Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 3889–3895
Bioorganic & Medicinal Chemistry

Novel morpholin-3-one derivatives induced apoptosis and elevated the level of P53 and Fas in A549 lung cancer cells

Qiuxia He,[a,c] Xingshang Zhu,[a,c] Mei Shi,[a,c] Baoxiang Zhao,[b,*] Jing Zhao,[a,c] Shangli Zhang[a,c] and Junying Miao[a,c,*]

[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]*The key Laboratory of Experimental Teratology Ministry of Education, Jinan, Shandong 250012, China*

Received 6 January 2007; revised 2 March 2007; accepted 3 March 2007
Available online 28 March 2007

Abstract—Previously, we found that nine kinds of new morpholin-3-one derivatives could inhibit the growth of A549 lung cancer cells in a dose-dependent manner, but how they performed their function remained unknown. In this paper, we studied the effects of the three more effective morpholin-3-one derivatives {4-(4-chlorophenyl)-6-((4-nitrophenoxy) methyl) morpholin-3-one (1); 6-(4-chlorophenoxy)-4-(4-methoxyphenyl) morpholin-3-one (2); and 6-((4-nitrophenoxy) methyl)-4-phenylmorpholin-3-one (3)} on the cell cycle distribution, apoptosis, and the level of P53 and Fas that are two kinds of important proteins in the regulation of A549 cell growth and apoptosis. According to the results of cell viability, we selected 40 μg/ml of morpholin-3-one derivatives as the most appropriate concentration for the following study. The results showed that the morpholin-3-one derivatives partly blocked the cells at G1 phase, induced apoptosis, and elevated the level of P53 and Fas proteins significantly. The effect of the morpholin-3-one derivates was associated with translocation of P53 and clustering of Fas. Our data suggested that the morpholin-3-one derivates might be promising tools for elucidating the molecular mechanism of lung cancer cell apoptosis and they will be very potential candidates for developing anti-cancer drugs.

1. Introduction

Cancer disease is one of the serious clinical problems and poses significant social and economic impacts on the healthcare system.[1–3] Lung cancer is the leading cause of cancer death in the world, causing more than one million deaths each year.[4,5] Current therapeutic interventions have little impact on the epidemic proportions of the disease, and the case fatality rate remains at 85–90%.[6] Current challenges in lung cancer treatment include the search of the best promising new agents which can be integrated into current methods of treatment and the clarification of the mechanism by which lung cancer cells undergo growth, proliferation, differentiation, metastasis and apoptosis.[7]

It has been noted that chemical genetics, using small molecules to induce changes of cell phenotype, has made a significant impact in diverse areas of cell biology.[8] It provides a powerful tool for screening specific small molecules that target specific proteins indispensable to the survival or proliferation of cancer cells.[8,9] The majority of these biologically active small molecules are effective, specific inhibitors of some known intracellular signaling pathways. Moreover, the identification of small molecules with specific biological properties, but unknown intracellular targets, can also reveal significant insights into various biological questions.[10]

Morpholin-3-one derivatives have attracted considerable interest owing to their biological and pharmacological activity. For example, morpholinone-based P1/P2 derivatives have been discovered to provide a new and promising scaffold toward potent mimetics of the HIV-1 protease inhibitor, Amprenavir.[11] The benzomorpholinone was found to be PPARγ agonist agent, that has application to treating type II diabetes,[12] and 6-hydroxymorpholin-3-one derivatives were identified as cornea permeable calpain inhibitor.[13] Although a

Keywords: Morpholin-3-one derivate; A549 lung cancer cell; P53; Fas; Apoptosis.
* Corresponding authors. Tel.: +86 531 88364929; fax: +86 531 88565610 (J.M.); e-mail addresses: bxzhao@sdu.edu.cn; miaojy@sdu.edu.cn

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.03.008

large number of compounds with much more potent effects have been reported, there is no report about apoptosis-inducing effects of morpholinone derivatives on lung cancer cells. In connection with the apoptosis-inducing program of our laboratory, we are interested in extending our small molecules library to meet the requirement of our research. Recently, we synthesized a series of new 4-aryl-6-aryloxymethylmorpholin-3-one derivatives in which the commercially available compounds were used as starting material and they suppressed A549 lung cancer cell growth.[14] But, the mechanism by which the morpholin-3-one derivatives performed their functions remained unknown (Fig. 1).

Apoptosis, a universal genetic program of cell death in higher eukaryotes, is a basic process involved in cellular development and differentiation.[15] 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.[16] P53 can directly activate the transcription of genes known to promote apoptosis.[17,18] P53 is the most extensively studied tumor suppressor and acts in response to diverse forms of cellular stress to mediate a variety of anti-proliferative processes (review in Ref. 19). The Fas antigen is a cell surface protein that can mediate apoptosis and that belongs to the tumor necrosis factor receptor family.[20] Fas is a key protein in apoptosis of cells and tissues.[21] Fas/APO-1 is expressed in human airway epithelial cells and has a critical role in the pathophysiology of various pulmonary disorders.[22] Upregulation of Fas/APO-1 expression has been demonstrated to induce apoptosis in hydrogen peroxide-treated A549 cells.[22] However, the role of Fas in controlling apoptosis of the lung cancer is controversial. Some of the anti-cancer agents have been observed that their induction of apoptosis in lung cancer cells is not mediated by Fas.[23] The aim of this study was to investigate whether the morpholin-3-one derivatives (**1**, **2**, and **3**) can induce apoptosis and regulate the level of P53 and Fas in A549 lung cancer cells.

Here, we report that the morpholin-3-one derivatives could induce apoptosis and elevate the level of P53 and Fas in the lung cancer cells. Among the three derivatives, the most effective one is compound **3**. The findings provided evidence for elucidating the molecular mechanism of lung cancer cell apoptosis.

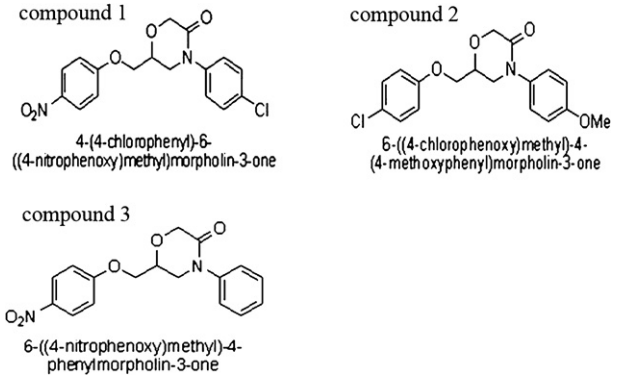

Figure 1. Chemical structures of morpholin-3-one derivatives.

2. Results

2.1. Inhibitory effect of the morpholin-3-one derivatives on the proliferation of A549 lung cancer cells

We first tested the anti-proliferative effect of morpholin-3-one derivatives (compounds **1**, **2**, and **3**) on A549 cell growth by the MTT assay. As shown in Figure 2, the proliferative inhibitory effect of morpholin-3-one derivatives were in a dose-dependent manner. IC_{50} of them was 38.02, 22.91, and 16.60 μg/ml, respectively. According to the results of cell viability, we selected 40 μg/ml of morpholin-3-one derivatives as the most appropriate concentration for the following study.

2.2. Effect of the morpholin-3-one derivatives on cell cycle distribution

The results that morpholin-3-one derivatives inhibited the proliferation of A549 cell led us to further study the effects of them on cell cycle distribution. After the cells were treated with 40 μg/ml of compound **1** for 48 h, the percentage of the cells in S phase obviously decreased to 14.82% and that in G1 and G2/M phases increased to 63.25% and 21.93% in comparison with the control group (G0–G1 53.34%; S 29.07%; G2-M 16.59%). The results indicated that compound **1** partly arrested cell cycle at G1 and G2/M phases (Fig. 3). After the cells were incubated with 40 μg/ml of compounds **2** and **3** for 48 h, the percentage of the cells in S and G2/M phases obviously decreased to 10.99% and 18.64%, and that in G1 phase obviously increased to 79.44% and 73.3% in comparison with the control group (G0–G1 53.34%; S 29.07%; G2-M 16.59%). The results indicated that compounds **2** and **3** made the cells partly arrest at G1 phase (Fig. 3).

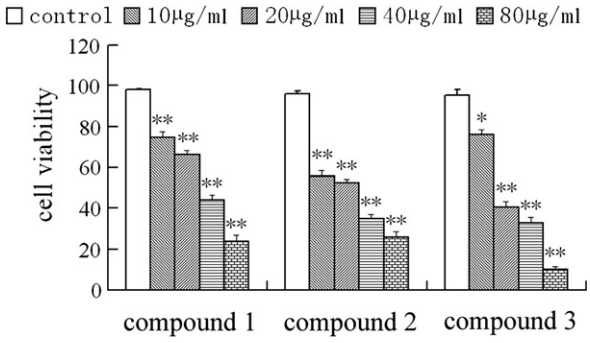

Figure 2. Effect of morpholin-3-one derivatives on cell viability of A549 lung cancer cells. We cultured A549 cells in the medium and treated with or without morpholin-3-one derivatives (10, 20, 40, 80 μg/ml) for 48 h, respectively. The viability of cells was determined by MTT assay as described in Section 4. $*p < 0.05$, $**p < 0.01$ vs. DMSO. Mean values were derived from three independent experiments.

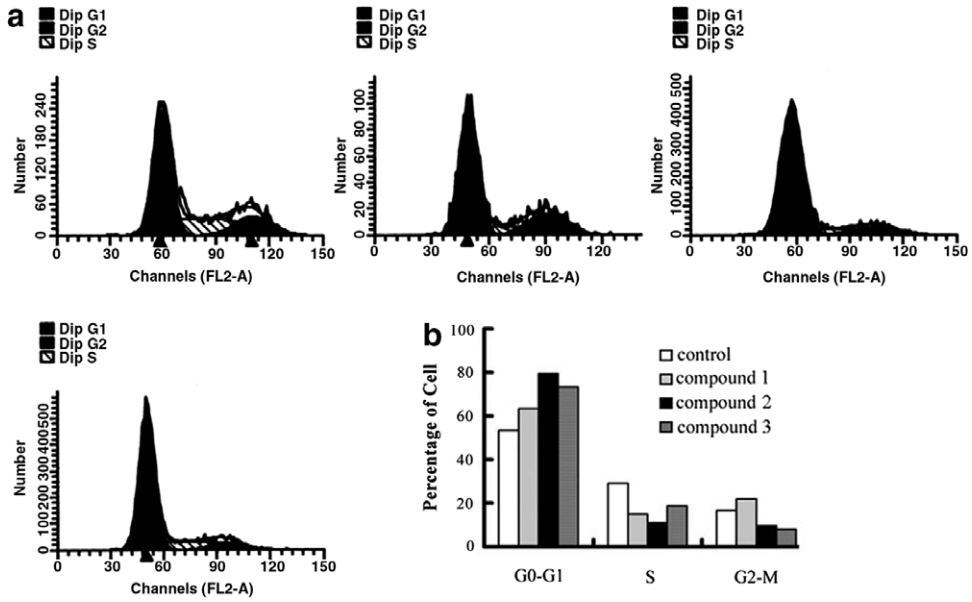

Figure 3. Morpholin-3-one derivatives arrested the cell cycle at G1 phase. A549 cells were cultured in the medium in the presence or in the absence of morpholin-3-one derivatives (40 μg/ml) for 48 h. Cell cycle distribution assay was performed by using flow cytometry as described in Section 4. Data presented are representatives of three independent experiments.

2.3. Effect of the morpholin-3-one derivatives on the morphology and DNA fragmentation of A549 cells

Loss of cell cycle controls may initiate the apoptotic program. Therefore, we investigated the effects of the morpholin-3-one derivatives on A549 cell apoptosis. First we observed the morphological changes of the cells treated with the derivatives under a phase contrast microscope. The results showed that the morphological changes associated with apoptosis occurred. Many apoptotic bodies were formed in the treated cells, especially in the cells treated with compound **3** (Fig. 4). To further demonstrate the cell death was due to apoptosis, we examined the DNA fragmentation of the treated cells by acridine orange assay. Treated with three morpholin-3-one derivatives at 40 μg/ml for 48 h, the cells presented chromatin condensation and DNA fragmentation (Fig. 5). We found compound **3** was the most effective apoptosis inducer (Fig. 5d). The results showed that the morpholin-3-one derivatives inhibited A549 cells' proliferation by inducing apoptosis.

2.4. Morpholin-3-one derivatives elevated the level of P53 and Fas

P53 and Fas are two kinds of very important proteins in controlling cell growth and apoptosis, so, we investigated the effects of the derivatives on the level of these proteins. As shown in Figure 6a, when the cells were treated with 40 μg/ml of the morpholin-3-one derivatives for 48 h, the relative level of P53 protein increased significantly from 13.82 to 23.98, 22.41, and 33.73 ($p < 0.05$ and $p < 0.01$) and P53 was accumulated in nucleus in comparison with the control group. As shown in Figure 6b, the relative level of Fas was remarkably elevated from 6.96 to 21.53, 23.45, and 40.97 in the cells treated with 40 μg/ml of the morpholin-3-one derivatives for 48 h ($p < 0.01$). At the same time, Fas protein distribution on cell membrane was altered. In the control group, Fas was diffusely distributed on the cell surface. However, in the morpholin-3-one derivatives-treated cells, Fas was assembled into patches, and some patches were found to migrate toward one pole of the cell and showed cap-like forms, especially in compound **3**-treated group. The results indicated that the morpholin-3-one derivatives might trigger A549 cell apoptosis through elevating P53 and Fas level and changing their distribution.

3. Discussion

There are some reports about the multiactivity of morpholin-3-one derivatives.[11–13] However, the effect of them on lung cancer cell apoptosis is not known. In our previous study, nine kinds of new morpholin-3-one derivatives were synthesized and their effects on the growth of A549 lung cancer cells were examined to find new small molecules which could inhibit the cell growth.[14] We found that compounds **1**, **2**, and **3** were the more effective small molecules in suppressing A549 lung cancer cell growth.[14] In this study, we further investigated the possible mechanism by which morpholin-3-one derivatives performed their actions in A549 cells. The results showed that the morpholin-3-one derivatives induced apoptosis and elevated the level of P53 and Fas in A549 lung cancer cells.

The P53 tumor suppressor protein is a sequence-specific DNA-binding transcription factor that induces cell cycle arrest or apoptosis in response to genotoxic stress.[24] It has been also reported that P53 induces apoptosis and

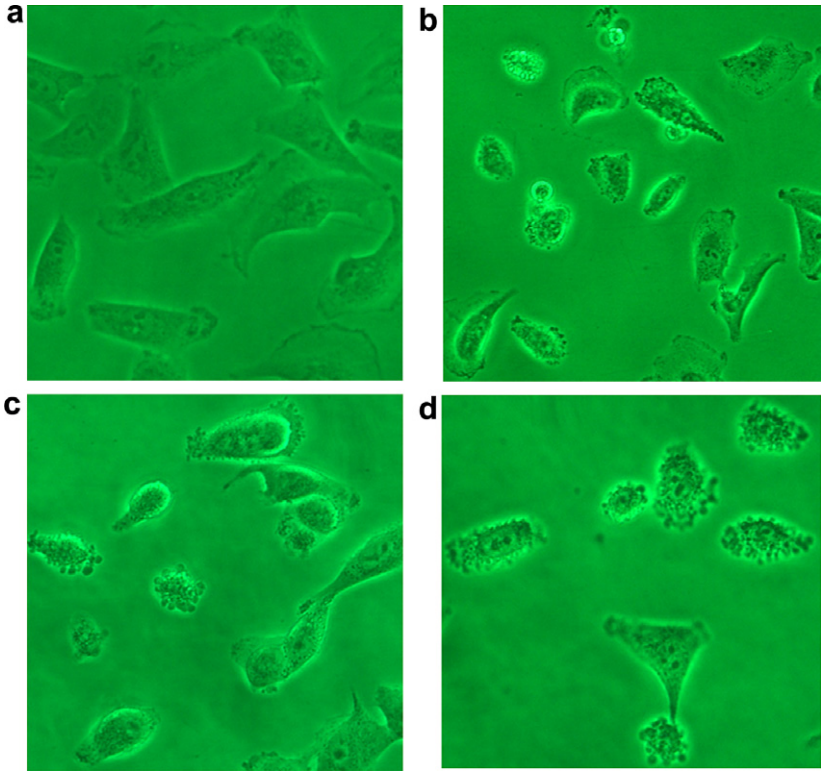

Figure 4. Effect of morpholin-3-one derivatives on A549 cell morphology. (a) The cells cultured in the medium without morpholin-3-one derivatives. (b, c, and d) The cells treated with 40 μg/ml of compounds **1**, **2**, and **3** for 48 h, respectively. The photos were obtained by using a phase contrast microscope.

cell cycle arrest in HeLa, INBL, CaSki, C33A, and ViBo cell lines to respond to neocarzinostatin.[25] Our results showed that morpholin-3-one derivatives not only elevated the level of P53 but also changed its localization. P53 was shown to be actively transported through the nuclear membrane in both directions. Nuclear localization is essential for its growth-suppressing activity.[26] The results indicated that these compounds induced apoptosis probably through increasing the level of P53 and accumulating the protein in nucleus. The result was partly consistent with our previous report in which we showed that safrole oxide could induce A549 cell apoptosis probably by up-regulating the expression of P53 protein, but safrole oxide did not change the localization of P53.[27] Here, we reported compound **3** was more effective than safrole oxide in triggering apoptosis of A549 cells because the IC_{50} of compound **3** was only 16.60 μg/ml.

Fas (also called Apo-1 or CD95), a death domain, has a central role in the physiological regulation of programmed cell death.[28] Previous report shows that synthetic retinoid CD437 promotes Fas expression and induces apoptosis in three human lung cancer cell lines with wt p53.[29] Our data showed that morpholin-3-one derivatives not only elevated the level of Fas but also induced Fas patching in A549 lung cancer cell with wt p53. Cremesti et al. showed that in Jurkat cells Fas capping rapidly followed patching, ultimately, the cells underwent apoptosis.[30] Clustering of Fas induces the formation of death-inducing signaling complex (DISC). The formation of DISC results in autoproteolytic processing of the initiator caspases and the effector caspases.[31] Thus, our data suggested that morpholin-3-one derivatives induced apoptosis likely through Fas in A549 cells.

In summary, the results showed that the morpholin-3-one derivatives (40 μg/ml for 48 h) could induce apoptosis, arrest the cell cycle partly at G1 phase, elevate the level of P53 and Fas, and change the distribution of the two kinds of proteins in A549 lung cancer cells. The data suggested that the morpholin-3-one derivatives might be used as apoptosis inducers to find new molecule target for the cure of lung cancer and they will be very potential candidates for developing anti-cancer drugs.

4. Materials and methods

4.1. Reagents and chemicals

Acridine orange (AO) was purchased from Shandong Chemical Industries (Jinan, China). Anti-P53 and anti-Fas monoclone antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Secondary antibodies (FITC-IgG) were obtained from Santa Cruz Biotechnology. 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 from Shanghai Sangon Biological Engineering Technology and Services Company.

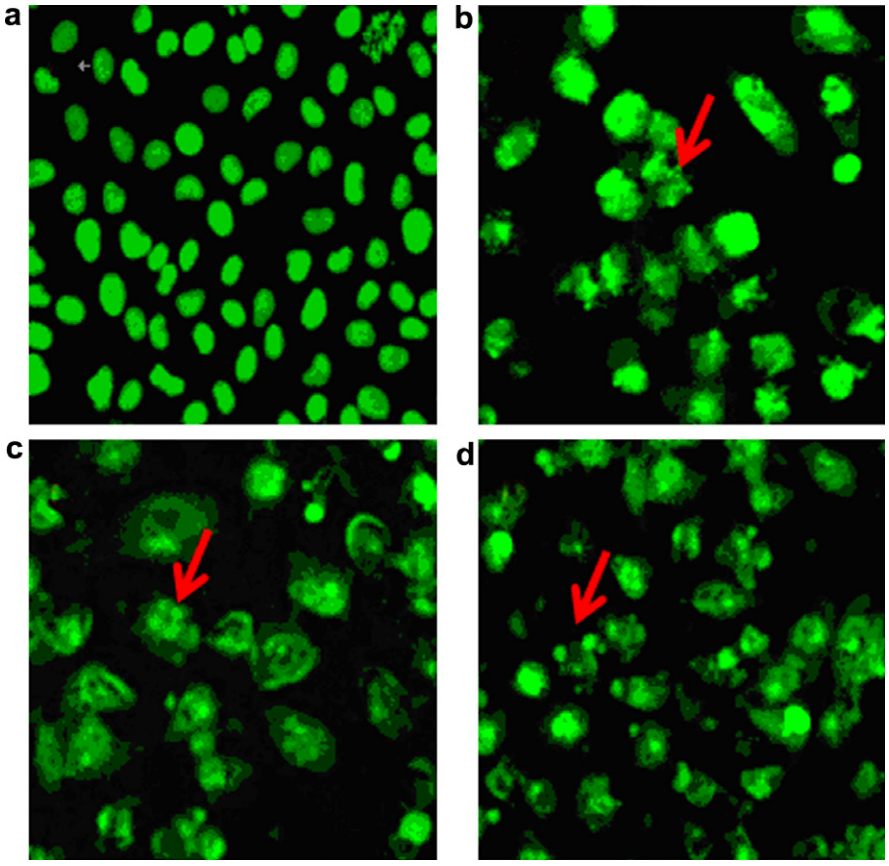

Figure 5. Morpholin-3-one derivatives induced DNA fragmentation in A549 cells. (a) The nuclei of the cells cultured in the medium without morpholin-3-one derivatives. (b, c, and d) The nuclei of the cells treated with 40 μg/ml of compounds **1**, **2**, and **3** for 48 h, respectively. Data presented are representatives of three independent experiments.

4.2. General synthetic procedure of morpholin-3-one derivatives (1, 2, and 3)

Compounds **1**, **2**, and **3** were synthesized by adopting new strategy.[14] In short, the total process was divided into two main steps where universally available aromatic amines and epoxides were used as starting materials to afford β-amino alcohols. And then, 2-chloroacetyl chloride reacted with β-amino alcohols to produce the desired *N*-aryl-6-aryloxymethyl-morpholin-3-one derivatives. The reaction of aminoalcohol with 2-chloroacetyl chloride in the presence of potassium carbonate at 0 °C afforded the corresponding hydroxyamide in good yield (72–87%). The reaction of hydroxyamide with sodium hydride in THF at −25 °C gave the corresponding alkoxide, to result in a spontaneous intramolecular ring closure to form morpholin-3-one in moderate to high yield (76–92%). The structures of all the newly synthesized compounds were assigned by IR, NMR, and mass spectral data.

4.3. 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.

4.4. Cell viability assay

Cells were seeded onto 96-well plates and treated with compounds **1**, **2**, and **3** at 20, 40, and 80 μg/ml for 48 h, respectively. Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay according to Price et al.[32] The light absorption was measured at 570 nm using Spectra MAX 190 microplate spectrophotometer (GMI Co., USA).

4.5. Cell cycle distribution assay

The cells incubated with compounds **1**, **2**, and **3** (40 μg/ml) for 48 h were processed according to Sugiyama et al.[33] DNA content of cells was analyzed using a FACSCalibur flow cytometry (Becton Dickinson, USA). Cell cycle distribution was calculated by Modifit-3 program.

4.6. DNA fragmentation assay

The cells were incubated with compounds **1**, **2**, and **3** (40 μg/ml) 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 confocal scanning laser microscope (TCS-SP2, Leica, Germany).

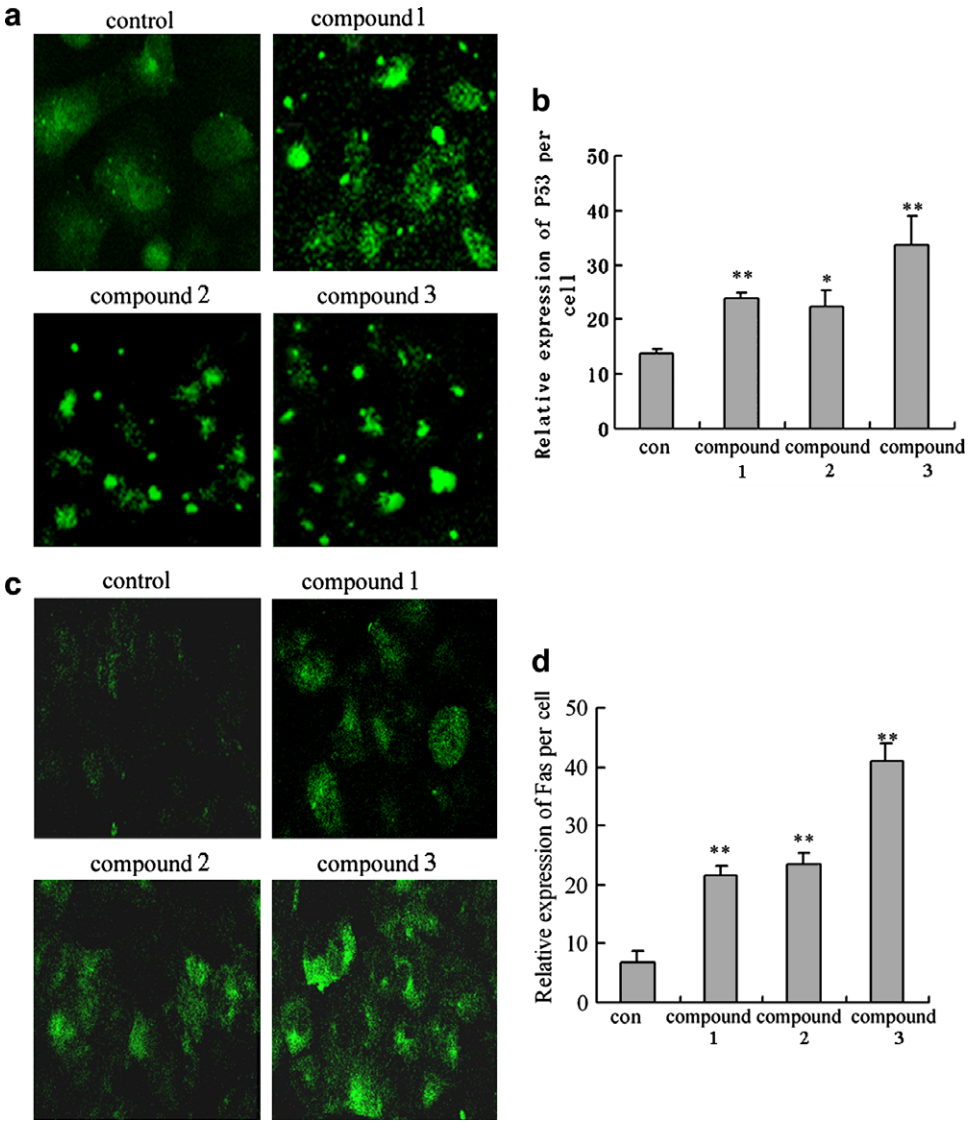

Figure 6. Effect of morpholin-3-one derivatives on the level of P53 and Fas in A549 lung cancer cells. (a) A549 cells were cultured in the medium in the presence or in the absence of morpholin-3-one derivatives (40 μg/ml) for 48 h. The level of P53 was determined using immunofluorescence assay as described in Section 4. Data presented are representatives of three independent experiments. (b) The quantity of P53 level in the four groups mentioned above. The value of the figure represented the relative fluorescent intensity per cell determined by laser scanning confocal microscopy. $^{**}p < 0.01$ compared to control. (c) A549 cells were treated with or without morpholin-3-one derivatives (40 μg/ml). After 48-h treatment, the level of Fas was determined by using immunofluorescence assay as described in Section 4. Data presented are representatives of three independent experiments. (d) The quantity of Fas level in the four groups mentioned above. The value of the figure represented the relative fluorescent intensity per cell determined by laser scanning confocal microcopy. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control.

4.7. Immunofluorescence assay

After the incubation of compounds **1**, **2**, and **3** (40 μg/ml) for 48 h, the Fas and P53 level were analyzed according to Zhao et al.[34] After adding the primary antibodies (rabbit anti-human Fas and P53 primary antibody) and secondary antibodies (FITC-IgG) in turn, samples were photographed using laser scanning confocal microscope (Leica, Germany). The immunofluorescence technique allows semiquantitative evaluation of protein expression.[35] The value of relative fluorescent intensity per cell equals to the total value of sample in scan zoom divided by the total number of cells (at least 100 cells) in same zoom. This assay was repeated at least three times. Here, a representative result from three similar experiments is shown.

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 three independent experiments.

Conflict of interest statement

None declared.

Acknowledgment

This study was supported by the Foundation of the Ministry of Education (104112).

References and notes

1. Raez, L. E.; Rosenblatt, J. D.; Podack, E. R. *Expert Opin. Emerg. Drugs* **2006**, *11*, 445.
2. Modlich, O.; Prisack, H. B.; Bojar, H. *Expert Opin. Pharmacother.* **2006**, *7*, 2069.
3. Lebedeva, I. V.; Sarkar, D.; Su, Z. Z.; Gopalkrishnan, R. V.; Athar, M. *Cancer Res.* **2006**, *66*, 2403.
4. Rivera, M. P. *Semin. Respir. Crit. Care Med.* **2004**, *1*, 3.
5. Koizumi, K. *J. Nippon Med. Sch.* **2006**, *73*, 116.
6. Ries, L. A. *Semin. Surg. Oncol.* **1994**, *10*, 21.
7. Thatcher, N. *Lung Cancer* **2006**, *Sup 2*, S25.
8. Spring, D. R. *Chem. Soc. Rev.* **2005**, *34*, 472.
9. Walsh, D. P.; Chang, Y. T. *Chem. Rev.* **2006**, *106*, 2476.
10. Smukste, I.; Stockwell, B. R. *Annu. Rev. Genomics Hum. Genet.* **2005**, *6*, 261.
11. Kazmierski, W. M.; Furfine, E.; Spaltenstein, A.; Wright, L. L. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5226.
12. Rybczynski, P. J.; Zeck, R. E.; Dudash, J. J.; Combs, D. W.; Burds, T. P.; Yang, M.; Osborne, M. C.; Chen, X.; Demarest, K. T. *J. Med. Chem.* **2004**, *47*, 196.
13. Nakamura, M.; Miyashita, H.; Yamaguchi, M.; Shirasaki, Y.; Nakamura, Y.; Inoue, J. *Bioorg. Med. Chem.* **2003**, *11*, 5449.
14. Tan, W.; Zhao, B.; Shi, M.; Sha, L.; Miao, J.; Shin, D. *Chinese J. Chem.* **2006**, *24*, 396.
15. Raff, M. C. *Nature* **1992**, *356*, 397.
16. Thompson, C. B. *Science* **1995**, *267*, 1456.
17. Yu, J.; Zhang, L.; Hwang, P. M.; Rago, C.; Kinzler, K. W.; Vogelstein, B. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14517.
18. Sax, J. K.; el-Deiry, W. S. *Cell Death Differ.* **2003**, *10*, 413.
19. Fridman, J. S.; Lowe, S. W. *Oncogene* **2003**, *22*, 9030.
20. Itoh, N.; Nagata, S. *J. Biol. Chem.* **1993**, *268*, 10932.
21. Nagata, S.; Golstein, P. *Science* **1995**, *267*, 1449.
22. Fujita, T.; Maruyama, M.; Araya, J.; Sassa, K.; Kawagishi, Y.; Hayashi, R.; Matsui, S.; Kashii, T.; Yamashita, N.; Sugiyama, E.; Kobayashi, M. *Am. J. Respir. Cell Mol. Biol.* **2002**, *27*, 542.
23. Ferreira, C. G.; Tolis, C.; Span, S. W.; Peters, G. J.; van Lopik, T.; Kummer, A. J.; Pinedo, H. M.; Giaccone, G. *Clin. Cancer Res.* **2000**, *6*, 203.
24. Waterman, M. J.; Stavridi, E. S.; Waterman, J. L.; Halazonetis, T. D. *Nat. Genet.* **1998**, *19*, 175.
25. Banuelos, A.; Reyes, E.; Ocadiz, R.; Alvarez, E.; Moreno, M.; Monroy, A.; Gariglio, P. *Pharmacol. Exp. Ther.* **2003**, *306*, 671.
26. Liang, S. H.; Clarke, M. F. *Eur. J. Biochem.* **2001**, *268*, 2779.
27. Du, A.; Zhang, S.; Miao, J.; Zhao, B. *Exp. Lung Res.* **2004**, *30*, 419.
28. Krammer, P. H. *Nature* **2000**, *407*, 789.
29. Sun, S. Y.; Yue, P.; Hong, W. K.; Lotan, R. *Cancer Res.* **2000**, *60*, 6537.
30. Cremesti, A.; Paris, F.; Grassmé, H.; Holler, N.; Tschopp, J.; Fuks, Z.; Gulbins, E.; Kolesnick, R. *J. Biol. Chem.* **2001**, *276*, 23954.
31. Shi, Y. *Cell* **2004**, *117*, 855.
32. Somlyo, A. P.; Garfield, R. E.; Chacko, S.; Somlyo, A. V. *J. Cell Biol.* **1975**, *66*, 425.
33. Sugiyama, K.; Akiyama, T.; Shimizu, M.; Tamaoki, T.; Courage, C.; Gescher, A.; Akinaga, S. *Cancer Res.* **1999**, *59*, 4406.
34. Zhao, Q. T.; Araki, S.; Zhang, S. L.; Miao, J. Y. *Toxicon* **2004**, *44*, 161.
35. Babcock, G. F. *Methods Enzymol.* **1999**, *307*, 319.