



Antroquinonol inhibits NSCLC proliferation by altering PI3K/mTOR proteins and miRNA expression profiles

V. Bharath Kumar^a, Ta-Chun Yuan^a, Je-Wen Liou^b, Chih-Jen Yang^c,
Ping-Jyun Sung^d, Ching-Feng Weng^{a,*}

^a Department of Life Science and Institute of Biotechnology, National Dong Hwa University, Hualien, Taiwan

^b Department of Biochemistry, School of Medicine, Tzu-Chi University, Hualien, Taiwan

^c Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan

^d Graduate Institute of Marine Biotechnology, Department of Life Science, National Dong Hwa University, Pingtung, Taiwan

ARTICLE INFO

Article history:

Received 25 September 2010

Received in revised form

11 December 2010

Accepted 19 December 2010

Available online 24 December 2010

Keywords:

Antroquinonol

PI3K/mTOR

miRNA

cdc2 proteins

PARP

Caspase cleavage

ABSTRACT

Antroquinonol a derivative of *Antrodia camphorata* has been reported to have antitumor effects against various cancer cells. However, the effect of antroquinonol on cell signalling and survival pathways in non-small cell lung cancer (NSCLC) cells has not been fully demarcated. Here we report that antroquinonol treatment significantly reduced the proliferation of three NSCLC cells. Treatment of A549 cells with antroquinonol increased cell shrinkage, apoptotic vacuoles, pore formation, TUNEL positive cells and increased Sub-G1 cell population with respect to time and dose dependent manner. Antroquinonol treatment not only increased the Sub-G1 accumulation but also reduced the protein levels of cdc2 without altering the expression of cyclin B1, cdc25C, pcdc2, and pcdc25C. Antroquinonol induced apoptosis was associated with disrupted mitochondrial membrane potential and activation of Caspase 3 and PARP cleavage in A549 cells. Moreover, antroquinonol treatment down regulated the expression of Bcl2 proteins, which was correlated with the decreased PI3K and mTOR protein levels without altering pro apoptotic and anti apoptotic proteins. Results from the microarray analysis demonstrated that antroquinonol altered the expression level of miRNAs compared with untreated control in A549 cells. The data collectively suggested the antiproliferative effect of antroquinonol on NSCLC A549 cells, which provides useful information for understanding the anticancer mechanism influenced by antroquinonol and is the first report to suggest that antroquinonol may be a promising chemotherapeutic agent for lung cancer.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Lung cancer remains the leading cause of cancer-related death in the worldwide. Among them, 80% of lung cancers are non-small cell lung cancer (NSCLC). Adenocarcinoma is a subgroup of NSCLC and some other tumour histotype shows resistance to radiation, chemotherapy and platinum-based doublet chemotherapy while other tumour subtypes display very low response rate for the chemotherapy [1–5]. One of every three cancer-related deaths accounts for lung cancer with an overall survival of 5-years, having less than 15% survival probability [6,7]. Similarly in two thirds of cases the cancer might have start spreading during the time of diagnosis, due to limited therapeutic options [8,9]. Till now numerous new cytotoxic agents have been introduced in NSCLC treatment, only small improvements have been observed in the survival of patients with advanced or metastatic lung cancer [10]. Thus it

is important to consider a new bioactive compound from natural products, such as phytochemicals that can selectively inhibit tumour cells growth without affecting normal cells [11,12].

Phytochemicals represent a new class of compounds with anti-carcinogenic properties that are gaining attention in the treatment of human cancers, thus offering promised new options for the development of effective chemopreventive and chemotherapeutic strategies, particularly those that can be administered as dietary supplements [13]. *Antrodia camphorata* (*A. camphorata*) is a new basidiomycete in the Polyporaceae (Aphyllphorales), causing brown heart rot in *Cinnamomum kanehirai* hay (Lauraceae) in Taiwan has been identified as distinct species of the genus *Antrodia* [14,15]. *A. camphorate* is used for the treatment of diarrhoea, abdominal pain, hypertension, itching of the skin, and liver cancer [16]. Previous studies have shown that growth inhibitory effects of *A. camphorate* in prostate cancer, bladder carcinoma, and breast cancer cells [17–19]. Furthermore, ethanol extracts of *A. camphorata* mycelia (SACE and Fraction-6) can induce apoptosis of NSCLC cells by down regulating the synthesis of galectin-1, RhoGDI- α , human calpain small (regulatory) subunit and eIF5A

* Corresponding author. Tel.: +886 3 8633637; fax: +886 3 8630255.

E-mail address: cfweng@mail.ndhu.edu.tw (C.-F. Weng).

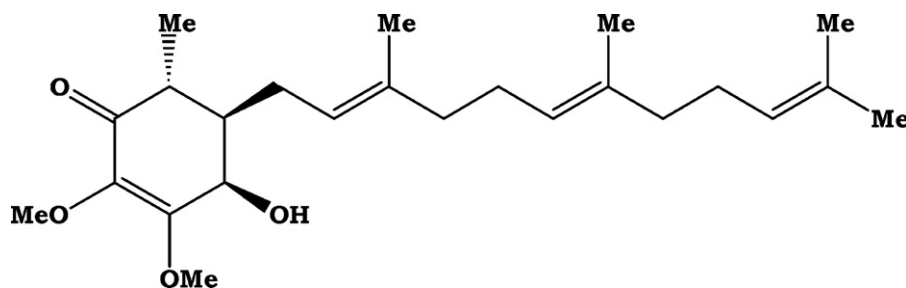


Fig. 1. Chemical structure of antroquinonol.

[20,21]. Most of the biological activity of *A. camphorata* is carried out by the high amounts of polysaccharides, terpenoids, maleic and succinic acid derivatives [22]. All the tested extracts of *A. camphorata* possess anti-inflammatory effects by suppression of nitric oxide (NO) through reducing inducible nitric oxide synthase (iNOS) expression in activated macrophages or microglia [23]. Previous study has shown that antroquinonol, a major compound found in *A. camphorata*, exhibits growth inhibitory effect against Hepatocellular Carcinoma (HCC) cell lines, and the underlying molecular mechanism is associated with the activation of AMPK and the inhibition of mTOR signalling [24]. Accumulating evidence has suggested that mammalian target of rapamycin (mTOR) acts as a downstream target of phosphatidylinositol 3 kinase (PI3K) pathway and is phosphorylated (or activated) in response to stimuli that activate the PI3K/Akt pathway [25,26]. Drug resistance is also associated with activation of PI3K/Akt signalling pathway [27], thus activation of PI3K/Akt pathway by external agents plays a key role in cell growth, survival, proliferation and tumorigenesis [28,29]. Activated PI3K can phosphorylate Akt and thus promotes cell survival by inhibiting apoptosis through its ability to phosphorylate/inactivate Bad, a pro-apoptotic proteins and GSK-3 β [30,31].

MicroRNA, a small non-coding RNA has been demonstrated to play a crucial role in modulating the growth and survival of different cells, including cancers. Further study showed that inhibition of miRNAs by chemotherapeutic agents induced apoptosis in cancer cells [32]. These miRNA play an important role in regulating cell proliferation and apoptosis, whereas aberrant expressions of miRNAs are strongly implicated in carcinogenesis and tumour progression [33,34]. On the basis of the early findings, in this study we investigated the potential mechanism by which antroquinonol may regulate cell proliferation and apoptosis in NSCLC cells. Eventually, the role of antroquinonol on miRNA expression profiles using miRNA microarray analysis was also investigated. These studies could provide us crucial information in designing antroquinonol-based therapeutic strategies for treating NSCLC that are being resistant to commercially available drugs.

2. Materials and methods

2.1. Chemicals and antibodies

All the chemicals were purchased from Sigma (MO, USA.), unless stated otherwise. Antroquinonol, which chemical structure shown in Fig. 1, was obtained from Golden Biotechnology Corporation (Taipei, Taiwan). The NMR data of the antroquinonol were checked for consistency with previous reports [22]. Further, the purity was checked on HPLC using chiral OD-H (0.46 cm id \times 25 cm) column by eluting with 5% IPA/Hex at a flow rate of 0.25 ml/min and detected at 254 nm. Stock solution of Antroquinonol (25 mM) and paclitaxel (1 mM) were prepared by dissolving them in ethanol, and appropriate working dilutions were prepared with the cell culture medium prior to the experiments.

2.2. Cell culture

Three human lung cancer cell lines were used for the experiments, including A549 type II human lung alveolar epithelial cell carcinoma, H441 lung adenocarci-

noma and H661 lung large cell carcinoma, which was obtained from Dr. Chih-Jen Yang at the Department of Internal Medicine, Kaohsiung Medical University Hospital (Kaohsiung, Taiwan). Each cell line was cultured in DMEM (A549) or RPMI 1640 (H441 and H520) medium (GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (BIOCHROM AG, Leonorenstr, Berlin) and 1% penicillin streptomycin (GIBCO) and incubated at 37 °C in 5% CO₂. Antibodies against Cyclin B1, Cdc2, Cdc25c, p-Cdc2 (Thr14) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against PI3K, AKT, pAKT, mTOR, Caspase 3, Bad, Bak, Bax, pcdc25C, Bcl2 and PARP were purchased from Upstate® (Lake Placid, NY, USA) and Cell Signalling (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies, was purchased from Leinco Technologies (St Louis, MO, USA).

2.3. MTT assay

A549 (1×10^4 cells per well), H441 (1×10^4 cells per well) and H520 (1×10^4 cells per well) cells were seeded in 96 well plate (Corning Inc, NY, USA) and treated with ethanol (control), antroquinonol or paclitaxel for desired time points and dosages. After incubation, the media were replaced with 20 μ l of MTT reagent (5 mg/ml) and incubated in 5% CO₂ at 37 °C for 4 h. DMSO was then added to solubilise the MTT tetrazolium crystal. Absorbance was measured at 540 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.4. Cell cycle analysis

For cell cycle distribution analysis, A549 cells were treated with ethanol (control), antroquinonol (10 and 25 μ M) or paclitaxel (0.5 and 1 μ M) for 12 h. The cells were then fixed in ice-cold 70% ethanol for overnight. After an additional wash with PBS, the cell pellets were stained with 1 ml of propidium iodide (PI) staining solution containing 200 μ g of PI in 1 ml of PBS containing 2 mg of DNase free RNase for 30 min. Acquisition and analysis were performed by Cytomics FC500 flow cytometry (Beckman Coulter, Fullerton, CA, USA) with excitation at 488 nm.

2.5. Apoptotic morphology under light and electron microscopy bio-atomic force microscopy (Bio-AFM)

A549 cells were plated at a density of 2×10^4 on a cover slip were grown on 12 well plates and incubated for 24 h. After treatment with respective concentration of antroquinonol or paclitaxel for 12 h, media were discarded, washed with PBS and the morphological changes were observed under an inverted microscope (Nikon TS100, Nikon, Tokyo, Japan). After fixation with 1% glutaraldehyde in PBS for 5 min at 37 °C, cells were imaged under bio-atomic force microscope (Bio-AFM, Nanowizard, JPK, Germany) that was mounted on an inverted microscope, TE-2000-U (Nikon, Tokyo, Japan). The silicon nitride non-sharpened probes with a nominal cantilever force constant of 0.06 Nm⁻¹ (DNP-20, Veeco, CA, USA) were used. Imaging was performed using contact mode. Line scan rates varied from 0.5 to 2 Hz.

2.6. Terminal nucleotidyl transferase-mediated nick end labeling assay

A549 cells were seeded at a density of 5×10^3 cells per chamber on poly-L-lysine-coated Lab-Tek II Chamber slides (Nalge Nunc International, Rochester, NY, USA) and following the treatment of ethanol control, antroquinonol (10 and 25 μ M), or paclitaxel (0.5 and 1.0 μ M) for 12 h. After treatment, the cells were washed with PBS, fixed in 4% formaldehyde for 25 min at room temperature (RT), and permeabilized in 0.2% Triton® X-100 for 5 min at RT. Subsequently the fixed cells were incubated with Equilibration Buffer, Nucleotide Mix and rTdT Enzyme for 60 min at 37 °C in a humidified atmosphere and then immersed in stop buffer for 15 min at RT. DNA fragmentation was detected immuno-histochemically using the DeadEnd™ Fluorometric TUNEL System (Promega Corporation, Madison, WI) according to the manufacturer's instructions.

2.7. Assessment of mitochondrial membrane potential (MMP)

Briefly, after paclitaxel and antroquinonol treatment, A549 cells were loaded with Rhodamine 123 (1 μ g/ml) and incubated at 37 °C for 30 min in the dark. Cells

were then harvested, washed and resuspended in PBS and analyzed immediately using flow cytometry assay with the excitation wavelength at 488 nm and the emission wavelength at 525 nm.

2.8. Western blot analysis

Cells were collected and washed twice in $1 \times$ PBS, then lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 1 mg/ml aprotinin, 2 μ g/ml pepstatin A, and 2 μ g/ml leupeptin) for 5 min. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF (Millipore, Bedford, MA, USA) membrane. The blots were blocked with 5% non-fat milk in TBST saline (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.05% Tween-20) at RT for 1 h and incubated with the appropriate primary antibody at 4 °C. After wash, the blots were incubated with peroxidase-conjugated secondary antibody for 1 h. Bands were monitored using Western blot chemiluminescence reagent (Amersham Biosciences Corp., Piscataway, NJ, USA).

2.9. miRNA microarray analysis

The miRNA microarray analysis was performed by Phalanx Biotech Group (Hsinchu, Taiwan). Total RNA was extracted from A549 cells using TRIzol reagent (Invitrogen, Burlington, ON, USA) according to the manufacturer's instructions. 2.5 μ g of total RNA was labelled with Cy5 fluorescent dyes using miRNA ULSTM Labeling Kit (Kreatech Diagnostics, Amsterdam, Netherlands). Labelled miRNA targets enriched by NanoSep 100K (Pall Corporation, NY, USA) were hybridized to the Human & Primate miRNA OneArray™ (PmiOA v1.0), which contains triplicate 1040 unique miRNA probes from human (miRBase Release 12.0), in technical replicates. After overnight hybridization at 37 °C, non-specific binding targets were washed away, and the slides were dried by centrifugation and scanned by Axon 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). The Cy5 fluorescent intensities of each spot were analyzed by GenePix 4.1 software (Molecular Devices). The signal intensity of each spot was processed by R program. The fine signals (flag=0) were extracted and preprocessed by log₂ transformation, quantile normalization method and ANOVA test. All the experiments were saved as Microsoft Excel files.

2.10. Statistical analysis

Data were expressed as means \pm SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($*p < 0.05$), ($**p < 0.01$) and ($***p < 0.001$) between the means of control and the treatment were analyzed by Dunnett's test.

3. Results

3.1. Antiproliferative effect of antroquinonol and paclitaxel in H661, H441 and A549 cells

We examined the anti-proliferative effect of antroquinonol on H661 (Large cell lung carcinoma), H441 and A549 cells (Adenocarcinoma) using MTT assay. As shown in Fig. 2a except H661, both the adenocarcinoma cells showed the resistance to paclitaxel treatment. The paclitaxel concentration used in this experiment was chosen from the previous studies [35]. However, antroquinonol treatment decreased the cell viability in all three cell lines in a dose dependent manner. The EC₅₀ values of antroquinonol for all three cell lines were 25 μ M for 12 h treatment (Fig. 2b). To examine the molecular mechanism of antroquinonol mediated antiproliferative effect, we will use A549 cells for further experiments.

3.2. Effect of antroquinonol on the cell cycle distribution and cell cycle proteins

We initially perform flowcytometric analysis for determining the mechanism responsible for antroquinonol-mediated cell growth inhibition and cell cycle. As shown in Fig. 3a, paclitaxel used as a positive control caused a significant inhibition of cell cycle proteins in A549 cells at 12 h, resulting in a G₂/M phase accumulation compared with the control. Interestingly, antroquinonol treatment for 12 h induced sub-G₁ accumulation in a dose dependent manner as compared with the paclitaxel treatment and control cells (Table 1).

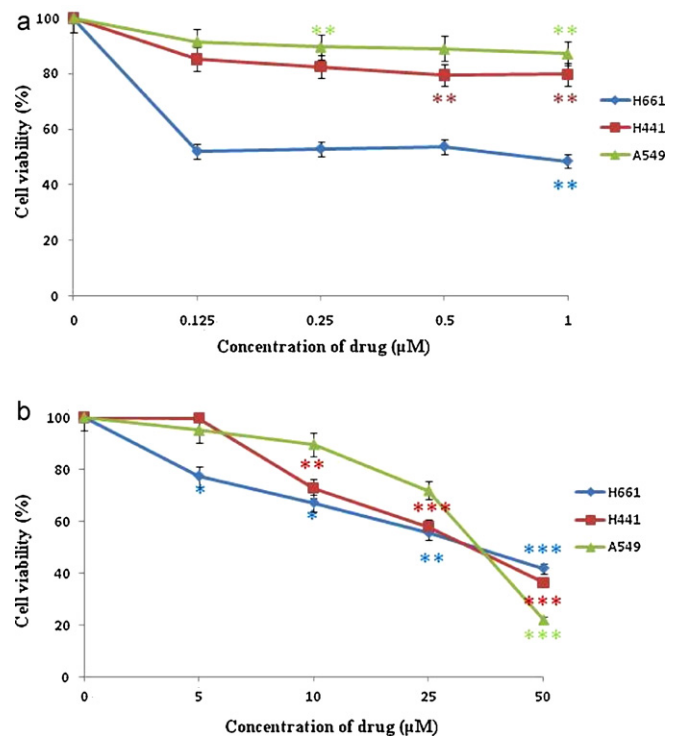


Fig. 2. The anti-proliferative effect of antroquinonol, paclitaxel on H661, H441 and A549 cells in a dose dependent manner. The cells were treated with (a) paclitaxel (P) at concentration of 0.25, 0.50, 1.0 μ M, (b) antroquinonol (A) at concentration of 5, 10, 25 and 50 μ M for 12 h, respectively. The surviving cells were determined and presented as a percentage of the untreated cells as a control. The shown data are the mean from three independent experiments. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, compared with the untreated control.

To understand the effect caused by antroquinonol and paclitaxel on cell cycle machinery, next we assessed the effects of antroquinonol and paclitaxel on the expression of cell cycle-regulating proteins by immunoblotting analysis. Cells exposed to paclitaxel treatment caused no effect on the expression of cyclin B1, cdc2, and cdc25C at 6 h, but led to increased expression of those proteins at 12 h (Fig. 3b). Importantly, paclitaxel-treated cells resulted in an increase in levels of inactive phospho-cdc2 (Tyr¹⁵) in a time and dose dependent manner. Exposure of cells to antroquinonol did not alter the expression profile of cyclin B1, cdc2, cdc25C and the phosphorylation level of cdc2 significantly inhibited cdc2 protein expression in a dose-dependent manner. We suggest that inhibition of cdc2 by antroquinonol terminated the entry of cell cycle at G₂-M phase and thus caused cell cycle arrest at sub-G₁ phase as compared with the paclitaxel treated cells.

Table 1

Changes of cell cycle progression by antroquinonol and paclitaxel in A549 cancer cells.

Dosage (μ M)	sub G ₁ (%)	G ₁ (%)	S (%)	G ₂ -M (%)
Control	2.5 \pm 0.3	62.7 \pm 2.7	15.1 \pm 1.1	19.6 \pm 2.6
P 0.5	2.3 \pm 0.8	5.6 \pm 8.2 ^{**}	37.1 \pm 1.9 [*]	55.7 \pm 3.8
P 1.0	2.4 \pm 0.7	9.4 \pm 4 ^{**}	34.4 \pm 0.2	56.3 \pm 0.4 ^{**}
A 10	9.4 \pm 1.2 [*]	15.7 \pm 1.3 [*]	46.9 \pm 1.5	24 \pm 1.4
A 25	29.3 \pm 0.4	35.1 \pm 2.7 ^{**}	19.1 \pm 5.7	15.6 \pm 2

A549 cells were treated with increasing concentration of antroquinonol and paclitaxel for 12 h. The distribution of cells at each phase of cell cycle was analyzed by flow cytometry as described in Section 2. The shown data are the mean from three independent experiments. $*p < 0.05$, $**p < 0.01$ compared with the untreated control.

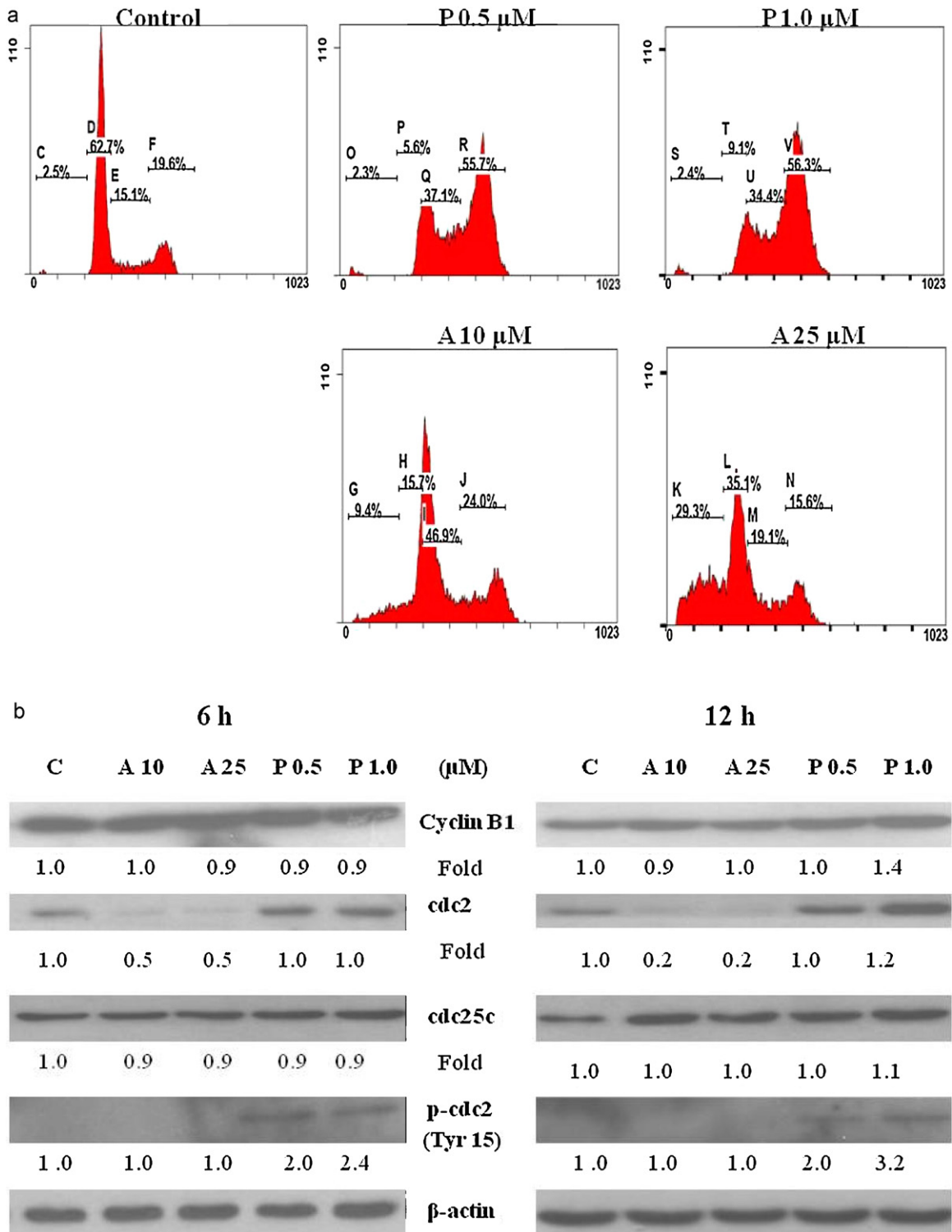


Fig. 3. Effect of antroquinonol and paclitaxel on cell cycle progression of A549 (a) A549 cells were treated with antroquinonol (10 and 25 μM) and paclitaxel (0.5 and 1.0 μM) for 12 h, respectively. After treatment, cells were collected, fixed with methanol, stained with propidium iodide, and analyzed by flow cytometry. Data on each sample represent the percentage of cells in the sub-G1, G1, S, G2/M phases of the cell cycle, respectively. (b) Immunoblotting results of G2/M phase-related proteins in antroquinonol/paclitaxel-treated A549 cells. After treatment with paclitaxel (P 0.5, 1 μM) or antroquinonol (A 10, 25 μM) for indicated time periods, the cells were harvested and the total cell lysates were prepared. The expression of Cyclin B1, cdc2, p-cdc2 (Tyr15) and cdc25C, were determined by immunoblotting analysis. β-Actin was used as the loading control in this study. Experiments are repeated for three times and similar results were obtained.

3.3. Apoptotic effect of antroquinonol on A549 cells

Antroquinonol-induced apoptotic effect was further validated using fluorometric TUNEL staining assay in A549 cells. As shown in Fig. 4a, antroquinonol treatment increased the accumulation

of green fluorescence apoptotic cells, which is in consistent with the cell cycle data. Surprisingly, 0.5 and 1 μM of paclitaxel-treated A549 cells showed apoptotic cells in a dose dependent manner, which was different from the cell cycle data (Fig. 3a). Previous study showed that detached cells and associated cell death by

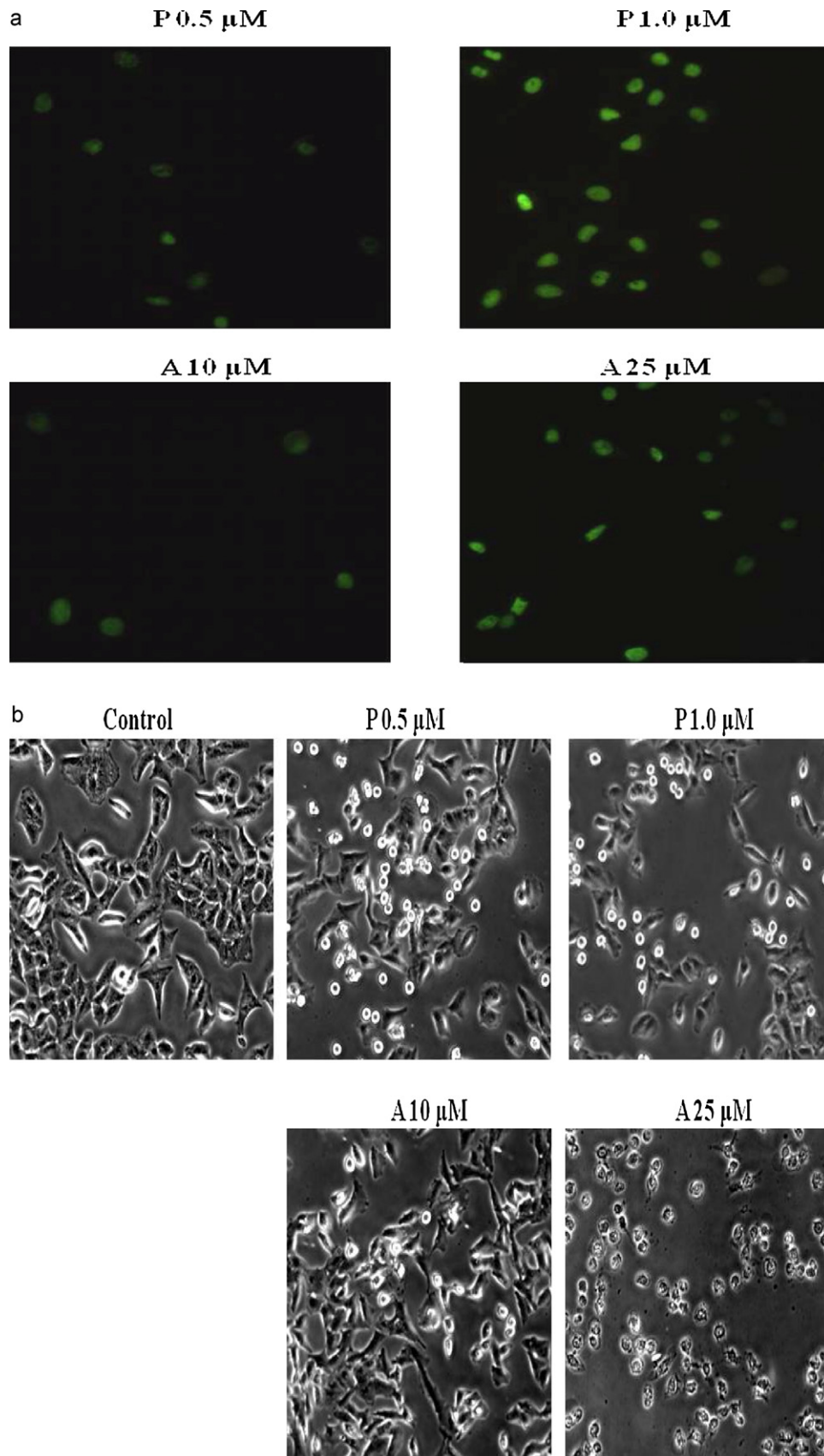
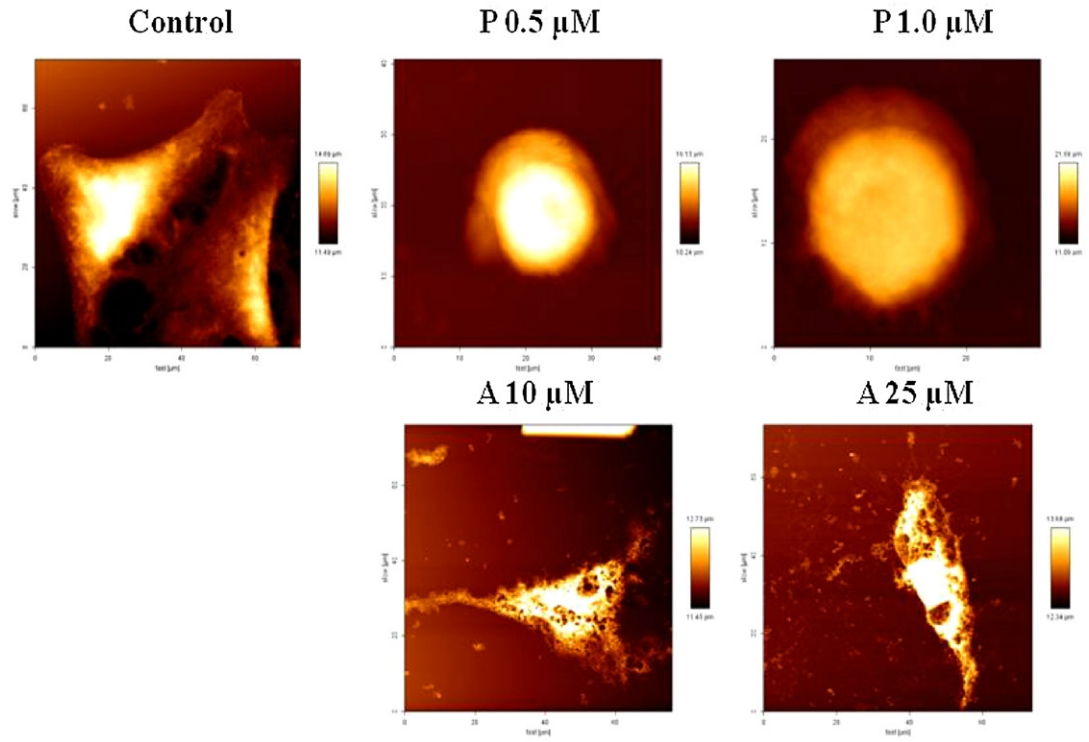


Fig. 4. A549 cells were treated in the presence or absence of vehicle or paclitaxel (P 0.5, 1 μM) or antroquinonol (A 10, 25 μM) for 12 h. The morphological features were analyzed with (a) optical microscope with a 100-fold magnification, (b) morphology changes induced by antroquinonol and paclitaxel were detected by AFM, both the height measured images (upper panel) and the deflection images (lower panel) were illustrated, (c) determination of apoptotic cells by TUNEL staining, as visualised by green fluorescence. The detailed procedures were described in Section 2.

c Height measured image



Deflection Image

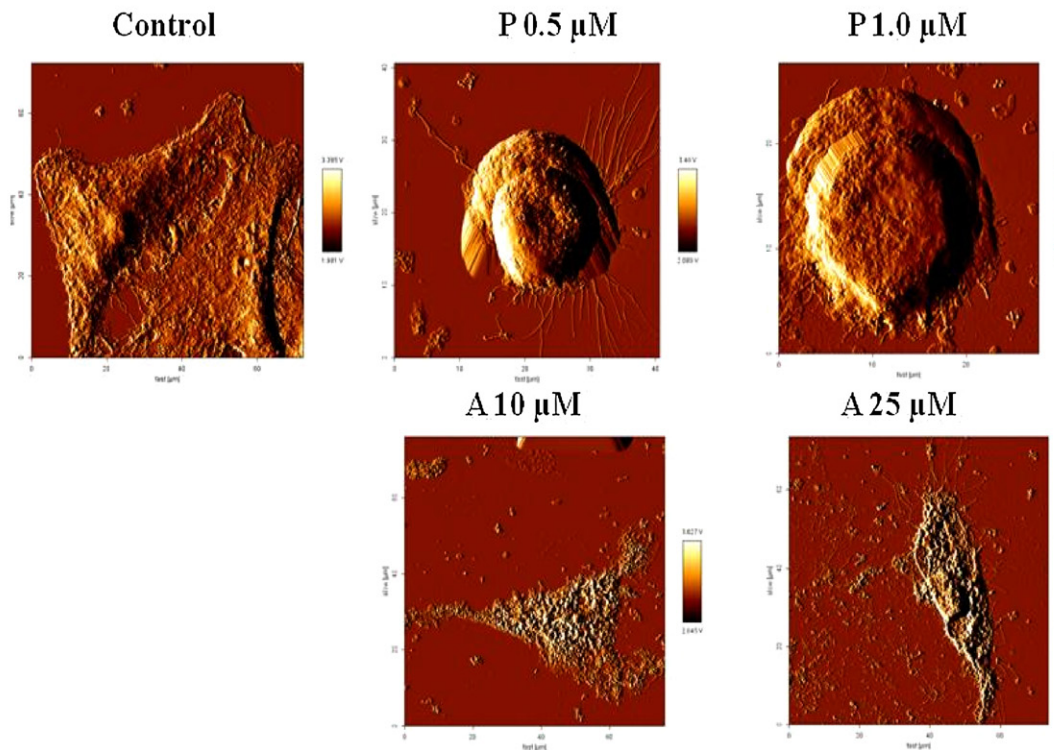


Fig. 4. (Continued)

paclitaxel was the result of a mitotic arrest in the G2/M phase [36]. From this data, we speculate that TUNEL experiments are more sensitive in detecting the apoptotic cells than propidium iodide staining studies.

To further prove the above hypothesis, morphological changes of A549 cells were analyzed using inverted microscope after antroquinol and paclitaxel treatment. Twelve hours after induction with 0.5 and 1 μM of paclitaxel, most cells did not show any apop-

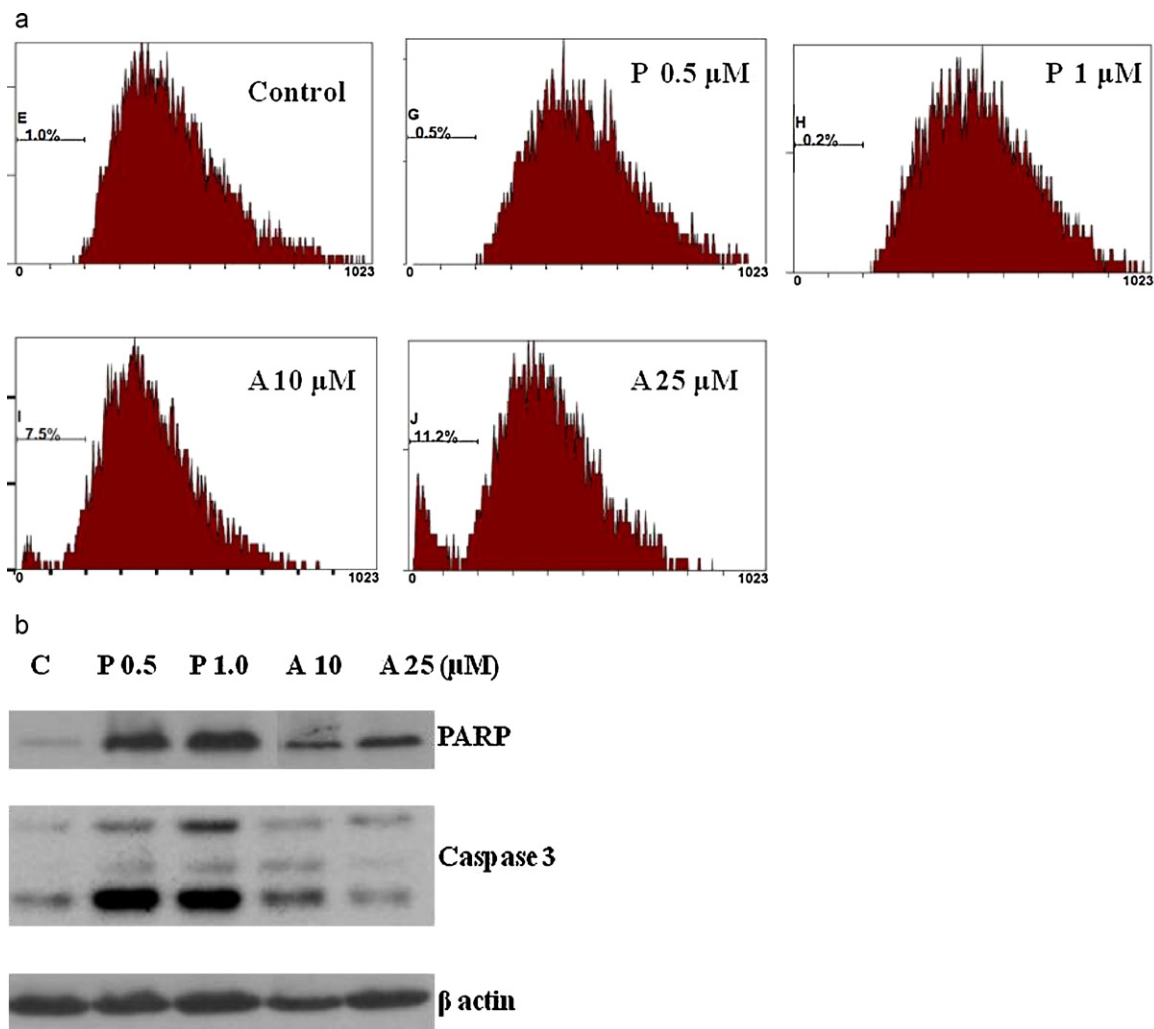


Fig. 5. Antroquinonol induces mitochondria membrane potential (MMP) leakage, Caspase-3 and PARP cleavage in A549 cells. Cells were exposed to varying concentrations of antroquinonol for 12 h. (a) MMP changes were measured by flow cytometry. Data were shown as means plus SD from three independent experiments with representative plots of one set of the experiments, (b) Effects of antroquinonol on cleavage of Caspase-3 and PARP in A549 cells. A549 cells were cultured as described in Section 2. Cells were treated with 0.1% ethanol or indicated concentrations of antroquinonol/paclitaxel for 12 h. Experiments were repeated three times with similar results.

totic alteration despite small proportion of cells having detached from the culture dish (Fig. 4b). In contrast, antroquinonol induced cell shrinkage and apoptotic vacuole formation in a dose dependent manner.

The effect of antroquinonol on the morphology of A549 cells was further analyzed by utilizing AFM. From the height image (upper panel) and deflection image (lower panel) of the AFM, it was seen that in paclitaxel treated cells began to change gradually over the duration of the reaction period, whereas antroquinonol treatment increased the surface roughness of the cells and pores were found on cells following a 12-h exposure with respect to concentrations used in Fig. 4c. Both the bright field and the AFM data demonstrated that property changes in the cell membrane may be an early damage response of tumour cells to these anti-cancer agents.

3.4. Antroquinonol disrupts mitochondrial membrane potential (MMP) by downregulating caspase-3 and PARP cleavage

To determine whether antroquinonol-induced apoptosis is mediated by mitochondrial pathway, the changes of mitochondria membrane potential (MMP) were measured. As shown in Fig. 5a, a decrease in MMP was found in antroquinonol treated cells in a dose dependent manner, but not in paclitaxel treated cells. We further

analyzed the effect of antroquinonol on the expression of Caspase and PARP, two apoptosis associated enzymes. As shown in Fig. 5b, there were strong western blot bands indicating Caspase 3 (17 kDa) and PARP cleavage (85 kDa) after 12 h of antroquinonol treatment. The cells treated with paclitaxel as a positive control, which showed a significant increase in Caspase 3 and PARP cleavage. These results indicate that both paclitaxel and antroquinonol can induce apoptosis by activating Caspase 3 and PARP. These results collectively suggested that antroquinonol treatment caused loss of MMP and leads to activation of Caspase 3 and PARP cleavage in A549 cells.

3.5. Antroquinonol inhibits Bcl2 proteins without altering other antiapoptotic proteins

Bcl-2 family proteins control mitochondria-dependent apoptosis with the balance of the anti- and pro-apoptotic members. Whether the expression levels of pro-apoptotic proteins Bax, Bak and Bad and anti-apoptotic protein Bcl2 are altered on antroquinonol treatment were determined. Compared to control and paclitaxel treated cells, antroquinonol treatment significantly decreased Bcl2 protein expression level in A549 cells with slight increase of Bax expression in a dose dependent manner, while other proteins remain to be unaffected, at least 25 μ M of antroquinonol

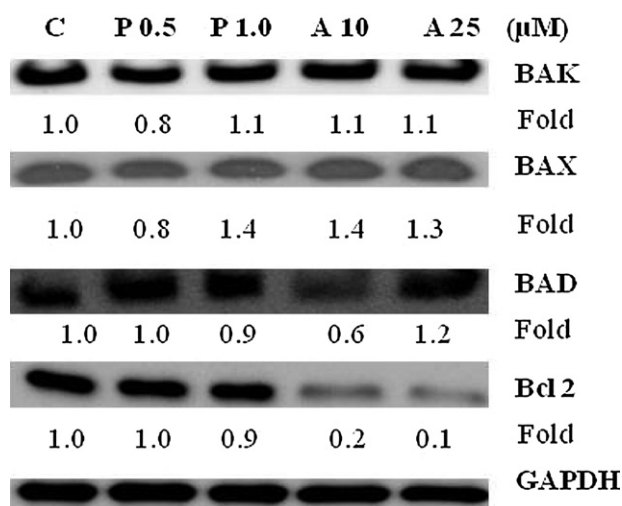


Fig. 6. Effects of antroquinonol on the levels of Bak, Bax, Bad and Bcl2 in A549 cells. A549 cells were cultured as described in Section 2. Cells were treated with 0.1% ethanol or indicated concentrations of antroquinonol/paclitaxel in for 12 h. Cells were harvested and lysed for western blot. Changes in the levels of Bak, Bax, Bad, and Bcl-2 proteins after being normalized to the levels of GAPDH are shown below each blot. Experiments were repeated three times with similar results.

treatment (Fig. 6). Taken together, these data demonstrated that antroquinonol induced apoptosis through mitochondrial pathway by down-regulating the Bcl2 protein.

3.6. PI3K/mTOR are down regulated by antroquinonol treatment

Since the Bcl-2 family proteins are regulated by PI3K mediated signalling [37], we further investigated whether antroquinonol induced apoptosis through PI3K/AKT/mTOR pathway. The result showed a dose dependent decrease protein levels of PI3K and mTOR in A549 cells as compared with that of control and paclitaxel treated cells. By contrast the protein levels of AKT and pAKT remained to be unaffected by antroquinonol treatment (Fig. 7). Taken together, the results suggested that antroquinonol induce apoptosis by specifically targeting PI3K/mTOR pathway.

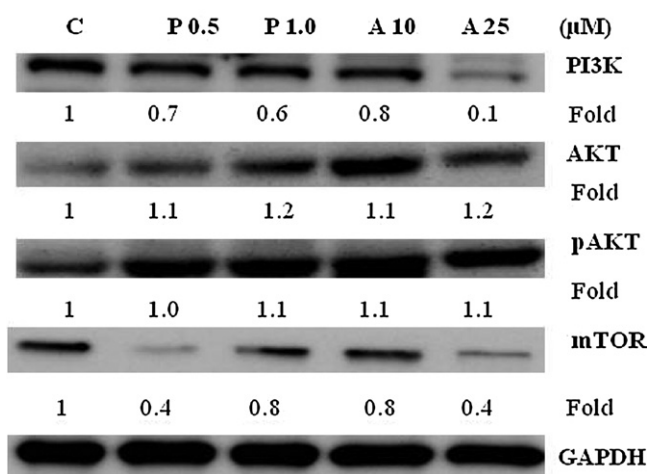


Fig. 7. Reduction of PI3K/mTOR levels correlates with antroquinonol-induced apoptosis. A549 cells were treated with antroquinonol/paclitaxel for 12 h. PI3K, total Akt (t-Akt) or phospho-Akt (p-Akt) and mTOR. GAPDH blot was included as loading control.

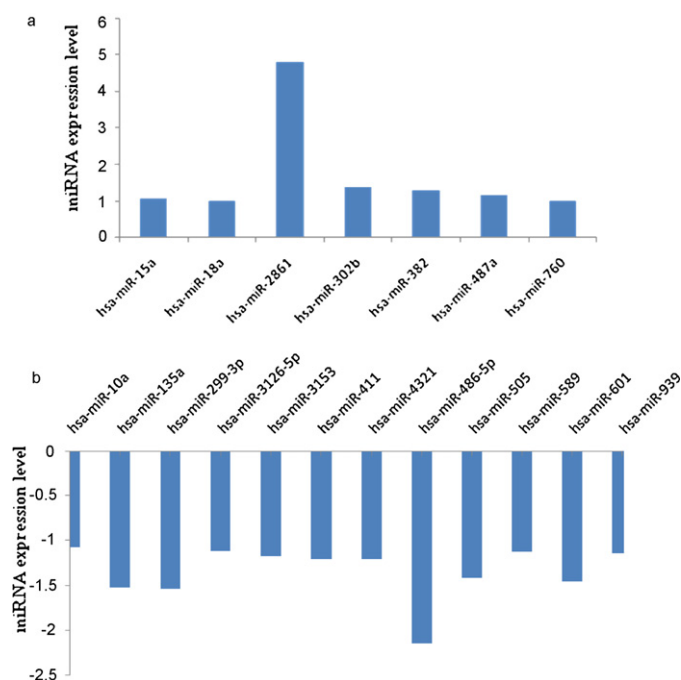


Fig. 8. A549 cells were treated with antroquinonol (A 25 μ M) for 12 h and their miRNA expression profile was analyzed using microarray analysis. miRNAs showed >2-fold change in comparison with each group after antroquinonol treatment were chosen. (a) Up-regulated miRNAs and (b) Down-regulated miRNAs were shown.

3.7. Regulation of miRNA expression profile after antroquinonol treatment

Using Agilent human miRNA microarray version 2 kit, miRNA expression profiles was compared between control and antroquinonol (25 μ M) treated A549 cells. A total of 851 human miRNAs were selected to analyze miRNA profile. Those human miRNA were continually filtered with flags to obtain more defined data. Total 21 human miRNAs were selected as a result of flag filtration (data not shown). To confirm whether miRNA expression patterns are affected by the antroquinonol treatment, 21 flagged miRNAs were analyzed and miRNAs which did not demonstrate a 2-fold change in expression levels between treatment and control were removed. Based on the expression profile analysis of 21 miRNAs, we found that 7 miRNAs (miR-15a, 18a, 2861, 302b, 382, 487a and 760) showed higher expression while 12 miRNAs (miR-10a, 135a, 299-3p, 3126-5p, 3153, 411, 4321, 486-5p, 505, 589, 601 and 939) were down-regulated in antroquinonol treated A549 cells (Fig. 8a and b). The data collectively supported that miRNAs could regulate the proliferative and apoptotic activities of A549 cells through oncogenes or tumour suppressor genes.

4. Discussion

Apoptosis resistance is known to be a hallmark of various cancers by developing resistance to chemotherapy and radiotherapy [38]. Currently, it appears that platinum-based chemotherapy shows placebo effect towards the NSCLC patients. Hence to eradicate this issue, new forms of treatment are needed to overcome this major obstacle [39]. Antroquinonol ($C_{24}H_{38}O_4$; mol. wt = 413.2668), a component purified from *A. camphorate* is a well known traditional Chinese medicine used for the treatment of liver diseases, showed effective anti cancer activity against both HBV DNA positive and negative cell [24]. Previously antroquinonol showed moderate cytotoxic effect against MCF-7, MDA-MB-231,

HepG2, DU-145 and LnCaP cancer cells, compared with the relevant chemotherapeutic drugs [22]. However, anti-tumour effect and mechanism of antroquinonol in human lung cancer has not yet been well reported. In this study, our data demonstrated the antiproliferative effect of antroquinonol in human NSCLC A549 cells.

Following DNA damage, cell cycle plays a critical role in regulating cell proliferation, growth as well as cell division. A series of studies demonstrated that proteins involved in cell cycle progression can be an appropriate target for tumour therapy [40,41]. Cyclin dependent kinases (cdks) cdk1, cdk2 complex with specific regulatory proteins cyclins A and B during G2-M phase progression and drive the cell through the cell cycle [42,43]. At this stage, cyclin B1/cdk1 is kept inactive by phosphorylation of T14 and Y15 by Wee and Myt kinases. Subsequently, cyclin B1-cdk1 can be activated by cdc25 phosphatases [44]. cdc25C a protein phosphatase responsible for dephosphorylating and activating Cdc2, is one of the key process in regulating the entry of all eukaryotic cells into the M phase of the cell cycle [45]. The cell cycle analysis showed that at short duration antroquinonol can induce cell cycle arrest at G2-M phase and simultaneously increasing the sub-G1 population, whereas 12 h antroquinonol incubated cells displayed decrease in the G2-M phase accumulation, and thereby induced more accumulation of sub-G1 phase. Furthermore in consistent to our cell cycle data, antroquinonol treatment could not inhibit cyclin B1, cdc25C expression, and thus failed to induce the cdc2 phosphorylation. By contrast it could decrease the expression of cdc2 in a dose and time dependent manner. Thus we assume that antroquinonol may prove to be a valuable tool for inhibition of Cdc2/cyclin A complex.

Cells proliferation becomes out of control when the cell surface receptors are mutated or damaged and leads to tumour formation [46]. Thus it's more important to analyze the nano scale changes happening during the drug-cell interaction [47]. Antroquinonol treatment induced a significant accumulation of apoptotic portion sub-G1 peaks as well as increased green TUNEL positive apoptotic bodies in A549 cells. In order to see the sub-G1 area, a cell must lost enough DNA to appear there; so if cells enter apoptosis from the S or G2/M phase of the cell cycle, they may not appear in the sub-G1 peak (<http://science.cancerresearchuk.org>). Consistent with this observation in our study, the paclitaxel treatment did not show sub-G1 phase accumulation whereas TUNEL-positive (apoptotic) cells were observed in TUNEL assay. Furthermore apoptosis induced by antroquinonol treatment was confirmed using AFM experiment, which gave a detailed understanding of the drug interaction with cells. We observed lots of pore formation in the cytoskeleton of antroquinonol treated A549 cells. A positive control, paclitaxel induced apoptotic cells in cell membrane changes from polygonal shape to round shape. From this data, we speculated that the observed changes in the cell membrane ultrastructure may be an early injury response of tumour cells to this anti-cancer drug.

Mitochondria play a crucial role in controlling apoptosis, which can be activated by a variety of signals to trigger loss of MMP. Signals like Bax oligomerization and translocation to mitochondria is essential step in inducing apoptosis through mitochondrial dependent pathway [48]. The ratio between pro-apoptotic Bax and the anti-apoptotic factor Bcl2 determines whether a cell responds to an apoptotic signal. These apoptosis pathway is tightly synchronized under the control of several signalling pathways. Bcl2 members (e.g., Bcl2) is one of the major gene groups that inhibit apoptosis by protecting cells from causing cell death, whereas Bax members (e.g., Bax, Bad) induce apoptosis, indicating that the Bcl2 family regulates a common cell death pathway and functions at a point where various signals unite [49–51]. Following the treatment of A549 cells with antroquinonol, we observed that antroquinonol treatment increased Bax expression without altering Bak and Bad expression, and a significant decrease of Bcl2 suggesting that changes in the ratio of pro-apoptotic and anti-apoptotic Bcl2 protein family might

contribute to the apoptosis-promotion activity by antroquinonol. PARP is a 116-kDa nuclear protein is a well-characterized substrate for Caspase 3, involved in DNA repair, genome stability, replication, transcription and apoptosis. Activated Caspase 3 cleaves PARP, generating 89- and 24-kDa inactive fragments and cause apoptosis [52]. Our results indicated that antroquinonol induced Caspase 3 a key executioner of apoptosis further caused cleavage and inactivation of key cellular protein PARP. The results showed that antroquinonol caused marked changes in the Bax/Bcl2 ratio and induced the MMP leakage and thus caused Caspase-3 activation. Similarly, previous studies have shown that antroquinonol treatment caused marked reduction in the mitochondrial membrane potential [24].

Another additional mechanism by which cancer cells were killed may be related to the pro-survival signalling PI3K pathway; once PI3K is activated it synthesizes the second messenger phosphatidylinositol triphosphate, which is necessary for phosphorylation of the serine/threonine kinase Akt [53]. Aberrant activation of the PI3K/Akt/mTOR pathway correlates with a resistance to gefitinib and a poor prognosis in NSCLC patients [54]. Recent studies have indicated that, in HCC cells, antroquinonol caused translation inhibition neither through blockade of PI3K/Akt pathway nor by p38 MAPK activity [24]. Contrarily in A549 cells, antroquinonol induced apoptosis by down-regulating PI3K/mTOR proteins without altering Akt levels. These results suggest that PI3K and mTOR are mediators of antroquinonol-induced apoptosis.

miRNAs are short RNA molecules, which function to regulate gene expression by inhibiting translation and/or triggering degradation of their target mRNAs and thus can be used as a biomarker [55–58]. Suberoylanilide hydroxamic acid (SAHA) treatment in A549 cells shows alteration in the expression of miRNA that has several targets related to angiogenesis, apoptosis, chromatin modification, cell proliferation and differentiation [59]. From our result, miR-15a, miR-18a and miR-486 may act as tumour suppressor/oncogenes. miR-15a located at chromosome 13q14, have been implicated in cell cycle control and apoptosis and are frequently deleted or down-regulated in squamous cell carcinomas and adenocarcinomas of the lung [60]. miR-15a are tumour suppressors and proven to be important regulators of Bcl2 expression [61]. miR-18a may function as a potential tumour suppressor through its targeting effect on K-Ras. miR-18a located at chromosome 13q31.3. When transfected in A431 cells, it suppressed the K-Ras expression, cell proliferation and anchorage-independent growth in this cells [62]. miR-486 located at 8p11.21 was predicted to regulate PTEN, PIK3R1 and Cyclin D2 (<http://www.targetscan.org/index.html>). The up-regulated PTEN can function towards cell cycle arrest and apoptosis through several regulatory axes of the signalling pathway, for instance, PTEN has been shown to inhibit PI3K/Akt signalling pathway. We also reported miR-2861 and 486-5p showed high level of expression profiles than other miRNAs. These present data clearly demonstrate that all the miRNAs were expressed or down-regulated after antroquinonol treatment found to play a key factor in regulating apoptosis pathway in A549 cells.

In summary the present study indicated that inhibition of cell cycle progression and induction of apoptotic cell death contribute to the anti-proliferative effects of antroquinonol in human lung adenocarcinoma A549 cells. Inhibition of cell cycle proteins cdc2 and modulation of PI3K and mTOR signalling activation might be considered as a possible mechanism of action for antroquinonol in A549 cells. Moreover we also found that antroquinonol induces cell death by altering the miRNA expression profiles. Nevertheless, the role of miRNA in inducing the apoptosis pathway after antroquinonol treatment is not fully understood. However, additional studies are in progress to identify the possible gene targets of miR-15a, 18a, 486, 2861 and 486-5p and general cellular changes influenced by these miRNAs after antroquinonol treatment. With

this basic understanding and puzzle. We would like to further evaluate the sensitising effect of antroquinonol on paclitaxel resistant cells using low concentration. Taken all data together suggest that antroquinonol could be developed as a novel anticancer agent.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by the grants from the National Science Council (NSC 98-2321-B-259-001 and 98-2622-B-259-001-CC3), Taiwan. We sincerely thank Miss Pei-Hui Wang for her technical assistance in Western blot.

References

- [1] N. Shivapurkar, J. Reddy, P.M. Chaudhary, A.F. Gazdar, Apoptosis and lung cancer: a review, *J. Cell. Biochem.* 88 (2003) 885–898.
- [2] S.C. Erridge, H. Moller, A. Price, D. Brewster, International comparisons of survival from lung cancer: pitfalls and warnings, *Nat. Clin. Pract. Oncol.* 4 (2007) 570–577.
- [3] N. Cordes, C. Beinke, L. Plasswilm, D. Van Beuningen, Irradiation and various cytotoxic drugs enhance tyrosine phosphorylation and β 1-integrin clustering in human A549 lung cancer cells in a substratum-dependent manner in vitro, *Strahlenther. Onkol.* 180 (2004) 157–164.
- [4] C.Y. Chen, C.H. Chen, Y.C. Lo, B.N. Wu, H.M. Wang, W.L. Lo, C.M. Yen, R.J. Lin, Anticancer activity of isobutylsilactone A from *Cinnamomum kotoense*: involvement of apoptosis, cell-cycle dysregulation, mitochondria regulation, and reactive oxygen species, *J. Nat. Prod.* 71 (2008) 933–940.
- [5] G.J. Peters, C.H. Smorenburg, C.J. Van Groenigen, Prospective clinical trials using a pharmacogenetic/pharmacogenomic approach, *J. Chemother.* 16 (2004) 25–30.
- [6] R.N. Proctor, Tobacco and the global lung cancer epidemic, *Nat. Rev. Cancer* 1 (2001) 82–86.
- [7] A. Jemal, T. Murray, E. Ward, E.A. Samuels, R.C. Tiwari, A. Ghafoor, E.J. Feuer, M.J. Thun, Cancer statistics 2005, *CA Cancer J. Clin.* 55 (2005) 10–30.
- [8] D.E. Maziak, B.R. Markman, J.A. MacKay, W.K. Evans, Cancer Care Ontario Practice Guidelines Initiative Lung Cancer Disease Site Group, Photodynamic therapy in non-small cell lung cancer: a systematic review, *Ann. Thorac. Surg.* 77 (2004) 1484–1491.
- [9] P.C. Hoffman, A.M. Mauer, E.E. Vokes, Lung cancer, *Lancet* 355 (2000) 479–485.
- [10] D.N. Carney, Lung cancer – time to move on from chemotherapy, *New Engl. J. Med.* 2 (2002) 126–128.
- [11] K. Krishnan, S. Campbell, F. Abdel-Rahman, S. Whaley, W.L. Stone, Cancer chemoprevention drug targets, *Curr. Drug Targets* 4 (2003) 1389–4501.
- [12] R.P. Singh, S. Dhanalakshmi, R. Agarwal, Phytochemicals as cell cycle modulators: a less toxic approach in halting human cancers, *Cell Cycle* 1 (2002) 56–161.
- [13] S. Akhtar, S.M. Meeran, N. Katiyar, S.K. Katiyar, Grape seed proanthocyanidins inhibit the growth of human non-small cell lung cancer xenografts by targeting insulin-like growth factor binding protein-3, tumour cell proliferation, and angiogenic factors, *Clin. Cancer Res.* 15 (2009) 821–831.
- [14] M. Zang, C.H. Su, *Ganoderma camphoratum*, a new taxon in genus *Ganoderma* from Taiwan, China, *Acta Bot. Yunnanica* 12 (1990) 395–396.
- [15] S.H. Wu, L. Ryvardeen, T.T. Chang, *Antrodia cinnamomea* (niu-chang-chih), new combination of a medicinal fungus in Taiwan, *Bot. Bull. Acad. Sin.* 38 (1997) 273–275.
- [16] C.C. Chen, Y.J. Shiao, R.D. Lin, Y.Y. Shao, M.N. Lai, C.C. Lin, L.T. Ng, Y.H. Kuo, Neuroprotective diterpenes from the fruiting body of *Antrodia camphorata*, *J. Nat. Prod.* 69 (2006) 689–691.
- [17] K.C. Chen, C.C. Peng, R.Y. Peng, C.H. Su, H.S. Chiang, J.H. Yan, H.M. Hsieh-Li, Unique formosan mushroom *Antrodia camphorata* differentially inhibits androgen-responsive LNCaP and -independent PC-3 prostate cancer cells, *Nutr. Cancer* 57 (2007) 111–121.
- [18] C.C. Peng, K.C. Chen, R.Y. Peng, C.C. Chyau, C.H. Su, H.M. Hsieh-Li, *Antrodia camphorata* extract induces replicative senescence in superficial TCC, and inhibits the absolute migration capability in invasive bladder carcinoma cells, *J. Ethnopharmacol.* 109 (2007) 93–103.
- [19] H.L. Yang, C.S. Chen, W.H. Chang, F.J. Lu, Y.C. Lai, C.C. Chen, T.H. Hseu, C.T. Kuo, Y.C. Hseu, Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*, *Cancer Lett.* 231 (2006) 215–227.
- [20] H. Wu, C.L. Pan, Y.C. Yao, S.S. Chang, S.L. Li, T.F. Wu, Proteomic analysis of the effect of *Antrodia camphorata* extract on human lung cancer A549 cell, *Proteomics* 6 (2006) 826–835.
- [21] Y.Y. Chan, C.S. Chang, L.H. Chien, T.F. Wu, Apoptotic effects of a high performance liquid chromatography (HPLC) fraction of *Antrodia camphorata* mycelia are mediated by down-regulation of the expressions of four tumour-related genes in human non-small cell lung carcinoma A549 cell, *J. Ethnopharmacol.* 127 (2010) 652–661.
- [22] T.H. Lee, C.K. Lee, W.L. Tsou, S.Y. Liu, M.T. Kuo, W.C. Wen, A new cytotoxic agent from solid-state fermented mycelium of *Antrodia camphorata*, *Planta Med.* 73 (2007) 1412–1415.
- [23] D.Z. Liu, H.J. Liang, C.H. Chen, C.H. Sub, T.H. Lee, C.T. Huang, W.C. How, S.Y. Lin, W.B. Zhong, P.J. Lin, L.F. Hung, Y.C. Liang, Comparative anti-inflammatory characterization of wild fruiting body, liquid-state fermentation, and solid-state culture of *Taiwanofungus camphoratus* in microglia and the mechanism of its action, *J. Ethnopharmacol.* 113 (2007) 45–53.
- [24] P.C. Chiang, S.C. Lin, S.L. Pan, C.H. Kuo, I.L. Tsai, M.T. Kuo, W.C. Wen, P. Chen, J.H. Guh, Antroquinonol displays anticancer potential against human hepatocellular carcinoma cells: a crucial role of AMPK and mTOR pathways, *Biochem. Pharmacol.* 79 (2010) 162–171.
- [25] M.A. Bjornstj, P.J. Houghton, The TOR pathway: a target for cancer therapy, *Nat. Rev. Cancer* 4 (2004) 335–348.
- [26] N. Hay, The Akt-mTOR tango and its relevance to cancer, *Cancer Cell* 8 (2005) 179–183.
- [27] H.M. Coley, Mechanisms and strategies to overcome chemotherapy resistance in metastatic breast cancer, *Cancer Treat. Rev.* 34 (2008) 378–390.
- [28] T.F. Franke, C.P. Hornik, L. Segev, G.A. Shostak, C. Sugimoto, PI3K/Akt and apoptosis: size matters, *Oncogene* 22 (2003) 8983–8998.
- [29] T.F. Franke, S.I. Yang, T.O. Chan, K. Datta, A. Kazlauskas, D.K. Morrison, D.R. Kaplan, P.N. Tsichlis, The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase, *Cell* 81 (1995) 727–736.
- [30] T.F. Franke, L.C. Cantley, Apoptosis. A bad kinase makes good, *Nature* 390 (1997) 116–117.
- [31] M. Pap, G.M. Cooper, Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway, *J. Biol. Chem.* 273 (1998) 19929–19932.
- [32] P.E. Blower, J.H. Chung, J.S. Verducci, S. Lin, J.K. Park, Z. Dai, C.G. Liu, T.D. Schmittgen, W.C. Reinhold, C.M. Croce, J.N. Weinstein, W. Sadee, MicroRNAs modulate the chemosensitivity of tumour cells, *Mol. Cancer Ther.* 7 (2008) 1–9.
- [33] C.M. Croce, G.A. Calin, miRNAs, cancer, and stem cell division, *Cell* 122 (2005) 6–7.
- [34] R.I. Gregory, R. Shiekhattar, MicroRNA biogenesis and cancer, *Cancer Res.* 65 (2005) 3509–3512.
- [35] J. Zhao, Q.Q. Li, B. Zou, G. Wang, X. Li, J.E. Kim, C.F. Cuff, L. Huang, E. Reed, K. Gardner, In vitro combination characterization of the new anticancer plant drug beta-elemene with taxanes against human lung carcinoma, *Int. J. Oncol.* 31 (2007) 241–252.
- [36] F. Impens, P. Van Damme, H. Demol, J. Van Damme, J. Vandekerckhove, K. Gevaert, Mechanistic insight into taxol-induced cell death, *Oncogene* 33 (2008) 4580–4591.
- [37] X. Tang, C.P. Downes, A.D. Whetton, P.J. Owen-Lynch, Role of phosphatidylinositol 3-kinase and specific protein kinase B isoforms in the suppression of apoptosis mediated by the Abelson protein-tyrosine kinase, *J. Biol. Chem.* 275 (2000) 13142–13148.
- [38] F. Zhang, T. Zhang, T. Jiang, R. Zhang, Z.H. Teng, C. Li, Z.P. Gu, Q. Mei, Wortmannin potentiates roscovitine-induced growth inhibition in human solid tumour cells by repressing PI3K/Akt pathway, *Cancer Lett.* 286 (2009) 232–239.
- [39] G. Giuseppe, Targeted therapy in non-small cell lung cancer, *Lung Cancer* 38 (2002) 29–32.
- [40] S. Shukla, S. Gupta, Molecular mechanisms for apigenin induced cell-cycle arrest and apoptosis of hormone refractory human prostate carcinoma DU145 cells, *Mol. Carcinog.* 39 (2004) 114–126.
- [41] T. Jacks, R.A. Weinberg, Cell-cycle control and its watchman, *Nature* 381 (1996) 643–644.
- [42] P.M. O'Connor, D.K. Ferris, M. Pagano, G. Draetta, J. Pines, T. Hunter, D.L. Longo, K.W. Kohn, G2 delay induced by nitrogen mustard in human cells affects cyclin A/cdk2, and cyclin B1/cdc-2 kinase complexes differently, *J. Biol. Chem.* 268 (1993) 8298–8308.
- [43] D.J. Lew, S. Kornbluth, Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control, *Cell. Biol.* 8 (1996) 795–804.
- [44] C.J. Yang, C.S. Wang, J.Y. Hung, H.W. Huang, Y.C. Chia, P.H. Wang, C.F. Weng, M.S. Huang, Pyrogallol induces G2-M arrest in human lung cancer cells and inhibits tumour growth in an animal model, *Lung Cancer* 66 (2009) 162–168.
- [45] E.K. Shibuya, G2 cell cycle arrest—a direct link between PKA and Cdc25C, *Cell Cycle* 2 (2003) 39–41.
- [46] C.F. Gao, Q. Xie, Y.L. Su, J. Koeman, S.K. Khoo, M. Gustafson, B.S. Knudsen, R. Hay, N. Shinomiya, G.F.V. Woude, Proliferation and invasion: plasticity in tumor cells, *Proc. Natl. Acad. Sci. U.S.A.* 30 (2005) 10528–10533.
- [47] J. Wang, Z. Wan, W. Liu, L. Li, L. Ren, X. Wang, P. Sun, L. Ren, H. Zhao, Q. Tu, Z. Zhang, N. Song, L. Zhang, Atomic force microscope study of tumour cell membranes following treatment with anti-cancer drugs, *Biosens. Bioelectron.* 4 (2009) 721–727.
- [48] A. Antignani, R.J. Youle, How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane, *Curr. Opin. Cell Biol.* 18 (2006) 685–689.
- [49] M.O. Hengartner, The biochemistry of apoptosis, *Nature* 407 (2000) 770–776.
- [50] C. Jiang, Y.F. Yang, S.H. Cheng, Fas ligand gene therapy for vascular intimal hyperplasia, *Curr. Gene Ther.* 4 (2004) 33–39.
- [51] X. Cai, S. Gao, J. Cai, Y. Wu, H. Deng, Artesunate induced morphological and mechanical changes of Jurkat cell studied by AFM, *Scanning* 31 (2009) 83–89.

- [52] Y.A. Lazebnik, S.H. Kaufmann, S. Desnoyers, G.G. Poirier, W.C. Earnshaw, Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE, *Nature* 371 (1994) 346–347.
- [53] V.M. Adhami, I.A. Siddiqui, N. Ahmad, S. Gupta, H. Mukhtar, Oral consumption of green tea polyphenols inhibits insulin-like growth factor-I-induced signalling in an autochthonous mouse model of prostate cancer, *Cancer Res.* 64 (2004) 8715–8722.
- [54] M.L. Janmaat, F.A.E. Kruyt, J.A. Rodriguez, G. Giaccone, Response to epidermal growth factor receptor inhibitors in non small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways, *Clin. Cancer Res.* 9 (2003) 2316–2326.
- [55] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, *Cell* 116 (2004) 281–297.
- [56] X. Chen, Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, Y. Zhang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, J. Wang, K. Zen, J. Zhang, C.Y. Zhang, Characterization of miRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases, *Cell Res.* 18 (2008) 997–1006.
- [57] C.H. Lawrie, S. Gal, H.M. Dunlop, B. Pushkaran, A.P. Liggins, K. Pulford, A.H. Banham, F. Pezzella, J. Boulton, J.S. Wainscoat, C.S. Hutton, A.L. Harris, Detection of elevated levels of tumour associated microRNAs in serum of patients with diffuse large B-cell lymphoma, *Br. J. Haematol.* 141 (2008) 672–675.
- [58] P.S. Mitchell, R.K. Parkin, E.M. Kroh, B.R. Fritz, S.K. Wyman, E.L. Pogosova-Agadjanian, A. Peterson, J. Noteboom, K.C. O'Brian, A. Allen, D.W. Lin, N. Urban, C.W. Drescher, B.S. Knudsen, D.L. Stirewalt, R. Gentleman, R.L. Vessella, P.S. Nelson, D.B. Martin, M. Tewari, Circulating microRNAs as stable blood-based markers for cancer detection, *Proc. Natl. Acad. Sci. U.S.A.* 105 (2008) 10513–10518.
- [59] E.M. Lee, S. Shin, H.J. Cha, Y. Yoon, S. Bae, J.H. Jung, S.M. Lee, S.J. Lee, I.C. Park, Y.W. Jin, S. An, Suberoylanilide hydroxamic acid (SAHA) changes microRNA expression profiles in A549 human non-small cell lung cancer cells, *Int. J. Mol. Med.* 24 (2009) 45–50.
- [60] N. Bandi, S. Zbinden, M. Gugger, M. Arnold, V. Kocher, L. Hasan, A. Kappeler, T. Brunner, E. Vassella, miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer, *Cancer Res.* 13 (2009) 5553–5559.
- [61] A. Cimmino, G.A. Calin, M. Fabbri, M.V. Iorio, M. Ferracin, M. Shimizu, S.E. Wojcik, R.I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C.G. Liu, T.J. Kipps, M. Negrini, C.M. Croce, miR-15 and miR-16 induce apoptosis by targeting BCL2, *Proc. Natl. Acad. Sci. U.S.A.* 102 (2005) 13944–13949.
- [62] W.P. Tsang, T.T. Kwok, The miR-18a* microRNA functions as a potential tumour suppressor by targeting on K-Ras, *Carcinogenesis* 6 (2009) 953–959.