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Shikonin Synergistic with Doxorubicin to Antagonize the Fine Particulate matter (PM2.5) Induced Lung Cancer Cell Growth by Regulating Apoptosis and Cell Cycle Progression.

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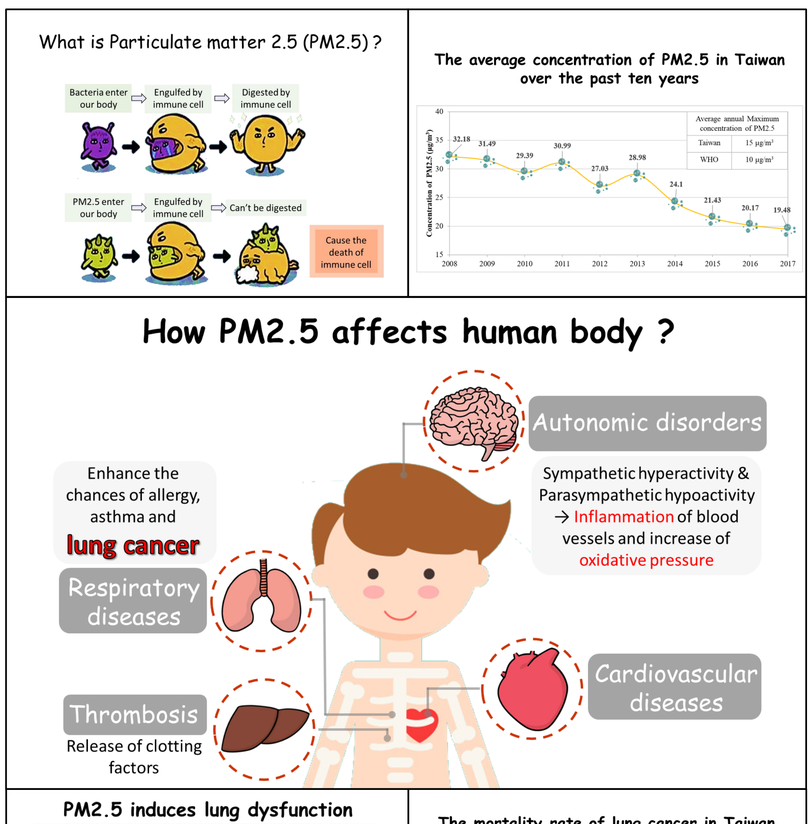
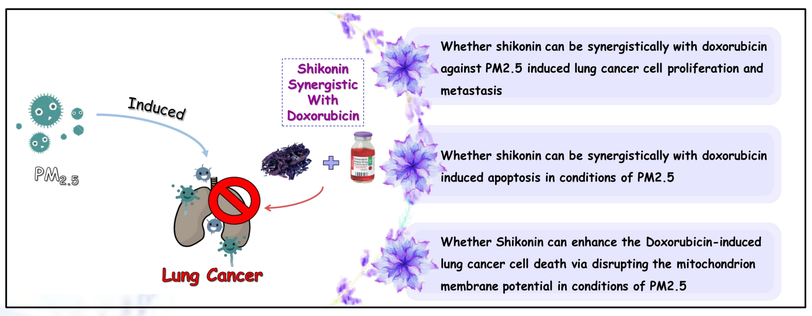
Abstract

Multi-drug resistance remain an unsolved major problems in cancer therapy. More seriously, the Particulate Matter 2.5 ($PM_{2.5}$) has been implicated in accelerating lung cancer cell growth and metastasis. Shikonin (SHK), a natural naphthoquinone isolated from Lithospermum erythrorhizon, has been proposed to enhance the doxorubicin (DOX) antitumor effects in lung cancer cell. The aim of the current study was to investigate whether SHK synergized with DOX could control the $PM_{2.5}$ induced cellular responses in human lung adenocarcinoma cell A549 and high-metastatic PC-9 cells by microscopic, biochemical, and flow cytometric analyses. Compared with A549 cells, $PM_{2.5}$ significantly induced PC-9 cell proliferations when doses are less than 450 μg/mL while higher concentration (1000 μg/mL) precipitated the cell death in both cells. Notably, pretreatment with lower dose of SHK (0.5 μg/mL) and DOX (0.5 μg/mL) showed significantly decreased cell viability, increased early apoptosis, induced cell cycle arrest in G2/M phase, and exhibited obvious loss of mitochondrial membrane potential compared with treated with high-dose of DOX (1.0 μg/mL) alone in PC-9 cells. Specially, the effects of $PM_{2.5}$-induced PC-9 cell proliferation, increased late apoptosis, alterations of cell-cycle, and mitochondrial membrane damage could be antagonized by co-treatment with SHK and DOX. In conclusions, our data indicated the $PM_{2.5}$ has a strong ability to promote cancer metastasis whereas SHK could specifically augment the antitumor effects of DOX to change the biological behaviors of metastatic cells and reduce lung cancer drug resistant. Therefore, SHK has the potential to be used as a promising complementary agent toward the personalized medicine for treatment of lung cancer in clinical practice.

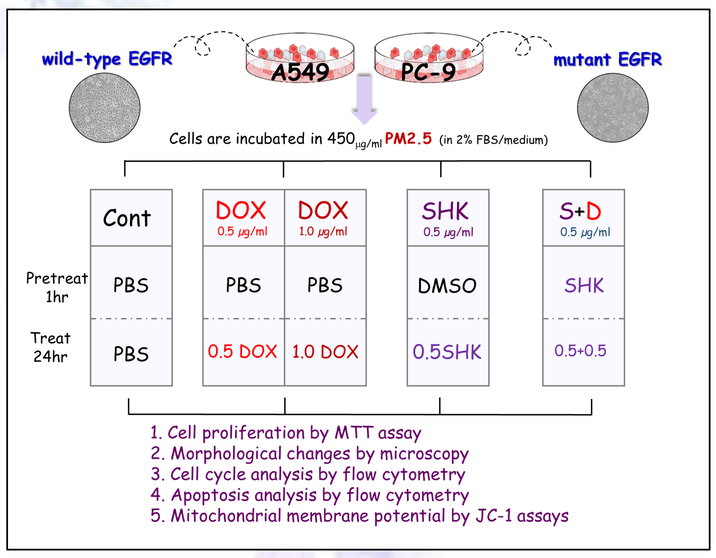
Background



Specific Aims



Experimental Design



Results

Figure 1: Cell proliferation by MTT assay

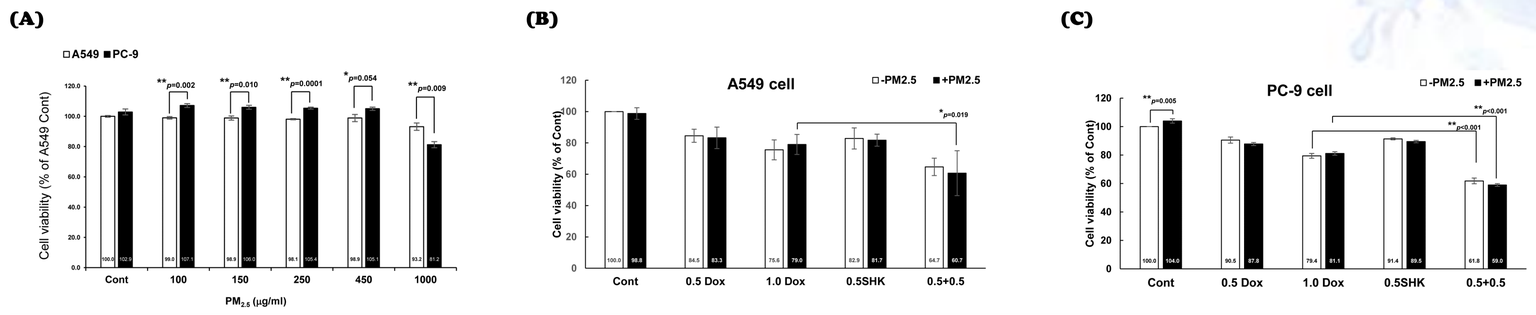


Figure 1. Effects of $PM_{2.5}$, Doxorubicin (DOX), and Shikonin (SHK) on lung cancer cell viability. (A). A549 and PC-9 lung cancer cell lines were exposure to vehicle (Cont) or various doses of $PM_{2.5}$ (100, 150, 250, 450, or 1000 μg/ml) for 24 h and the cell viability was determined by MTT assay. The viability of the control cells (Cont) in A549 exposed to PBS only was set as 100%. There were no significant differences among the $PM_{2.5}$-treated groups (concentration up to 450 μg/ml), whereas higher concentration of $PM_{2.5}$ (1000 μg/ml) cause the cell death. (B) & (C) Cells were pre-exposure to vehicle or with $PM_{2.5}$ at concentration of 450 μg/ml and then subjected to vehicle (Cont) or DOX (0.5 or 1.0 μg/ml), SHK (0.5 μg/ml), or DOX (0.5 μg/ml) plus SHK (0.5 μg/ml, 1 h prior to DOX). The viability of the cells pretreated with vehicle (Cont) was set as 100%. After 24 h, the MTT cell viability of the co-treatment with SHK and DOX decreased to 60.8% of the control level in A549 cells and 59% in PC-9 cells when pretreated with $PM_{2.5}$. Data were collected at least from three independent experiments and represented as mean±S.E.M. *p<0.05 and **p<0.01 compared with the differences between a Cont group.

Figure 2: Flow-cytometry in analysis of cell apoptosis

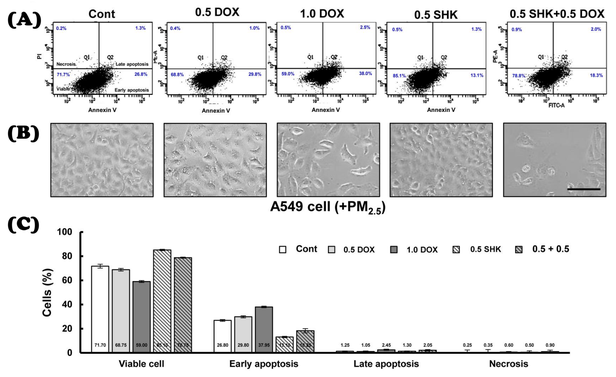


Figure 2. Effects of $PM_{2.5}$, SHK and DOX on apoptosis and necrosis of A549 and PC-9 cells. (A) & (D) Representative flow cytometry results. After exposure to $PM_{2.5}$ (450 μg/ml) and then treated with DOX, SHK, or DOX+SHK, cells were stained with Annexin V/PI and then subjected to flow cytometry. The dotted plot shows Annexin V-FITC in the x-axis and PI in the y-axis. Viable cells (scattered in left lower panel), early-apoptotic cells (right lower panel), late-apoptotic cells (right upper panel), and necrotic cells (left upper panel) are shown in the quadrant of the graphs. (B) & (E) Representative photomicrographs of cells were observed with phase-contrast light microscope. Scale bar indicate 40 μm. (C) & (F) Quantitative results from flowcytometry. Data are from at least three independent experiments and represented as mean±S.E.M. *p<0.05 and **p<0.01.

Figure 3: Flow-cytometry in analysis of cell cycle

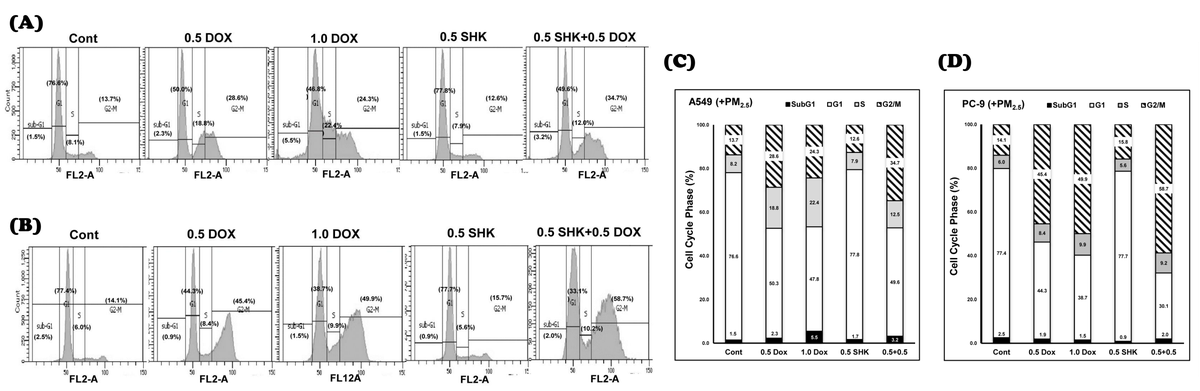


Figure 3. Effects of $PM_{2.5}$, SHK and DOX on cell cycle progression of A549 and PC-9 cells. (A) & (B) Representative DNA distribution histogram of A549 and PC-9 cells by flowcytometric analysis. Cells were pre-exposure to $PM_{2.5}$ (450 μg/ml), and then subjected to DOX, SHK, and DOX+SHK for 24 h and then cells were harvested and stained with PI for 30 min at 37°C. Fluorescence intensities (FL2-A channel) are presented in arbitrary units on a logarithmic scale as a measure of the amount of staining per cell. (C) & (D) Summary of the percentage of A549 and PC-9 cells at different cell cycle stages by flow cytometric data. Data are from three independent experiments performed in triplicate.

Figure 4: Detection of apoptosis by the mitochondrial membrane potential

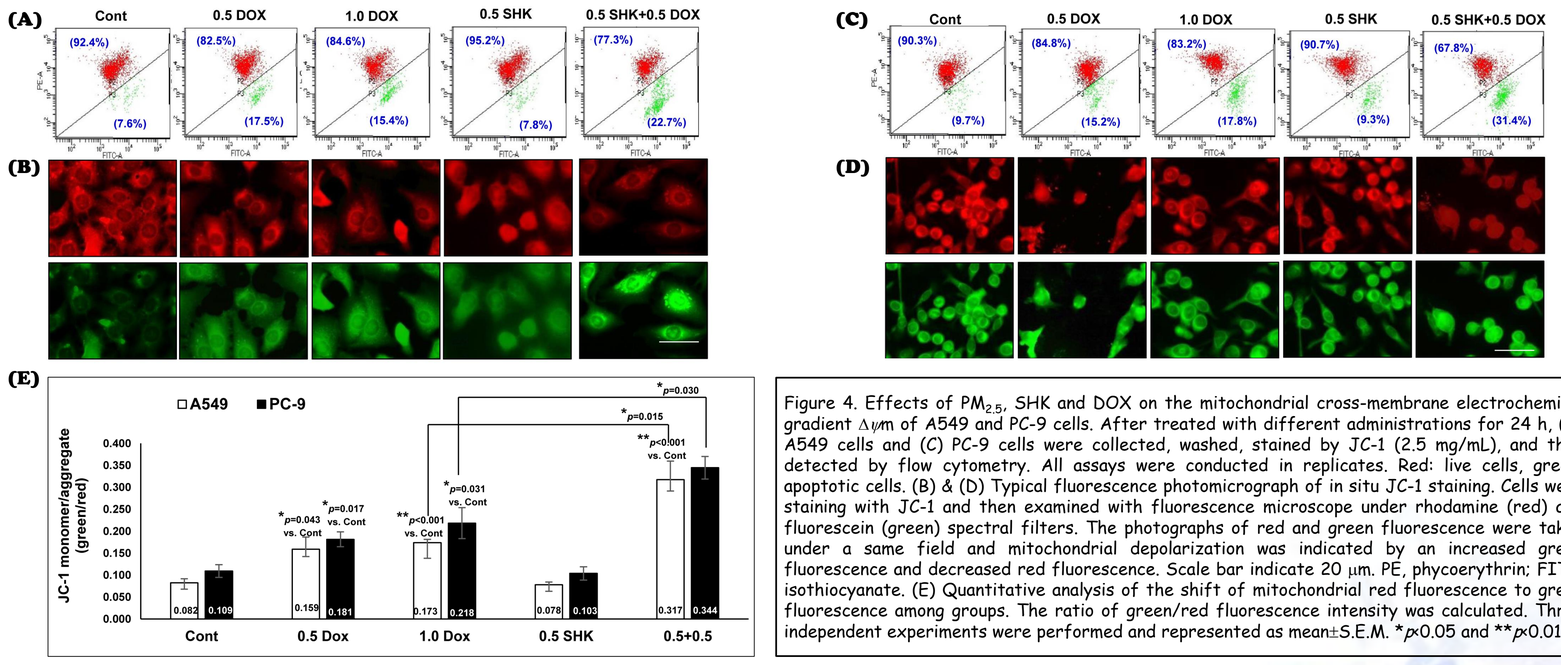


Figure 4. Effects of $PM_{2.5}$, SHK and DOX on the mitochondrial cross-membrane electrochemical gradient $\Delta\psi$m of A549 and PC-9 cells. After treated with different administrations for 24 h, (A) A549 cells and (C) PC-9 cells were collected, washed, stained by JC-1 (2.5 mg/mL), and then detected by flow cytometry. All assays were conducted in replicates. Red: live cells, green: apoptotic cells. (B) & (D) Typical fluorescence photomicrograph of in situ JC-1 staining. Cells were staining with JC-1 and then examined with fluorescence microscope under rhodamine (red) and fluorescein (green) spectral filters. The photographs of red and green fluorescence were taken under a same field and mitochondrial depolarization was indicated by an increased green fluorescence and decreased red fluorescence. Scale bar indicate 20 μm. PE, phycoerythrin; FITC, isothiocyanate. (E) Quantitative analysis of the shift of mitochondrial red fluorescence to green fluorescence among groups. The ratio of green/red fluorescence intensity was calculated. Three independent experiments were performed and represented as mean±S.E.M. *p<0.05 and **p<0.01.

Conclusions

1. Effects of PM2.5 exposure on lung cancer cell proliferations:

(1) $PM_{2.5}$ can inhibit proliferation of both A549 and PC-9 cells at higher dose (1000 μg/ml), while the growth rates in PC-9 cells are significantly higher than in A549 cells at relatively lower doses (0-450 μg/ml).

(2) Synergistic treatment of SHK and DOX at lower dose (0.5 μg/ml) shows significant growth-inhibitory actions on PC-9 cells when compare to the cells treated with DOX only (1.0 μg/ml), no matter with or without pre-exposure to $PM_{2.5}$.

(3) SHK can enhance the DOX-induced cell death and morphological alterations via inducing the cell cycle arrest at G2/M phase in $PM_{2.5}$-treated A549 cells; furthermore, SHK can stimulate early apoptosis and weaken late apoptosis and necrosis induced by DOX in $PM_{2.5}$-treated PC-9 cells correspondingly.

2. Combined regimen of SHK and DOX improves disrupting mitochondrial membrane potentials

(1) SHK has no adverse side effect on $\Delta\psi$M and it appears to increase the activity of DOX induced disruption of mitochondrial membrane potential in both $PM_{2.5}$-treated A549 and PC-9 cells.

(2) Taken together, our results suggest that a most efficient strategy of using SHK and DOX in combination could be helpful to modulate the PM2.5 induced adverse effects and enhance the efficiency of DOX and to augment anticancer synergism by reduction of the toxicity.

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