**, compound **8f** ($^{*}p < 0.05$ vs control siRNA group, $n = 3$).

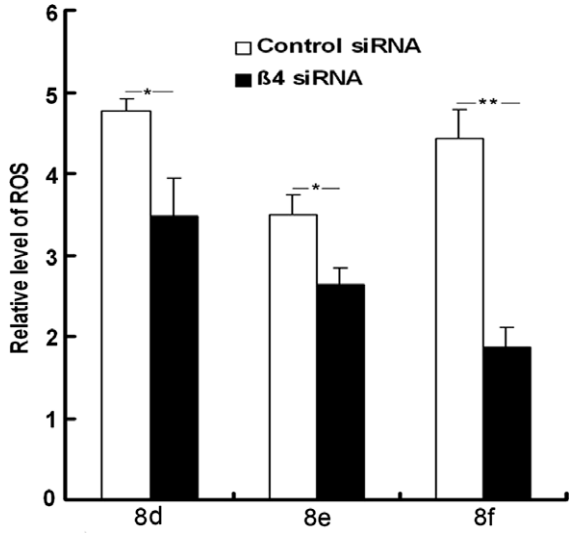

Figure 8. Effects of compounds **8d**, **8e** and **8f** on ROS level when integrin β4 was knockdown in A549 cells. The cells were pretreated with 40 nM integrin β4 specific siRNA (β4 siRNA) or scramble siRNA (control siRNA) for 24 h, then treated with compounds **8d**, **8e** or **8f** (20 μM) for another 24 h, respectively. The relative quantity of ROS level was examined by ROS assay. **8d**, compound **8d**; **8e**, compound **8e**; **8f**, compound **8f** ($^{*}p < 0.05$; $^{**}p < 0.01$ vs control siRNA group, $n = 3$).

sample in scan zoom divided by the total number of cells (at least 200 cells) in same zoom.

4.8. Intracellular ROS assay

The levels of intracellular ROS were detected in the cells treated with or without 20 μM compounds **8d**, **8e** and **8f** using a fluorescent probe, 2′,7′-dichlorodihydrofluorescin (DCHF, Sigma, USA), which can be oxidized into fluorescent 2′,7′-dichlorofluorescin (DCF) by intracellular ROS. This assay is a reliable method for measuring the intracellular ROS.[33] The fluorescence was monitored with laser scanning confocal microscopy (Leica, Germany). The amount of ROS was quantified as the relative fluorescence intensity of DCF per cell in the scanned area.

4.9. RNA interference (RNAi)

RNAi was performed as described[27,34] with the use of the specific integrin β4 siRNA, a pool of 3 target-specific, 20–25 nt siRNAs (sc-35678; Santa Cruz Biotechnology), which is available for down-regulation of integrin β4.[34] RNAi experiments followed the manufacturer's protocols. Integrin β4 siRNA (20–80 nM) was transfected into A549 cells with RNAiFect™ Transfection Reagent (Qiagen, USA). Then, we monitored the effect of gene silencing using western immunoblotting assay. Furthermore, to evaluate siRNA-mediated gene silence, rhodamine-labeled siRNA and scramble siRNA were used as controls (Qiagen & Santa Cruz Biotechnology, USA).

4.10. 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.

Acknowledgments

This study was supported by the Natural Science Foundation of Shandong Province (Z2006D02) and by the National 973 Research Project (No. 2006CB503803) and by National Natural Science Foundation of China (No. 90813022).

References and notes

1. Jemal, A.; Siegel, R.; Ward, E.; Hao, Y.; Xu, J.; Murray, T.; Thun, M. J. *CA Cancer J. Clin.* **2008**, *58*, 71.
2. Hu, W.-P.; Yu, H.-S.; Chen, Y.-R.; Tsai, Y.-M.; Chen, Y.-K.; Liao, C.-C.; Chang, L.-S.; Wang, J.-J. *Bioorg. Med. Chem.* **2008**, *16*, 5295.
3. Kemnitzer, W.; Jiang, S.; Wang, Y.; Kasibhatla, S.; Crogan-Grundy, C.; Bubenik, M.; Labrecque, D.; Denis, R.; Lamothe, S.; Attardo, G.; Gourdeau, H.; Tseng, B.; Drewe, J.; Cai, S. X. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 603.
4. Lee, E.-R.; Kang, Y.-J.; Choi, H.-Y.; Kang, G.-H.; Kim, J.-H.; Kim, B.-W.; Han, Y. S.; Nah, S.-Y.; Paik, H.-D.; Park, Y.-S.; Cho, S.-G. *Biol. Pharm. Bull.* **2007**, *30*, 2394.
5. Silvestri, R.; Cascio, M. G.; Regina, G. L.; Piscitelli, F.; Lavecchia, A.; Brizzi, A.; Pasquini, S.; Botta, M.; Novellino, E.; Marzo, V. D.; Corelli, F. *J. Med. Chem.* **2008**, *51*, 1560.
6. Szabó, G.; Fischer, J.; Kis-Varga, Á.; Gyires, K. *J. Med. Chem.* **2008**, *51*, 142.
7. Graneto, M. J.; Kurumbail, R. G.; Vazquez, M. L.; Shieh, H.-S.; Pawlitz, J. L.; Williams, J. M.; Stallings, W. C.; Geng, L.; Naraian, A. S.; Koszyk, F. J.; Stealey, M. A.; Xu, S. D.; Weier, R. M.; Hanson, G. J.; Mourey, R. J.; Compton, R. P.; Mnich, S. J.; Anderson, J. D.; Monahan, J. B.; Devraj, R. *J. Med. Chem.* **2007**, *50*, 5712.
8. Menozzi, G.; Fossa, P.; Cichero, E.; Spallarossa, A.; Ranise, A.; Mosti, L. *Eur. J. Med. Chem.* **2008**. doi:10.1016/j.ejmech.2008.01.043.
9. Farag, A. M.; Mayhoub, A. S.; Barakatb, S. E.; Bayomi, A. H. *Bioorg. Med. Chem.* **2008**, *16*, 4569.
10. Wei, F.; Zhao, B.-X.; Huang, B.; Zhang, L.; Sun, C.-H.; Dong, W.-L.; Shin, D.-S.; Miao, J.-Y. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 6342.
11. Wei, F.; Zhao, B.-X.; Dong, W.-L.; Hua, Z.; Shin, D.-S.; Wang, D.-W.; Xia, Y.; Ge, Y.-Q. *Synth. Commun.* **2007**, *37*, 4415.
12. Ding, X.-L.; Meng, N.; Xia, Y.; Wang, L.-J.; Zhang, X.-F.; Zhao, B.-X.; Miao, J.-Y. *Chin. J. Org. Chem.* **2007**, *27*, 1542 (in Chinese).
13. Xia, Y.; Dong, Z.-W.; Zhao, B.-X.; Ge, X.; Meng, N.; Shin, D.-S.; Miao, J.-Y. *Bioorg. Med. Chem.* **2007**, *15*, 6893.
14. Xia, Y.; Fan, C.-D.; Zhao, B.-X.; Zhao, J.; Shin, D.-S.; Miao, J.-Y. *Eur. J. Med. Chem.* **2008**. doi:10.1016/j.ejmech.2008.01.021.
15. Fouda, M. F. R.; Abd-Elzaher, M. M.; Abdelsamaia, R. A.; Labib, A. A. *Appl. Organometal. Chem.* **2007**, *21*, 613.
16. Kelly, P. N.; Prêtre, A.; Devoy, S.; O'Rielly, I.; Devery, R.; Goel, A.; Gallagher, J. F.; Lough, A. J.; Kenny, P. T. M. *J. Organomet. Chem.* **2007**, *692*, 1327.
17. Biot, C.; Delhaes, L.; Maciejewaski, L. A.; Mortuaire, M.; Camus, D.; Dive, D.; Brocard, J. S. *Eur. J. Med. Chem.* **2000**, *35*, 707.
18. Sun, H.; Wang, Q.; Huang, R.; Li, H.; Li, Y. *J. Organomet. Chem.* **2002**, *655*, 182.
19. Huang, R.; Wang, Q. *J. Organomet. Chem.* **2001**, *637–639*, 94.
20. Cottineau, B.; Toto, P.; Marot, C.; Pipaud, A.; Chenault, J. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2105.
21. Yu, H.; Shao, L.; Fang, J. *J. Organomet. Chem.* **2007**, *692*, 991.
22. Zora, M.; Görmen, M. *J. Organomet. Chem.* **2007**, *692*, 5026.
23. Xie, Y.-S.; Pan, X.-H.; Zhao, B.-X.; Liu, J.-T.; Shin, D.-S.; Zhang, J.-H.; Zheng, L.-W.; Zhao, J.; Miao, J.-Y. *J. Organomet. Chem.* **2008**, *693*, 1367.
24. Thompson, C. B. *Science* **1995**, *267*, 1456.
25. Giancotti, F. G. *Trends Pharmacol. Sci.* **2007**, *28*, 506.

26. Lipscomb, E. A.; Simpson, K. J.; Lyle, S. R.; Ring, J. E.; Dugan, A. S.; Mercurio, A. M. *Cancer Res.* **2005**, *65*, 10970.
27. Lv, X.; Su, L.; Yin, D.-L.; Sun, C.-H.; Zhao, J.; Zhang, S.-S.; Miao, J.-Y. *Int. J. Biochem. Cell Biol.* **2008**, *40*, 689.
28. Zhao, J.; Miao, J.-Y.; Zhao, B.-X.; Zhang, S.-L. *FEBS Lett.* **2005**, *579*, 5809.
29. Miao, J.-Y.; Araki, S.; Kaji, K.; Hayashi, H. *Biochem. Biophys. Res. Commun.* **1997**, *233*, 182.
30. Zhao, B.-X.; Zhang, L.; Zhu, X.-S.; Wan, M.-S.; Zhao, J.; Zhang, Y.; Zhang, S.-L.; Miao, J.-Y. *Bioorg. Med. Chem.* **2008**, *16*, 5171.
31. Price, P.; McMillan, T. J. *Cancer Res.* **1990**, *50*, 1392.
32. Gavrieli, Y.; Sherman, Y.; Ben-Sasson, S. A. *J. Cell Biol.* **1992**, *119*, 493.
33. Suematsu, N.; Tsutsui, H.; Wen, J.; Kang, D.; Ikeuchi, M.; Ide, T.; Hayashidani, S.; Shiomi, T.; Kubota, T.; Hamasaki, N.; Takeshita, A. *Circulation* **2003**, *107*, 1418.
34. Liu, X.; Yin, D.; Zhang, Y.; Zhao, J.; Zhang, S.; Miao, J. *FEBS Lett.* **2007**, *581*, 5337.