

Suppression of growth of A549 lung cancer cells by waltonitone and its mechanisms of action

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Abstract. Natural compounds are a great source of cancer chemotherapeutic agents. Our investigation indicates that waltonitone, a triterpene extracted from medicinal plants, inhibits the proliferation of A549 cells in a concentration- and time-dependent manner. Waltonitone induced apoptosis of A549 cells in a concentration-dependent manner, as determined by fluorescence microscopy and flow cytometry. The apoptosis was accompanied by increased Bax protein levels and decreased Bcl-2 protein levels in A549 cells. Furthermore, the treatment of A549 cells with waltonitone altered the expression of miRNAs. We found that 27 miRNAs were differentially expressed in waltonitone-treated cells, of which 8 miRNAs target genes related to cell proliferation and apoptosis. In summary, our results demonstrate that waltonitone has a significant inhibitory effect on the proliferation of A549 cells. It is possible that upregulation of Bax/Bcl-2 and regulation of expression of specific miRNAs play a role in inhibition of proliferation and induction of apoptosis in waltonitone-treated cells. Waltonitone can be applied to lung carcinoma as a chemotherapeutic candidate.

Introduction

Lung cancer is one of the most common human cancers and the leading cause of cancer death worldwide (1,2). At present, the treatment of lung cancer mainly includes surgery, radiotherapy and chemotherapy. Chemotherapy plays an important role in the treatment of lung cancer, but it is limited to a significant extent by its toxicities, side effects and drug resistance (3,4).

The mortality in lung cancer patients remains high and the 5-year overall survival in many countries generally is <15% (5). Therefore, it is necessary to search for drugs capable of preventing and treating lung cancer and other malignancies. Recently, the use of natural compound as anticancer agents has gained a great deal of attention for their possible therapeutic qualities with minimal toxicity (6-9).

Some natural agents suppress the proliferation of cancer cells by inducing apoptosis (10). The expression of several genes has been demonstrated to be critical for the regulation of apoptosis such as Bcl-2 family proteins. The members of the Bcl-2 family play an important role in the regulation of the apoptotic pathway (11). In the Bcl-2 family members, Bcl-2 is the most important anti-apoptotic protein, whereas Bax is a pro-apoptotic protein (12). The ratio of Bax/Bcl-2 is critical for the induction of apoptosis and this ratio determines whether cells will undergo apoptosis (13).

MicroRNAs (miRNAs) are a kind of non-coding small RNAs that can post-transcriptionally regulate the expression of hundreds of their target genes (14,15). miRNAs play an important role in diverse cellular processes, such as development, proliferation, differentiation and apoptosis (16,17), some miRNAs act as oncogenes or tumor suppressors in tumorigenesis (18,19). Recent evidence indicates that some anticancer agents suppress the proliferation of malignant cells by regulation expression of miRNAs (20-22).

Waltonitone (Fig. 1), an ursane-type pentacyclic triterpene isolated from Chinese medical plant *Gentiana waltonii* Burkil, has been shown to inhibit cell growth and induces cell apoptosis in human hepatocellular carcinoma (23). However, since the anticancer effect of waltonitone in lung cancer is unknown, we explored the effect of waltonitone treatment in lung cancer A549 cells and investigated the mechanisms underlying the inhibitory effect of waltonitone in lung cancers.

Materials and methods

Reagents and chemicals. RPMI-1640, penicillin, streptomycin, and trypsin were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Fetal bovine serum (FBS) was purchased from Solarbio (Beijing Solarbio Science and Technology, China). 3-(4,5-Dimethyl thiazol-2yl)-2,5-diphe-

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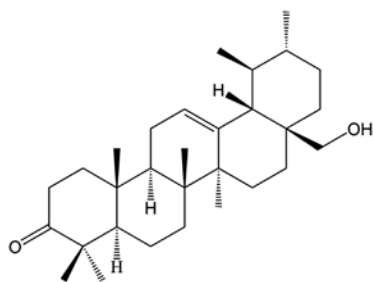


Figure 1. The structure of waltonitone.

nyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC and PI double staining kit were purchased from KeyGene (Nanjing, China). Waltonitone (HPLC purity $\geq 98\%$) was purchased from Shanghai R&D Centre for Standardization of Chinese Medicines (Shanghai, China). A 100-mM stock solution of waltonitone was prepared in DMSO and stored at -20°C . Antibodies against Bcl-2 and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibody was obtained from Santa Cruz Biotechnology. TRIzol reagent was from Invitrogen (CA, USA). All other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shenyang, China).

Cell culture. A human lung cancer A549 cell line was obtained from the China Center for Type Culture Collection (Wuhan, China). A549 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in a humidified atmosphere of 5% CO_2 . Culture medium was changed every day. Cells for assays were detached by a solution of 0.125% trypsin and 0.02% EDTA.

Cell viability assay. Viability was assessed by MTT assay. A549 cells were plated at a density of 1×10^4 cells per well in 96-well plates overnight. Cells were treated with different concentrations of waltonitone (5, 10, 20, 30, 40 and 50 $\mu\text{mol}/\text{l}$). After exposure to waltonitone for 24, 48, 72 h, 25 μl of MTT solution (2 mg/ml in PBS) was added to each well and the plates were incubated for an additional 4 h at 37°C . Then the medium was totally removed and 150 μl of DMSO were added to each well to solubilize the formazan crystals formed in viable cells. Finally, the plates were shaken and the optical density was determined at 570 nm (OD570) using ELISA plate reader (Model 550, Bio-Rad, Laboratories, Hercules, CA, USA). This procedure was replicated three times.

Fluorescent microscopy. The morphology of apoptotic cells was detected by nuclear staining with Hoechst 33342. A549 cells (5×10^5) were grown on coverslips placed into 6-well plates overnight. Cells were treated with different concentrations of waltonitone (0 and 20 $\mu\text{mol}/\text{l}$). After 24 h, the cells were washed twice with cold PBS, then fixed with cold methanol and acetic acid (3/1, v/v) at 4°C overnight and stained with Hoechst 33342 for 30 min in the dark, washed again in PBS and finally mounted in mounting medium (80% glycerol

in PBS). Processed cells were observed with a fluorescence microscope (Nikon, Japan).

Annexin V/PI flow cytometric analysis. The number of apoptotic cell death induced by waltonitone was measured by flow cytometry using Annexin V-FITC Apoptosis kit. Briefly, following treatment with waltonitone (0, 10, 20 and 30 $\mu\text{mol}/\text{l}$) for 24 h, 1×10^6 cells were harvested by centrifugation (1,000 rpm/min), washed twice with cold PBS. The cell pellet was re-suspended in 1X binding buffer at a concentration of 1×10^6 cells/ml. Cell suspension (100 μl) was transferred into FCM tube. Annexin V-FITC (5 μl) and 10 μl of PI were added into the cell suspension, followed by gentle vortexing. The cells were incubated at room temperature for 15 min in the dark. An additional 400 μl of 1X binding buffer was added to each tube. The cells were analyzed using a FACScan flow cytometer and analyzed using CellQuest software (Becton-Dickinson, Redlands, CA).

Western blot analysis. The expression of apoptosis-related proteins was evaluated by western blot analysis. In brief, 3×10^7 cells were incubated with the designated doses of waltonitone (0, 10, 20 and 30 $\mu\text{mol}/\text{l}$) for 24 h. The cells were then washed in PBS and suspended in five volumes of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP40, 0.5 mM DTT, 1% protease inhibitor cocktail). Lysates were then collected and stored at -20°C until further use. Supernatant protein concentration was determined by the Bradford method. Supernatant samples containing 40 μg of total protein were resolved by SDS-PAGE gel depending on the target protein sizes, transferred to PVDF membranes by electroblotting, and probed with anti-Bax, anti-Bcl-2 and anti-actin. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Blots were developed using an ECL kit.

Microarray analysis of miRNA profiles. miRNA microarray was used to investigate the differentially expressed miRNAs in A549 cells after waltonitone treated. A549 cells were treated with waltonitone (0 and 30 $\mu\text{mol}/\text{l}$). Total RNA was extracted from cells using TRIzol reagent 24 h after waltonitone treatment. Total RNA samples were analyzed by CapitalBio Corp. for miRNAs microarray experiments. The GeneChip miRNA Array containing probes complementary to 847 human miRNAs registered in miRBase 11.0. Experimental procedures were performed as described in detail on the website of CapitalBio (<http://www.capitalbio.com>). A miRNA was determined as differentially expressed if its expression change was >1.5 -fold, and it was identified as significantly changed using the Significance Analysis of Microarrays method with $\text{FDR} < 0.05$.

Classification and target prediction of miRNAs. The candidate target genes of miRNAs are predicted and analyzed by miRanda (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>), PicTar (<http://pictar.mdc-berlin.de/>) and TargetScan (<http://www.targetscan.org/>), and we chose the genes found by all the three software as the miRNA targets. Then, human genes related to cell proliferation and apoptosis were selected from the Gene Ontology database (<http://>

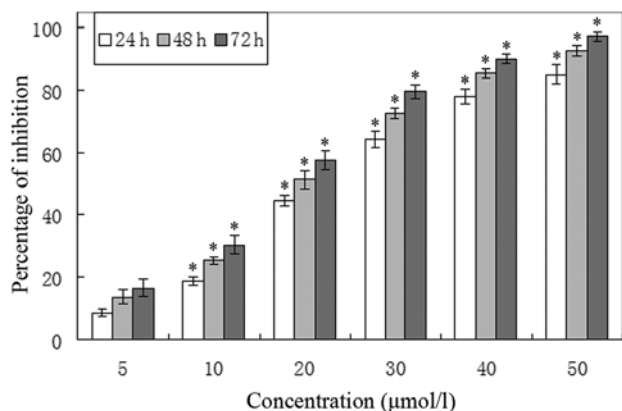


Figure 2. The proliferative inhibition effects of waltonitone on human lung cancer A549 cells. * $P < 0.05$ vs. control group.

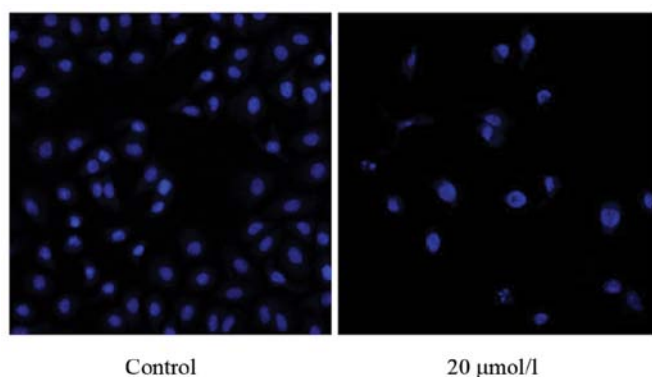


Figure 3. Cell apoptosis observed by Hoechst 33342 staining. A549 cells treated with (0 and 20 µmol/l) of waltonitone for 24 h. Apoptotic cells exhibited chromatin condensation, nuclear fragmentation and apoptotic bodies.

www.geneontology.org/), and the candidate genes predicted as targets of the miRNAs and those selected based on gene ontology were aligned by their gene names. Genes appearing in both lists were chosen and listed.

Statistical analysis. All experiments were conducted three times. Data were expressed as mean \pm SD. Statistical correlation of data was checked for significance by ANOVA and Student's t-test. The statistical significance was defined as $P < 0.05$. These analyses were performed using SPSS 11.0 software.

Results

Waltonitone inhibits A549 cell proliferation. In order to evaluate the growth inhibition effects of waltonitone on A549 cells, the cells were treated with various concentrations of waltonitone for 24, 48 and 72 h. As shown in Fig. 2, waltonitone had significant growth inhibitory effects on A549 cells in a concentration- and time-dependent manner.

Waltonitone induces A549 apoptosis. Fluorescent microscopy analysis exhibited different morphological alterations in A549 cells after treatment with waltonitone (0 and 20 µmol/l)

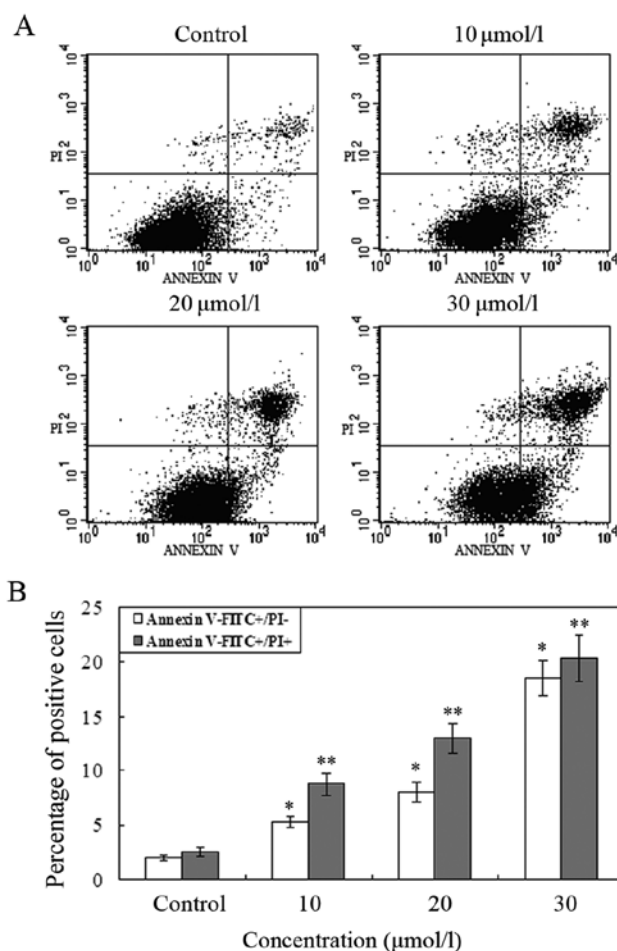


Figure 4. (A) Waltonitone-induced apoptosis in A549 cells as assayed by Annexin V/PI staining. A549 cells were treated with (0, 10, 20 and 30 µmol/l) of waltonitone for 24 h. The cells were then harvested and were stained with Annexin V/PI and flow cytometric analysis was performed to analyze apoptosis. (B) Summaries of the apoptosis data in histograms. * $P < 0.05$ vs. the control group (Annexin V positive); ** $P < 0.05$ vs. the control group (Annexin V and PI positive).

for 24 h. As showed in Fig. 3, condensation of chromatin, nuclear fragmentations and apoptotic bodies were found clearly in treated A549 cells using Hoechst 33324 staining. The results showed that when exposed to waltonitone (20 µmol/l), A549 cells underwent the typical morphologic changes of apoptosis.

The number of apoptotic cell death induced by waltonitone was measured by Annexin V/PI staining. A549 cells were treated with different concentrations of waltonitone (0, 10, 20, and 30 µmol/l) for 24 h and were analyzed by flow cytometry. As displayed in Fig. 4, the numbers of early and late apoptotic cells were significantly increased compared to control group. The results show that when treated with waltonitone for 24 h, the ratio of apoptotic cells significantly increased in a concentration-dependent manner.

Waltonitone induced expression of apoptosis-related Bcl-2 family proteins. In order to understand the molecular basis of waltonitone-induced apoptosis, we investigated the expression levels of Bcl-2 and Bax by western blot analysis. The western blot analysis showed that waltonitone treatment leads to

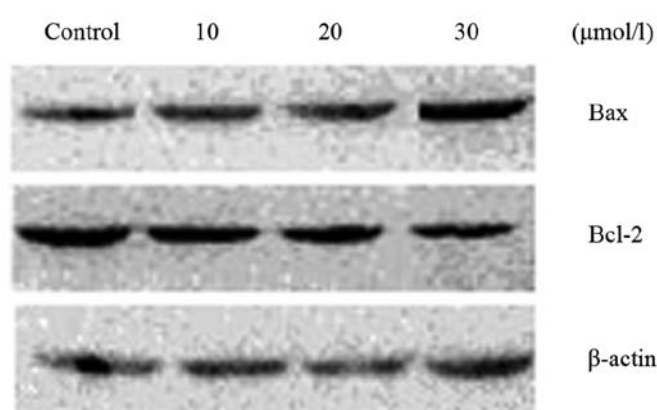


Figure 5. Effect of waltonitone on Bcl-2 family proteins by western blot analysis. A549 cells were treated with (0, 10, 20 and 30 $\mu\text{mol/l}$) of waltonitone for 24 h. Proteins were extracted, then Bax, Bcl-2 and β -actin expressions were analyzed by western blot analysis.

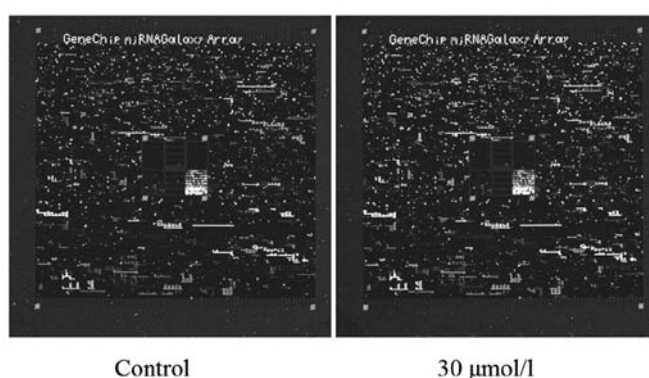


Figure 6. miRNA GeneChip showing changes after waltonitone exposure.

decrease in Bcl-2 levels; increase in Bax levels as compared to control cells (Fig. 5). The ratios of Bax/Bcl-2 increased as the concentration of waltonitone increased.

Waltonitone affects the expression of miRNAs. We investigated whether the treatment of cells with waltonitone could affect the expression of miRNAs. Using miRNA microarray analysis, we found 27 miRNAs with >1.5 -fold expression changes in waltonitone-treated A549 cells (Fig. 6), of which 15 were upregulated, and 12 downregulated. A total of 27 miRNAs with expression levels regulated by waltonitone treatment (Table I) were selected to identify potential target genes related to cell proliferation and apoptosis.

Latent targets of each miRNA were predicted using miRanda, PicTar and TargetScan. We investigated related targets from the Gene Ontology database that were separated into two groups: cell proliferation and apoptosis. We found 26 genes associated with cell proliferation, and 26 genes associated with apoptosis (Table II) (Fig. 7).

Discussion

Lung cancer is the leading cause of cancer-related deaths in the world. It accounts for 17.6% of cancer death, and its 5-year

Table I. The 27 miRNAs showing 1.5-fold expression changes after waltonitone exposure.

miRNA name	Change-fold
Upregulation	
hsa-miR-1246	3.461
hsa-miR-663	2.513
hsa-miR-1308	2.504
hsa-miR-1225	1.844
hsa-miR-21	1.764
hsa-miR-346	1.739
hsa-miR-29a	1.728
hsa-miR-720	1.624
hsa-miR-1228	1.602
hsa-miR-126	1.562
hsa-miR-149	1.554
hsa-miR-500	1.542
hsa-miR-221	1.532
hsa-miR-638	1.519
hsa-miR-22	1.51
Downregulation	
hsa-miR-455-3p	1.501
hsa-miR-371-5p	1.524
hsa-miR-10a	1.526
hsa-miR-324-3p	1.536
hsa-miR-18b	1.538
hsa-miR-186	1.543
hsa-let-7c	1.689
hsa-miR-886-3p	1.697
hsa-miR-516a-5p	1.745
hsa-miR-411	1.754
hsa-miR-18a	1.919
hsa-miR-324	2.481

survival rate is only 8.9-15% (1,2,5). Lung cancer is generally classified into two histological types, small cell lung cancer and non-small cell lung cancer. Non-small cell lung cancer accounts for approximately 85% of the cases and it is further divided into squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and others (24,25). Despite advances in early detection and standard treatment, non-small cell lung cancer is often diagnosed at an advanced stage and has a poor prognosis (26). Chemotherapy plays an important role in the treatment of non-small cell lung cancer, but it is limited to a significant extent by its toxicities, drug resistance and significant side effects, including myelosuppression, neutropenia and thrombocytopenia (3,4). One possible way to increase the efficacy of anticancer drugs and to decrease toxicities or side effects is to develop natural compounds, especially from medicinal plants.

Waltonitone, an ursane-type pentacyclic triterpene isolated from Chinese medical plant *Gentiana waltonii* Burkill, has been used for centuries in treatment of patients with various autoimmune diseases in China. Study has shown that it

Table II. Classification and target prediction of miRNAs.

miRNA name	Functions of target genes	
	Cell proliferation	Apoptosis
hsa-let-7c	UHRF2, TUSC2, CDC25A, CBFA2T3	DPF2, PLAGL2, BCL2L1
hsa-miR-10a	MAPRE1, BCL6, PURB	BCL6, PURB
hsa-miR-126	-	-
hsa-miR-149	PURB, BAI1, RAP1B	PURB, BCL2L2
hsa-miR-186	PURB, DLG5, PDGFC	EP300, PURB
hsa-miR-21	JAG1, PURB, TGFB1, PITX2	PURB, NTF3, RHOB
hsa-miR-22	PURB, CUL3, PTEN, FOSL1	LGALS1, PURB, SIRT1, PLAGL2, CUL3, YARS, PTEN
hsa-miR-221	IRF2	MAP3K10, PDCD10
hsa-miR-29a	PPM1D, PDGFB, PMP22, LAMC1	FEM1B, RYBP, BCL2L2, SGK, PPP2CA

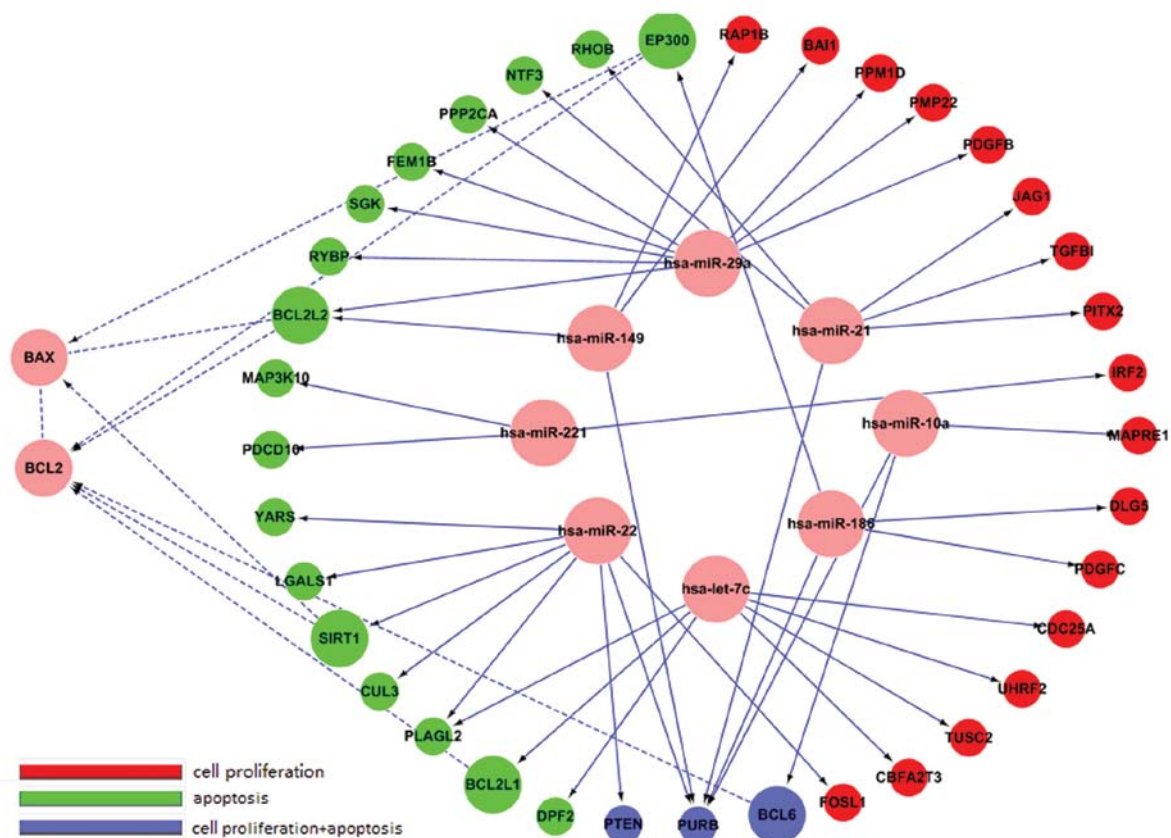


Figure 7. The relation of Bax/Bcl-2 and potential target genes.

inhibits growth of cultured cells and induces cell apoptosis in cancer cells (23). In this study, we first demonstrated that A549 cells treated with waltonitone showed a concentration- and time-dependent inhibition of the proliferation. Nuclear fragmentation, chromosome condensation and formation of apoptotic bodies were also observed by fluorescent microscopy. Flow cytometric analysis revealed that waltonitone treatment results in an increase of apoptotic cells. We observed the molecular basis of waltonitone-induced apoptosis by downregulation of Bcl-2 protein levels and upregulation of Bax protein expression. Furthermore, the mechanism of

waltonitone-inhibited proliferation involves the regulation of miRNAs.

The Bcl-2 family regulates the apoptotic pathway and can be divided into two types: anti-apoptotic proteins and pro-apoptotic proteins (11-13). Many anticancer agents extracted from Chinese herbs induce cancer cell apoptosis targeting the balance of pro- and anti-apoptotic proteins (27-29). Bcl-2 is an anti-apoptotic protein of the Bcl-2 family, and Bax is a pro-apoptotic protein. The ratio of Bax/Bcl-2 is a decisive factor and plays an important role in determining whether cells will undergo apoptosis. Our results showed that expression of Bcl-2

was downregulated by waltonitone, whereas Bax expression was upregulated, leading to upregulation of the ratio between Bax and Bcl-2. This might contribute to the apoptosis promotion activity of waltonitone.

miRNAs are small non-coding RNA molecules that modulate the post-transcriptional regulation of gene expression in multi-cellular organisms by complementary interaction with the 3'-untranslated regions (3'-UTR) of target mRNA (14,30,31). miRNA interaction influences protein expression by promoting the degradation or suppressing mRNA translation (15). The expression of miRNAs has been recognized as integral components of many biologic processes including cell proliferation, development, apoptosis and differentiation (16,17). Recently, many studies have shown that miRNAs play an important role in the development and progression of cancers (18,19). The miRNAs expression patterns could be potential biomarkers used for diagnosis, prognosis, and personalized therapy in cancer include non-small cell lung cancer (32-35). It is likely, therefore, that some anticancer agents suppress the proliferation of malignant cells by regulation expression of miRNAs (20-22). While altered expression of miRNAs has been the important application for discovering effective targets and mechanisms in treatment of cancer with natural compound.

To further understand the mechanisms of action of waltonitone against the growth of human lung cancer cells, we investigated the effects of waltonitone on the expression of miRNAs. Using miRNA microarray analysis, we found 27 miRNAs with >1.5-fold expression changes in waltonitone-treated A549 cells, 15 upregulated and 12 downregulated miRNAs. Furthermore, we identified potential target genes of these miRNAs related to cell proliferation and apoptosis. We found 26 genes associated with cell proliferation and 26 genes associated with apoptosis. Thus, waltonitone may inhibit proliferation and induce apoptosis of human lung cancer A549 cells through regulation of expression of miRNAs.

In conclusion, we demonstrated that waltonitone inhibited proliferation and promoted apoptosis in lung cancer A549 cells. This apoptotic response is associated with the upregulation of the ratio of Bax/Bcl-2. Moreover, modulation of miRNA expression may be an important mechanism underlying the biological roles of waltonitone. Based on the outcome of this study, we suggest that waltonitone, at least in part, inhibited proliferation of human lung cancer A549 cells through regulating expression of miRNAs. Waltonitone shows potential for development as an agent for the management of lung cancer.

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