

## Review Article

## Sulforaphane Induces Cell Cycle Arrest, Migration, Invasion, and Apoptosis in Epithelial Ovarian Cancer Cells

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### Abstract

**Objectives:** Isothiocyanates (ITC) has long been shown to demonstrate chemopreventive properties. Sulforaphane (SFN) is a major ITC present in broccoli and other cruciferous vegetables. We reviewed the current literatures of SFN on ovarian carcinoma cell lines.

**Methods:** Studies were conducted on the effects of SFN on the growth of the OVCAR-3, MDAH 2774 and SKOV-3 ovarian carcinoma cell lines. Chuang *et al.* evaluated the effect of SFN on ovarian cancer cell cycles. Subsequently Chaudhuri *et al.* determined the specific pathway that was affected and Bryant *et al.* explored the signaling mechanisms through which SFN influences the cell growth and proliferation in ovarian cancer cell lines.

**Results:** Chuang *et al.* showed a concentration dependent decrease in cell density. Analysis of cell cycle phase progression revealed a decrease in the cell populations in S and G2M phases, with an increase of G1 cell population, indicating a G1 cell cycle arrest. The degree of decrease in the replicating population was concentration and time dependent. These results clearly demonstrated an effect of SFN in inducing growth arrest and apoptosis in ovarian carcinoma cell lines. Chaudhuri *et al.* investigated the effects of sulforaphane on Akt signal transduction pathway. Both total Akt protein and active phosphorylated levels of Akt and phosphoinositide 3-kinase were significantly decreased in sulforaphane-treated ovarian cancer cell lines. Utilizing gene expression profile analysis, Bryant *et al.* showed SFN treatment resulted in G1 cell cycle arrest through down modulation of RB phosphorylation and by protecting the RB-E2F-1 complex.

**Conclusions:** SFN induced growth arrest and cell death in ovarian cancer cells in G1 cell cycle arrest. The Akt pathway was identified as the possible target for SFN. SFN suppresses growth of ovarian cancer cells in vitro by modulating cell cycle regulatory proteins and by enhancing apoptosis. Inhibition of retinoblastoma (RB) phosphorylation and reduction in levels of free E2F-1 appear to play an important role in ovarian cancer growth arrest, migration, and invasion.

**Keywords:** Sulforaphane; apoptosis; epithelial ovarian cancers; Paclitaxel

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Ovarian cancer is the most common cause of death from gynecologic cancers in the US. In 2013, 22,240 new cases will be diagnosed, and 14,030 women will die of ovarian cancer[1]. Ovarian cancer accounts for 5% of all cancer deaths in women in the United States. The etiology of ovarian cancer has not been clearly identified. Endocrine, genetic, and environmental factors are implicated in ovarian cancer carcinogenesis. Ovarian cancer can originate from epithelial, germ or stromal cells in the ovaries; epithelial ovarian cancer (EOC) is the commonest histologic type and represents 90% of ovarian cancer [2]. EOC can be categorized into four major subtypes, including serous, mucinous, endometrioid, and clear cell carcinomas. The serosal surface of ovary has long been regarded as the cell of origin for EOCs. It has its highest incidence in industrialized Western and Asian countries. In previous studies, a diet high in animal fat has been reported to be associated with an increased risk of ovarian cancer[2]. However, other case-control studies failed to show that fat, protein, fiber, vitamins A and C elevated the risk of ovarian cancer [3]. In prevention of the development of ovarian cancers, factors that induce anovulation, including oral contraceptives, pregnancy, and breast feeding, are associated with a reduced risk of ovarian cancer [4]. A recent study suggested that fenretinide, a synthetic retinoid, might protect women from development of ovarian cancers. Additionally, synthetic retinoids, such as CD437 and AHPN, were found to inhibit the growth of ovarian carcinoma via retinoic acid receptors [5]. However, cancer cells have often showed resistance to retinoids. Non-steroid anti-inflammatory agents, such as acetylsalicylic acid (ASA), acetaminophen and COX-2 inhibitor (NS-398) were among those tested using OVCAR, SK-OV-3, and CAOV-3 cell lines. Recently, the focus has been on cancer prevention by natural constituents from cruciferous vegetables. The intake of cruciferous vegetables, such as cabbage, cauliflowers, broccoli, kale and brussels sprouts, has been shown to reduce the risk of lung, pancreas,

prostate, colon, thyroid, bladder, skin and stomach cancers in rodent models[6]. Isothiocyanates (ITC) has long been shown to demonstrate chemopreventive properties. Sulforaphane (SFN) is a major ITC present in broccoli and other cruciferous vegetables. We conducted a review on the current literatures studying the effect of Sulforaphane (SFN) on ovarian cancer cell lines. The cell lines that were studied included OVCAR (high resistant to cisplatin), SK-OV-3 (Akt over-expressing estrogen ovarian cancer cell line), and CAOV-3 cell lines (ovarian mucinous adenocarcinoma cancer cell line) [7]. Other cell lines tested in studies included A2780 (cisplatin resistant cell line), MDAH 2774 (ovarian endometrioid adenocarcinoma cell line), C3, and T3 (mouse ovarian cancer cell line) cell lines.

### **Sulforaphane induces growth arrest and apoptosis in human ovarian cancer cells**

The protective effects of these cruciferous vegetables against cancer are thought to be at least partially due to their high content of glucosinolates[8]. Isothiocyanates (ITCs) are among the hydrolysis products of the glucosinolates. Myrosinase, an enzyme found in plants, in a separate compartment from glucosinolates, catalyses the hydrolysis of glucosinolates. When the plant cells are cut or chewed, myrosinase comes in contact with the glucosinolates, and hydrolysis takes place.

Sulforaphane (1-isothiocyanate-4-methyl-sulfonyl butane) (SFN) is an ITC that broccoli is rich in. The chemopreventive properties of SFN are thought to involve the inhibition of phase 1 enzymes, such as P450s, and the induction of phase 2 detoxifying enzymes, hence, facilitating carcinogen removal[9]. An increasing body of evidence has indicated that these dietary factors are not only chemopreventive, but also capable of regulating the growth of

malignant cells, and thereby inhibiting the progression of carcinogenesis[10]. Chiao *et al.* were the first to report that SFN is a strong mediator of growth arrest and apoptosis in prostate cancer cells [8]. SFN has since been demonstrated to induce apoptosis and growth arrest in other cancer cells [10, 11]. Tang and Zhang demonstrated that ITCs induce apoptosis by damaging the mitochondrial membrane in the transformed cell, resulting in the release of cytochrome C and caspase-9 activation [11]. In addition, modulation of the bcl-2 family proteins also contributes to mitochondrial membranes damage. To determine if SFN and its major metabolite is effective against ovarian cancer cells, the effects of SFN in ovarian cancer cell lines, OVCAR-3 and SKOV-3 were studied[12]. Sulforaphane (SFN) was cultured with OVCAR-3 and SKOV-3 cell lines at a concentration of  $1 \times 10^6$

cells/ml. Apoptotic cells were determined by their characteristic morphology, and by the presence of DNA strand breaks with terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labelling (TUNEL). Analysis of cell cycle phases was performed using a Becton-Dickinson FACScan flow cytometer.

Chuang *et al.* found a significant reduction in the OVCAR-3 and SKOV-3 cell density after exposure to SFN for 2 days. Figure 1 shows that in contrast to the control culture without SFN, which increased from  $1 \times 10^6$  to  $1.8 \times 10^6$  ml, the OVCAR-3 cells exposed to  $2 \mu\text{MOL/L}$  SFN decreased to  $0.82 \times 10^6 \pm 0.01$ , approximately 50% of the control. Similarly, the number of cells in cultures treated with 10 and  $50 \mu\text{mol/l}$  SFN showed  $58 \pm 2$  and  $66 \pm 1.1\%$  decrease in cell densities compared to control cultures ( $p < 0.0001$ ).

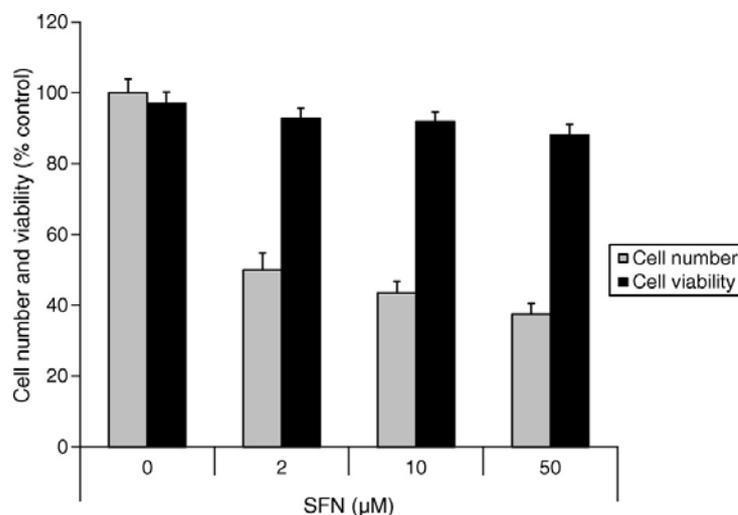


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The inhibition of proliferation of SFN on ovarian cancer cell lines analyzed showed 60% reduction in cells in the S-phase. Concomitantly, an accumulation of cells in G1-phase was observed,

indicating that the transition from G1- to S-phase was inhibited. The TUNEL method was used to determine the presence of apoptosis. The presence of apoptotic cells could be detected within 4 h of

SFN exposure. Figure 2(A) demonstrates that the effect of SFN is concentration dependent. Two-day exposure of OVCAR-3 cells to 2, 10 and 50  $\mu\text{mol/l}$  SFN in suspension cultures showed that  $6.5\pm 0.5$ ,  $8.7\pm 0.9$  and  $17.4\pm 1.8\%$ , respectively, of the cells were apoptotic ( $p<0.0001$ ). When the OVCAR-3 cells were incubated with 10  $\mu\text{mol/l}$  SFN for various time periods, the percentage of apoptotic cells

increased with incubation time. In this regard, Figure 2(B) shows that after 2- and 3-day exposure to 10  $\mu\text{mol/l}$  SFN, approximately  $18.7\pm 2$  and  $42.5\pm 5\%$  of the cell population were apoptotic, respectively ( $p<0.0001$ ). These results indicate that an increased quantity of apoptotic cells was detected after a longer culture period with SFN at lower dosages.

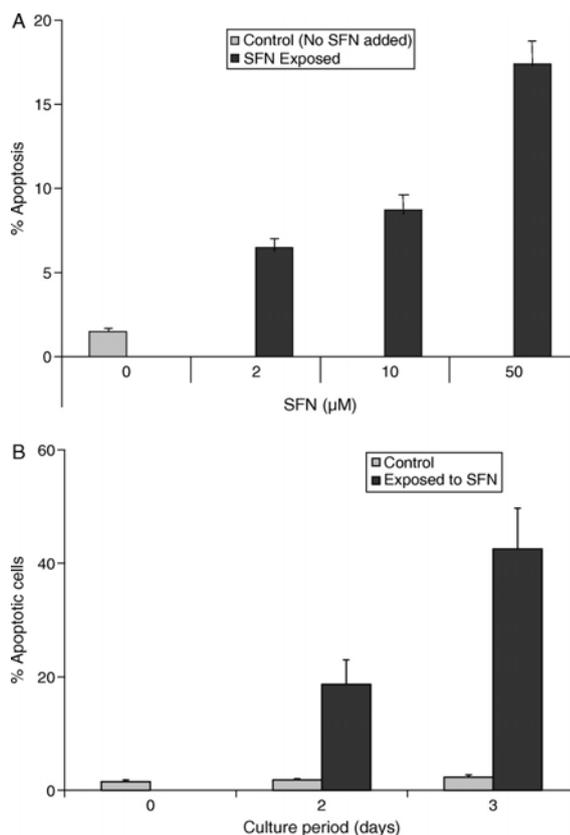


Figure 2 (A) The effect of SFN is concentration dependent. Two-day exposure of OVCAR-3 cells to 2, 10 and 50  $\mu\text{MOL/L}$  SFN in suspension cultures showed that  $6.5\pm 0.5$ ,  $8.7\pm 0.9$  and  $17.4\pm 1.8\%$ , respectively, of the cells were apoptotic ( $p<0.0001$ ). (B) After 2- and 3-day exposure to 10  $\mu\text{MOL/L}$  SFN, approximately  $18.7\pm 2$  and  $42.5\pm 5\%$  of the cell population were apoptotic, respectively ( $p<0.0001$ ).

The results of this study demonstrated that SFN inhibited two different ovarian cancer cell lines, OVCAR-3 and Akt-overexpressing SKOV-3 cell lines. There was a concentration dependent decrease in ovarian cancer cell density. On the analysis of cell cycle phase progression, the decrease was in the cell populations in S and G2M phases, with an increase of G1 cell population. This demonstrated a G1 cell cycle arrest. The degree of decrease in the

replicating population correlated to the concentration and time of incubations with SFN. As much as 17% of apoptosis was observed on OVCAR-3 cells with 50  $\mu\text{mol/l}$  of SFN. In addition, when OVCAR-3 cells were exposed to SFN for various time periods, the percentage of cells undergoing apoptosis was directly proportional to the incubation period. In this regard, while 18% of the cells underwent apoptosis after 2 days, 42% of

the cells showed apoptosis after 3 days of incubation.

In mammalian cells, it has been reported that a concentration of 20  $\mu\text{mol/l}$  of SFN was measured in the urine samples of human ingested broccoli [13]. This result indicated that SFN is bio-available. It has been estimated that approximately 20  $\mu\text{mol/l}$  SFN can be derived from consumption of 50 g of broccoli. This level exceeds the concentrations of SFN utilized in this study. The concentrations of SFN used may be utilized in vivo assays [14].

In this paper, Chuang *et al.* presented the first report of the effects of SFN on two different human ovarian cancer cell lines, OVCAR-3 and SKOV-3. They suggested that SFN might be one of the most popular chemopreventive agent derived from natural sources in ovarian cancers.

### **Antiproliferative activity of sulforaphane in Akt-overexpressing ovarian cancer cells**

The etiology of epithelial ovarian cancer is thought to be a result of the neoplastic transformation of the ovarian epithelium or the ampullary portion of the fallopian tubes. Overexpression of proto-oncogenes and signaling pathways such as ras are mutated, and Akt, epidermal growth factor receptor, platelet-derived growth factor/vascular endothelial growth factor receptor, and vascular endothelial growth factor are seen in ovarian cancer [15]. Signal transduction pathways are major target for treatment in ovarian cancer, and the serine-threonine kinase Akt has generated great interest. Akt has wide-ranging receptors and is activated by phosphatidylinositol-3,4,5,-triphosphates, which are generated by phosphoinositide 3-kinase (PI3K) and mediates downstream events regulated by PI3K [16-20]. Akt phosphorylates many different molecules that are important in the cell cycle, glycogen synthesis, cell death, and survival. Components of the PI3K/Akt pathway are

frequently overexpressed in ovarian cancer and are thought to play major roles in ovarian cancer carcinogenesis [21]. The overexpression of the Akt pathway is frequently associated with poor prognosis and more aggressive phenotype in patients with ovarian cancers [22]. Consequently, agents that target on Akt pathway and the downstream molecules may mitigate the adverse oncogenic effects of Akt.

Chaudhuri *et al.* examined the effects of sulforaphane on SKOV3, a human ovarian cancer cell line that is highly resistant to cytotoxic agents, such as cisplatin, adriamycin, and the tumor necrosis factor [23]. The effects of sulforaphane on mouse ovarian cancer cell lines C3 and T3, which were engineered to constitutively overexpress activated Akt were studied. Antiproliferative effects of SFN were studied as a function of time and dose. After treating the cell lines with 1, 5, 10, 20, and 40  $\mu\text{mol/L}$  of SFN, 0.025% Coomassie brilliant blue R250 (in 50% methanol and 10% acetic acid) was used for the clonogenic assay. The steady-state levels of cellular proteins were evaluated by Western Bolt Analysis using the antibodies including cuclin D1, cyclin-dependent kinase 4 (cdk4), cdk6, Akt, pAkt, PI3K p85 subunit, and actin. The Akt kinase assay was done using the nonradioactive Akt kinase assay kit in SKOV3 and C3 cell extracts  $\pm$  SFN. ELISA kit was used to detect the apoptosis induced by SFN on the cell lines. Finally the changes in cell morphology were evaluated using the inverted Zeiss Axiovert 200 microscope using Axiovision 4.3 software. Chaudhuri showed sulforaphane down-regulates PI3K, Akt, and phosphorylated Akt (pAkt). The consequent loss in the kinase activity leads to potent antiproliferative effects and induction of apoptosis in ovarian cancer cell lines.

In this study, antiproliferative effects of sulforaphane were tested on SKOV3, C3, and T3 cell lines by the XTT assay. Cell survival decreased with increasing doses of sulforaphane, and near total loss of cell viability was observed at 100  $\mu\text{mol/L}$  sulforaphane (Figure 3). The IC50 was determined

to be 40  $\mu\text{mol/L}$  for SKOV3 cells and 25  $\mu\text{mol/L}$  for C3 and T3 cell. The results show sulforaphane as a potent antiproliferative agent against ovarian cancer cells. As Akt is constitutively expressed in SKOV3, C3, and T3 cell lines, the treatment of SKOV3, C3, and T3 cells with sulforaphane resulted in down-regulation in the steady-state levels of total Akt protein as well as the active pAkt (Ser473) at 24 h (Figure 4). A dose-dependent down-regulation of PI3K was also observed in all three cell lines upon treatment with sulforaphane at 24 h. A decrease in total Akt protein levels was observed at 40  $\mu\text{mol/L}$  sulforaphane, with no significant changes observed in PI3K levels in C3 and T3 cell lines. In the case of SKOV3 cells, 12-h treatment of sulforaphane led to a decrease in pAkt levels, but no effects were observed on total Akt and PI3K levels. The manifestation of this activity occurs between 12 and 24 h of sulforaphane treatment, with significant changes in protein levels only at 24 h. The results show that sulforaphane inhibits the PI3K/Akt pathway by down-regulating the key enzymes in this

pathway, indicating the Akt pathway as a possible target of sulforaphane. A significant reduction in the kinase activity of Akt was observed in SKOV3 and C3 cells treated for 24 h with 10, 20, and 40  $\mu\text{mol/L}$  sulforaphane. Their results show that sulforaphane inhibits the kinase activity of Akt and may account for the antiproliferative effects of sulforaphane in ovarian cancer. Sulforaphane affects critical proteins involved in the G1-S transition of the cell cycle. SKOV3, C3, and T3 cells were treated with 10, 20, and 40  $\mu\text{mol/L}$  sulforaphane for 12 and 24 h, and the whole-cell lysates were used to detect the steady-state levels of cyclin D1, cdk4, and cdk6. A down-regulation of cyclin D1, cdk4, and cdk6 levels was detected with increasing concentration of sulforaphane for 24 h in all three cell lines, leading to a possible cell cycle arrest and decrease in proliferation (Figure 5). This corroborates the antiproliferative effects of sulforaphane on three cell lines, which is manifested by the induction of programmed cell death (Figure 6).

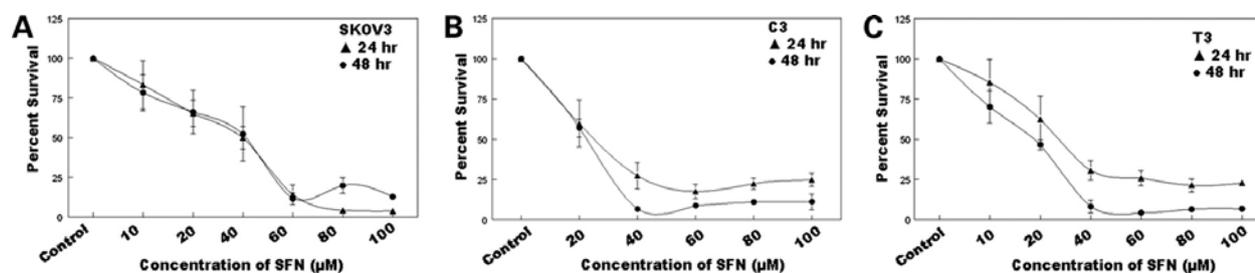


Figure 3 Dose- and time-dependent antiproliferative effect of sulforaphane (SFN) on SKOV3 (A), C3 (B), and T3 (C) cells by the XTT assay. Cells were treated with varying concentrations (10, 20, 40, 60, 80, and 100  $\mu\text{mol/L}$ ) of sulforaphane were added to each well for 24 h ( $\blacktriangle$ ) and 48 h ( $\bullet$ ). Percentage cell survival was determined by XTT assay and plotted as a function of time and dose of sulforaphane.

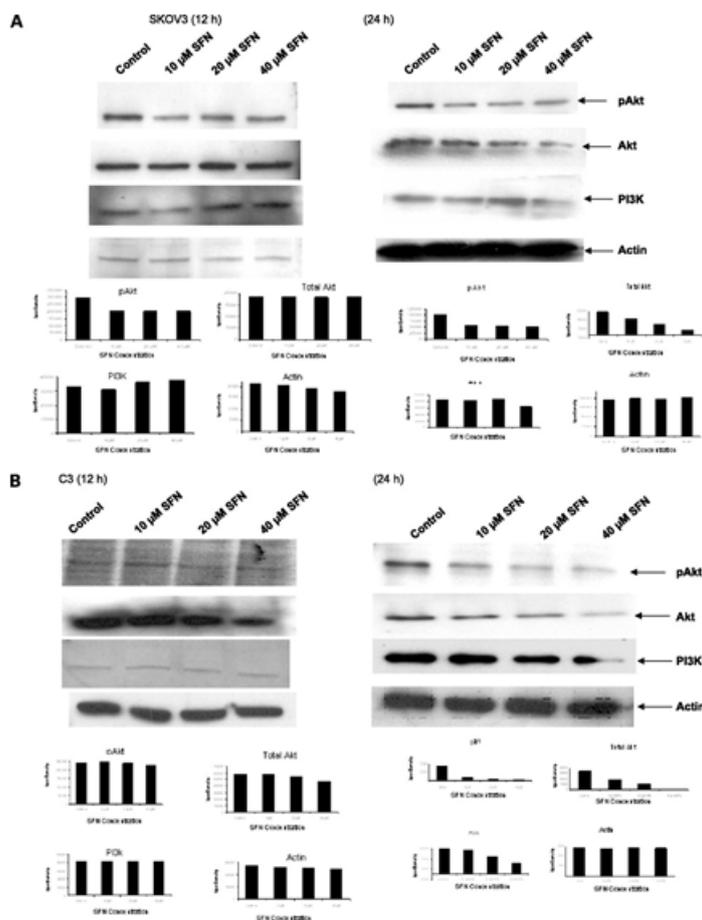


Figure 4 Effect of sulforaphane on the PI3K/Akt pathway. SKOV3 (A), and C3 (B) cells were either untreated or treated with 10, 20, and 40 μmol/L sulforaphane for 12 and 24 h, and the steady-state protein levels of Akt, pAkt (Ser473), and PI3K were determined by Western blot analysis. β-Actin was used as loading control. Representative of three independent experiments

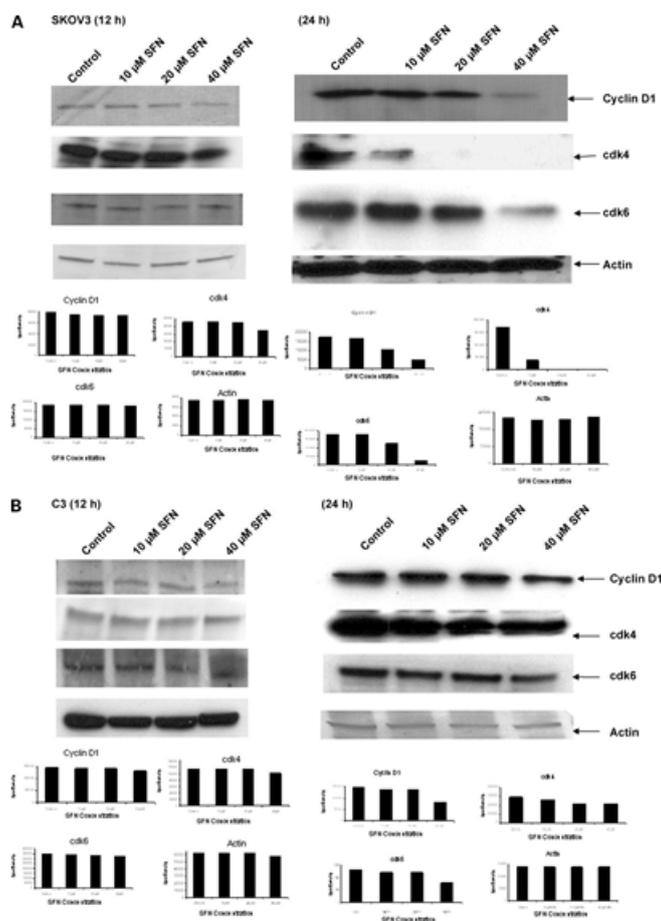


Figure 5 Effect of sulforaphane on steady-state levels of cell cycle proteins. SKOV3 (A), and C3 (B) cells were either untreated or treated with 10, 20, and 40 μmol/L sulforaphane for 12 and 24 h, and the whole-cell lysates were used for Western blot analysis of G1-S cell cycle transition markers, cyclin D1, cdk4, and cdk6. β-Actin was used as loading control. Representative of three independent experiments

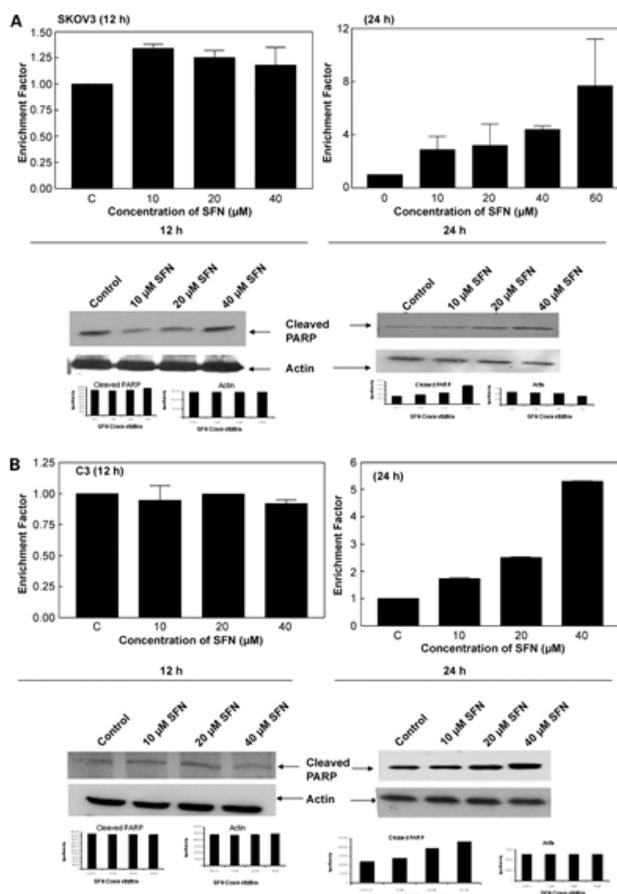


Figure 6 Induction of apoptosis by sulforaphane in SKOV3 (A), and C3 (B) cells. Cell death ELISA was done and expressed as enrichment factor compared with untreated control (*top*). Columns, mean in treated cells ( $n = 3$ ) of three experiments; bars, SD. Induction of apoptosis was also measured by cleavage of poly(ADP)ribose polymerase (PARP) using a specific antibody (*bottom*).

Based on this study, the authors demonstrated that sulforaphane targets and inhibits the Akt pathway with a subsequent decreased functional kinase activity of Akt, thereby demonstrating its anticancer effects. The pAkt levels are down-regulated at 12 hours in all the cells studied with minimal changes in total Akt and no changes in PI3K with 12 hours of sulforaphane treatment. A more pronounced

down-regulation in pAkt, total Akt, and PI3K is observed only at 24 hours of treatment. This indicates that there is an early effect of sulforaphane on the phosphorylated form of Akt with a more pronounced suppressive effect at a later point on the pathway. These results are significant as Akt is constitutively expressed and active in SKOV3 ovarian cancer cells, and C3 and T3 overexpressed

Akt because they were generated by the overactivation of the Akt oncogene. The significant finding is that the Akt pathway was identified as a target of sulforaphane in ovarian cancer. The PI3K/Akt pathway is considered for chemotherapy resistance in ovarian cancer. Mutations in the regulatory subunit of PI3K and the tumor-suppressor gene PTEN, a phosphoprotein/phospholipid phosphatase and negative regulator of Akt, have been observed in various cancers, including ovarian cancer. However, this study showed a down-regulation in the Akt levels along with a decrease in the functional kinase activity, thus identified Akt as an ideal target of sulforaphane.

### **Sulforaphane induces cell cycle arrest by protecting RB-E2F-1 complex in epithelial ovarian cancer cells**

As SFN inhibits epithelial ovarian cancer cell (EOC) line SkOV-3 by down-regulating AKT activity [23]. Similar compounds such as synthetic isothiocyanate derivative ethyl 4-isothiocyanatobutanoate (E-4IB) and phenylisothiocyanates have been shown to induce cell death in A2780 and OVCAR-3 EOC cell lines [24]. Indole-3-ethyl isothiocyanate (NB7 M) has also shown cytotoxic activity in SkOV-3 by the inhibition of PIK3/AKT pathway [25]. The inhibition of PIK-3 and AKT pathways was reported in the earlier studies by the upstream events at the cell surface, various downstream signaling pathways

responsible for SFN activity are not well studied. It is known retinoblastoma protein (RB) regulates G1-S phase cell cycle transition [26]. Negative regulation of the cell cycle is secondary to the ability of active, under phosphorylated RB to bind the transcription factor E2F-1 and repress transcription required for S phase progression [27]. In the study by Bryant *et al.*, the authors conducted cell proliferation assays using two human ovarian cancer cell lines, MDAH-2774 and SkOV-3. Cells were grown as sub-confluent monolayer cultures and propagated under standard conditions. Cell lines were treated with serial dilutions of SFN dissolved in DMSO. Human fibroblasts were similarly treated to display differential cytotoxicity (data not shown). SFN treatment resulted in a concentration-dependent inhibition of the proliferation of MDAH 2774 with an IC<sub>50</sub> of ~8 µmol/L (Figure 7). The extent of growth inhibition increased with longer interval of treatments and higher concentrations ranging from 5 to 20 µmol/L. The extent of cell death with SFN increased as a function of dosage in 48 h as observed by phase contract microscopy in MDAH-2774 cell line. Current chemotherapy treatment of ovarian cancer includes paclitaxel with platinum based regimens, the authors evaluated the impact of the combination of SFN and paclitaxel in treating MDAH-2774 cell line. Cells treated with paclitaxel with or without SFN and viability was assessed for 72 hours. As shown in their study, 8 µmol/L SFN which is IC<sub>50</sub> for MDAH-2774 or 2 µmol/L paclitaxel resulted in ~40% and ~55% cell death, respectively; the combination treatment resulted in increased cell death of ~70%.

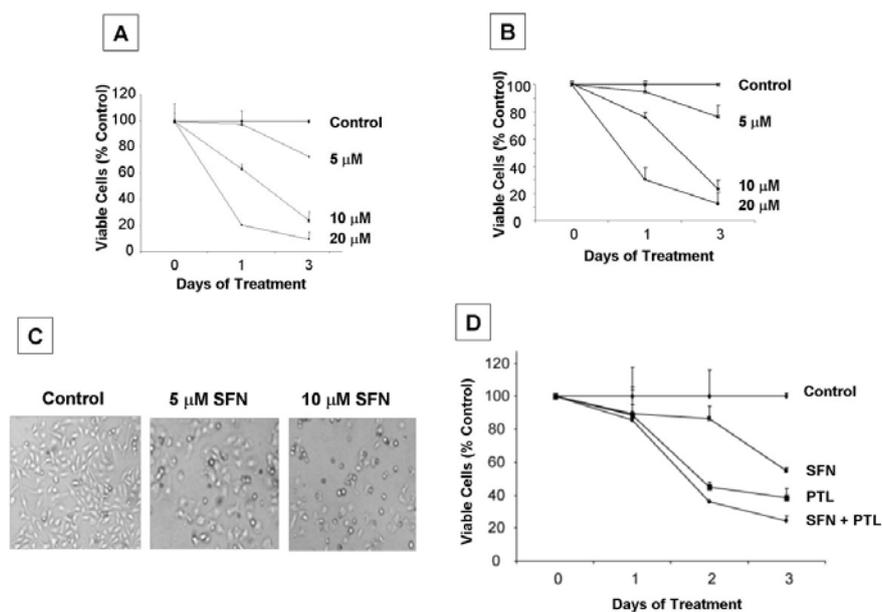


Figure 7 Cells were cultured for indicated time intervals with various concentrations of the SFN. Cell growth was assessed by CCK-8 cell proliferation assay method. A and B are MDAH-2774 and SkOV-3 cell lines respectively. Graph is expressed as a percentage of control (DMSO treated cells) and represents the mean of triplicate cultures. C. Phase contrast pictures taken at 100 $\times$  magnification after indicated concentrations of drug treatment of MDAH-2774 cells. D. Paclitaxel mediated cytotoxicity with or without SFN on MDAH-2774 cells.

Inhibition of cell cycle progression in tumor cells may be associated with a concomitant activation of apoptosis. Cell deaths subsequently induces changes on the cell surface, which results in translocation of phosphatidylserine (PS) from the inner layer of the plasma membrane to the outer layer [28]. Annexin V is a Ca<sup>2+</sup> -dependent protein with high affinity for PS that can be used to assess apoptotic cells. The quantification of apoptotic changes was evaluated by flow-cytometry analysis after treatment with SFN. Control and treated cells were prepared for bivariate analysis using annexin V stain that detects cells undergoing apoptosis and propidium iodide (PI) to detect nonviable cells. The number of pro-apoptotic

cells after 48 h of 10  $\mu$ mol/L SFN treatment was significantly higher as compared with control. It was noted that the ratio of apoptotic cells increased with the increased dose of SFN treatment: approximately 8% (5  $\mu$ mol/L treatment) and 20% (10  $\mu$ mol/L treatment) of the cells over 48-hour period. Further analysis was carried out by immunofluorescence staining with Annexin V for apoptosis after SFN treatment. After 48 hr exposure to 10  $\mu$ mol/L SFN, 60% of the cells stained positive for annexin V, whereas untreated were less than 5% annexin V positive.

The causes of the growth inhibition by SFN were

determined by cell proliferation assays. Serum starved MDAH-2774 cells were treated with either control or 10  $\mu\text{mol/L}$  SFN for 12 or 24 hrs. The cells were then washed, fixed, and cell cycle phase determination was performed utilizing flow cytometry and a cell cycle phase determination kit. SFN was found to induce G1 arrest in a time-dependent manner as noted in the two previous studies [12, 23]. There is a non-significant increase of S phase cells after 12 hrs (0.7%) and 24 hrs (2.7%) with SFN treatment compared with 40% of cells in S phase for control cells at 24 hrs. There was a substantial increase in the cells at G1 phase of the cell cycle after treatment with SFN.

The genes that influence the cell cycle progression into S phase were studied. Tumor suppressor retinoblastoma proteins (RB, p107 and p130) sequesters cell cycle promoting E2F-1 transcription factors [29]. Gene expression analysis of RB proteins revealed a 1.5 and 2.0 fold decrease of RB and p130, respectively; as well as, a 2 folds increase in p107 levels. The E2F family of proteins, E2F-1, 2 and 3 which interact with RB, demonstrated 1.0 and 1.5 fold decrease in the expression levels of E2F-1 and 2, respectively, and 1.0 x increase in the levels of E2F-3. Cyclins and

cyclin dependent kinases (CDKs) and their inhibitors exhibit distinct expression patterns, which contribute to the temporal coordination of each event in cell cycle progression. SFN treatment resulted in the lower expression of G1 phase cyclins and CDKs while increasing the expression of cyclin dependent kinase inhibitors (CKIs), which bind and inhibit the activity of cyclin/Cdk complexes and negatively regulate cell cycle progression.

The progressive conversion of phosphorylated RB to non-phosphorylated RB after 48 hrs when MDAH-2774 cells were treated with 5 to 15  $\mu\text{mol/L}$  SFN treatment was reported (Fig 8). It is known that E2F-1 is elevated in many tumor cell lines and is responsible for cycle progression by the expression of S phase genes. Decrease in the E2F-1 protein levels was observed with SFN treatment. Cyclins and cyclin-dependent kinases (CDKs) regulate the activity of RB by phosphorylation resulting in control of progression through G1 phase to S phase [27]. Lower levels of the CDK4 and CDK6, were responsible for phosphorylation of RB in response to SFN was observed in a dose dependent manner. The authors also reported a dose dependent increase in the RB levels in E2F-1 immunoprecipitates [30].

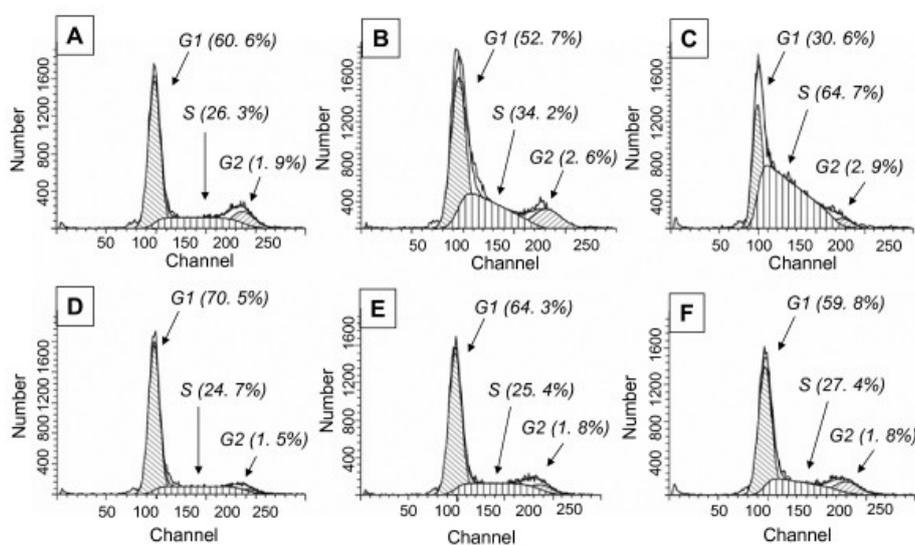


Figure 8 A. Cells were seeded at 106 cells and grown serum-free medium for 24 hr for cell cycle synchronization.

Cells were then treated with either vehicle DMSO (i, ii and iii) or 10  $\mu\text{mol/l}$  SFN (iv, v and vi) for 0 hours (i and iv), 24 hours (ii and v) and 48 hours (iii and vi), respectively. Cells were fixed, stained and flow cytometric analysis was conducted using a cell cycle phase determination kit (Cayman chemical company, Ann Arbor, MI). B. Tabular representation of the % changes in the cell cycle phases. The results demonstrated that SFN induces G1 arrest in a time-dependent manner (D, E, F) in comparison with control cells (A, B, C).

SFN has been reported to inhibit cell mobility and invasiveness. Cell motility following wound generation showed a greater cell migration in control cells compared with SFN treated cells. After 20 hrs, Bryant *et al.* observed almost complete closure of the wound in control but was inhibited by 50% and 75% by 5  $\mu\text{mol/l}$  and 10  $\mu\text{mol/l}$  SFN in cancer cell lines, respectively (Fig 9). SFN treatment also resulted in inhibition of cell migration in a modified Boyden chamber.

Bryant *et al.* showed that SFN activates the RB-E2F-1 interaction with MDAH-2774 ovarian cancer cell line resulting the upstream AKT inhibition to the downstream blocking of cell cycle [30]. They also showed the inhibition of invasiveness of cells and subsequent migration.

## Conclusions

SFN mediating cell growth arrest has been documented in colon, prostate and several other cancers [23, 31-33]. Little is known on its effects in gynecologic malignancies. Only three had reported on its effects on ovarian cancer cells [23, 30, 31, 34]. At a concentration of 20  $\mu\text{mol/l}$  of SFN was measured in the urine samples of human ingested broccoli [13]. This result indicated that SFN is bio-available in human. It has been estimated that consumption of 50 g of broccoli can generate 20  $\mu\text{mol/l}$  of SFN. As this level exceeds the concentration of SFN utilized in in-vitro studies, the concentrations of SFN used in the study may be utilized in vivo assays. Chuang *et al.* presented the first report of the effects of SFN on two different human ovarian cancer cell lines, OVCAR-3 and SKOV-3 [31]. Consequently, this might make SFN as an attractive chemopreventive and

chemotherapeutic agent derived from natural sources in ovarian cancers.

Chaudhuri *et al.* demonstrated that SFN inhibits the growth of the ovarian cancer cells and the inhibition of the AKT pathway is one of the upstream molecular events [23]. They observed potent antiproliferative effects of sulforaphane in SKOV3, C3, and T3 cells. Sulforaphane was found to inhibit the clonogenic ability of ovarian cancer cells. The clonogenic ability is considered a marker of neoplastic propensity. This is significant as sulforaphane is able to target a small percentage of cells in a tumor that can survive and proliferate using autocrine factors and may be associated with up-regulated prosurvival pathways. Bryant *et al.* investigated downstream molecular mechanisms at the level of cell cycle control in the nucleus. Combination therapy with paclitaxel is known to increase overall survival [35], but contributes to the development of resistance phenotype resulting in eventual relapse of the disease. Unlike traditional chemotherapy drugs, natural therapeutics like SFN may not contribute to the development of chemo-resistant phenotype. Bryant's results indicated a synergistic effect of cell death when SFN was used in combination with paclitaxel. Phosphorylated RB cannot interact with E2F-1, thus leaving large pool of free E2F-1 transcription factors driving the G1/S cell cycle transition. E2F-1 has been shown to have growth promoting activity in EOC and is over expressed in roughly half of the ovarian cancers [34]. Invasion followed by degradation of basal membrane is hallmark of tumor metastasis where proliferating tumor cells infiltrate into other tissues. Wound healing assays and Boyden chamber assays provide evidence that the cell motility and invasiveness are inhibited by SFN. These findings suggested that SFN has very good

potential for use in the treatment against invasion and metastasis of EOC. SFN induces growth arrest and apoptosis in EOC cells by inhibiting RB phosphorylation and reduction in the levels of free E2F-1.

### Future Directions

A subset of ovarian cancer cell lines and tumors harbors genetic alterations activated Akt in the PI3K/Akt pathways, which is poor prognostic biomarker. Thus, selective, allosteric Akt inhibitors in patients with ovarian cancer are a prominent focus for clinical utility. Other subsets with RB1 loss or RAS or RAF mutation were however often resistant to Akt inhibition, irrespective of pathway activation. It is also reported the selective Akt1 inhibition was sufficient for maximal antitumor effects in a subset of ovarian cancer cell lines whereas pan-Akt inhibition was required in those expressing Akt3. Current literatures reported antitumor effects on ovarian cancer cell lines (SKOV3 and OVCAR3) overexpressing Akt, and retain RB and PTEN. In order to reach the definitive conclusion of SFN effect in Akt or RB, further analysis is needed of ovarian cancer cell lines without Akt activation, with RB loss or Kras activation. It is also needed to evaluate other subsets of ovarian cancer cell lines with RAS or RAF mutations, which is resistant to Akt inhibitors, such as SKOV8 cell line. Mutant p53 depletion has been reported as an important target for cancer chemoprevention and therapy by natural and synthetic ITCs and a critical mechanism induce apoptosis in various types of cancers. In such circumstances, SFN did not show repression of mutant p53 expression unless it is at a high concentration [36]. Addition studies are needed to evaluate in p53 expressing ovarian cancer cell lines. As refractory and recurrent ovarian cancers are often refractory to cisplatin and paclitaxel treatments, further study is required to evaluate combination of SFN and other agents to overcome resistance to treatments of these chemotherapeutic agents.

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