Sulforaphene Synergistically Sensitizes Cisplatin *via* Enhanced Mitochondrial Dysfunction and PI3K/PTEN Modulation in Ovarian Cancer Cells

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Abstract. *Aim: To explore if a natural isothiocyanate, sulforaphene (SFE), sensitizes ovarian cancer cells to the chemotherapy drug cisplatin (CDDP). Materials and Methods: We studied reactive oxygen species (ROS), mitochondrial membrane depolarization and cell-cycle distribution in two ovarian cancer cell lines SKOV3 and SNU 8 treated with SFE and cisplatin. We further analyzed the expression of caspases 3, 8, and 9, Phosphoinositide 3-kinase (PI3K) and Phosphatase and tensin homolog (PTEN) by western blotting. Results: SFE sensitized cells to cisplatin by enhancing ROS and mitochondrial membrane depolarization that released cytochrome c and activated caspase 9 and caspase 3 in the mitochondrial pathway. It also inhibited extrinsic pathway protein caspase 8, growth-related protein PI3K and further activated PTEN in combination with cisplatin. Conclusion: SFE synergistically inhibited proliferation and induced apoptosis of SKOV3 and SNU8 cells in combination with cisplatin by activating multiple apoptotic pathways. Therefore, we suggest sulforaphene as a chemo-enhancing adjuvant to improve the efficacy of cisplatin in ovarian cancer treatment.*

Ovarian cancer is one of the most common gynecological malignancies and accounts for an alarming number of women deaths from gynecological disorders worldwide (1, 2). At early stages, its non-specific symptoms often mislead to diagnosis of ovarian cancer as benign conditions and it remains undetected until it spreads to the other parts of the body. At this late stage, it becomes more difficult to treat. Cisplatin, which induces DNA cross-links and results in apoptosis, is one of the commonly used chemotherapies for ovarian cancer (3). Patients who initially respond to platinum-based chemotherapy, however, do not respond after a few months and generally experience relapse within next 6 months (4, 5). This resistance to platinum-based drugs in women with advanced-stage ovarian cancer has become a major concern to clinicians. Multiple signaling pathways have been reported to contribute to the resistance of cancer cells (6, 7). Recent reports suggest that deregulation of Phosphoinositide 3-kinase (PI3K) and inhibition of Phosphatase and tensin homolog (PTEN) is commonly involved in ovarian cancer. This activation of PI3K contributes to cellular growth and proliferation. In contrast, suppression of PTEN which negatively regulates PI3K also helps in cellular proliferation (8, 9). Thus, this activation of PI3K and suppression of PTEN contribute to the cisplatin resistance in ovarian cancer.

The approach of combination therapy in cancer management is now being used as an effective treatment modality to achieve higher therapeutic efficacy as well as reducing drug dosages. Moreover, it also helps in overcoming resistance to chemotherapy drugs. Several agents have been introduced in combination with platinum-based drugs to enhance efficacy and overcome drug resistance (10-12). Natural agents are one of the promising options that showed better chemotherapeutic efficacy in combination with conventional chemotherapy drugs (13, 14). Recently a novel class of isothiocyanate, sulforaphene, was isolated from cruciferous vegetables. Previous studies reported that sulforaphene can induce apoptosis in different types of cancer cells with minimum effect on normal lymphocytes (15, 16). However, little is known on its synergistic anticancer efficacy in combination therapy.

In the present study, we investigated whether combination of sulforaphene with cisplatin can produce an enhanced inhibitory effect on human ovarian cancer cells. This study

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carried-out a relevant *in vitro* analysis using sulforaphene in combination with cisplatin for the treatment of ovarian cancer.

Materials and Methods

Cell line and reagents. Two human ovarian cancer cell lines, SKOV 3 and SNU 8, were obtained from the Korean Cell Line Bank and maintained in RPMI-1640 media 10% heat-inactivated bovine serum. Sulforaphene was obtained from LKT Laboratories (St. Paul, MN, USA). Cisplatin was obtained from Korea United Parma. Inc., Seoul, South Korea. Antibodies to cytochrome *c*, poly ADP ribose polymerase (PARP), caspase-8, Phosphoinositide 3-kinase (PI3K),Phosphatase and tensin homolog (PTEN) and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and caspase 9, caspase 3 were purchased from Calbiochem (San Diego, CA, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay. SKOV 3 and SNU 8 cells (5×10^3 cells/well) were treated for 24 h with increasing concentrations (0-100 μg/ml) of cisplatin and sulforaphene individually. For combination treatment, cells were treated with 1 μg/ml of sulforaphene followed by cisplatin (0-100 μg/ml). Control cells received no treatment and were grown in RPMI-1640 media. After 24 h, MTT solution (0.2 mg/ml) (Sigma, St. Louis, MO, USA) was added to each well and cells were incubated for 4 h. The resulting formazan crystals were dissolved in 150 μl of dimethylsulfoxide and absorbance of the each well was measured at 570 nm using Asys UVM340 Microplate Reader, Biochrom (Cambridge, UK). Cell viability was expressed as the percentage absorbance compared with control cells.

Determination of synergism. To understand the efficacy of sulforaphene in inducing a synergistic effect in combination treatment, data were analyzed according to the Chou and Talalay method (17). Briefly, SKOV 3 and SNU 8 cells were seeded in 96-well plates and treated with increasing concentrations of cisplatin and a constant concentration of sulforaphene (1 μg/ml). Control cells did not receive any treatment. After 24 h, the fractions of cells affected were calculated by dividing the viability of the treated cells with that of the control. Synergy analysis was performed using CompuSyn version 1.0 software (ComboSyn Inc., NJ, USA) to calculate the combination index (CI). Synergy was quantified by CI with CI <1 indicating synergism, CI >1 indicating antagonism, and CI=1 indicating additivity. An isobologram was also prepared with the fraction affected by combination treatment to confirm the synergism between sulforaphene and cisplatin.

Hoechst and Propidium Iodide (PI) staining for apoptosis assay. To study morphological changes of the nuclear chromatin, cells were treated with cisplatin and sulforaphene, as described and stained with Hoechst (1 μg/ml) and PI (1 μg/ml) for 15 min. Cells were then washed with Dulbecco's Phosphate-Buffered Saline (DPBS) and observed under a laser scanning confocal microscope Zeiss LSM 510 Meta(Carl Zeiss, Jena, Germany). Images of the control and treated cells were taken and a histogram was prepared by counting early and late apoptotic cells in control and treated groups.

Determination of intracellular reactive oxygen species (ROS) generation. Formation of ROS in treated cells was monitored using 2',7'-dichlorofluorescin diacetate (DCFDA) (Molecular Probes Inc., Eugene, OR, USA). Briefly, after treatment, SKOV3 and SNU 8 cells were incubated with 10 μM DCFDA for 15 min at 37˚C. Cells were washed with DPBS before images of the green fluorescent cells were taken using a confocal microscope.

Detection of changes in mitochondrial membrane potential ($\Delta\Psi m$). Changes in Ψm in treated cells were studied using rhodamine 123 (Molecular Probes Inc.). Cells were stained with rhodamine 123 at 37˚C for 15 min after treatment. Cells were then washed and images of the rhodamine 123 stained control and treated cells were collected using a confocal microscope.

Flow cytometric analysis of mitochondrial membrane potential. Changes in Ψm were also analyzed by flow cytometry. After treatment, SKOV 3 and SNU 8 cells were incubated with 10 μM of rhodamine 123 at 37˚C,washed with DPBS and 10,000 cells were analysed by BD Accuri C6 flow cytometer and by BD Accuri C6 software (BDBioscience, San Jose, CA, USA).

Cell-cycle analysis. After the treatment, SKOV 3 and SNU 8 cells were harvested by centrifugation at approximately 225 × *g* (Hanil Scientific Industrial Co Ltd., Gyeyangku, Inchun, South Korea) for 5 min and fixed with ice-cold 70% (v/v) ethanol. Cell pellets were washed with DPBS (pH 7.4) and suspended in PBS containing PI (50 μg/ml) (Sigma). The DNA content of control and treated cells was determined by BD Accuri C6 flow cytometer through FL-2 filter and analyzed by BD Accuri C6 software for three different sets of data.

Western blot. SKOV 3 and SNU 8 cells were washed with cold DPBS and proteins were extracted in RIPA buffer (Sigma) containing protease and phosphatase inhibitor (Sigma). Equivalent amounts of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and were transferred to a PVDF membrane (BioRad Laboratories, Hercules, CA, USA). The membranes were probed with primary antibodies to cytochrome c, caspase 9, caspase 3, PARP, caspase 8, PI3K, PTEN and β- actin overnight at 4˚C with a gentle shaking. The membranes were then incubated with the respective horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The protein bands were detected by ECL western blotting detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Statistical analysis. All data are expressed as the mean±S.E. for at least three independent experiments. Statistical analysis was performed and the differences between cisplatin and combination treatment groups were analyzed using the two-tailed Student's *t*-test. Values of $p<0.01$ were considered to be statistically significant.

Results

Viability of SKOV3 and SNU 8 cells was reduced with combination treatment. The effect of sulforaphene on cisplatin-mediated cytotoxicity in human ovarian cancer cells

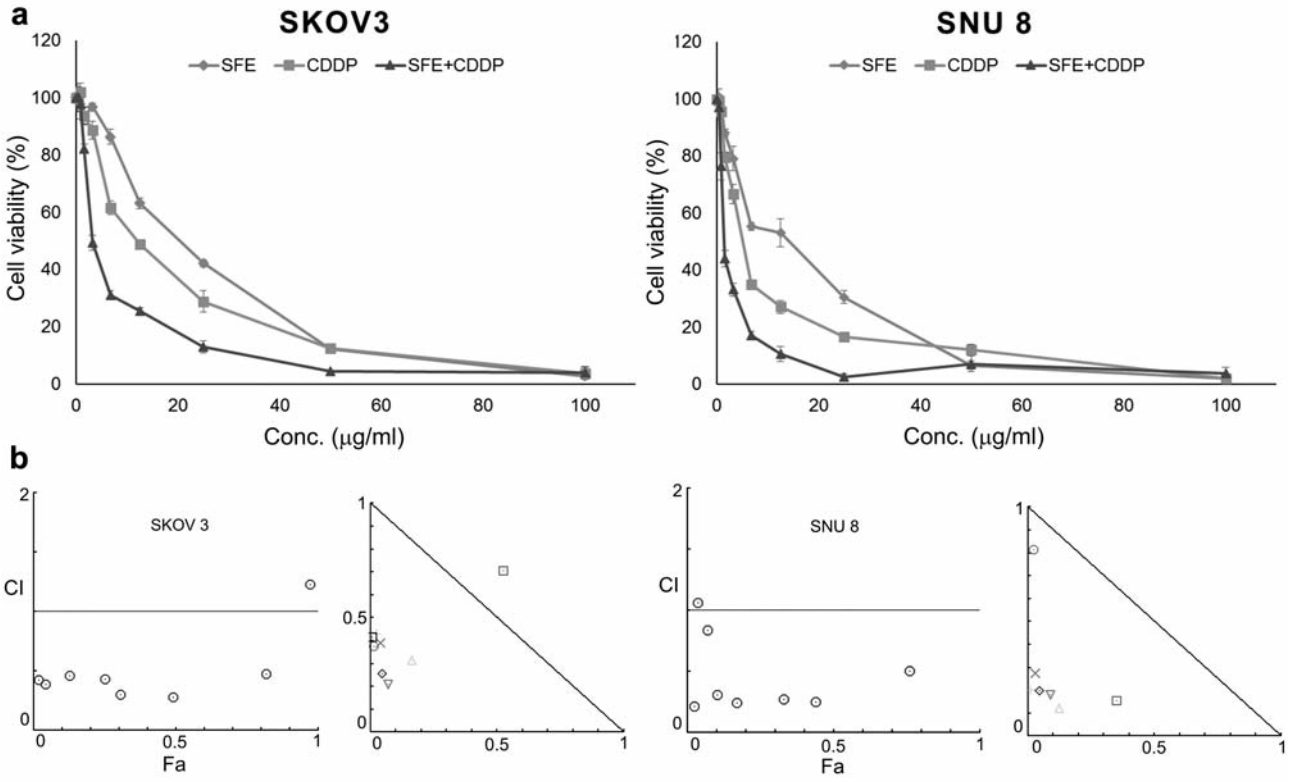

Figure 1. *a: Cell viability study by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Cell viability was reduced by increasing concentrations of sulforaphene and cisplatin. It was reduced more by the combination treatment. b: Graph of the combination index (CI) vs. fraction affected (Fa) for CDDP combined with SFE. The most pronounced synergism in both cell lines (CI<1) was demonstrated when the two drugs were combined. Isobologram analysis showed a SKOV 3 and SNU 8 cell-growth inhibition by simultaneous combination of CDDP and SFE.*

were preliminarily screened by the MTT assay. Figure 1a shows that the viability of SKOV3 and SNU 8 cells decreased gradually with cisplatin concentration. Cell viability was also reduced in sulforaphene-treated cells in a dose-dependent manner. No significant change in cell viability was observed in either cell line at 1 μg/ml of sulforaphene. However, a noticeable change in cell viability was observed when SKOV and SNU 8 cells were treated with 1 μg/ml of sulforaphene with increasing concentrations of cisplatin (0-100 μg/ml). The half-maximal inhibitory concentration IC_{50} values of cisplatin for SKOV 3 and SNU 8 cells were measured as 24.8 μg/ml and 12.9 μg/ml, respectively. However, in the presence of 1 μg/ml of sulforaphene, the (IC_{50}) values of cisplatin were reduced to 3.1 μg/ml and 1.4 μg/ml respectively. Although the viability of SNU 8 cells was slightly lower than that of SKOV 3 cells at the same concentrations of each drug alone, the combination of sulforaphene and cisplatin had a higher efficacy in both cases. From this result, 1 μg/ml of sulforaphene and 5 μg/ml of cisplatin were selected for our further studies.

Sulforaphene in combination with cisplatin showed synergism. The combination index (CI) was plotted against the fraction of affected cells (Fa) for the different doses of drugs. In Figure 1b, simultaneous exposure of SKOV 3 and SNU 8 cells to 1 μg/ml sulforaphene and different concentrations of cisplatin for 24 h led to strong and moderate synergism indicated by a CI<1. Although some concentrations of cisplatin showed no synergism, maximum synergy was observed between 1.6-25 μg/ml of cisplatin. A representative isobologram of SKOV 3 and SNU 8 cells exposed to low concentrations of sulforaphene and different concentrations of cisplatin for 24 h also showed clear synergism.

Apoptotic cells increased with cisplatin and sulforaphene combination as shown by Hoechst/PI staining. To study the apoptotic cell morphology of the treated cells, SKOV3 and SNU 8 cells were double-stained with Hoechst 33342 and PI and observed under a confocal laser scanning microscope. Figure 2a shows that control SKOV 3 and SNU 8 cells exhibited normal cell morphology with a healthy nucleus. The number of brightly stained apoptotic cells was increased

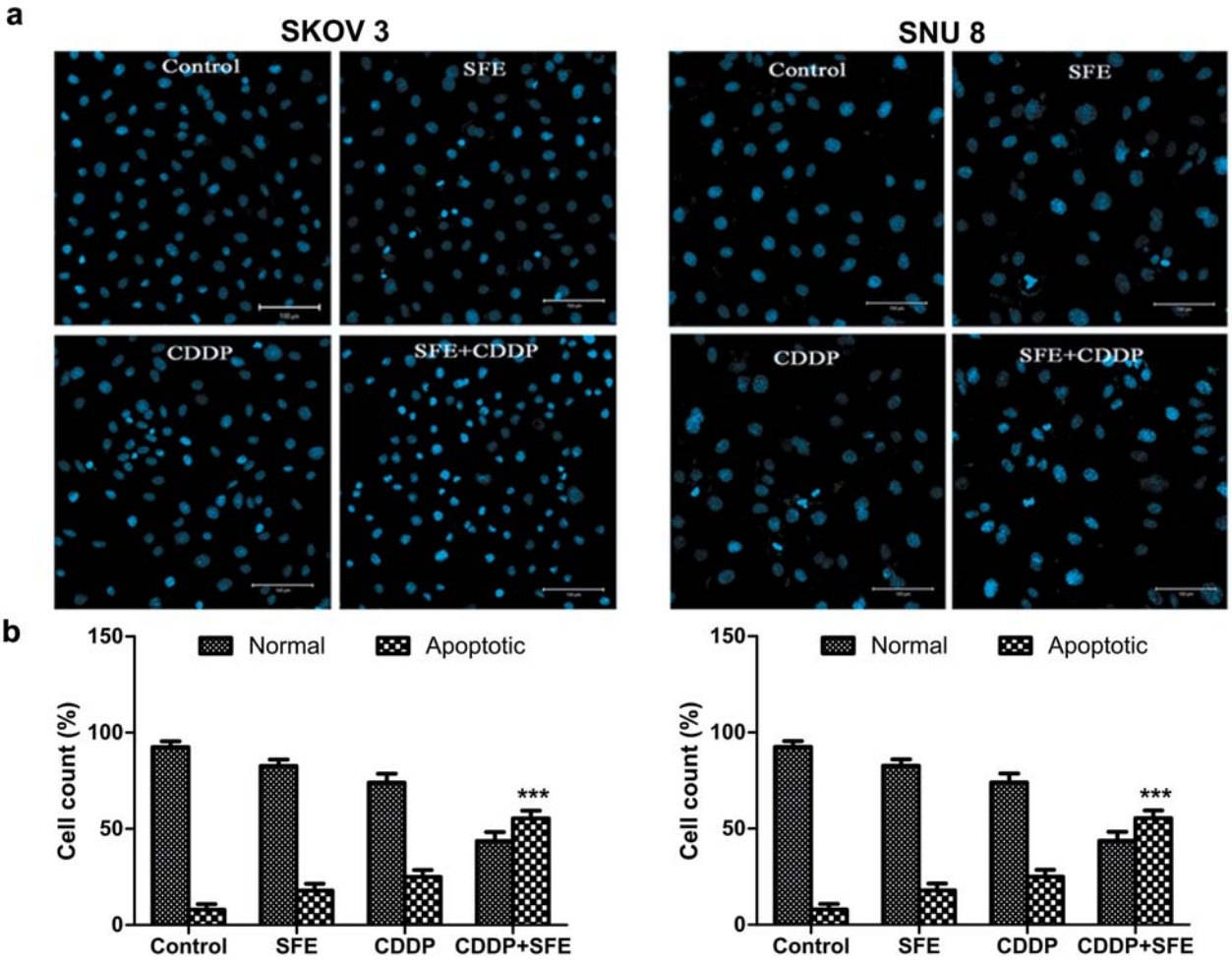

Figure 2. *a: Apoptotic cell morphology study with Hoechst and Propidium Iodide (PI) staining. The number of brightly-stained apoptotic cells was slightly increased with sulforaphene and cisplatin treatment. Apoptotic cells was increased more by the combination treatment. b: The histogram shows the number of normal cells with regular cell morphology decreased and the number of apoptotic cells increased significantly with SFE and CDDP combination treatment.*

slightly with separate cisplatin and sulforaphene treatments. However, the combination treatment led to an increased number of brightly-stained apoptotic cells compared to that of cisplatin and sulforaphene alone. The histogram in Figure 2b shows that this enhanced apoptosis with combination treatment was statistically significant ($p<0.001$).

Intracellular ROS increased with combination treatment. From the confocal microscopic images in Figure 3, it can be seen fluorescence intensity, indicating generation of ROS, was slightly increased in SKOV3 and SNU 8 cells with cisplatin and sulforaphene treatment. Fluorescence intensity was distinctly enhanced with combination treatment. Both the SKOV3 and SNU 8 cells in the combination group exhibited brightly-stained green fluorescence, indicating enhanced ROS generation.

Combination of sulforaphene and cisplatin reduced the mitochondrial membrane potential. As shown in Figure 4a, treatment with sulforaphene and cisplatin slightly reduced the fluorescence intensity, showing a slight decrease in mitochondrial membrane potential. However, the fluorescence intensity of the treated SKOV3 and SNU 8 cells was more greatly reduced with combination treatment in both SKOV 3 and SNU 8 cells.

The depolarization of mitochondrial membrane was confirmed by flow cytometry. The histogram in Figure 4b shows that both sulforaphene and cisplatin slightly reduced the mitochondrial membrane potential. Loss of Ψm was observed approximately in 18% and 31% of sulforaphene-treated cells respectively. Approximately 48% and 50% cells had lower Ψm after cisplatin treatment in SKOV 3 and SNU 8 cells respectively. However, the mitochondrial membrane

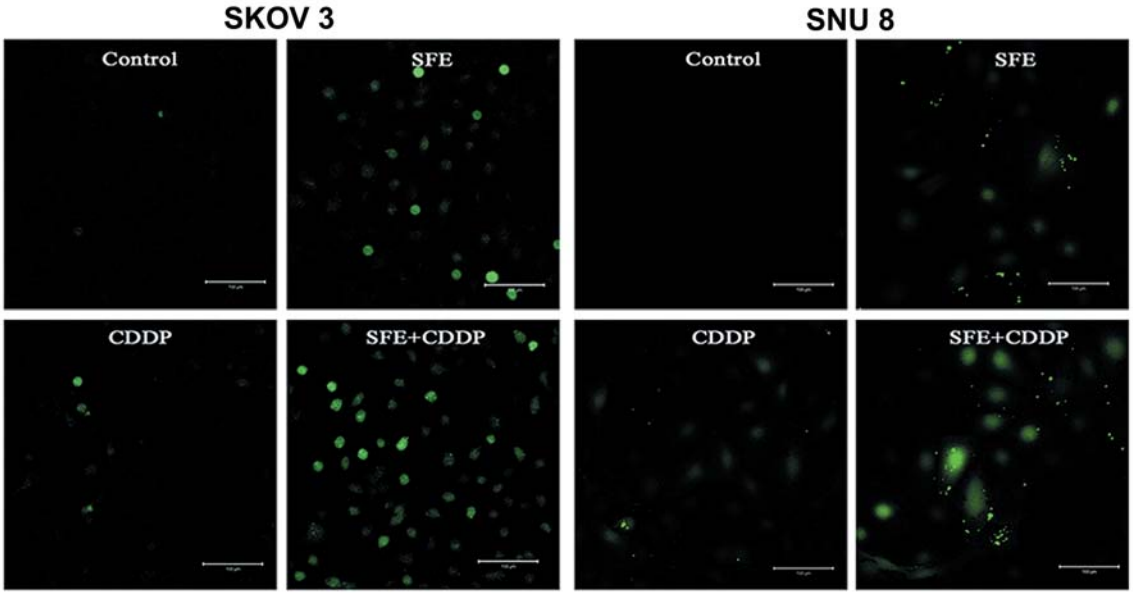

Figure 3. *Confocal microscopic study of the generation of reactive oxygen species (ROS). Cells were stained with 2',7'-dichlorodihydrofluorescein diacetate to study the generation of ROS. Combination of sulforaphene and cisplatin led to higher ROS generation compared to the treatments with CDDP and SFE alone.*

potential was reduced to 68% and 70%, respectively, after combination treatment of SKOV 3 and SNU 8 cells.

Combination treatment resulted in a higher number of sub-G_1 cells in cell-cycle analysis. From the flow cytometric data (Figure 5), the percentage of cells in the sub-G_1 region in control groups was 3.9±0.23% and 5.2±0.36% for SKOV 3 and SNU 8 cells, respectively. After sulforaphene and cisplatin treatment, the percentage of apoptotic cells in sub-G_1 region increased to 19.1±0.30% and 23.7±0.42% for SKOV 3 and 25.6±0.45% and 37.8±0.37% for SNU 8 cells, respectively. However, this percentage further increased significantly to 47.2±0.36% for SKOV 3 cells and 53±0.40% for SNU 8 cells with combination treatment. Statistical analysis further indicated that number of apoptotic cells in the sub-G_1 phase was significantly ($p<0.001$) higher in the combination group compared to the individual treatments.

Caspases were expressed at higher levels with sulforaphene and cisplatin combination. From Figure 6, it can be seen that expression of cytochrome *c* was increased with combination treatment of SKOV 3 and SNU 8 cells. Expression of the downstream proteins caspases 3 and 9 in the mitochondrial pathway of apoptosis were increased with sulforaphene and cisplatin treatment after 24 h. But this activation of caspase 3 and 9 was found to be more greatly enhanced with combination treatment. Moreover, expression of external pathway protein caspase 8 was also up-regulated with combination treatment.

Expression of PI3K was reduced and PTEN was activated with the combination treatment. Both sulforaphene and cisplatin lowered the expression of PI3K in SKOV3 and SNU 8 cells after 24 h of treatment (Figure 6). Expression of PI3K was distinctly lowered after the cells were treated with cisplatin in combination with sulforaphene. In addition to this, expression of PTEN in sulforaphene and cisplatin-treated groups were slightly increased in both cell lines. However, the expression of PTEN in the combination-treated group was more activated than by their respective individual treatments.

Discussion

The aim of combination treatment is to achieve synergistic therapeutic efficacy with a lower drug dose so as to reduce adverse effects and minimize the induction of drug resistance. Ovarian carcinoma often exhibits resistance to platinum-based drugs, limiting their efficacy. Activation of phosphatidylinositol-3-kinase (PI3K) and suppression of phosphatase and tensin homolog (PTEN) may play an important role in acquiring drug resistance and tumor progression in ovarian cancer. Therefore, targeting PI3K/PTEN pathways may be a promising strategy to circumvent resistance to platinum-based chemotherapy (18, 19). On the other hand, natural agents usually have no or less adverse effects and hence using natural bioactive compounds with chemotherapy may be one strategy to overcome drug resistance with minimum side-effects (20-24).

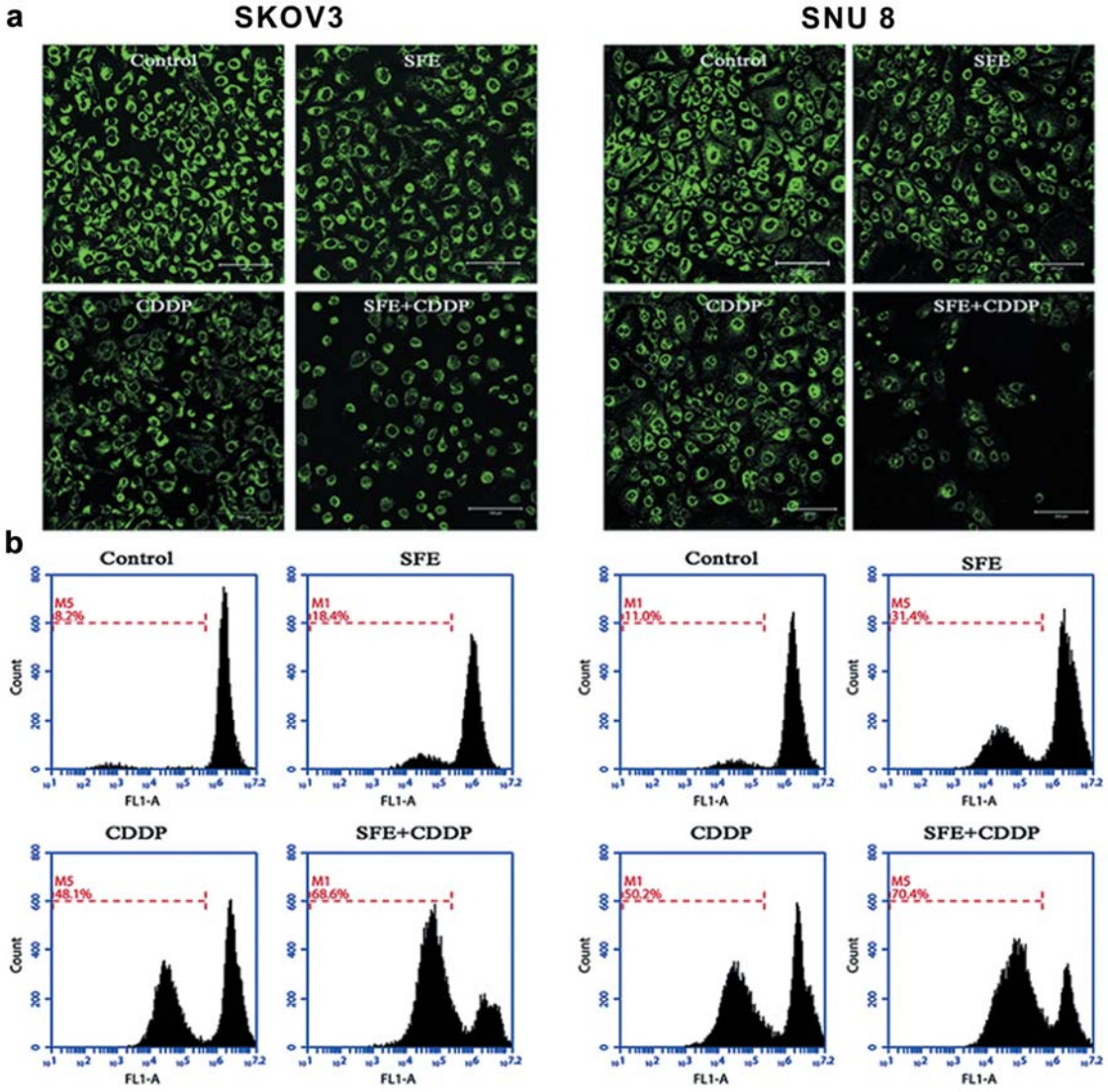

Figure 4. *a: Confocal microscopic study of change in mitochondrial membrane potential (ΔΨm). Cell fluorescence was reduced in both sulforaphene- and cisplatin-treated cells. Reduced green fluorescence i.e. depolarization of mitochondrial membranes, was observed more with the combination treatment. b: Flow cytometric analysis of ΔΨm. Both SFE and CDDP treatment led to slight depolarization of mitochondrial membrane. The combination treatment led to greater mitochondrial dysfunction in cells.*

Isothiocyanates play a very promising role in combating various types of cancers (25, 26). The main objective of the present study was to analyze the role of the isothiocyanates in enhancing the chemotherapeutic efficacy of cisplatin in ovarian cancer. Our study suggests that sulforaphene can synergistically enhance the efficacy of cisplatin in human ovarian cancer cells SKOV 3 and SNU 8. This combination treatment led to a higher number of apoptotic cells with enhanced ROS that leads to dysfunction of mitochondrial membrane potential and release of cytochrome *c* into the cytosol. Thus, the mitochondrial pathway of apoptosis was activated by activating downstream proteins caspase 3 and 9. The external pathway of apoptosis *via* caspase 8 was also activated by combination treatment. Furthermore, cisplatin treatment with sulforaphene reduced PI3K activity in SKOV 3 and SNU 8 cells. PTEN, which negatively regulates PI3K activity, was increased with combination treatment. Therefore, different signaling pathways were modulated in this combination treatment. Firstly, the mitochondrial pathway of apoptosis was activated *via* caspases 3 and 9. Secondly, the external pathway was activated *via* caspase 8 that potentiated the apoptotic efficacy of combination treatment. Finally, PI3K was suppressed and PTEN was activated with combination treatment that contributed in cellular growth inhibition.

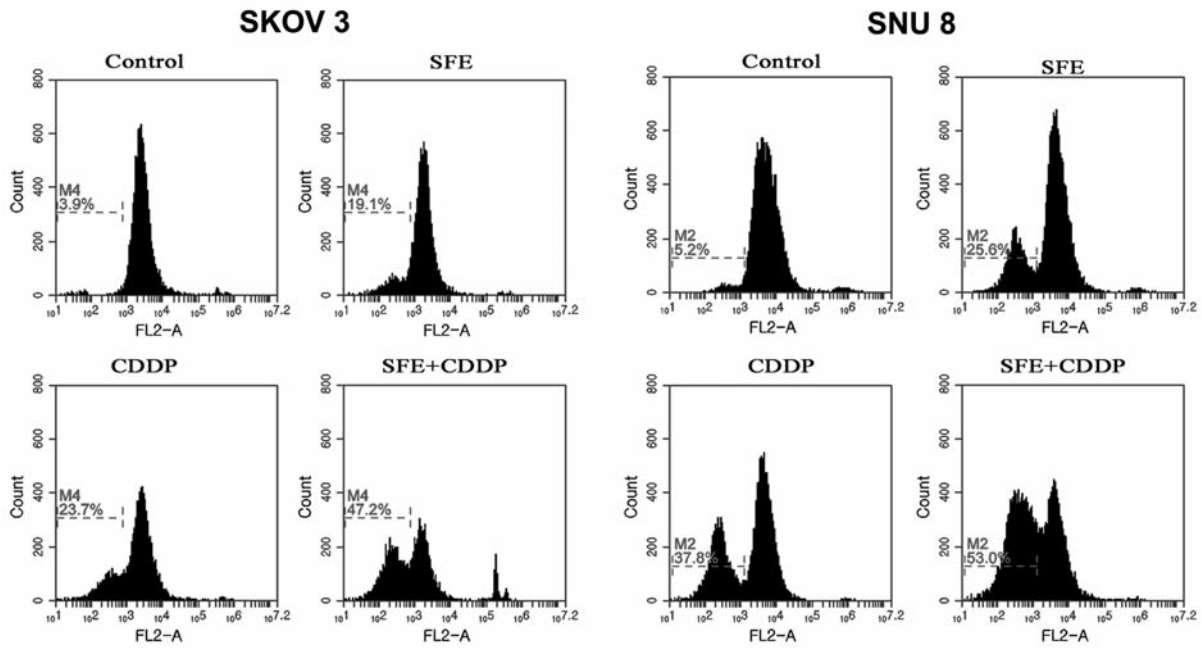

Figure 5. *Analysis of apoptotic cell distribution by flow cytometry. Sulforaphene and cisplatin treatment increased the apoptotic cells in the sub-G_1 region. The number of apoptotic cells in the combination group was increased, compared to those of the individual treatments with CDDP and SFE.*

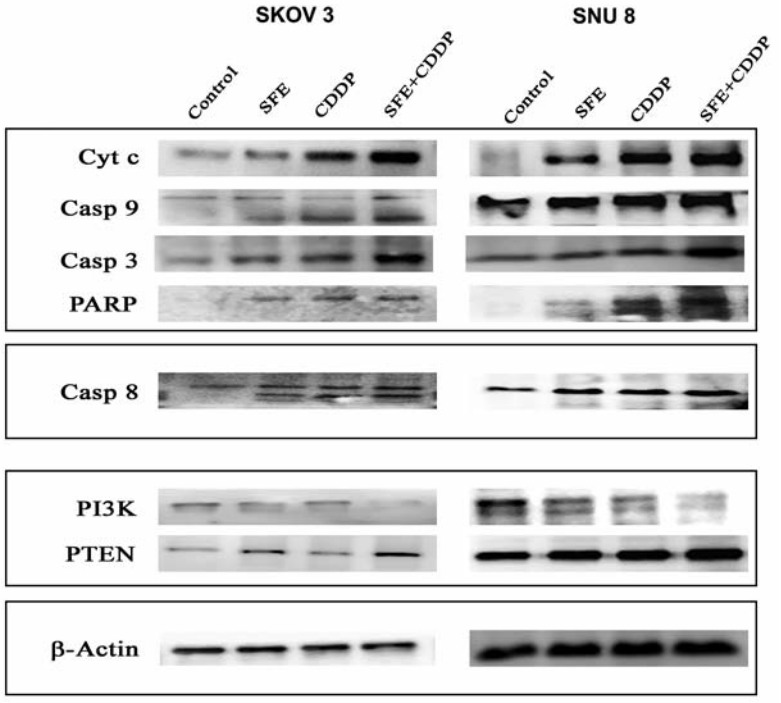

Figure 6. *Western blot analysis of apoptotic proteins in SKOV3 and SNU 8 cells. Activity of mitochondrial pathway-related proteins cytochrome c, caspase 3, and caspase 9 were up-regulated in combination treatment. Caspase 8 was also activated more in the combination group. The expression of phosphoinositide 3-kinase (PI3K) was inhibited in the SFE- and CDDP-treated groups, but PI3K was inhibited more with combination treatment. The expression of phosphatase and tensin homolog (PTEN) also was increased more with combination treatment.*

In conclusion, we suggest that sulforaphene potentiates cisplatin efficacy by simultaneous activation of mitochondrial and external pathway of apoptosis. It also inhibits cell proliferation by modulating PI3K and activates PTEN when used in combination with cisplatin. This simultaneous activation of several apoptotic pathways and inhibition of cellular growth may synergistically potentiate cisplatin activity. Therefore, sulforaphene might be a useful chemo-enhancing adjuvant for the treatment of human ovarian cancer.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Acknowledgements

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