

RESEARCH ARTICLE

Sulforaphane Inhibits Growth of Human Breast Cancer Cells and Augments the Therapeutic Index of the Chemotherapeutic Drug, Gemcitabine

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Abstract

Phytochemicals are among the natural chemopreventive agents with most potential for delaying, blocking or reversing the initiation and promotional events of carcinogenesis. They therefore offer cancer treatment strategies to reduce cancer related death. One such promising chemopreventive agent which has attracted considerable attention is sulforaphane (SFN), which exhibits anti-cancer, anti-diabetic, and anti-microbial properties. The present study was undertaken to assess effect of SFN alone and in combination with a chemotherapeutic agent, gemcitabine, on the proliferative potential of MCF-7 cells by cell viability assay and authenticated the results by nuclear morphological examination. Further we analyzed the modulation of expression of Bcl-2 and COX-2 on treatment of these cells with SFN by RT-PCR. SFN showed cytotoxic effects on MCF-7 cells in a dose- and time-dependent manner via an apoptotic mode of cell death. In addition, a combinational treatment of SFN and gemcitabine on MCF-7 cells resulted in growth inhibition in a synergistic manner with a combination index (CI) < 1. Notably, SFN was found to significantly downregulate the expression of Bcl-2, an anti-apoptotic gene, and COX-2, a gene involved in inflammation, in a time-dependent manner. These results indicate that SFN induces apoptosis and anti-inflammatory effects on MCF-7 cells via downregulation of Bcl-2 and COX-2 respectively. The combination of SFN and gemcitabine may potentiate the efficacy of gemcitabine and minimize the toxicity to normal cells. Taken together, SFN may be a potent anti-cancer agent for breast cancer treatment.

Keywords: Sulforaphane - gemcitabine - chemoprevention - apoptosis - breast cancer

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Introduction

Despite rapid advances in diagnosis and prognosis of breast cancer, it still remains the most common malignancy among women worldwide (Youlten et al., 2012). Several modifiable and non-modifiable risk factors have been associated with the incidence of breast cancer thereby providing compelling basis for development of breast cancer prevention and treatment strategies. The purpose of such strategies should be inhibition or reversal of breast carcinogenesis while producing minimal to no side effects, consequently targeting even the asymptomatic women (Gabriel and Jatoi, 2012).

Sulforaphane (SFN), a dietary component found in abundance in many cruciferous vegetables including broccoli, cabbage and cauliflower, is one such widely studied agent. Its chemopreventive effects have assigned to multiple mechanisms such as modulatory action on

phase 2 enzymes via Keap1-Nrf2 signaling and antioxidant response element (ARE)-driven gene expression as well as by induction of cell cycle arrest and apoptosis and inhibition of histone deacetylase (HDAC) (Clarke et al., 2011; Rajendran et al., 2011). Conspicuously, SFN has been shown to selectively target the precancerous and cancerous cells (Clarke et al., 2011).

In order to avoid the complications associated with standard cancer treatments for instance chemotherapy, combinational treatment strategies involving chemopreventive agents have been proposed for achieving a therapeutic synergy between individual drugs, while minimizing systemic toxicity caused by these therapies (Notarbartolo, 2005; Kumi-Diaka et al., 2010; Doudican et al., 2012). The present study was undertaken to analyze the chemopreventive activity of SFN alone and its interaction with a chemotherapeutic drug, gemcitabine on human breast cell line (MCF-7).

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Materials and Methods

Cell culture

The human breast cancer cell line, MCF-7 was kindly gifted by Dr. Tahir A Rizvi, Department of Microbiology and Immunology, Faculty of Medicine and Health Science, UAE University, Al-Ain, United Arab Emirates. It was maintained in DMEM (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA) and 100X Pen-strep (Sigma, USA) in a humidified atmosphere of 5%CO₂ in air at 37°C.

Preparation of drug solutions

SFN was purchased from Sigma (Sigma Aldrich, U.S.A.). A stock solution of 10 mM SFN was prepared in DMSO (Sigma, USA). The solution was stored in aliquots at -20°C. Further dilutions were made in complete medium to required concentrations between 0.01-75 µM for the treatment of MCF-7 cells.

A stock solution of gemcitabine (Intas Biopharmaceuticals, India) at a concentration of 133 mM (40 mg/ml) was prepared in DMSO (Sigma, USA) and further dilutions were made in complete medium at concentrations of 0.1-100 mM.

Cell viability assay

The antiproliferative activity of sulforaphane (0.01-75µM) or gemcitabine (0.1-100 mM) and combinations of both was evaluated on MCF-7 cells by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as previously described (Hussain et al., 2012). Briefly, ~8000 cells/well are plated in 96 well plates and allowed to grow for 24h in complete medium at 37°C in order to obtain semi-confluent cultures. This was followed by treatment with varying concentrations of sulforaphane or gemcitabine at 24 and 48h and the effect of combination of SFN and gemcitabine treatment was analysed at 24h. After appropriate incubation, MTT (Sigma, USA) (final concentration 0.5 mg/ml) was added to each well and further incubated for 2-4h at 37°C. Thereafter, DMSO was added to dissolve the formazan crystals and absorbance was read at 570 nm using an Absorbance Microplate Reader (BioTek, USA). Cell viability was calculated as percent of control (untreated cells), and averaged from three independent experiments.

Calculation of combination effects of SFN and gemcitabine

Combination index (CI), which provides qualitative information on the nature of drug interaction, was determined as described previously by Chou and Talalay (Chou and Talalay, 1984). A CI <, =, or >1 represent synergy, additivity, and antagonism of SFN and gemcitabine, respectively.

Morphological analysis of MCF-7 cells before and after treatment with SFN

Morphological changes in MCF-7 cells elicited by sulforaphane were documented using normal inverted microscope (Labomed, USA). MCF-7 cells were treated at a concentration of 25 µM for 24 h. Comparison of SFN treated cells were made with the untreated cells.

Nuclear morphological analysis of MCF-7 cells treated with sulforaphane

The nuclear morphological changes associated with cells undergoing apoptosis were studied using propidium iodide (PI) staining after the treatment with sulforaphane for different time intervals (0, 6 and 24 h). Briefly, cells (~10⁶ Cells/ml) were seeded on glass coverslips and left overnight to attach in complete medium at 37 °C, followed by treatment with 25 µM SFN for varying time points (0, 6 and 24 h). After treatment with SFN, cells were fixed in a mixture of acetone: methanol (1:1) at -20°C for 10 min washed with 1× PBS (pH 7.4) twice and stained with propidium iodide (10 µg/ml in PBS) for 30 s in dark at room temperature. The coverslips were thoroughly washed with PBS and placed upturned onto a glass slide with mounting media (DPX). Slides were viewed at 515nm under the Progress Fluorescent Microscope (Olympus, USA). The images were captured at 40× magnification.

Expression analysis of Bcl-2 and COX-2 by RT-PCR

Reverse transcription-PCR was used to detect transcriptional regulation of Bcl-2 and COX-2 in response to treatment with 25 µM SFN. Total RNA extraction from untreated and SFN-treated MCF-7 cells was carried out as per the manufacturer's instructions (GenElute Mammalian Genomic Total RNA Kit, Sigma, USA) at various time intervals (6, 24 and 48h). Further, total RNA was subjected to first strand synthesis as per manufacturer's protocol (ProtoScript M-MuLV Taq RT-PCR Kit, New England Biolabs, USA) followed by PCR using gene-specific primers for β-actin, Bcl-2 and COX-2 (Bondesen et al., 2004; Wang et al., 2005). The PCR cycle was as follows: initial denaturation at 95°C for 5 min, followed by 35 amplification cycles (denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 45s), with final extension at 72°C for 7 min. Amplified products were visualized on a 2% agarose gel containing ethidium bromide. β actin was taken as an internal control.

Statistical analysis

All data are expressed as means±SD of at least 3 experiments. Fisher's exact test was adopted for statistical evaluation of the results. Significant differences were established at p<0.05.

Results

Cell growth inhibitory effect of SFN and gemcitabine on human breast cancer cells, MCF-7

The effect of SFN on cell growth of human breast cancer cells, MCF-7 was determined by treating the cells with varying concentrations of SFN (0.01-75µM) for 24h and 48h respectively. Treatment with increasing concentrations of SFN (0.01-75µM) resulted in inhibition of cell growth in a dose and time-dependent manner compared with the untreated controls (Figure 1). SFN at a dose of 25µM for 24h and 20µM at 48h induced approximately 50% decrease in cell viability (EC₅₀) in MCF-7 cells (Figure 1). The experiment was repeated at least three times.

MCF-7 cells showed growth inhibition in a dose- and

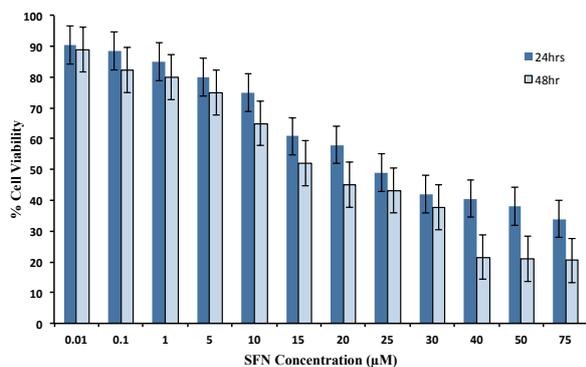


Figure 1. Effect of SFN on MCF-7 Cells. SFN treatment at varying concentrations (0.01-75µM) result in dose and time dependent growth inhibition of MCF-7 Cells at 24 and 48h time intervals. EC₅₀ of Sulforaphane was found to be 25µM at 24h and 20µM at 48h. Values are means±SD of three independent experiments. Each value with SFN treatment differs from the control value (p<0.05)

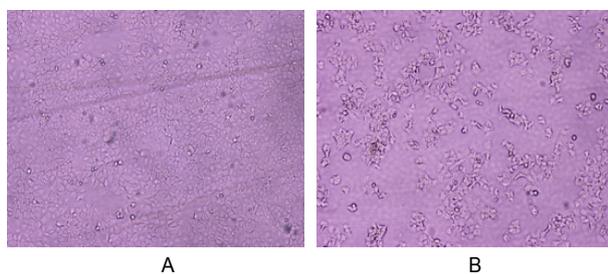


Figure 2. SFN may Induce Cell Death in MCF-7 Cells via Apoptosis. **A)** Microscopic examination of untreated MCF-7 cells showed their proper morphological characteristics; **B)** After exposure of MCF-7 cells to 25 µM SFN for 24h, typical rounding off of these cells was observed which is a feature of cell death through the apoptotic pathway (Magnification 100×)

time-dependent manner (Figure 4) when treated with gemcitabine at concentrations ranging from 0.1-100 mM for 24 and 48 h. The EC₅₀ value was found to be 35mM and 1mM for 24 and 48 h respectively.

Morphological studies of SFN treated human breast cancer Cell line, MCF-7

Morphological changes in MCF-7 cells elicit by SFN treatment were documented using normal inverted microscope. A characteristic indicator of cell death, rounding off of cells, was observed in 25µM SFN treated MCF-7 cells for 24 h as compared to untreated cells which showed proper morphology of attached healthy cells. This indicates that the cell death induced by SFN may be through the apoptotic pathway (Figure 2).

SFN induced cell death in MCF-7 Cells via Apoptosis

To verify whether SFN induces apoptotic cell death in MCF-7 cells, propidium iodide (PI) staining was used to detect nuclear morphological changes. Results show that MCF-7 cells treated with SFN for 24h had undergone remarkable morphological changes such as chromatin condensation and nuclear debris, which could be identified as apoptotic bodies. On the other hand, the nuclei of untreated cells intact and appeared uniform in chromatin density (Figure 3). Thus, this study confirms

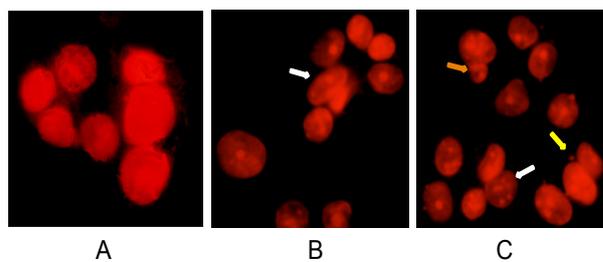


Figure 3. SFN Treatment Induces Nuclear Morphological Changes in MCF-7 Cells. **A)** Untreated MCF-7 cells show large and prominent nuclei; **(B) and (C)** On treatment with 25µM SFN for 6 and 24 h respectively, the nuclei of these cells showed various characteristics associated with apoptosis such as nuclear condensation and fragmentation (white arrow), apoptotic bodies (yellow arrow) and nuclear blebbing (orange arrows) which accumulated with increased time of exposure. (Magnification 400×)

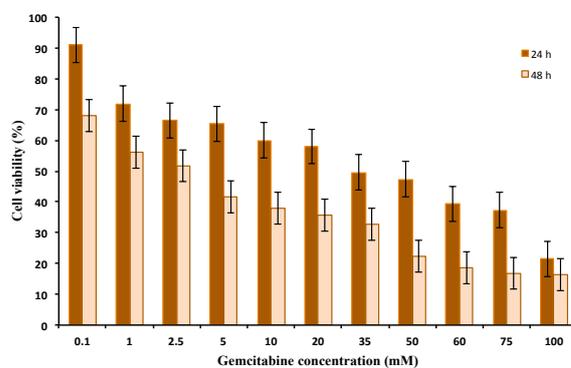


Figure 4. Effect of Gemcitabine Treatment on MCF-7 Cells. Treatment of MCF-7 cells with varying concentrations (0.1-100mM) of gemcitabine for 24 and 48h resulted in decrease in cell viability in a dose and time dependent manner. The EC₅₀ of gemcitabine was determined to be 35mM at 24h and 1mM at 48h. Values are means±SD of three independent experiments. Each value with gemcitabine treatment differs from the control value (p<0.05)

that SFN-treated cell death in MCF-7 cells is mediated by apoptosis.

Lower dose combinations of SFN and gemcitabine may act synergistically to suppress growth of MCF-7 cells

The combination effects were evaluated by selecting two sub-lethal doses of both SFN and gemcitabine. The sub-lethal doses of SFN i.e., 5µM and 10µM resulted in 17 and 24% decrease in cell viability (Figure 5). Similar decrease in cell viability (34-39%) was also observed at the sub-lethal doses of gemcitabine (5mM to 10mM). Then, the effect of different combinations of SFN and gemcitabine was evaluated. 5µM of SFN used in combination with 5mM and 10mM gemcitabine resulted in significant decrease in cell viability (54 and 65% respectively) compared to either of the compounds alone. Also, 10 µM sulforaphane when combined with 5mM and 10mM gemcitabine resulted in significant decrease in cell viability (56 and 58% respectively) in comparison to the individual doses. The combination index (CI) was calculated for all the combination and was found to be<1 thereby indicating a synergistic interaction of these drugs. These results confirm the hypothesis that low doses of SFN

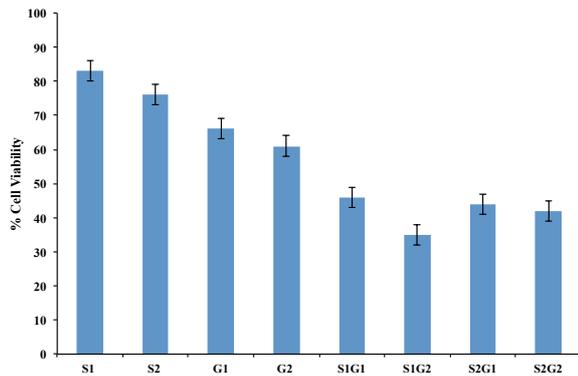


Figure 5. Low-dose Combinations of SFN and Gemcitabine Synergistically Induce Cell Death in MCF-7 Cells. Simultaneous treatment of MCF-7 cells with sub-lethal doses of SFN (S1=5 μ M and S2=10 μ M) and gemcitabine (G1=5mM and G2=10mM) induced synergistic decrease in cell viability of MCF-7 cells (combination index [CI]<1). Each value is a ratio of the level in the treated cells to that in the control cells. Values are means \pm SD of 3 independent experiments. Each value with SFN and gemcitabine treatment differs from the control value ($p<0.05$)

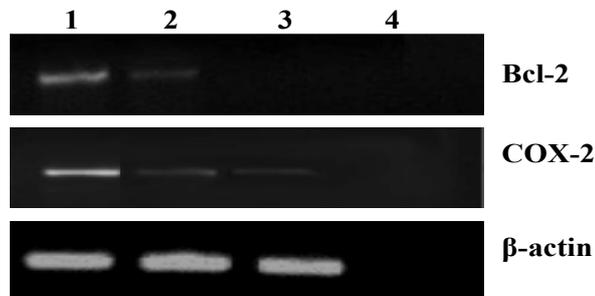


Figure 6. SFN Downregulates the Expression of Bcl-2 and COX-2. 25 μ M SFN treatment significantly reduced the expression of Bcl-2 and COX-2 in a time dependent manner in MCF-7 cells compared to untreated cells. Lane 1 shows untreated MCF-7 cells; lanes 2 and 3 show SFN treated MCF-7 cells for 6 and 24 h respectively; lane 4 shows negative control for RT-PCR. β -actin was used as an internal control

can potentiate the effect of gemcitabine at lower doses.

SFN treatment downregulates the expression of Bcl-2 and COX-2

The effect of sulforaphane on the expression of Bcl-2 (B-cell lymphoma), an anti-apoptotic gene, was analyzed before and after treatment at various time-points. Densitometric analysis of the transcript levels was performed and normalized against β -Actin (Figure 6). Untreated MCF-7 cells showed high level of Bcl-2 expression indicative of evasion of apoptosis in these cells. However, on treatment with SFN, its expression was found to be significantly downregulated in a time dependant manner compared to the untreated control cells (Figure 6). Thus SFN may induce apoptosis in these cells by downregulating Bcl-2 gene.

Further, COX-2 expression was analysed in SFN treated MCF-7 cells which has been shown to be involved in inflammation and its upregulation has been reported in various cancers. The untreated MCF-7 cells showed a high level of expression of COX-2, whereas SFN treatment

resulted in decrease in COX-2 expression in MCF-7 cells in a time dependant manner (Figure 6).

Discussion

Efficacy of chemoprevention stems from the ability of the dietary agents to counteract the various stages of carcinogenesis that lead to DNA damage, generation of free radicals and other forms of cellular stress. Therefore it is coming up as an economical, easily available, and novel approach to cancer treatment and management (Karikas, 2010). Sulforaphane is an isothiocyanate, found in cruciferous vegetables, such as broccoli, kale, and cauliflower displays significant chemopreventive activity resulting from its inhibition of carcinogen-activating enzymes and induction of detoxification enzymes affecting carcinogen metabolism and disposition and by modulating epigenetic machinery (Barcelo et al., 1996; Basten et al., 2002; Myzak et al., 2006; Gibbs et al., 2009).

In the present study, treatment with SFN at varying concentrations resulted in dose and time-dependent decrease in cell viability of MCF-7 cells. The effective concentration (EC_{50}) was found to be 25 μ M SFN for duration of 24h (Figure 1). Other studies also showed that SFN treatment significantly reduces cell viability of various cancer cells i.e., human leukemia T-cells, breast, pancreatic, lymphoblastoid and glioma cancer cells in a dose-dependent manner (Fimognari et al., 2002; Misiewicz et al., 2004; Meeran et al., 2010; Huang et al., 2012; Li et al., 2012). Interestingly, SFN has a safe cytotoxicity profile towards the normal cells thus it can be utilized in the development of safe cancer treatment strategies (Sharma et al., 2012).

Further, in order to evaluate whether the cytotoxic effect of SFN on MCF-7 cells is via the apoptotic pathway, microscopic examination and nuclear morphologic analysis using propidium iodide were carried out. Microscopic examination revealed that SFN treated cells appeared rounded compared to the untreated MCF-7 cells indicative of apoptotic cell death (Figure 2). In continuation, nuclear morphological analysis confirmed that SFN induces apoptosis in these cells as SFN treated cells showed the characteristic features of apoptosis such as nuclear condensation and fragmentation as well as formation of apoptotic bodies which accumulated in a time-dependent manner (Figure 3). Hence, our results confirm that decrease in MCF-7 cell viability by SFN was due to its induction of apoptosis in these cells. These results are in line with other studies which have also shown that SFN induces cell death in cancer cells via apoptosis (Kanematsu et al., 2011; Huang et al., 2012; Li et al., 2012).

Apoptosis is a desirable mode of cell death by different cancer treatment strategies and is regulated by various genes such as Bcl-2, bax etc. The present study investigated the effect of sulforaphane on expression of the anti-apoptotic gene, Bcl-2, in MCF-7 cells by RT-PCR. Our RT-PCR results showed the high expression of Bcl-2 in untreated MCF-7 cells which significantly decreased in response to the sulforaphane treatment in time dependent manner (Figure 6). These results are consistent with

previous studies involving use of chemopreventive agents to induce apoptosis by downregulation of Bcl-2 (Pledge-Tracy et al., 2007; Kalra et al., 2008; Kim et al., 2009; Kumar et al., 2012). Considering that increased expression of Bcl-2 may be related to chemoresistance, reduction in the level of Bcl-2 by chemopreventive drugs such as SFN may increase the sensitivity to anticancer drugs.

Gamut reports indicate that conventional cancer treatment strategies have important but limited scope as reflected in the poor life style of the cancer patients. In this study, gemcitabine, a chemotherapeutic drug and SFN were used in combination at lower doses. MCF-7 cells treated with gemcitabine alone showed a dose and time-dependent decrease in cell viability (Figure 4). The EC₅₀ of gemcitabine on MCF-7 cells was found to be 35mM at 24h treatment. Various studies have also shown its cytotoxicity towards various cancer cells (Pacini et al., 1999; Strouch et al., 2009; Hastak et al., 2010). However, it was found to have cytotoxic effects against normal cells also (Sharma et al., 2011). Therefore, combinational treatment modalities using conventional therapies such as chemotherapy and newer strategies like chemoprevention are gaining attention.

Our results on the combination of SFN and gemcitabine show that SFN enhances the growth inhibitory effects of gemcitabine at sub-lethal doses (Figure 5). When the sub-lethal doses of SFN (5µM and 10µM) were used in various combinations with lower doses of gemcitabine (5mM and 10mM), the decrease in cell viability induced by these combinations was more pronounced than either of the compound alone and the CI index was found to be <1 for each combination, thus indicating a synergistic interaction between these drugs (Figure 5). This implies that sulforaphane enhances the efficiency of gemcitabine against the cancer cells, especially at lower doses, thereby minimizing its cytotoxicity to normal cells resulting in better survival. Other studies have also reported a synergistic interaction between different treatment strategies (Liu et al., 2001; Kuhar et al., 2007; Kumi-Diaka et al., 2010; Wang et al., 2011). Other studies of concurrent curcumin and chemotherapeutic drugs such as cisplatin, doxorubicin, and taxol showed that curcumin enhances the antitumor activities of the latter in various cancer cells (Chan et al., 2003; Notarbartolo et al., 2005). SFN when used in combination with arsenic trioxide and doxorubicin augmented their growth inhibitory properties (Fimognari et al., 2007; Doudican et al., 2012). Kotowski et al. (2011) also found that SFN can induce radiosensitization in head and neck cancer cells (Kotowski et al., 2011). Thus, combining SFN with conventional treatment strategies may allow minimizing their adverse effects.

Interestingly, a study on curcumin found that its combination with celecoxib reduced the cell growth in a synergistic manner which was in turn correlated with the inhibition of COX-2 pathway (Lev-Ari et al., 2005). Additionally it is well established that COX-2 is a promising molecular target for cancer chemoprevention owing to its involvement in inflammation (Khan et al., 2012; Nadda et al., 2012). Based on these findings, we analyzed the effect of SFN treatment on COX-2 expression in MCF-7 cells. The untreated MCF-7 cells

showed a high level of expression of COX-2 while on SFN treatment it was found to be significantly downregulated in MCF-7 cells in a time dependant manner (Figure 6). Several dietary components including curcumin, green tea catechins, genistein etc have been shown to suppress COX-2 expression (Plummer et al., 1999; Gerhäuser et al., 2003; Hwang et al., 2009). Hence, it can be inferred that reduction in COX-2 expression may be an important target for chemoprevention by agents like SFN.

Conclusively, SFN exhibits anti-carcinogenic effects and act as a biological response modifier in breast cancer treatment. It can also be used in combination with chemotherapeutic drugs to increase their efficacy and reducing their side effects. Further comprehensive in vitro and in vivo studies and clinical trials are requisite to make SFN available for therapeutic purposes.

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