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Investigation of anticancer mechanism of thiadiazole-based compound in human non-small cell lung cancer A549 cells

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Abstract

In this study, we have synthesized several compounds and examined their cytotoxic effects on human non-small cell lung cancer A549 cells. We found that GO-13 ((*E,E*)-2,5-bis[4-(3-dimethyl-aminopropoxy)styryl]-1,3,4-thiadiazole) is the most effective one by the MTT assay. Furthermore, the GO-13-induced apoptotic reaction was identified based on several criteria, such as negative release reaction of lactate dehydrogenase and positive labeling of annexin V and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) techniques. GO-13 induced the apoptosis in A549 cells in a concentration- and time-dependent manner. The data demonstrate that the regulations of p38 mitogen-activated protein kinase and protein kinase C was not involved in the GO-13-mediated mechanism. However, GO-13 significantly induced a down-regulation of Bcl-X_L expression in a short-term treatment (less than 3 hr), whereas stimulated up-regulation of Bax expression in a long-term treatment (24 hr) indicating their involvement in GO-13 action. GO-13-mediated apoptosis is also positively correlated with the increase in caspase-3 activity. Worth noting is the fact that GO-13 did not modify the phosphorylation level of Akt/protein kinase B (PKB) until a 24-hr exposure was carried out indicating that the inhibition of Akt/PKB activation was involved in the late-phase apoptosis. Besides the anticancer activity, GO-13 also showed equivalent anti-angiogenic activity in the nude mice angiogenesis model.

In summary, we conclude that GO-13 is the most effective anticancer compound in our screening tests. It induced the early-phase apoptosis in A549 cells via the Bcl-X_L down-regulation, and that of the late-phase through up-regulation of Bax expression as well as inhibition of Akt/PKB activation.

Keywords: Thiadiazole; Apoptosis; Bcl-X_L; Bax; Phosphoinositide 3-kinase

1. Introduction

Lung cancer accounts for more solid tumor deaths than colorectal, breast, prostate, and pancreatic cancers in Taiwan in recent years. Over the past decade, the mortality of lung cancer has risen dramatically for both men and women. More than 75% of lung cancers are non-small cell lung cancers (NSCLC) and chemotherapy is the standard treatment for patients who have a comparatively good performance status (0, 1, and select 2) advanced NSCLC (stage III B and IV). It has been reported that chemotherapy significantly improves symptoms and the quality of life of patients with advanced NSCLC; however, only modest increase in survival rate can be achieved [1–5]. Hence, the therapeutic treatment of advanced NSCLC requires approaches aspiring to improve survival and optimize symptom palliation. The development of chemotherapeutic agents to maximize the antitumor activity and minimize the toxicity is probably the way to go in this management.

The Bcl-2 family proteins play important roles in the regulation of apoptosis. The effects of the Bcl-2, Bcl-X_L and Bax proteins on the regulation of cancer cell apoptosis induced by chemotherapy have been recently investigated [6–8]. It has been suggested that in a lot of human cancer cell lines, the levels of Bcl-2 and Bcl-X_L expressions demonstrate a positive correlation with the prevention of apoptosis induced by various cytotoxic drugs [9]; additionally, overexpression of Bax protein sensitizes cancer

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cells to several chemotherapy agents [9]. These evidences reveal that the modulation of the intrinsic targets, such as Bcl-2 family proteins, might be a potential strategy for the development of anticancer agents.

The neovasculature results in a supplement and exchange of oxygen and nutrients, providing a paracrine stimulus to cancer cells and stimulation of tumor growth, invasion and metastasis. Therefore, the angiogenesis is a crucial step in the tumorigenesis. There are several reports revealing that the increased tumor angiogenesis is associated with poor clinical outcome in a variety of solid malignancies, including NSCLC. In addition, numerous studies have demonstrated a significant correlation between tumor angiogenesis, metastasis and less survival in NSCLC patients [10–12]. The inhibition of tumor angiogenesis becomes a potential strategy in anticancer therapy.

We have synthesized several compounds based on several key structures, such as pyridine, quinoline, quinazolin, pyrimidine, pyrone, thiadiazole and thiadiazine. We have carried out a screening test on the cytotoxic effects of these compounds in human NSCLC A549 cells using the MTT assay. We found that pyrone (GO-12)- and thiadiazole (GO-13)-based compounds showed more potent cytotoxic effect; furthermore, the necrotic and apoptotic cell death were distinguished between these two compounds, respectively. The thiadiazole-based compound also showed anti-angiogenic activity in an *in vivo* angiogenesis model. Therefore, this compound appears to be the preferred candidate in our anticancer development. The apoptotic mechanisms are defined in this study.

2. Materials and methods

2.1. Materials

RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and all other tissue culture reagents were obtained from GIBCO/BRL Life Technologies. Human non-small cell lung cancer A549 cells were obtained from ATCC. EGTA, EDTA (disodium salt), leupeptin, dithiothreitol, phenylmethylsulfonyl fluoride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), SB203580, 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine (H7) and β-isopropanol were purchased from Sigma Chemical. Annexin V-FITC and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) apoptosis detection kits were from R&D Systems and Promega, respectively. Monoclonal antibodies of Bcl-2, Bcl-X_L and Bax were from Transduction Laboratories; polyclonal antibodies of Akt/protein kinase B (PKB) and phosphorylated Akt/PKB were from New England Biolabs Inc. Anti-mouse and anti-rabbit IgGs were from Calbiochem-Novabiochem. The caspase-3 colorimetric assay kit was purchased from R&D Systems Inc.

The GO-compounds were designed and synthesized by Dr. Ji-Wang Chern. They are named from GO-1 to GO-15 in this study, and their chemical names are (*E,E*)-2,4-bis-(3,5-dibromo-4-hydroxystyryl)pyridine, (*E,E*)-2,4-bis(3,4-dihydroxystyryl)-pyridine, (*E*)-2-(3,4-dihydroxystyryl)-8-hydroxyquinoline, (*E*)-2-(3,4-diacetoxystyryl)-8-acetoxyquinoline, (*E*)-2-(3,5-dibromo-4-hydroxystyryl)-8-hydroxyquinoline, (*E*)-2-(3,5-dibromo-4-acetoxystyryl)-8-acetoxyquinoline, 1-benzyl-4-phenyl-1*H*-quinazolin-2-one, 1-benzyl-4-(4-methoxyphenyl)-1*H*-pyrido[4,3-*d*]pyrimidine-2-one, 1-(4-chlorobenzyl)-4-phenyl-1*H*-quinazolin-2-one, (*E*)-4-(3,4-dihydroxystyryl)pyridine, (*E*)-4-(3,5-dibromo-4-hydroxystyryl)pyridine, (*E,E*)-2,6-bis[4-(3-dimethylaminopropoxy)styryl]-4-pyrone, (*E,E*)-2,5-bis[4-(3-dimethylaminopropoxy)styryl]-1,3,4-thiadiazole, 2-(3-4-(2-methoxy-phenyl)-piperazinyl)ethylaminoquinoline-4-carboxamide, and (*E*)-4-(3,4-dihydroxy-styryl)quinoline, respectively. GO-12 and GO-13 (Fig. 1) were compared and studied in this study.

2.2. Cell cultures

Human NSCLC A549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (v/v) and penicillin (100 units mL^{-1})/streptomycin (100 μg mL^{-1}). Cultures

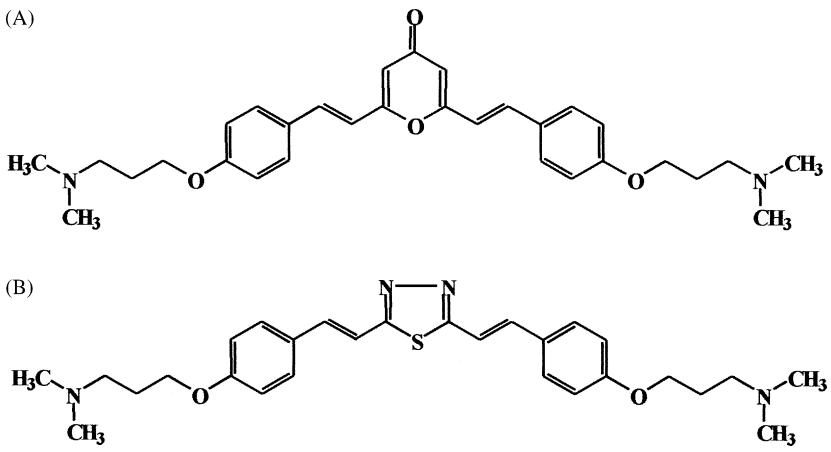

Fig. 1. The chemical structures of GO-12 (A) and GO-13 (B).

were maintained in a humidified incubator at 37° in 5% CO_2/95% air.

2.3. MTT assay method

Cells were pre-incubated in the absence or presence of the indicated inhibitors and then, the cytotoxic effect was assessed using the MTT assay method. The MTT was dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg mL^{-1} and filtered. From the stock solution, 10 μL per 100 μL of medium was added to each well, and plates were gently shaken and incubated at 37° for 1 hr. Treatment of living cells with MTT produces a dark blue formazan product, whereas no such staining is observed in dead cells. After the loading of MTT, the medium was replaced with 100 μL acidified β-isopropanol and left for 20–30 min at room temperature for color development, and then the 96-well plate was read by enzyme-linked immunosorbent assay (ELISA) reader (570 nm) to get the absorbance density values.

2.4. Assessment of cell necrosis

The necrotic cell death was measured by the release of lactate dehydrogenase (LDH) into the culture medium, which indicates the loss of membrane integrity and cell necrosis. LDH activity was measured using a commercial assay kit (Cytotoxicity assay kit, Promega), where the released LDH in culture supernatants is measured with a coupled enzymatic assay which results in the conversion of a tetrazolium salt into a red formazan product. The necrotic percentage was expressed as (sample value/maximal release) × 100%, where the maximal release was obtained following the treatment of control cells with 0.5% Triton X-100 for 10 min at room temperature.

2.5. In situ labeling of apoptotic cells

In situ detection of apoptotic cells was carried out using annexin V and TUNEL apoptosis detection methods. Annexin V-labeling is based on the apoptotic character of the flip of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane. The *in situ* TUNEL assay identifies apoptotic cells using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the free 3′-OH of cleaved DNA. The biotin-labeled cleavage sites were then visualized by reaction with fluorescein conjugated avidin (avidin-fluorescein isothiocyanate). Cells were cultured in chamber slides for 24 hr and then treated with GO-13 for the indicated time courses. After the incubation period, cells were washed twice with PBS, fixed (for TUNEL) or not (for annexin V) for 1 min with ice-cold ethanol/acetic acid (1:1) solution, and then washed three times with PBS. The fixed cells were permeabilized in ice-cold 0.2% Triton X-100 detergent for 5 min and then washed three times with PBS. Staining was carried out according to the protocol provided by the suppliers. Finally, the photomicrographs were obtained with a fluorescence microscope (Nikon).

2.6. Western blot analysis

After the exposure of cells to the indicated agents and time courses, cells were washed twice with ice-cold PBS and reaction was terminated by the addition of 100 μL ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin, and 1% Triton X-100). For the detection of phosphorylated Akt/PKB, 1 mM Na_3VO_4, 1 mM NaF, 50 mM tetrasodium pyrophosphate, 10 nM okadaic acid, 0.25% sodium deoxycholate were included in the lysis buffer. The cell lysates (25 μg/lane) were electrophoresized on 10–15% SDS-polyacrylamide gels and the Western blot analysis was carried out as we previously described [13]. Detection of signal was performed with an enhanced chemiluminescence detection kit.

2.7. Preparation of cytosolic extracts and measurement of caspase-3 activity

After the treatment of indicated agent, cells were washed twice with ice-cold PBS and then collected by centrifugation at 200 *g* for 5 min at 4°. The cell pellet was resuspended in lysis buffer (25 μL/10^6 cells) obtained from a commercial assay kit (Caspase-3 colorimetric assay kit, R&D Systems Inc.). After a 10-min incubation on ice, cell homogenates were centrifuged at 10,000 *g* for 1 min and supernatants were removed for the determination of caspase-3 activity. Proteolytic reactions were performed in a total volume of 100 μL reaction buffer containing 50 μL of cytosolic extracts and 5 μL DEVD-pNA obtained from the same commercial kit described above. The reaction mixture was incubated at 37° for 1–2 hr, and then the formation of *p*-nitroanilide was measured at 405 nm using an ELISA reader.

2.8. Nude mice angiogenesis model

The Matrigel (0.5 mL) containing vehicle, vascular endothelium growth factor (VEGF) or VEGF plus GO-13 was subcutaneously injected into the nude mice for the determination of anti-angiogenic activity of GO-13. After 6-day incubation, animals were euthanized with intraperitoneal administration of pentobarbital. The Matrigel plug was carefully clipped for histological examination and the determination of angiogenesis using a hemoglobin assay kit. Briefly, the plug was gently and shortly homogenized, and then 20 μL whole blood was added into 5 mL Drabkin's solution (from the commercial kit). The reaction was allowed for 15 min at room temperature, and then the optimal absorbance was obtained under the colorimetric

determination at 530–550 nm and the hemoglobin concentration was calculated based on the hemoglobin standard. For histological examination, the plug was put into 4% formaldehyde and embedded in paraffin. The embedded tissues were sectioned at 6-μm thick, stained with hematoxylin–eosin, and analyzed using a microscopy.

2.9. Statistics and data analysis

Data are presented as the mean ± SEM for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by a *t*-test and *P*-values less than 0.05 were considered significant.

3. Results

3.1. Effect of GO compounds on the cytotoxicity in human cancer cells

The cytotoxic effects of GO compounds were examined in several human cancer cells, such as the lung cancer A549, androgen-independent prostate cancer PC-3 and hepatoma HA22T cells. The data showed that GO-12 and GO-13 are more potent than the other compounds in the screening test (Table 1). In addition, the necrotic indication of cellular LDH release reaction was also examined after the exposure of GO compounds. The data showed that GO-12 (10 μM), but not GO-13, induced a profound increase of LDH release as early as 1-hr exposure to the cells (51.6 ± 4.0% of total release, N = 3) indicating the necrotic cytotoxicity to GO-12 action. The data showed that GO-12 was less potential than GO-13 and, therefore, the cytotoxic effect of GO-13 was further investigated in this study.

Table 1
Effect of GO compounds on the cytotoxicity in A549, PC-3 and HA22T cells by the MTT assay

Compound	IC_{50} (μM)		
	A549	PC-3	HA22T
1	33.2 ± 0.7	28.2 ± 0.6	49.0 ± 1.1
2	41.3 ± 0.9	40.8 ± 1.8	66.5 ± 3.4
3	67.8 ± 5.1	52.5 ± 2.2	79.6 ± 3.1
4	31.9 ± 1.8	37.7 ± 4.1	51.2 ± 2.7
5	89.4 ± 3.7	77.6 ± 4.2	>100
6	51.1 ± 2.5	40.2 ± 1.9	68.4 ± 2.8
7	>100	>100	>100
8	>100	>100	>100
9	>100	>100	>100
10	28.7 ± 1.1	33.9 ± 2.0	46.1 ± 1.8
11	21.2 ± 0.7	17.8 ± 2.6	36.4 ± 1.6
12	5.9 ± 0.8	6.6 ± 0.3	8.7 ± 1.2
13	6.8 ± 1.0	6.3 ± 0.6	9.7 ± 0.9
14	89.8 ± 2.6	76.5 ± 3.3	>100
15	>100	>100	>100

The data are expressed as mean ± SEM of three to six determinations (each in triplicate).

3.2. Identification of GO-13-induced apoptotic cell death in A549 cells

To determine whether the mode of GO-13-induced cytotoxicity was the apoptotic cell death, several identification techniques were carried out in this study. Morphological examination revealed that GO-13 caused A549 cells to round up and bleb (Fig. 2B), and further detach from the culture plates. These responses are consistent with the apoptotic characteristics. Additionally, we confirmed the apoptosis by annexin V-reaction techniques, since the flip of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane is the crucial event of apoptosis [14]. The results demonstrated the negative staining of annexin V in cells treated with vehicle (Fig. 2C), whereas the GO-13-treated cells showed the strong positive staining (Fig. 2D).

The hallmarks of apoptosis are nuclear chromatin condensation and fragmentation of DNA, which can be detected by labeling breaks in the DNA strand using *in situ* TUNEL-reactions. As demonstrated in Fig. 2E, the control cells showed negative staining, while GO-13 significantly induced the cell apoptosis with strong fluorescence staining (Fig. 2F). Furthermore, the cell cycle distribution of A549 cells was determined by FACScan analysis of propidium iodide staining cells, a measurement of DNA content. The data demonstrated that GO-13 did not significantly affect the number of cells in both S and G2/M phases of the cell cycle unless an increase of the distribution in sub-G0/G1 phase (5.2 ± 1.3%, 14.7 ± 3.2%, and 39.7 ± 5.1%, respectively for 1, 3, and 10 μM) with a concomitant decrease in that of G0/G1 phase of the cell cycle. Based on the above identification, it shows that the mode of GO-13-induced cell death is predominantly an apoptotic reaction.

3.3. Determination of GO-13-induced time-related apoptosis

To quantitatively determine the GO-13 action, the mitochondrial reduction of MTT assays was carried out. As demonstrated in Fig. 3, GO-13 (10 μM) induced the cytotoxic effect in a time-dependent manner. It showed two distinct time-related responses to GO-13 action, that is to say, a short-time (within 4 hr) and a longer-time effect (24 hr).

3.4. Examination of several inhibitors on the GO-13-induced effect

To investigate the functional mechanism of the GO-13-mediated effect, several apoptosis-related targets were examined in this study. The data showed that both H7 (a protein kinase C inhibitor) and SB203580 (a p38 mitogen-activated protein kinase (MAPK) inhibitor) had little influence on GO-13-induced effect (Fig. 4). However, trolox (an antioxidant) significantly inhibited GO-13

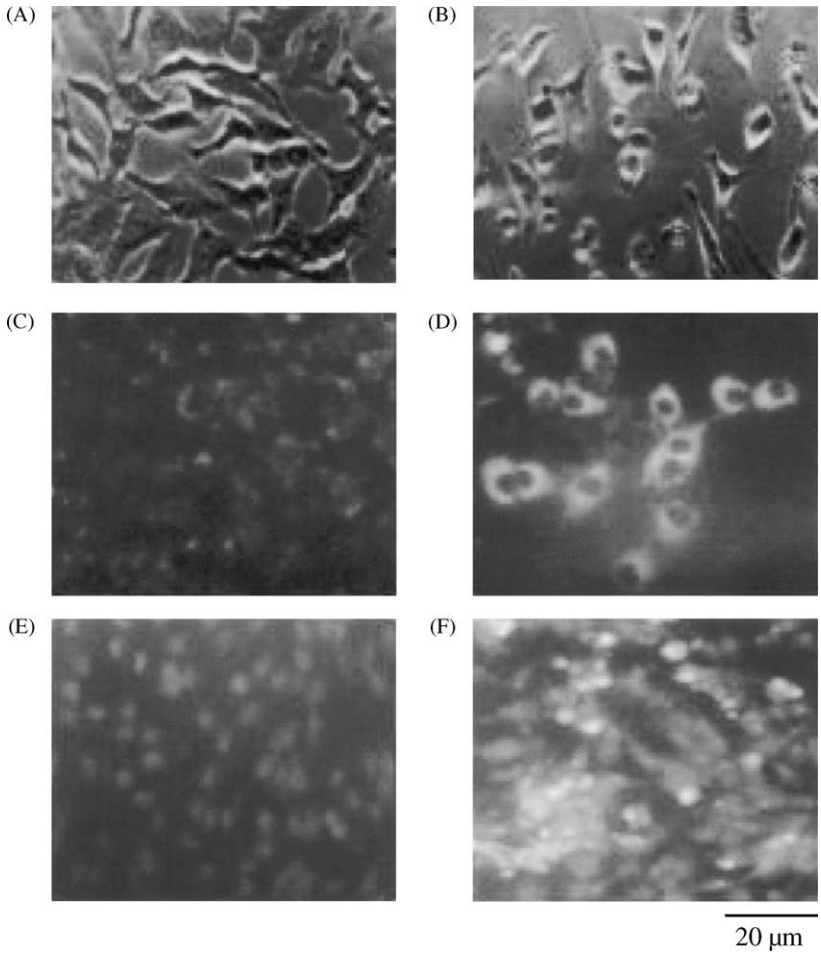

Fig. 2. Identification of the cytotoxic mode of GO-13 in A549 cells. Cells were cultured onto chamber slide and allowed to attach for 24 hr. Cells were incubated in the absence (A, C and E) or presence of 10 μM GO-13 (B, D and F) for further 16 hr. Then, the cell cytotoxicity was detected by microscopic morphological examination (A and B), annexin V immunoreaction (C and D) or TUNEL reaction (E and F) as described in Section 2.

(3 and 10 μM)-induced effect suggesting that reactive oxygen species (ROS) might functionally contribute to the apoptotic mechanism.

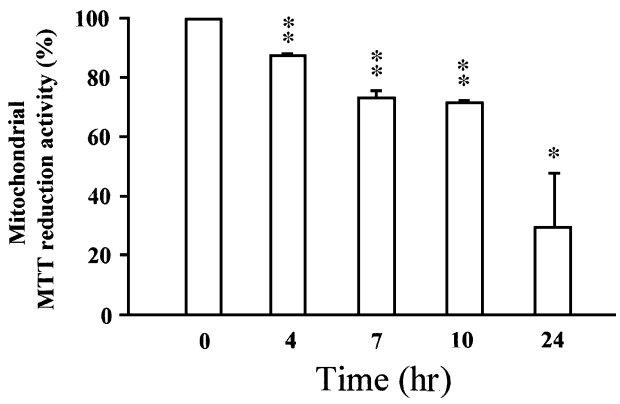

Fig. 3. Time-dependent effect of GO-13 on the cytotoxicity in A549 cells. Cells were incubated in the presence of GO-13 (10 μM) for different time courses. Then, the cytotoxic effect was detected by MTT assay method as described in Section 2. The cytotoxicity is expressed as the percentage of mitochondrial MTT reduction activity and the data are expressed as mean ± SEM of three determinations (each in triplicate). $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with control.

3.5. Effect of GO-13 on the regulation of phosphatidyl inositol (PI)-3 kinase activity

The lipid kinase PI3-kinase plays a crucial role in the intracellular signaling pathways in response to survival factors via generating phosphoinositol phospholipid signals and the subsequent activation of downstream effectors [15,16]. Among the downstream events regulated by PI3-kinase, the activation of the serine-threonine kinase Akt/PKB inhibits apoptosis induced by various apoptotic stimuli and regulates cell survival in a variety of cell types [15–17]. In our un-shown data, we found that the PI3-kinase inhibitors, LY294002 and wortmannin, induced the apoptosis in A549 cells in a concentration-dependent manner confirming the survival regulation by PI3-kinase activity. In this study, GO-13 showed little effect on serum-induced Akt/PKB phosphorylation indicating that GO-13 had no direct inhibition on PI3-kinase activity (Fig. 5A). Furthermore, the exposure of GO-13 to the cells for 1–7 hr also had little influence on the basal level of Akt/PKB phosphorylation (Fig. 5B), whereas it almost completely annihilated the basal after a 24-hr application (Fig. 5C).

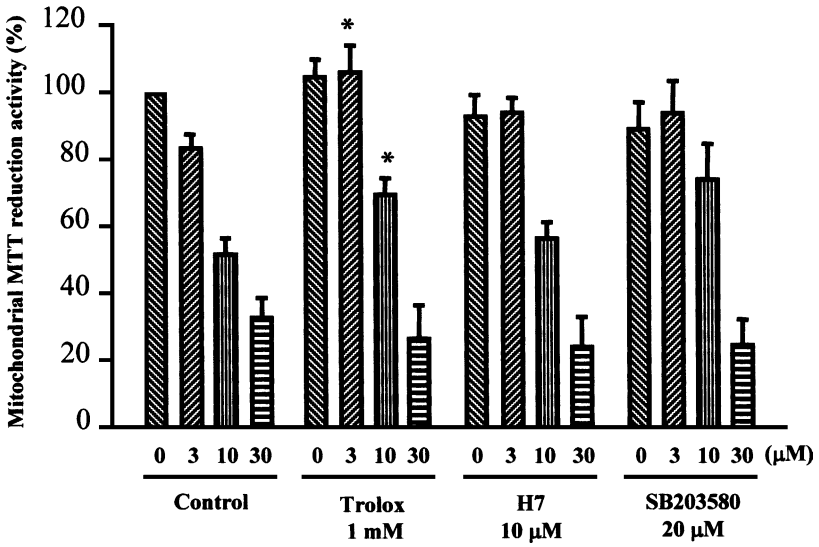

Fig. 4. Effect of several inhibitors on GO-13-induced cytotoxicity in A549 cells. Cells were incubated in the absence (basal and control) or presence of the indicated inhibitors for 30 min. Then, GO-13 ((▨), 3 μM; (▥), 10 μM; (▤), 30 μM) was added to the cells for 24 hr. The cytotoxic effect was detected by MTT assay method as described in Section 2. The cytotoxicity is expressed as the percentage of mitochondrial MTT reduction activity and the data are expressed as mean ± SEM of four determinations (each in triplicate). $^{*}P < 0.05$ compared with respective control.

3.6. Effect of GO-13 on the expressions of Bcl-2 family

The Bcl-2 family of proteins comprises a critical intracellular checkpoint of apoptotic response within a common cell death pathway. The over-expression of Bcl-2 or Bcl-X_L increased or prolonged the viability, while that of Bax accelerated the apoptosis, in response to numerous apoptotic stimuli [6–9]. In the present study, we examined the effect of GO-13 on the expression of Bcl-2 family proteins, such as Bcl-2, Bcl-X_L, and Bax expressions. However, we could barely detect the expression of Bcl-2 protein in A549 cells (data not shown). In contrast, Bcl-X_L was abundantly expressed in A549 cells. GO-13 profoundly down-regulated this anti-apoptotic protein within a 3-hr application (Fig. 6A). We also examined the Bax expression in this study. The data demonstrated that although GO-13 showed little influence on this protein expression during 1- to 7-hr incubation period (Fig. 6B), it caused the increase of Bax protein level after a 24-hr treatment in a concentration-dependent manner (Fig. 6C).

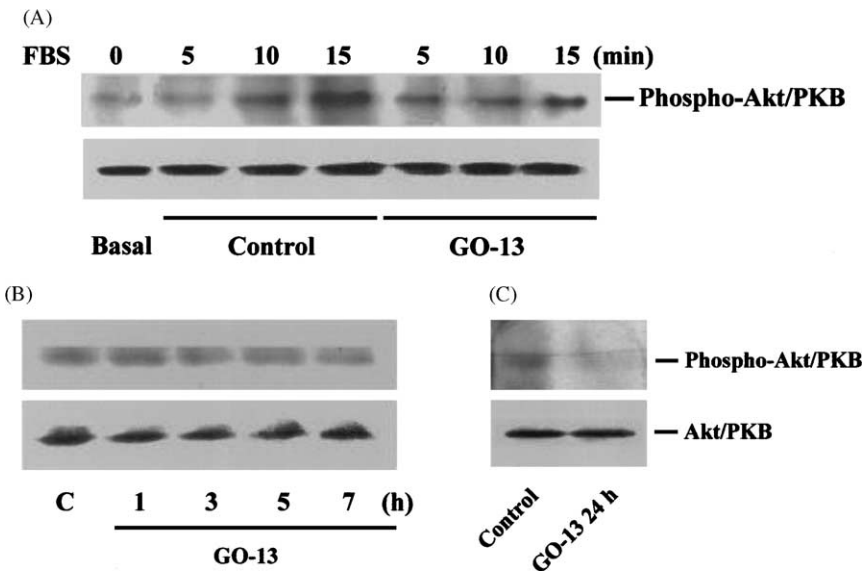

Fig. 5. Effect of GO-13 on Akt/PKB phosphorylation in A549 cells. (A) Cells were pre-incubated in the absence (basal and control) or presence of 30 μM GO-13 for 30 min and then 10% FBS was added to the cells for the indicated time courses; (B) and (C) cells were incubated without (control) or with 30 μM GO-13 for the indicated time courses. After the above treatment (A, B and C), the cells were harvested and lysed for the detection of Akt/PKB and phosphorylated-Akt/PKB by Western blot analysis as described in Section 2.

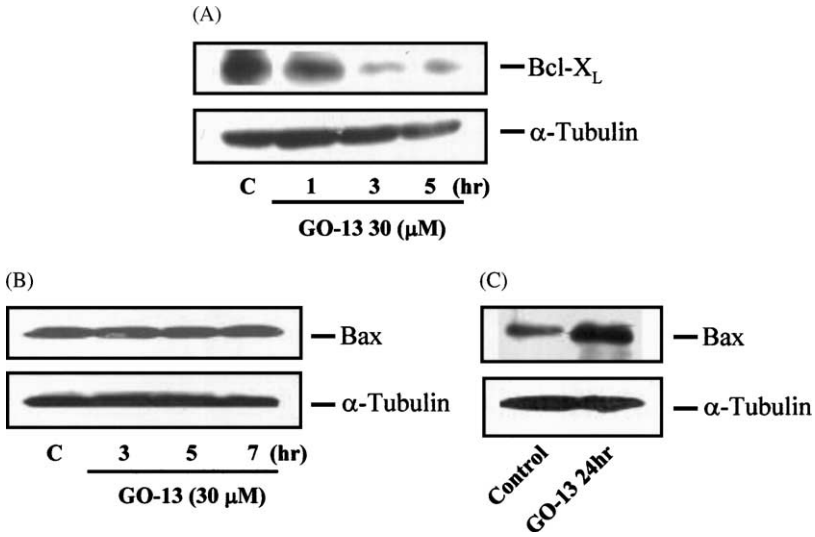

Fig. 6. Effect of GO-13 on Bcl-X_L and Bax expressions in A549 cells. Cells were incubated in the absence (control) or presence of 30 μM GO-13 for the indicated time courses. Then, the cells were harvested and lysed for the detection of Bcl-X_L and Bax protein expressions by Western blot analysis as described in Section 2.

3.7. Determination of the involvement of caspase-3 activation

The activation of caspases plays a pivotal role in the execution of cell apoptosis. Recent studies have demonstrated that caspase-3 is the major caspase activated in response to distinct apoptotic stimuli [18–21]. The data demonstrated that GO-13 (10 μM) induced the increase of caspase-3 activity in a time-dependent manner (Fig. 7); furthermore, trolox (1 mM) significantly attenuated this enzymatic activity to GO-13 action (Fig. 7).

3.8. Effect of GO-13 on VEGF-induced angiogenesis in the nude mice model

The nude mice models were used in this study to examine the effect of GO-13 on the regulation of the angiogenesis. As demonstrated in Fig. 8A, the subcutaneously inserted Matrigel plug showed a marked angiogenic effect after a 6-day incubation of VEGF (150 ng mL^{-1}); however, GO-13 significantly diminished the angiogenesis to VEGF action. The results were also detected by the histological examination (Fig. 8B) and quantitatively analyzed using the hemoglobin detection assay (Fig. 8C).

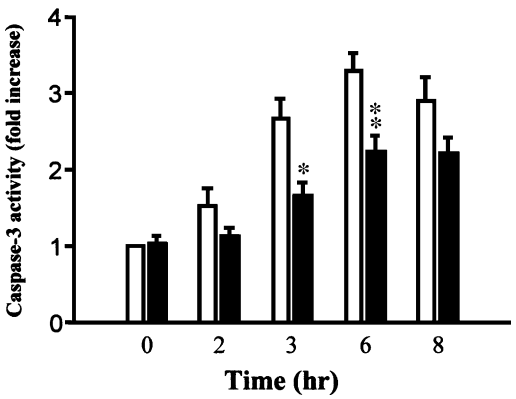

Fig. 7. Effect of GO-13 and trolox on the caspase-3 activity in A549 cells. Cells were pre-incubated in the absence (□) or presence (■) of trolox (1 mM), and then GO-13 (10 μM) was added to the cells for the indicated time courses. The caspase-3 activity was determined as described in Section 2. The data are expressed as mean ± SEM of three determinations. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the respective trolox-free control.

4. Discussion

Apoptosis is an energy requiring suicide program, which is usually activated following the death receptor ligation, the withdrawal of survival factors, or in response to cellular damage, such as the treatment with cytotoxic agents or ionizing radiation [22–24]. To date, the induction of apoptotic reaction has been widely used in the cancer chemotherapy. In this study, GO-12 and GO-13 were two effective agents showing the cytotoxic activities in A549 cells. However, GO-12 other than GO-13 profoundly induced the release of cellular lactate dehydrogenase, a necrotic index. Furthermore, based on several criteria to the GO-13 action, such as the morphological observation, positive annexin V-labeling and TUNEL response, we defined that GO-13-induced cytotoxic effect was the apoptotic reaction.

At present there are several studies searching for potential anticancer candidates in thiadiazole-based compounds. The cytotoxic effects to cancer cells were examined, but the anticancer potency was not satisfied and the mode of cytotoxicity and the action mechanism were not defined [25]. GO-13 is also a thiadiazole-based compound with two symmetric 4-(3-dimethylaminopropoxy)styryl side chains. It reveals that these two side chains might contribute to the cytotoxic potency since GO-12, which also

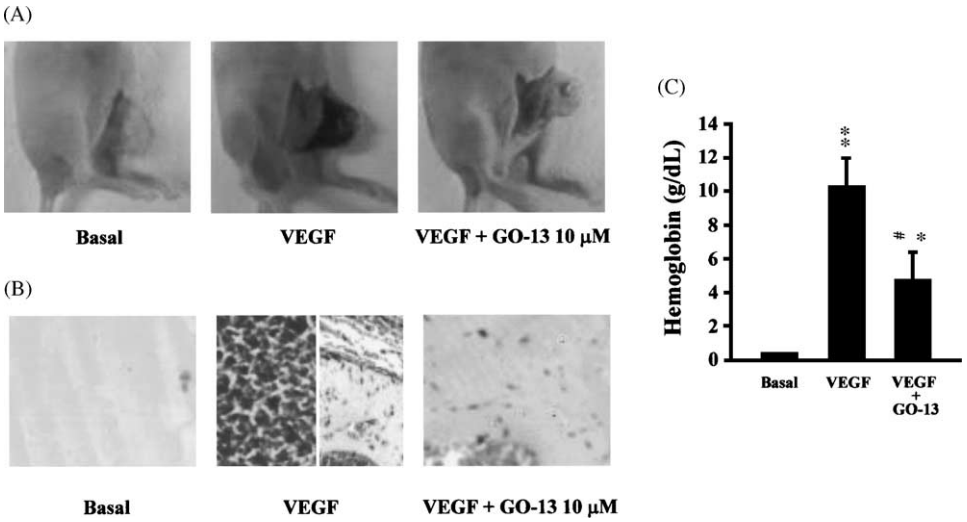

Fig. 8. Effect of GO-13 on anti-angiogenic effect in the nude mice models. The Matrigel plugs containing the indicated agents were subcutaneously injected into the nude mice. After a 6-day incubation, the animals were euthanatizedly sacrificed and the Matrigel plugs were carefully exposed for photographing (A). Then, the Matrigel plugs were carefully cut down, fixed, and embedded for section and H&E staining (B), or quantitative assessment for angiogenesis (C) as described in Section 2. Data are expressed as mean ± SEM of four animals in each group. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with basal; $^{\#}P < 0.05$ compared with VEGF control.

has the same side chains, shows similar cytotoxicity although the necrotic effect is present.

After examination of the time-dependent effect to the GO-13 action, we found that it induced the two-phase cytotoxicity in A549 cells. The first phase occurred within a 4-hr exposure of GO-13, it reached a plateau in a 7-hr treatment, and explained about 25% of cytotoxicity. After a longer incubation period of GO-13, the second phase happened, and accounted for a further 45% of the GO-13-mediated effect. These data implied that there might exist more than one apoptotic mechanism for the GO-13 action. By understanding the role that some factors of apoptosis play, either at the regulation or execution phases of cell death in cancer cells, we will be in a better position to design and explore new chemotherapeutic agents. Several factors, such as ROS, protein kinase C, and p38 MAPK have been suggested to play a role in the apoptotic mechanism in cancer cells. As to ROS, although diverse origins are relevant, it is frequently generated by redox reaction via electron transfer system. The oxidative stress is able to cause the cytochrome *c* release to cytosol, the caspase-3 activation and the apoptotic cell death [26]. In addition, the increase of ROS generation is frequently found in numerous anticancer agents or their metabolites [27]. In this study, the GO-13-induced effect was significantly reversed by trolox indicating the involvement of ROS generation to GO-13 action.

Protein kinase C, a family of serine/threonine protein kinases, is involved in signal transduction pathways that regulate cell proliferation, apoptosis, differentiation and numerous cellular responses [28]. Its central role in these processes, such as tumor initiation, progression, invasion, angiogenesis and the response to antitumor agents, makes it a potential therapeutic target in cancer. However, the fact that the protein kinase C inhibitor H7 had no influence on the cytotoxic effect induced by each concentration of GO-13, revealed that neither activation nor inhibition of protein kinase C involved in GO-13-mediated function. p38 MAPK, another serine/threonine protein kinase, belongs to the MAPK superfamily. Various extracellular stimuli, including pro-inflammatory cytokines, heat shock, ultraviolet light, irradiation, and some mitogens, trigger the stress-regulated p38 MAPK activation via phosphorylation on a TGY motif within the kinase activation loop [29]. p38 MAPK appears to play a crucial role in apoptosis, transcriptional regulation and cytokine production [30]. In this study, SB203580, a selective inhibitor of p38 MAPK, did not modify GO-13-induced apoptosis in A549 cells. In a parallel experiment by Western blot detection, GO-13 exhibited little effect on basal level of phosphorylated p38 MAPK expression (data not shown). These data indicate that GO-13-induced apoptosis is not through the activation of p38 MAPK.

The Bcl-2 family proteins play an important role in the regulation of apoptosis. It has been well suggested that the overall ratio of the death agonists (Bax, Bak and Bad) to antagonists (Bcl-2 and Bcl-X_L) determines the susceptibility to a death stimulus. Several members of Bcl-2 family of proteins were examined in this study. The data showed that A549 cells demonstrated a negligible expression of Bcl-2 protein. This was consistent with the other studies [31]. It has been suggested that the over-expression of Bcl-X_L is frequently detected in lung cancers, where it plays a central role in the tumor development, progression and drug resistance. In combination with our observation that Bcl-X_L protein expressed a profound level in A549 cells (Fig. 6A), we believe that Bcl-X_L is a predominant protein in the regulation of survival signaling in this type of

cells. In a short-term exposure to GO-13, the expression of Bcl-X_L protein was markedly down-regulated, and the activation of caspase-3 and induction of apoptosis occurred in A549 cells. It reveals that Bcl-X_L protein plays a role on GO-13-induced first-phase apoptosis. Interestingly, after a long-term treatment GO-13 significantly induced the increase of Bax protein level. This effect was associated with a nearly complete inhibition of Akt/PKB activation. It has been well established that Akt/PKB is a central downstream effector of PI3-kinase and regulates cell survival in response to apoptotic stimuli. One possible mechanism is that Akt/PKB inhibits a conformational change of Bax and its subsequent translocation to mitochondria, thus preventing apoptosis [32]. Although the study about Bax translocation needs further identification, we believe that the up-regulation of Bax expression and inhibition of Akt/PKB activation share the important role in GO-13-induced late-phase apoptosis in A549 cells.

Angiogenesis, the formation of new vasculature, is essential for tumor growth and metastasis. Accordingly, the inhibition of tumor angiogenesis by novel agents has become a very active field in the anticancer research. Bongartz *et al.* have carried out the anti-angiogenic screening tests based on thiadiazole-related compounds by the rat aortic ring assay [33]. In the present work, by means of the nude mice angiogenesis model system, we found that GO-13 also showed the anti-angiogenic activity with the similar potency of inducing apoptosis in A549 cells. However, we could not rule out the possibility that GO-13-induced anti-angiogenic activity is simply based on its cytotoxic activity.

Altogether, it is suggested that GO-13 is the most effective anticancer compound in our screening tests. It induces the early-phase apoptosis in A549 cells via the down-regulation of Bcl-X_L expression, and the late-phase effect through the up-regulation of Bax expression as well as the inhibition of Akt/PKB activation. However, the chemical optimization and the *in vivo* antitumor effect are still needed to obtain a much more potential candidate.

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