Bergapten induces G1 arrest of non-small cell lung cancer cells, associated with the p53-mediated cascade

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Received April 28, 2018; Accepted November 28, 2018

DOI: 10.3892/mmr.2019.9810

Abstract. The principal subtype of lung cancer, non-small cell lung cancer (NSCLC) is a life-threatening malignancy that causes high mortality rates. Bergapten (5-methoxypsoralen) has been identified to possess anticancer activity against a number of carcinomas. In the present study, the effects of bergapten on NSCLC cells were investigated. The cell viability was determined by MTT assay. Cell cycle distribution was analyzed using flow cytometry. Protein expression and kinase cascade were demonstrated using western blot analysis. The results demonstrated that treatment with bergapten (50 μM for 48 h) inhibited the viability of A549 and NCI-H460 NSCLC cells to 79.1±2.8% and 74.5±3.1%, respectively, compared with the controls. It was identified that bergapten induced G1 phase accumulation in A549 and NCI-H460 cells between ~58 and 75% ($P<0.01$). In addition, bergapten significantly increased the sub-G1 phase ratio to ~9% ($P<0.05$) in the two cell types. Further investigation demonstrated that bergapten upregulated the expression of cellular tumor antigen p53 (p53) and its downstream proteins cyclin-dependent kinase inhibitor 1 and cyclin-dependent kinase inhibitor 1B, whereas, it downregulated the expression of cyclin D1 and CDK4. Overall, these results suggested that bergapten may inhibit cell viability and trigger G1 arrest and apoptosis in A549 and NCI-H460 cells, which may be attributed to the activation of p53-mediated cascades. Therefore, bergapten may be beneficial for NSCLC treatment.

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Key words: bergapten, non-small cell lung cancer, cellular tumor antigen p53, G1 arrest, apoptosis

Introduction

Among the life-threatening cancer types, lung cancer is the most common cause of mortality worldwide. The principal clinical therapeutic regimens for non-small cell lung cancer (NSCLC) include surgery, radiotherapy and chemotherapy. Although a number of antitumor drugs have been approved and used for lung cancer therapy, the cancer-associated mortality rate remains high. This may primarily be attributed to late diagnosis and low response to chemotherapy (1,2). Therefore, at present, the 5-year survival rate of patients with lung cancer worldwide is ~15% (3-5). Lung cancer includes two principal subtypes, small cell lung cancer and NSCLC, the later accounts for ~85% of all cases and the outcomes for NSCLC remain unsatisfactory (6). Therefore, there is a necessity to develop compounds against lung cancer, particularly the NSCLC subtype.

Natural products have been regarded as important sources for the development of novel and potent antitumor drugs during the past decade. Bergapten (5-methoxypsoralen), a coumarine derivative, has been demonstrated to exhibit anti-proliferative activity against a number of malignant carcinoma cells, including breast cancer cells (7). Through photo-activation using UVA irradiation, it was determined that psoralen, structurally associated with coumarine, may be used to treat proliferative skin disorders; a human breast cancer model that overexpressed the erb-b2 receptor tyrosine kinase 2 oncogene was treated with psoralen to inhibit ErbB2 signaling (8). Panno *et al* (7) observed that bergapten inhibits the proliferation of MCF-7 cells and tamoxifen-resistant MCF7-TR1 cells by inducing the transforming growth factor-β/mothers against decapentaplegic homolog 4-associated degradation of estrogen receptor α (7). In addition, De Amicis *et al* (9) demonstrated that bergapten induces the phosphatase and tensin homolog (PTEN)-mediated autophagic cascade, including increased expression of PTEN, Beclin-1 and class III phosphatidylinositol 3-kinase, and microtubule-associated proteins 1A/1B light chain 3B conversion in MCF-7 and ZR-75 cells. These findings demonstrated that bergapten possesses potential anticancer activity by triggering different signaling pathways. However, the effects of bergapten on NSCLC cells require further examination.

Apoptosis, programmed cell death, is a common target of a number of treatment strategies and serves a crucial role in cancer treatment. Morphological alterations in cells undergoing apoptosis include cell shrinkage, membrane blebbing, organelle integrity loss, chromatin condensation and DNA fragmentation (10). Apoptosis may be categorized into two pathways: The extrinsic and mitochondria-mediated pathways (11). The two apoptotic pathways are associated with the activation of caspases, a family of cysteine proteases that mediate efficient and non-inflammatory cell destruction (12). A recent study demonstrated that bergapten, isolated from *Ruta angustifolia* L. Pers, has differential toxicity on A549 and MRC-5 cells (3). Therefore, the present study examined the anticancer effects of bergapten on NSCLC cells and the associated signaling cascade.

Materials and methods

Reagents and antibodies. MTT, 2-propanol, dimethyl sulfoxide (DMSO), deoxycholic acid, dithiothreitol, EDTA, bergapten (cat. no. 69664), glycerol, Igepal CA-630, phenylmethylsulphonyl fluoride (PMSF), NaCl, SDS, sodium phosphate, Tris-HCl, Tween-20, propidium iodide (PI), RNase A, Triton X-100 and trypsin/EDTA were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Primary antibodies against human apoptosis regulator Bcl-2-associated X protein (Bax, cat. no. 2772), B cell lymphoma-2 (Bcl-2, cat. no. 2872, caspase-3 (cat. no. 9662), cyclin D1 (cat. no. 2978), cyclin-dependent kinase 4 (CDK4, cat. no. 9662), cyclin-dependent kinase inhibitor 1 ($p21^{Cip1}$, cat. no. 2947), cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$, cat. no. 2552), cellular tumor antigen p53 (p53, cat. no. 9282) and GAPDH (cat. no. 2118), and horseradish peroxidase-conjugated secondary antibodies (cat. no. 7076 and 7074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and treatment with bergapten. The human NSCLC cell lines A549 and NCI-H460 and non-tumorigenic lung fibroblast MRC-5 were obtained from The American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% v/v fetal bovine serum (FBS), 1% nonessential amino acids, 1% L-glutamine (both Gibco; Thermo Fisher Scientific, Inc.) and 100 μg/ml penicillin/streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C in a humidified atmosphere with 5% CO_2, as previously described (13). Cells were seeded in a 6-well culture plate at an initial density of $2x10^5$ cells/ml and cultured until they reached ~80% confluence. For treatment with bergapten, cells were starved for 16 h in serum-free medium, and subsequently treated with different concentrations (10, 20, 30, 40 and 50 μM for cell viability assay and 10, 30, and 50 μM for the other analyses) of bergapten in DMEM for 24 or 48 h (cell viability assay) and 24 h (flow cytometry and western blot analysis). Following the treatments, the treated cells were washed with PBS (25 mM sodium phosphate; 150 mM NaCl; pH 7.2), and subsequently collected by centrifugation (800 x g; 5 min; 25˚C) for subsequent analyses.

Cell viability assay. Cell viability was determined by an MTT assay as previously described (14). Cells were seeded at a density of $4x10^4$ cells/well in a 24-well plate and cultured for 24 h. Subsequently, the cells were treated with bergapten at various concentrations (10, 20, 30, 40 and 50 μM) for 24 h. Each treatment was performed in triplicate for statistical analysis. Following the treatments, the medium was removed and the cells were washed with PBS. The washed cells were incubated with MTT solution (5 mg/ml) for 4 h. Subsequent to removing the supernatant, 2-propanol was added to solubilize the formazan for determination of absorbance at 563 nm. The percentage of viable cells was estimated by comparing with untreated cells.

Flow cytometric analysis. Cells were synchronized at the G0 phase by serum starvation for 24 h, and subsequently incubated with fresh serum containing medium to allow cell-cycle progression. Following serial treatments, cells were collected, fixed with 1 ml ice-cold 70% ethanol and incubated at -20˚C for 24 h, and centrifuged at 380 x g for 5 min at room temperature to spin down the cells. The cell pellets were treated with l ml cold staining solution containing 20 μg/ml PI, 20 μg/ml RNase A and 1% Triton X-100, incubated for 15 min in dark at room temperature, and subsequently analyzed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). CellQuest software (version 2.0; BD Biosciences) was used to determine cell cycle distribution (15). Representative data were acquired from three independent experiments.

Protein extraction. Cellular proteins were extracted as previously described (16). Cells were digested using trypsin/EDTA, and subsequently homogenized in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM dithiothreitol, 0.1 mM EDTA and 1 mM PMSF]. Following sonication at a frequency of 20 kHz at 4˚C for 30 min, the homogenate was centrifuged at 14,000 x g at 4˚C for 10 min, and the supernatant was subsequently transferred to a 1.5 ml-Eppendorf tube and stored at -70˚C until subsequent analysis. The protein concentration was quantified using the bicinchoninic acid (BCA) method (BCA Protein Assay kit; Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Western blot analysis. Cellular proteins (20 μg for each lane) were electrophoresed using 12.5% SDS-PAGE, and subsequently transferred onto a nitrocellulose membrane, as previously described (16). Following blocking with 5% nonfat milk at 25˚C for 1 h, the membrane was incubated with 1:1,000-diluted primary antibodies at 25˚C for 2 h, washed with PBS containing 0.5% Tween-20, and subsequently incubated with 1:2,000-diluted peroxidase-conjugated secondary antibody at 25˚C for 1 h. GAPDH was used as the loading control. Following the final wash, the signal was developed with enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA), and the relative density was quantified using the ImageQuant LAS-3000 image analysis system equipped with Multi Gauge software version 3.0 (Fujifilm, Tokyo, Japan).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. Statistical

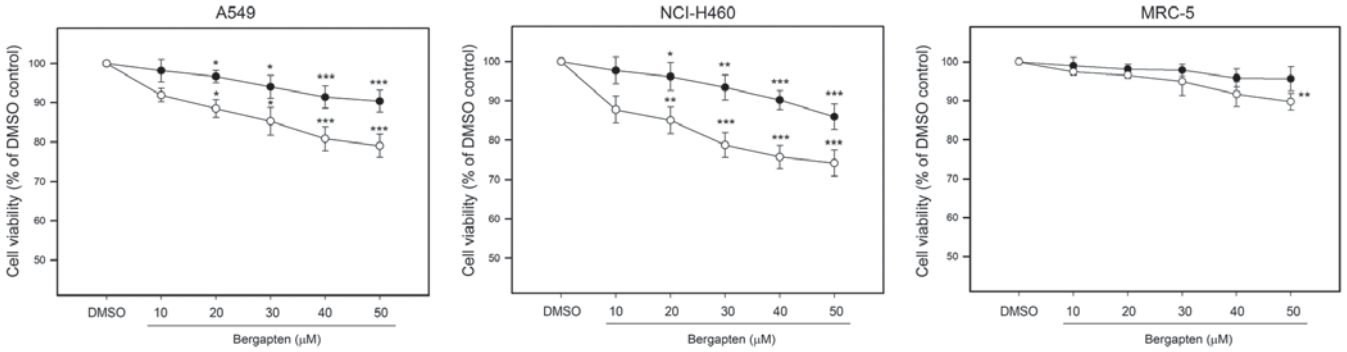

Figure 1. Bergapten reduces the cell viability of non-small cell lung cancer A549 and NCI-H460 cells; however, not the non-cancer lung fibroblast MRC-5 cells. Cells were treated with bergapten at 10, 20, 30, 40 and 50 μM for 24 (closed circle) or 48 h (open circle), and subsequently subjected to an MTT assay. Cell viability was presented as the percentage of the DMSO control. A total of three independent experiments were performed for statistical analysis. *P<0.05, **P<0.01, ***P<0.001 vs. respective DMSO control. DMSO, dimethyl sulfoxide.

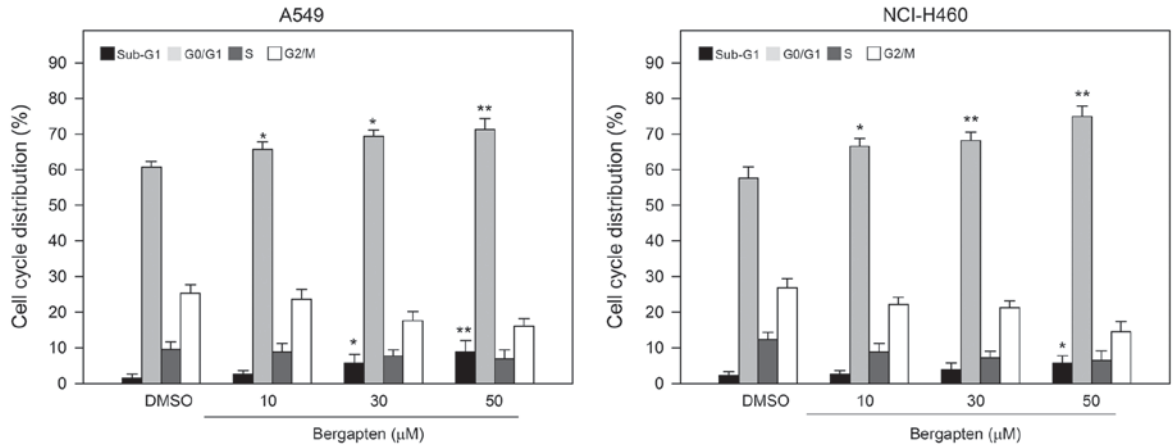

Figure 2. Bergapten induces G_0/G_1 arrest and increases the percentage of cells in the sub-G1 phase of non-small cell lung cancer A549 and NCI-H460 cells. Representative cell cycle distribution of A549 and NCI-H460 exposed to serial concentrations of bergapten for 24 h are presented. Percentage of different cell cycle phases, including sub-G1, G_0/G_1, sub-G1, S and G2/M, are presented. Data are presented as the mean ± standard deviation. A total of three independent experiments were performed for statistical analysis. *P<0.05, **P<0.01 vs. respective DMSO control. DMSO, dimethyl sulfoxide.

significance analysis was determined by one-way analysis of variance followed by Dunnett's test for multiple comparisons with the control using SPSS (version 17.0; SPSS Inc. Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Bergapten reduces the cell viability of NSCLC cell lines A549 and NCI-H460. The cytotoxic effects of bergapten on human NSCLC cell lines A549 and NCI-H460 were investigated by an MTT assay. As presented in Fig. 1, it was observed that the 24 h-treatments with bergapten dose-dependently and significantly decreased the viability of A549 and NCI-H460 cells to 90.2±3.4% and 87.3±3.9%, respectively, compared with the DMSO controls (50 μM; P<0.005). Whereas, treatments with the low-dose of bergapten (10 μM) did not significantly affect the viability of A549 and NCI-H460 cells (P=0.172 and 0.214, respectively). In addition, it was demonstrated that the 48 h-treatments with bergapten further decreased the viability of A549 and NCI-H460 cells to 79.1±2.8% and 74.5±3.1%, respectively, compared with the DMSO controls (50 μM; P<0.005). The cytotoxicity of bergapten on human non-cancer lung fibroblast MRC-5 was additionally evaluated, and the findings demonstrated that the 24-h treatments with bergapten did not significantly affect the viability of MRC-5; however, the 48-h treatment with bergapten at a high dose (50 μM) significantly decreased the viability of MRC-5 cells to 89.8±1.8%, compared with the control (P=0.008). Collectively, these findings demonstrated that bergapten exerted significant cytotoxic effects on the NSCLC cell lines A549 and NCI-H460; however, negligibly affected the viability of the non-cancerous MRC-5 cells.

Bergapten induces G_0/G_1 phase arrest of A549 and NCI-H460 cells. The effects of bergapten on cell cycle distribution in A549 and NCI-H460 cells were additionally examined. By flow cytometry, it was identified that treatment with bergapten (10, 30 and 50 μM) dose-dependently increased the percentage of cells in G_0/G_1 phase between 61.8±2.1% and 72.1±3.2% in A549 cells and between 57.2±2.8% and 76.4±3.3% in NCI-H460 cells, and the increase in the percentage of cells in the G_0/G_1 phase in response to the treatments with bergapten was significant (P<0.05; Fig. 2). Simultaneously, the percentage of cells in sub-G1 phase additionally increased to 9.1±2.6% in A549 cells and 5.8±2.2% in

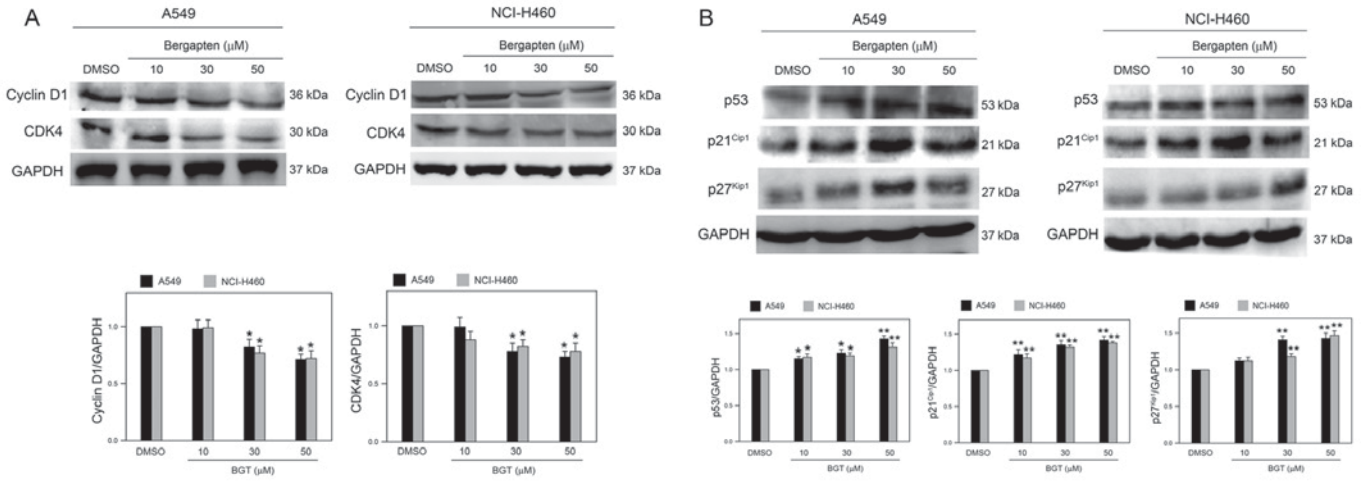

Figure 3. Bergapten downregulates cyclin D1 and CDK4, and increased the expression levels of p53, $p21^{Cip1}$ and $p27^{Kip1}$ in non-small cell lung cancer A549 and NCI-H460 cells. (A) Cells were exposed to serial concentrations of Bergapten (10, 30 and 50 μM) for 24 h, and subsequently western blot analysis was performed using specific antibodies against cyclin D1 and CDK4. (B) Cells were exposed to serial concentrations of Bergapten (10, 30 and 50 μM) for 24 h, and subsequently western blot analysis was performed using specific antibodies against p53, $p21^{Cip1}$ and $p27^{Kip1}$. GAPDH was used as the internal control. Approximate molecular weights for the immunodetected signals are indicated. CDK4, cyclin-dependent kinase 4; p53, cellular tumor antigen p53; DMSO, dimethyl sulfoxide; $p21^{Cip1}$, cyclin-dependent kinase inhibitor 1; $p27^{Kip1}$, cyclin-dependent kinase inhibitor 1B. The quantitative data were acquired from three independent experiments. *P<0.05, **P<0.01 vs. respective DMSO control.

NCI-H460 cells in the presence of bergapten at a concentration of 50 μM compared with the DMSO control (P<0.05; Fig. 2). The results suggested that bergapten induced the apoptosis of A549 and NCI-H460 cells.

Bergapten downregulates the expression of cyclin D1 and CDK4, and upregulates the expression of p53, $p21^{Cip1}$ and $p27^{Kip1}$ in A549 and NCI-H460 cells. Based on the observation that bergapten induced G_0/G_1 phase arrest, it was examined whether the treatments with bergapten regulated the expression levels of cyclins and CDKs associated with G_0/G_1 accumulation (17). The expression levels of cyclin D1 and CDK4 were determined by western blot analysis and quantified by densitometric analysis. It was observed that the 24 h treatments with bergapten (10, 30 and 50 μM) dose-dependently decreased the expression levels of cyclin D1 and CDK4 (Fig. 3A). The treatment with 50 μM bergapten decreased the expression levels of cyclin D1 and CDK4 to 21 and 33%, respectively, compared with the DMSO controls (Fig. 3A). p53 and its downstream proteins $p21^{Cip1}$ and $p27^{Kip1}$ are well-documented negative cell cycle regulators that contribute to cell cycle arrest (17). Therefore, the effects of bergapten on the p53/$p21^{Cip1}$/$p27^{Kip1}$ axis were subsequently examined. It was identified that bergapten increased the expression of p53, in addition to $p21^{Cip1}$ and $p27^{Kip1}$ (Fig. 3B). Collectively, the present results demonstrated that bergapten downregulated the expression levels of cyclin D1 and CDK4, and upregulated the expression levels of p53/$p21^{Cip1}$/$p27^{Kip1}$ in A549 and NCI-H460 cells.

Bergapten decreases the expression level of Bcl-2, and increases the expression level of Bax and cleavage of caspase-3 in A549 and NCI-H460 cells. As bergapten induced significant sub-G1 phase accumulation, apoptosis-associated components were subsequently investigated. By western blot analysis, the expression levels of anti-apoptotic Bcl-2 and pro-apoptotic Bax, and the cleavage of effector caspase-3 were determined. As presented in Fig. 4, treatments with bergapten decreased the Bcl-2 expression level; however, increased the Bax expression level and induced caspase-3 cleavage/activation in A549 and NCI-H460 cell lines. These observations suggested that bergapten decreased the anti-apoptotic signal and enhanced the apoptotic signals in A549 and NCI-H460 cells.

Discussion

In the present study, the anticancer effects of bergapten on the malignant human NSCLC cell lines A549 and NCI-H460 were examined and it was demonstrated that bergapten was able to inhibit the viability of the two cell types, which may be attributed to the induction of cell cycle arrest and apoptosis. It was previously identified that bergapten may inhibit the growth of a number of carcinoma cell types, including bladder transitional cell carcinoma T-24 (18), mucoepidermoid carcinoma cell MEC-1 (19) and hepatocellular carcinoma cell J5 (20). Similarly, the present results demonstrated that bergapten is able to suppress the viability of NSCLC cells, suggesting that bergapten has potential use in NSCLC treatment. However, although the *in vitro* findings indicated that bergapten has potential anticancer activity on NSCLC cells, further investigation is still needed to elucidate the *in vivo* anticancer effects of bergapten. p53 is a well-studied tumor suppressor protein in humans. It regulates cell fate in response to DNA damage, including cell cycle arrest, apoptosis and cellular senescence (21). Obstruction of p53 functions through mutations or deletions of p53 and p53-associated regulators has been permanently and widely discovered in human tumors (22). Recently, accumulating evidence demonstrated that p53 additionally serves an important role in regulating tumor metastasis and invasion in lung cancer (23,24). As a result, activation or

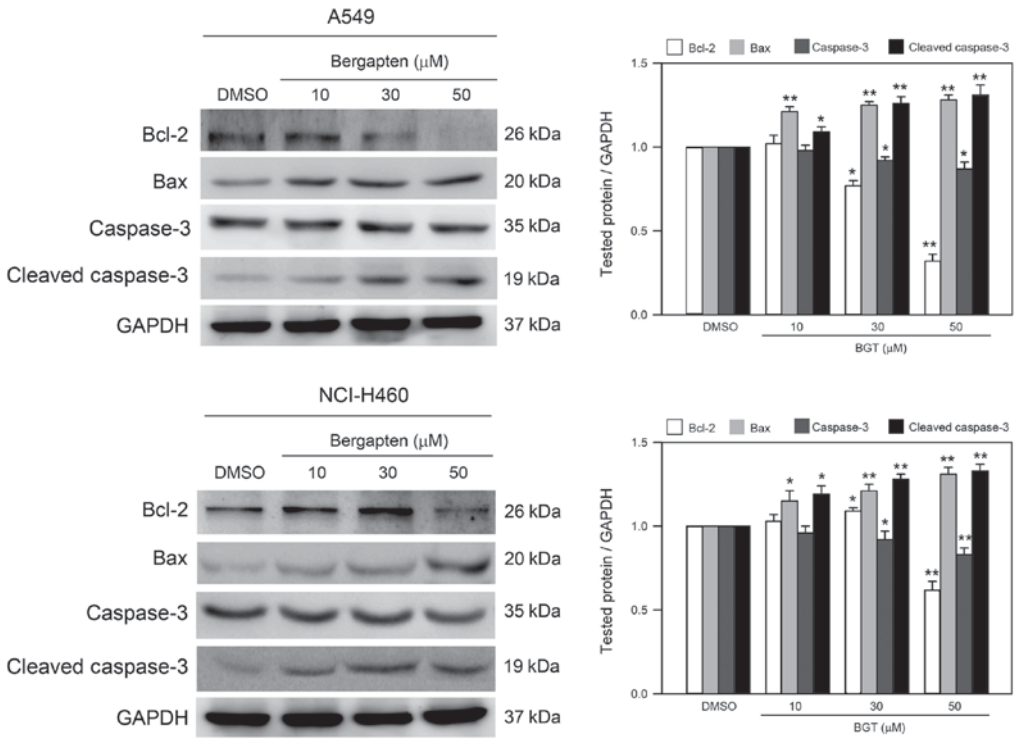

Figure 4. Bergapten decreases Bcl-2, increases Bax and induces caspase-3 activation in non-small cell lung cancer A549 and NCI-H460 cells. Cells were exposed to serial concentrations of Bergapten (10, 30 and 50 μM) for 24 h, and subsequently western blot analysis was performed using specific antibodies against Bcl-2, Bax, caspase-3 and cleaved caspase-3. GAPDH was used as the internal control. Approximate molecular weights for immunodetected signals are indicated. Bcl-2, B cell lymphoma 2; Bax, apoptosis regulator BAX; DMSO, dimethyl sulfoxide. The quantitative data were acquired from three independent experiments. *P<0.05, **P<0.01 vs. respective DMSO control.

gain-of-function of p53 is a potential anticancer treatment for numerous types of cancer. In the present study, A549 and NCI-H460 cell lines were used, which are derived from NSCLCs that express detectable p53 mRNA at expression levels comparable to normal lung tissue and exhibit no gross structural DNA abnormalities (25). Accordingly, the present findings demonstrated that bergapten increased the expression levels of p53 and downstream $p21^{Cip1}$ and $p27^{Kip1}$, suggesting that bergapten may exert its anticancer activity by inducing activation of the p53 cascade.

The disruption of cell cycle progression in cancer cells is considered an effective strategy to control tumor growth (26). The transition between a dormant quiescent stage (G0) to an active growing state is a prerequisite for the majority of cells entering the cell cycle, and it is a critical step for cancer cells (27). The progression of the cell cycle is regulated by a number of negative regulators termed CDK inhibitors, including $p21^{Cip1}$ and $p27^{Kip1}$ (27). $p21^{Cip1}$ is a universal cell cycle inhibitor that binds to cyclin-CDK complexes and proliferating cell nuclear antigen, thereby inducing cell cycle arrest at the G1 phase (28). In addition, the upregulation of $p21^{Cip1}$ and $p27^{Kip1}$ enhances the formation of complexes with G1-S CDKs and cyclins, thereby, inhibiting their activities (29-31). The results of the present study demonstrated that bergapten upregulated the expression levels of p53, $p21^{Cip1}$ and $p27^{Kip1}$; however, it downregulated the expression levels of CDK4 and cyclin D1. Overall, the results suggested that the bergapten-induced G1 phase arrest of NSCLC cells may be due to the upregulation of p53/$p21^{Cip1}$/$p27^{Kip1}$ and the consequent disruption of CDK4-cyclin D complexes.

A number of previous studies demonstrated that cell cycle arrest and apoptosis may be directly associated (32-34). For instance, the apoptotic cascade may be inhibited or induced via cell cycle manipulation depending on the cellular circumstance (35). In addition, the CDK inhibitors of the Cip/Kip family have been suggested to be indirectly involved in apoptosis. The upregulation of $p21^{Cip1}$ may be achieved via p53-dependent and p53-independent pathways following stress (36), and the overexpression of $p21^{Cip1}$ may trigger apoptosis (37). The present results demonstrated that treatment with bergapten increased the sub-G1 phase ratio in A549 and NCI-H460 cells, suggesting that bergapten not only induced G1 phase arrest; however, may additionally induce the apoptotic cascade in NSCLC cells.

Combination chemotherapy is a promising and effective treatment for cancer, which may maximize therapeutic efficacy, reduce side effects and overcome drug resistance (38). Previous clinical trials have additionally suggested that treatment with platinum-based combination chemotherapy may be considered as the first-line therapy for patients with advanced NSCLC and is superior to the single-agent treatments in terms of overall survival (39,40). The present study demonstrated that bergapten significantly decreases the viability of malignant human NSCLC cell lines A549 and NCI-H460, and induced G1 and sub-G1 phase accumulation, which may be attributed to the upregulation of p53, $p21^{Cip1}$ and $p27^{Kip1}$. The present findings suggested that bergapten exerts potential antitumor effects against NSCLC A549 and NCI-H460 cells and may be used in combination with chemotherapy to treat malignant human lung cancer.

Acknowledgements

The authors thank Dr Jing-Ting Tung (Doctor of Pharmacy, Monterey Park Hospital AHMC, Monterey Park, CA, USA) for her invaluable suggestions for the improvement of manuscript writing.

Funding

The present study was supported by the Intercollege grant from the Chung Shan Medical University (Taichung, Taiwan; grant no. CSMU-CMMC-105-02), Chi Mei Medical Center (Tainan, Taiwan, grant no. CMCSMU10503), and the China Medical University Hospital (Taichung, Taiwan; grant no. DMR-106-033; 2016).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

S-RC and S-HK conceived and designed the experiments, which were performed by C-SL, H-HL, and P-CS. S-RC, C-SL and S-HK analyzed the data. S-RC and S-HK contributed reagents, materials and analysis tools. S-HK wrote the paper. S-RC, C-SL, and H-HL provided additional technical assistance and contributed to interpretation of the data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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