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Anticancer Activity of a Broccoli Derivative, Sulforaphane, in Barrett Adenocarcinoma: Potential Use in Chemoprevention and as Adjuvant in Chemotherapy[1]

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Abstract

INTRODUCTION: The incidence of Barrett esophageal adenocarcinoma (BEAC) has been increasing at an alarming rate in western countries. In this study, we have evaluated the therapeutic potential of sulforaphane (SFN), an antioxidant derived from broccoli, in BEAC. *METHODS:* BEAC cells were treated with SFN, alone or in combination with chemotherapeutic, paclitaxel, or telomerase-inhibiting agents (MST-312, GRN163L), and live cell number determined at various time points. The effect on drug resistance/chemosensitivity was evaluated by rhodamine efflux assay. Apoptosis was detected by annexin V labeling and Western blot analysis of poly(ADP-ribose) polymerase cleavage. Effects on genes implicated in cell cycle and apoptosis were determined by Western blot analyses. To evaluate the efficacy *in vivo*, BEAC cells were injected subcutaneously in severe combined immunodeficient mice, and after the appearance of palpable tumors, mice were treated with SFN. *RESULTS:* SFN induced both time- and dose-dependent decline in cell survival, cell cycle arrest, and apoptosis. The treatment with SFN also suppressed the expression of multidrug resistance protein, reduced drug efflux, and increased anticancer activity of other antiproliferative agents including paclitaxel. A significant reduction in tumor volume was also observed by SFN in a subcutaneous tumor model of BEAC. Anticancer activity could be attributed to the induction of caspase 8 and p21 and down-regulation of hsp90, a molecular chaperon required for activity of several proliferation-associated proteins. *CONCLUSIONS:* These data indicate that a natural product with antioxidant properties from broccoli has great potential to be used in chemoprevention and treatment of BEAC.

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Introduction

Various epidemiologic studies have indicated that consumption of broccoli is associated with a lower risk of cancer [1], including breast [2], prostate [3], lung, stomach [4], and colon [5] cancers. The anticancer effect of broccoli has been attributed to sulforaphane (SFN), an isothiocyanate formed by hydrolysis of a precursor glucosinolate called "glucoraphanin" [1]. Although glucoraphanin is found in varying amounts in all cruciferous vegetables, the highest concentration of this compound is found in broccoli and its sprouts [6]. Among various parts of mature broccoli, the florets have the maximum amount of SFN. The amount of SFN in 1 g of dry broccoli florets ranges from 507 to 684 μg [7]. The sprouts of broccoli seem to have 20 to 30 times higher concentration of glucoraphanin, an SFN precursor [8,9], indicating that 1 oz of sprouts may have the amount of antioxidant present in 20 oz of mature broccoli. Glucoraphanin is converted to SFN by myrosinase, an enzyme released from broccoli during its consumption and also found in our stomach [1]. The reduced risk of cancer after consumption of broccoli is associated with the ability of SFN to inhibit phase 1 enzymes (implicated in the conversion of procarcinogens to carcinogens) and induce phase 2 enzymes (implicated in detoxification and excretion of carcinogens from body) [1,6].

However, the anticancer activity of SFN is not limited to its ability to promote detoxification and removal of carcinogens. SFN has been shown to inhibit cell cycle progression, induce apoptotic cell death, and inhibit angiogenesis in a variety of cancer cell types [1,6]. Exposure to SFN (20 μM) has been shown to induce a chk2 kinase–dependent arrest at the G_2/M phase of cell cycle in prostate cancer (PC3) cells [10]. A G_2/M arrest after treatment with SFN has also been demonstrated in human colon cancer (HCT116) cells [10]. Although cell cycle arrest at G_2M has been frequently observed after treatment of cancer cells with SFN, arrest at other phases of cell cycle has also been reported for many cell lines. For example, the treatment of colon cancer (HT-29) cells with this agent led to the induction of p21, down-regulation of cyclin D1 and cyclin A, and a G_1 cell cycle arrest [11], whereas prostate cancer (LnCap) cells arrested at G_1/S after treatment with 10 μM drug [12,13].

SFN has also been shown to induce apoptosis, or programmed cell death, in cancer cells. Treatment with 15 μM SFN induced apoptosis in both the p53-positive and p53-negative human colon cancer cell lines [14]. Similarly, exposure to 10 μM SFN caused apoptosis in prostate cancer DU145 cells [15]. The mechanisms of SFN-induced apoptosis in these cell lines include activation of caspase 7 and 9 [14] and/or release of cytochrome *C* from mitochondria [16].

Anticancer activity of SFN has also been demonstrated *in vivo*. SFN has been shown to inhibit growth of human pancreatic cancer [17], human prostate cancer [18], murine osteosarcoma xenografts [19], and prevent intestinal polyposis [11], UV light–induced skin tumors [20], carcinogen-induced skin tumors [21], and carcinogen-induced stomach tumors [22] *in vivo*. SFN has also been reported to increase natural killer cell activity and antibody-dependent cellular cytotoxicity in both the control and tumor-bearing mice [23]. These and other modulations of immune system by SFN have been shown to play important role in the inhibition of metastatic spread of melanoma in mice [24].

Barrett esophageal adenocarcinoma (BEAC) develops in Barrett esophagus, a precancerous condition associated with chronic esophageal reflux. Because the cancer starts to spread before onset of clinical symptoms, BEAC patients usually have a dreary outcome with a poor survival rate [25]. Moreover, the incidence of this cancer has been increasing at a disturbing rate in Europe and United States [26]. Because the effect of SFN on BEAC cells has not been demonstrated, we studied the effect of this natural food ingredient, either alone or in combination with other anticancer agents including paclitaxel, on cell cycle and cell viability in BEAC cell lines. We have shown that SFN induces cell cycle arrest and apoptosis in BEAC cells at concentrations (3-7 μM) lower than (10-20 μM) those of SFN required to kill other cancer cell lines. Moreover, the SFN also suppressed multidrug resistance protein (MRP), reduced drug efflux, and significantly enhanced the anticancer activity of telomerase inhibitors (MST-312, GRN163L) and paclitaxel, a chemotherapeutic currently used to treat BEAC, in BEAC cell lines tested. The treatment with SFN was also associated with induction of caspase 8 and p21 and suppression of hsp90, a molecular chaperone required for activity of several proliferation related proteins. A significant anticancer activity of SFN was also observed in a subcutaneous tumor model *in vivo*. These data indicate a marked anticancer activity of SFN in BEAC and provide a rationale for clinical evaluation.

Materials and Methods

Chemicals

L-Sulforaphane (SFN) and paclitaxel were purchased from Sigma-Aldrich (St Louis, MO) and dissolved in phosphate-buffered saline. MST-312 [*N*,*N*′-bis(2,3-dihydroxybenzoyl)-1,3-phenylenediamine] was purchased from Calbiochem/EMD Biosciences (Madison, WI) and dissolved in DMSO. GRN163L is a palmitoyl (C16) lipid—attached N3′-P5′ phosphoramidate oligonucleotide, targeting the template region of RNA subunit of telomerase (hTR) and was obtained from Geron Corporation (Menlo Park, CA). GRN140833 mismatch oligonucleotide was also obtained from Geron Corporation and used as a negative control.

Barrett Adenocarcinoma Cell Lines and Normal Cells

BEAC cell line FLO-1 and a lung adenocarcinoma cell line H460 have been described previously [27]. BEAC cell line OE33, from European Collection Of Cell Cultures, was purchased through Sigma-Aldrich and has been described previously [28,29]. Normal primary human esophageal epithelial cells (HEEC) were purchased from ScienCell Research Laboratories (Carlsbad, CA) and have been described previously [30]. FLO-1 cells were cultured in Dulbecco modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT). OE33 cells were cultured in RPMI-1640 supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Normal HEEC (human esophageal epithelial cells) were cultured in epithelial cell medium-2 (ScienCell Research Laboratories). Cells were maintained in a state of logarithmic growth at 37°C in humidified air with 5% CO_2, as described previously [30]. For RNA and protein analyses, cultures were harvested at the same final cell density (5×10^5/ml) and immediately processed.

Treatment and Growth of Cells

Cells (5×10^5) were plated in 100-mm dishes and treated with SFN alone or in combination with paclitaxel (PAC) and MST-312, at concentrations described in the figure legends. Substrate-attached viable cell number was counted, and cell viability was confirmed by trypan blue exclusion or cell proliferation assays at various time points. Cell proliferation assays were performed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc, Gaithersburg, MD) according to the manufacturer's protocol. The method provides a highly sensitive

colorimetric assay for the determination of viable cell number and is based on the production of a yellow product (formazan) after reduction of a highly water-soluble tetrazolium salt by dehydrogenases in viable cells. The amount of the formazan dye generated is measured by a plate reader and is directly proportional to the number of viable cells.

Rhodamine Efflux Assay

FLO-1 cells, plated in a six-well plate, were pretreated with SFN (3 or 5 μM) for 5 hours or with cyclosporin A (10 μM; a known inhibitor of MDR1 gene product) for 1 hour. Rhodamine 123 (3 μM) was then added into the same medium, and the cells were incubated for an additional 1.5 hours. Rhodamine-containing medium was then removed and cells were washed with PBS and incubated overnight at 37°C, 5% CO_2 with serum-free medium containing the same concentrations of drugs without rhodamine. Cells were harvested, washed with PBS, and evaluated for rhodamine 123 florescence by flow cytometry.

Cell Cycle Analysis

The effect of SFN and PAC on progression of the cell cycle was determined using the Cell Cycle Phase Determination Kit (Cayman Chemical Company, Ann Arbor, MI). Briefly, the control and treated cells were washed and resuspended in assay buffer at 10^6/ml. Fixative (1 ml) was then added, and cells were allowed to permeabilize for at least 2 hours. The cells were centrifuged, the fixative was removed, and cells were resuspended in 0.5 ml of staining solution containing propidium iodide. After 30 minutes of incubation at room temperature, the cells were analyzed in the FL2 channel of a flow cytometer with a 488-nm excitation filter.

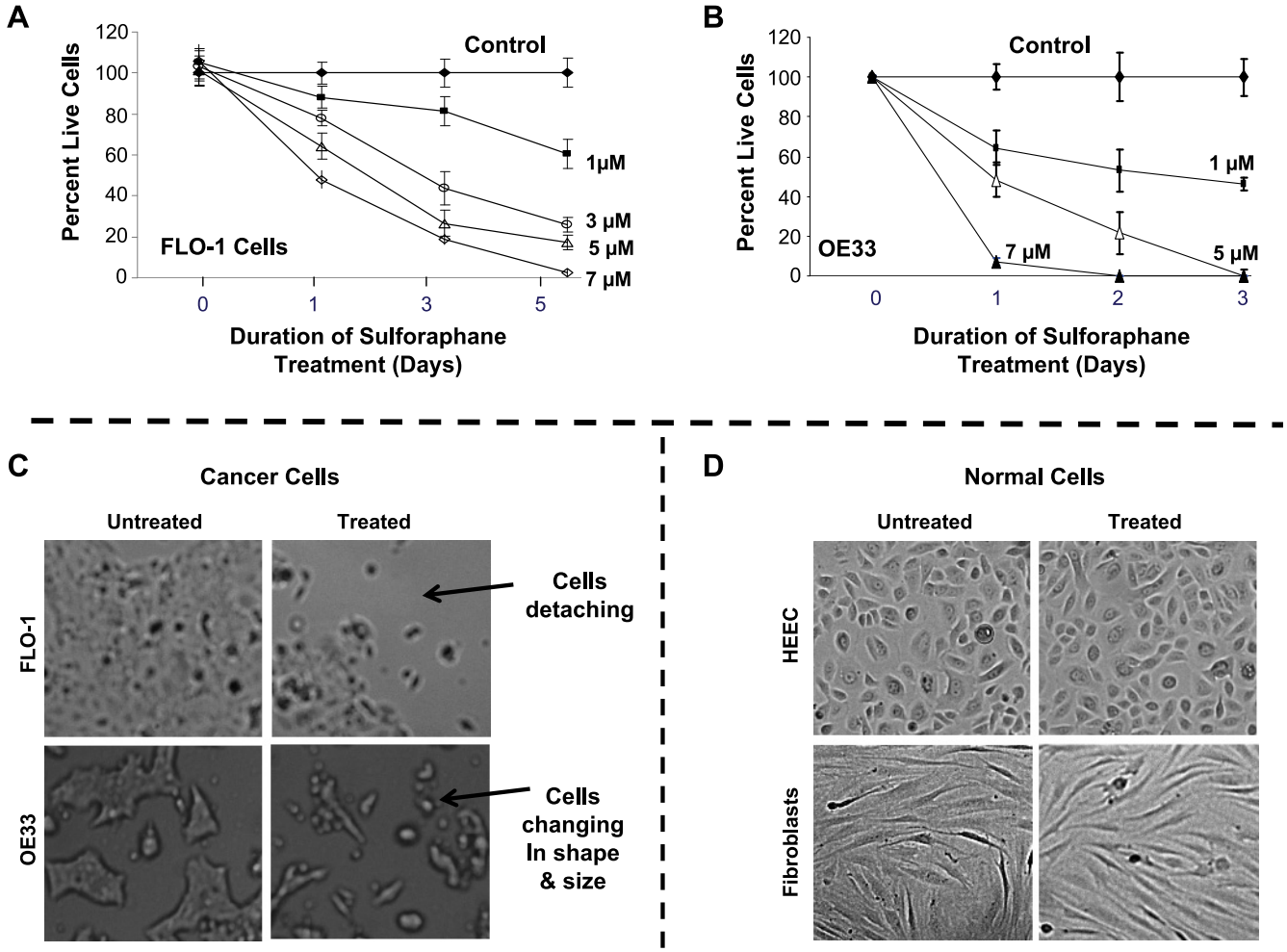

Figure 1. Effect of SFN on BEAC cell survival. BEAC cells were cultured in the medium containing no SFN or various concentrations of SFN. Cells were harvested at different time points as indicated and proliferative potential was assessed by trypan blue exclusion and/or proliferation assay, based on the production of a yellow product (formazan) after reduction of a highly water-soluble tetrazolium salt by dehydrogenases in viable cells. The growth curves show the mean of three independent experiments, with SEM. (A) Barrett adenocarcinoma (FLO-1) cells treated with various concentrations of SFN. (B) BEAC (OE33) cells treated with various concentrations of SFN. (C) Photomicrograph of BEAC (FLO-1 and OE33) cells treated with 3 μM SFN for 72 hours. (D) Photomicrograph of normal diploid fibroblasts and primary normal esophageal epithelial cells (ScienCell Research Laboratories) treated with 3 μM SFN for 72 hours. (E) FLO-1 cells were treated with SFN for 48 hours, detached floating cells from the medium and the attached cells (by trypsinization) were collected separately and evaluated for number and viability using trypan blue exclusion. The number of cells detached after treatment with various concentrations of SFN is expressed as percent of untreated FLO-1 cells. "Total" represents the total number of detached cells whereas "Dead" reflects the fraction of dead cells in detached cell population. (F) Panel (I): FLO-1 cells were incubated with various concentrations of SFN for 48 hours, and the expression of caspase 8 was detected by Western blot analysis, using anti–caspase 8 mouse monoclonal antibody (Cell Signaling, Danvers, MA). Panel (II): Bar graph showing caspase 8 expression relative to β-actin.

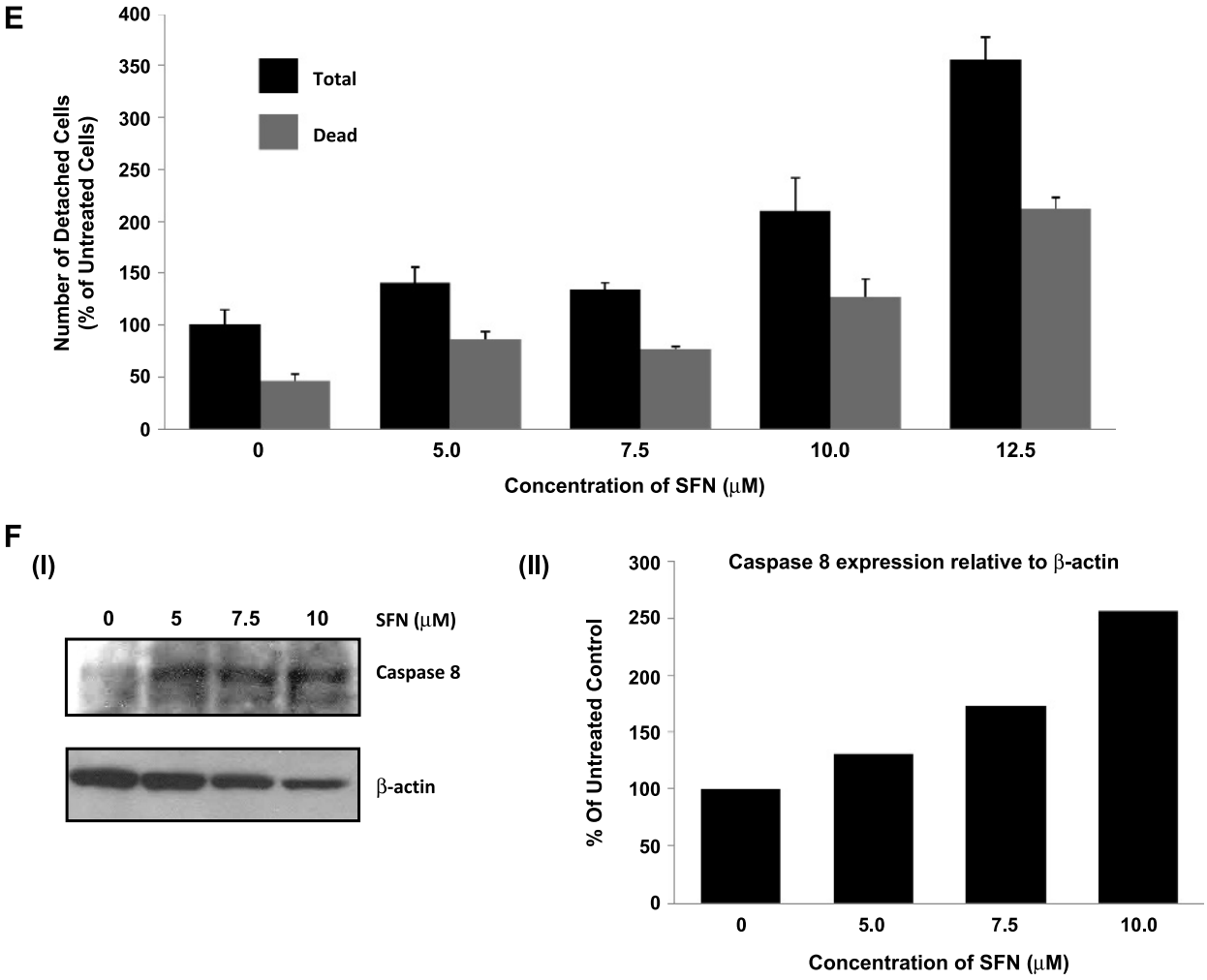

Figure 1. (*continued*).

Apoptosis Assay

Apoptotic cells were detected using the Annexin V–Biotin Apoptosis Detection Kit (Oncogene Research Products, San Diego, CA). Untreated or treated BEAC cells (1×10^6 cells/ml) were mixed with annexin V–biotin and medium-binding reagent and incubated in the dark for 15 minutes at room temperature. Cells were then centrifuged, and the medium was replaced with 1× binding buffer (Oncogene Research Products) containing fluorescein isothiocyanate (FITC)–streptavidin (Amersham Life Sciences, Inc, Arlington Heights, IL). A portion of the cell suspension (50 μl) was placed onto a glass slide, covered with a coverslip, and viewed immediately using a fluorescence microscope equipped with FITC (green) filter. Two hundred cells, representing at least five distinct microscopic fields, were analyzed to assess the fraction of FITC-labeled cells for each sample.

Western Blot Analysis

Approximately 50 mg of protein was suspended in Laemmli sample buffer (0.1 M Tris-HCl buffer pH 6.8, 1% SDS, 0.05% β-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue), boiled for 2 minutes, and electrophoresed on 4% to 20% glycerol gradient SDS–polyacrylamide gel for 4 hours at 120 V. Gels were electroblotted onto Trans-Blot nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) at 40 V for 3 hours in a Tris-glycine buffer system. Incubation with indicated antibodies was performed for 2 hours in PBS–Tween 20 (PBST) containing 1% BSA with constant rocking. Blots were washed with PBST and incubated in either antirabbit or antimouse horseradish peroxidase (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) conjugates for 2 hours in PBST containing 3% nonfat dry milk. After washing, specific proteins were detected using an enhanced chemiluminescence, according to the instructions provided in the manual (Amersham Life Sciences, Inc).

In Vivo *Study*

The *in vivo* efficacy of SFN was tested in a murine xenograft model of BEAC in which FLO-1 cells were injected subcutaneously in severe combined immunodeficient (SCID) mice. After detection of tumors, mice were treated with either 0.75 mg of SFN or 10% DMSO subcutaneously daily for 2 weeks. Tumor growth was measured in two perpendicular dimensions once every 3 days using a caliper and the following formula: $V = (a^2 \times b) / 2$, where a is the width of the tumor (smaller diameter) and b is the length (larger diameter).

Results

SFN Induces Time- and Dose-Dependent Decline in Survival of BEAC Cells

FLO-1 and OE33 cells were cultured in the presence or absence of SFN at various concentrations and for variable length of time, substrate-

attached viable cell number was counted, and cell viability was confirmed by trypan blue exclusion or CCK 8 assays (Figure 1, *A* and *B*). SFN induced both time- and dose-dependent decline in survival of BEAC cells (Figure 1). Exposure to 7 μM SFN led to a complete cell death in both BEAC cell lines tested, in a period of 3 to 5 days (Figure 1, *A* and *B*). A substantial antiproliferative activity was also seen by SFN at 5 μM, which killed 100% of OE33 cells in 3 days and 83% ± 4% of FLO-1 cells in 5 days. The treatment of FLO-1 cells with 3 μM SFN led to 74% ± 4% cell death in 5 days. At 1 μM, SFN was less affective and killed 54% of OE33 cells in 3 days and 39% of FLO-1 cells in 5 days (Figure 1). These data indicate that SFN inhibits the proliferation of BEAC cells. To evaluate the effect of SFN on the morphology of treated cells, both the cancer (FLO-1, OE33) and normal (fibroblasts, primary esophageal epithelial) cells were treated with 3 μM SFN for 72 hours and photographed under a phase-contrast microscope. As shown in Figure 1 (*C* and *D*), whereas the cancer (FLO-1 and OE33) cells started to detach and/or reduce in size and number, both types of normal cells (fibroblasts and esophageal epithelial cells) remained unaffected by the treatment.

To evaluate the nature of detached cells, FLO-1 cells were treated with SFN for 48 hours. Detached floating cells from the medium and the attached cells (by trypsinization) were collected separately and evaluated for the number and viability using trypan blue exclusion. Figure 1*E* shows the relative proportion of cells detached after treatment with various concentrations of SFN, expressed as percent of untreated FLO-1 cells. The total number of detached cells increased with increasing concentrations of SFN. Although majority (~60%) of detached cells were dead, approximately 40% appeared alive, indicating detachment before complete death (Figure 1*E*).

Consistent with decline in cell viability and attachment, Western blot analysis indicated that treatment of BEAC cells with SFN leads to a marked induction of caspase 8, an initiator caspase implicated in death receptor–induced apoptosis (Figure 1*F*).

SFN Increases Intracellular Accumulation of Drug in BEAC Cells

To evaluate if SFN can increase intracellular drug accumulation and chemosensitivity by inhibiting drug efflux, we used rhodamine efflux assay. Rhodamine 123 is a substrate of MRP and P-glycoprotein, a product of multidrug resistance gene (*MDR*), implicated in the extrusion of drugs outside the cell. We therefore exposed BEAC cells, pretreated with SFN or cyclosporin A (broad-spectrum inhibitor of multidrug resistance gene products), to rhodamine 123. Rhodamine-containing medium

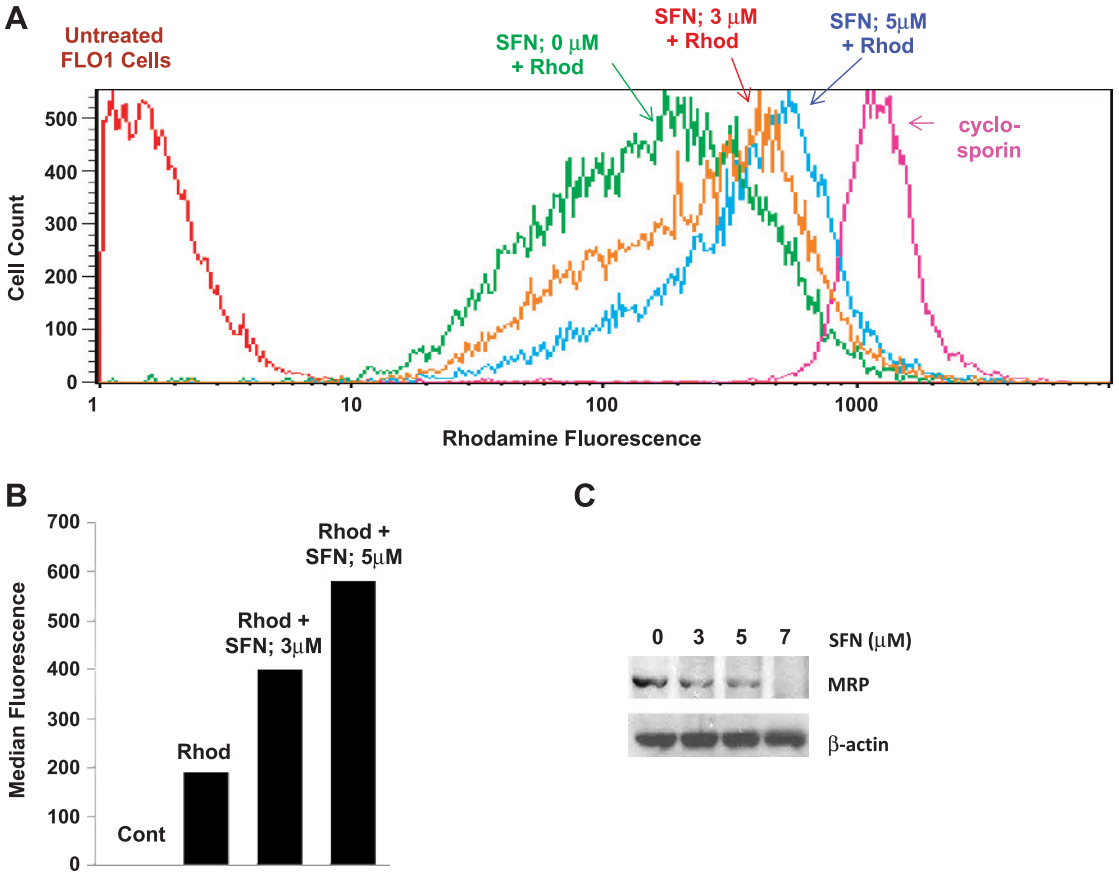

Figure 2. SFN increases intracellular accumulation of drug and enhances antiproliferative activity of other anticancer agents in BEAC cells. (A and B) Effect of SFN on intracellular drug accumulation. BEAC (FLO-1) cells, pretreated with SFN or cyclosporin A, were exposed to rhodamine 123. Rhodamine was then removed, and cells were incubated overnight in the presence of corresponding drugs as described. Cells were harvested, washed, and evaluated for rhodamine 123 florescence by flow cytometry. (A) The amount of rhodamine 123 fluorescence retained was measured using a fluorescence-activated cell flow analyzer. (B) Bar graph showing dose-dependent increases in the accumulation of intracellular rhodamine 123 in FLO-1 cells treated with different concentrations of SFN. (C) FLO-1 cells were treated with various concentrations of SFN for 24 hours, and the expression of MRP was monitored by Western blot analysis using MRP (E-19) antibody (Santa Cruz Biotechnology, Inc).

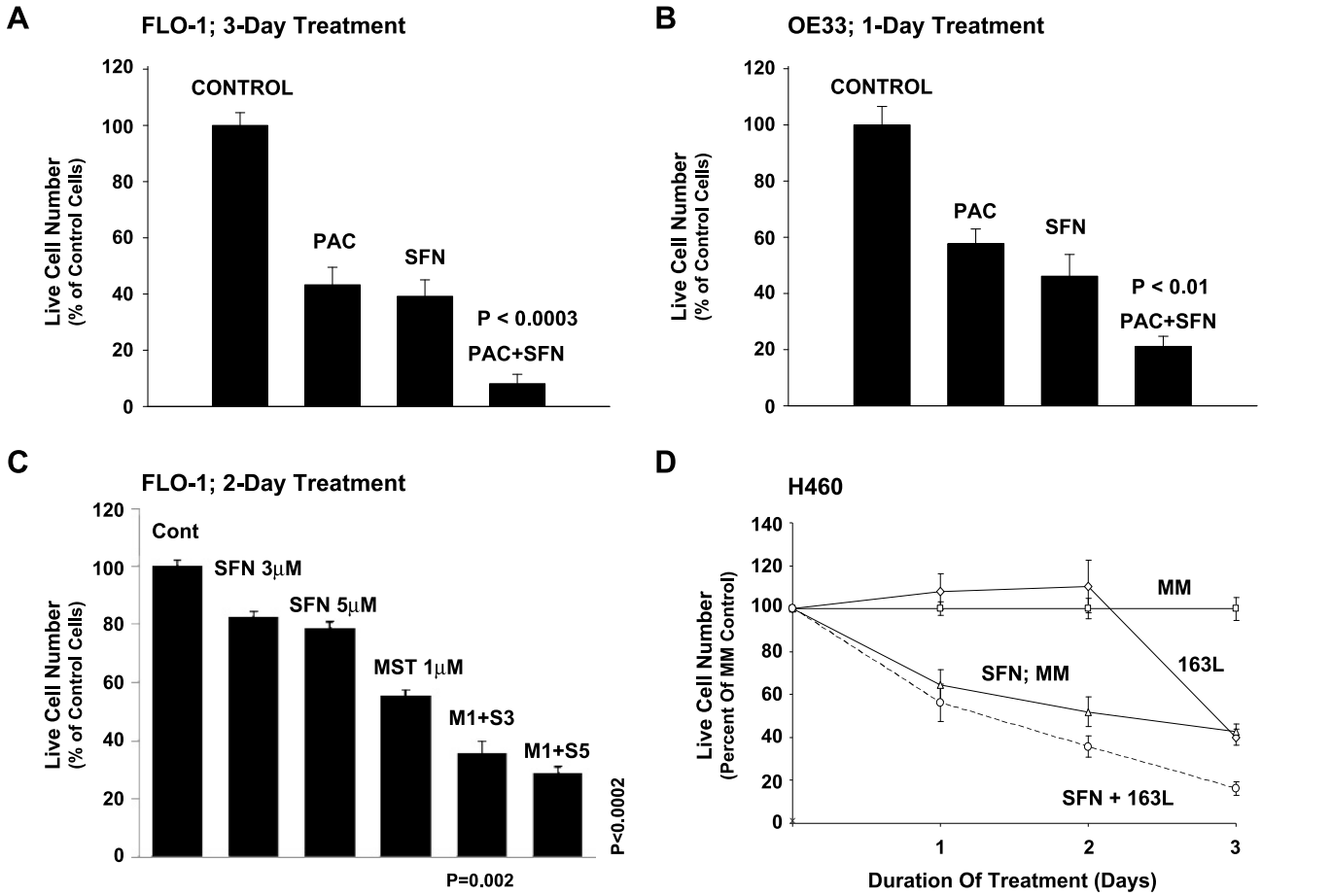

Figure 3. Effect of SFN on the antiproliferative activity of other agents. BEAC cells, FLO-1 (A) or OE33 (B), were cultured in the medium containing no drug, 1 μM paclitaxel, 3 μM SFN, or a combination of both 1 μM paclitaxel and 3 μM SFN. Cells were harvested at indicated time points, substrate-attached live cell number was determined, and cell viability was confirmed by trypan blue exclusion and/or proliferation assays described. Bar graphs show the mean of three independent experiments, with SEM. (C and D) Effect of SFN on antiproliferative activity of FLO-1 and H460 adenocarcinoma cells pretreated with telomerase inhibitors. FLO-1 cells (C), pretreated with MST-312 (1 μM) for 10 days, were treated with SFN (3 or 5 μM) for 48 hours, and live cell number was determined. H460 lung adenocarcinoma cells (D), treated with GRN163L or mismatch oligonucleotide at 2 μM for 10 days, were exposed to SFN (2 μM) and evaluated for live cell number everyday for the next 3 days. Error bars represent SEMs of triplicate experiments.

was then removed, and cells were washed with PBS and incubated overnight in the presence of corresponding drugs without rhodamine. Cells were harvested, washed with PBS, and evaluated for rhodamine 123 florescence by flow cytometry. Figure 2 (*A* and *B*) shows a dose-dependent increase in the accumulation of intracellular rhodamine 123 in FLO-1 cells treated with different concentrations of SFN. These data indicate that SFN inhibits the drug efflux, thus increasing the intracellular concentration and chemosensitivity. Consistent with these data, Western blot analysis showed down-regulation of MRP in SFN-treated, relative to untreated FLO-1 cells (Figure 2*C*).

SFN Significantly Enhances the Antiproliferative Effect of Chemotherapeutic and Telomerase-Inhibiting Agents in BEAC Cells

We next evaluated if SFN can enhance the antiproliferative affect of other anticancer agents. For this purpose, we chose paclitaxel (a chemotherapeutic agent used for treatment of BEAC), MST-312 (a chemical inhibitor of telomerase), and GRN163L (a lipidated oligonucleotide targeting RNA component of telomerase). For SFN-PAC combination study, we first cultured the FLO-1 and OE33 cells in the presence of paclitaxel at various concentrations and for variable length of time and evaluated for cell viability as described (not shown). Concentrations of SFN and paclitaxel inducing similar cell death at a given time point were used for combination study. As shown in Figure 3*A*, the treatment of FLO-1 cells with 1 μM paclitaxel and 3 μM SFN for 3 days led to 57% ± 6% and 61% ± 6% cell death, respectively. However, a combination of both drugs led to 92% ± 3% cell death, indicating a significant ($P > .0003$) 35% increase in cell death relative to paclitaxel alone (Figure 3*A*). Similarly, in OE33 cells, paclitaxel alone induced 42% ± 5% cell death, whereas a combination of paclitaxel and SFN led to 79% ± 4% cell death, showing a significant ($P < .01$) 37% increase in cell death relative to paclitaxel alone (Figure 3*B*). These data show that SFN can significantly enhance the antiproliferative effect of paclitaxel in BEAC cells.

To evaluate the effect of SFN on antiproliferative effect of telomerase inhibiting agents, FLO-1 cells were pretreated with a telomerase inhibitor MST-312 (1 μM) for 10 days to initiate telomere shortening and were then treated with SFN (3 or 5 μM) for 48 hours. As shown in Figure 3*C*, the treatment of FLO-1 cells pretreated with MST-312 to SFN led to a significant ($P \leq .002$) decrease in cell growth, relative

to cells treated with MST-312 or SFN alone. SFN at 3 and 5 μM increased the cell death by 36% and 48%, respectively, in FLO-1 cells pretreated with MST-312. Similar observations were also made in H460 lung adenocarcinoma cells treated with telomerase inhibitor GRN163L, a lipid-attached oligonucleotide-targeting RNA component of telomerase. H460, pretreated with GRN163L or mismatch oligonucleotide at 2 μM for 10 days, was exposed to SFN (2 μM) and evaluated for live cell number everyday for next 3 days. Addition of SFN to cells pretreated with 163L reduced the live cell number to 16.3% ± 3.2% in 3 days, whereas ~40% cells were still alive when drugs were used separately (Figure 3*D*). These data indicate that SFN significantly enhances the anticancer activity of chemotherapeutic and other antiproliferative agents.

SFN Inhibits Cell Cycle Progression and Enhances the Ability of Paclitaxel to Induce Cell Cycle Arrest

Paclitaxel is a microtubule inhibitor and is known to induce cell cycle arrest at G_2-M. In this study, we evaluated the effect of SFN on cell cycle progression and on paclitaxel-induced cell cycle arrest in BEAC cells. FLO-1 cells were treated with SFN (3 μM), paclitaxel (1 μM), or both for 3 days, and the effect on cell cycle progression was determined using the Cell Cycle Phase Determination Kit (Cayman Chemical Company). Briefly, the control and treated cells were fixed, stained with propidium iodide, and analyzed using a flow cytometer. Although 41% of control cells were in the S-phase, the fraction of cells in the S-phase after treatment with paclitaxel and SFN was only 18% and 24%, respectively (Figure 4*A*). Consistent with cell viability data, a more powerful inhibition of the S-phase was observed when paclitaxel was combined with SFN (Figure 4*A*, *panel IV*). Cells treated with paclitaxel were arrested at G_2-M (55%) or G_1 (27%), whereas those treated with SFN were mostly at G_1. However, when SFN was added along with paclitaxel, majority of cells were arrested at the G_1 phase of the cell cycle (Figure 4*A*).

Consistent with cell cycle arrest, the treatment with paclitaxel, SFN, and the combination of both drugs led to 5-fold, 9-fold, and

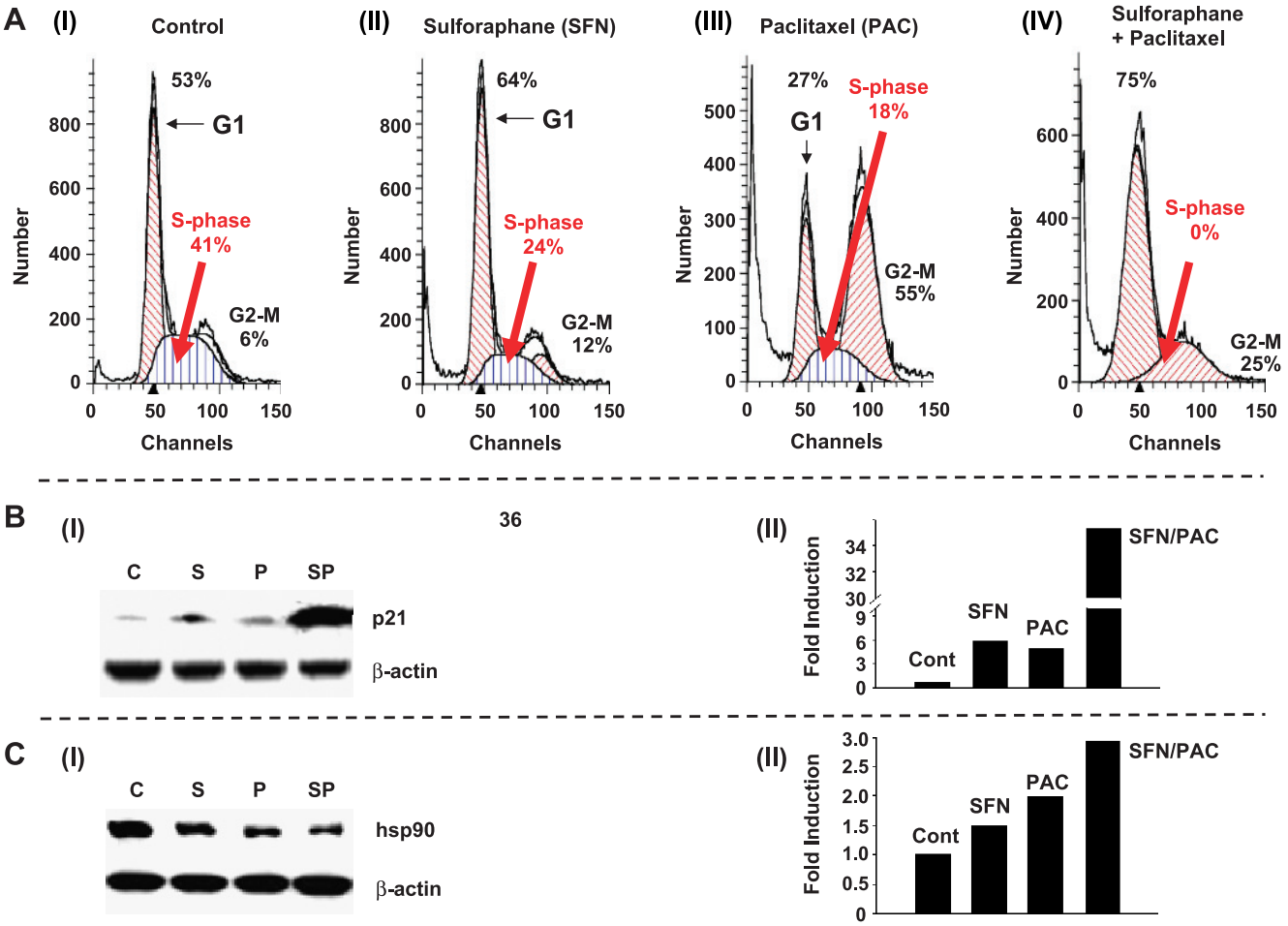

Figure 4. Effect of SFN, paclitaxel, and combination treatments on cell cycle in BEAC cells. (A) Barrett adenocarcinoma (FLO-1) cells, untreated or treated with SFN (3 μM), paclitaxel (1 μM), or both for 72 hours, were processed for evaluation of cell cycle using the Cell Cycle Phase Determination Kit (Cayman Chemical Company). Phases of cell cycle were analyzed in the FL2 channel of a flow cytometer with a 488-nm excitation filter. Panels: (I) untreated BEAC (FLO-1) cells, (II) BEAC (FLO-1) cells treated with SFN, (III) BEAC (FLO-1) cells treated with paclitaxel, and (IV) BEAC (FLO-1) cells treated with both the paclitaxel and SFN. (B) Induction of p21 by SFN. Panels: (I) Western blot showing protein levels of p21 after the above treatments and (II) bar graph showing fold induction in p21 protein levels in those treated relative to control cells, after normalization with β-actin. (C) Suppression of hsp90 by SFN: Transcript levels of hsp90 in FLO-1 cells after treatments described above. Panels: (I) Protein levels of hsp90 after the above treatments and (II) bar graph showing fold reduction in hsp90 protein levels in treated relative to control cells after normalization with β-actin.

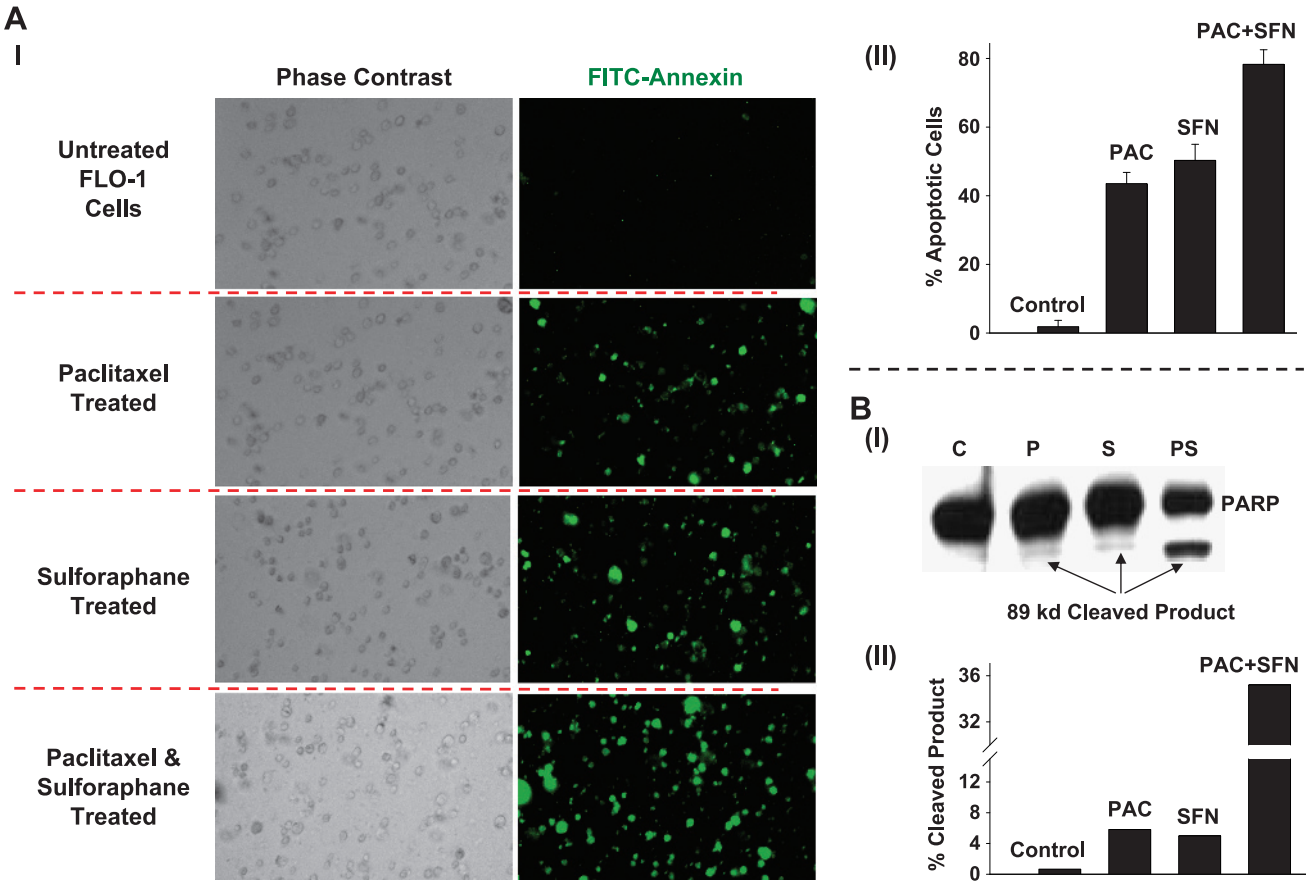

Figure 5. Effect of SFN, paclitaxel, and combination treatments on apoptosis in BEAC cells. (A) BEAC (FLO-1) cells, untreated or treated with SFN (3 μM), paclitaxel (1 μM), or both for 72 hours, were analyzed for apoptosis using the Annexin V–Biotin Apoptosis Detection Kit. Cells were sequentially treated with annexin V–biotin and FITC-streptavidin. FITC-streptavidin–labeled apoptotic cells within the same microscopic field were viewed and photographed by phase-contrast (PC) or by fluorescence emitted at 518 nm (FITC filter). Using the FITC filter, apoptotic cells appear bright green. Panels: (I) Annexin labeling of FLO-1 cells, untreated or treated as described and (II) bar graph showing percent apoptotic cells after each treatment. (B) FLO-1 cells were treated as described for panel A but for a duration of 48 hours and analyzed for cleavage of PARP, a marker for apoptosis. Panels: (I) PARP was identified by Western blot analysis using a rabbit polyclonal antibody against PARP (Santa Cruz Biotechnology, Inc) and (II) bar graph showing percentage of cleaved 89-kDa PARP fragment.

57-fold induction of cell cycle inhibiting protein, p21 (Figure 4*B*, *panels I* and *II*). These data not only confirm that p21 was induced after SFN treatment but also demonstrate a marked effect of the combination treatment in BEAC cells. SFN treatment also led to the down-regulation of hsp90, a molecular chaperone required for activity of several proliferation-associated proteins (Figure 4*C*). The suppression of hsp90 was also the strongest when SFN was used in combination with paclitaxel.

SFN Induces Apoptotic Cell Death and Enhances the Proapoptotic Activity of Paclitaxel

BEAC cells (FLO-1 and OE33) were treated with SFN (3 μM) and paclitaxel (1 μM) and analyzed for apoptotic cell death. Both untreated or BEAC cells were sequentially treated with annexin V–biotin and FITC-streptavidin and apoptotic cells were evaluated by a fluorescence microscope. Approximately 200 cells, representing at least five distinct microscopic fields, were analyzed to assess the fraction of annexin-positive cells for each sample. After a 3-day exposure, 43% ± 6% of cells treated with paclitaxel, 50% ± 7% of cells treated with SFN, and 80% ± 8% of cells treated with both paclitaxel and SFN were annexin V–positive (Figure 5*A*). These data indicate that both paclitaxel and SFN induce apoptosis in BEAC cells, and the addition of SFN significantly increases the fraction of cells undergoing apoptosis. To further evaluate the effect of these treatments and their combination on apoptosis, we analyzed poly(ADP-ribose) polymerase (PARP), a protein which undergoes caspase 3–mediated cleavage during apoptosis. At an earlier time point of 48 hours, the cells treated with paclitaxel or SFN alone had only 5% to 6% of cleaved PARP product, whereas a substantial fraction (35%) of PARP was found to be cleaved in the cells treated with combination of both drugs (Figure 5*B*).

In Vivo *Efficacy of SFN*

In vivo efficacy of SFN was demonstrated in a murine model in which SCID mice were subcutaneously inoculated in the interscapular area with human Barrett adenocarcinoma (BEAC; FLO-1) cells. After detection of tumors, mice were treated with either 0.75 mg of SFN or 10% DMSO subcutaneously daily, for 2 weeks. Tumor growth was measured in two perpendicular dimensions, once every 3 days, using a caliper. As shown in Figure 6*A*, the tumor size was

significantly reduced in the mice treated with SFN, relative to control mice. In Figure 6*B*, the tumor sizes in individual mice show that tumors in three of four treated mice were smaller than any control mice. These data show that SFN has significant antitumor activity *in vivo*.

Discussion

In this study, we have evaluated the therapeutic potential of SFN, an antioxidant derived from broccoli, in BEAC. We have found that: 1) SFN induces both the cell cycle arrest and apoptosis in BEAC cell lines tested and inhibits tumor growth in a subcutaneous model of BEAC *in vivo*. 2) The concentration of SFN (3-7 μM) required to induce cell cycle arrest and apoptosis in BEAC cells is lower than that (10-20 μM) reported for similar affects in several other cancer cell types. 3) Treatment of FLO-1 cells with SFN is associated with down-regulation of MRP and reduction in drug efflux. 4) SFN significantly enhances the antiproliferative activity of other antiproliferative agents (paclitaxel, a chemotherapeutic used in the treatment of BEAC) and telomerase inhibitors MST-312 and GRN163L. 5) Addition of SFN with paclitaxel leads to a marked increase in the expression of p21, cell cycle arrest, and apoptosis in BEAC cells. 6) Anticancer activity of SFN could be attributed, at least in part, to the induction of caspase 8 and p21 and the suppression of MRP and hsp90, a molecular chaperone required for activity of several proliferation associated proteins.

Anticancer effects of SFN have been demonstrated in several malignancies including human colon, bladder, prostate, ovarian, lymphoblastoid, pancreatic, cervical cancer, and lung cancers [15,17,31–36]. However, to our knowledge, this is the first report demonstrating the ability of SFN to induce apoptotic cell death, increase chemosensitivity, and significantly enhance the antiproliferative effects of chemotherapeutic and telomerase-inhibiting agents in BEAC cells. Moreover, the SFN could induce apoptosis in BEAC cell lines at 3 to 7 μM, a concentration lower than 10 to 40 μM, reported to induce apoptotic cell death in most other cancers including colon, ovarian, non–small lung, cervical, breast, lung adenocarcinoma, hepatoma, and prostate [10,15,18,31–39], indicating a greater sensitivity of this agent for BEAC.

SFN also significantly enhanced the antiproliferative activity of other anticancer agents including a chemotherapeutic agent, paclitaxel, used for the treatment of BEAC. A combination of SFN and paclitaxel led to a significant increase in the fraction of apoptotic cells, as indicated by annexin labeling (Figure 5). To further confirm the apoptosis and the increased efficacy of combination treatment, we analyzed the treated cells for PARP cleavage at an earlier time point of 48 hours. In the cells treated with paclitaxel, SFN, or both drugs, the fraction of PARP cleaved into the 89-kDa fragment was 6%, 5%, and 35%, respectively. These data indicate that the combination of both the drugs leads to a remarkable increase in apoptotic activity and is consistent with the significant increase in cell death and annexin labeling after exposure of BEAC cells to both the paclitaxel and SFN.

SFN also significantly enhanced the antiproliferative activity of a chemical inhibitor of telomerase (MST-312) in BEAC cells. A similar effect was also observed in lung adenocarcinoma H460 cells in which SFN significantly enhanced the antiproliferative activity of GRN163L, an oligonucleotide targeting the RNA component of telomerase. Consistent with these observations, we have found that SFN downregulates MRP and reduces drug efflux in BEAC cells. Because MRPs have been implicated in the export of chemotherapeutic drugs including paclitaxel [40], it is possible that SFN enhances the chemosensitivity

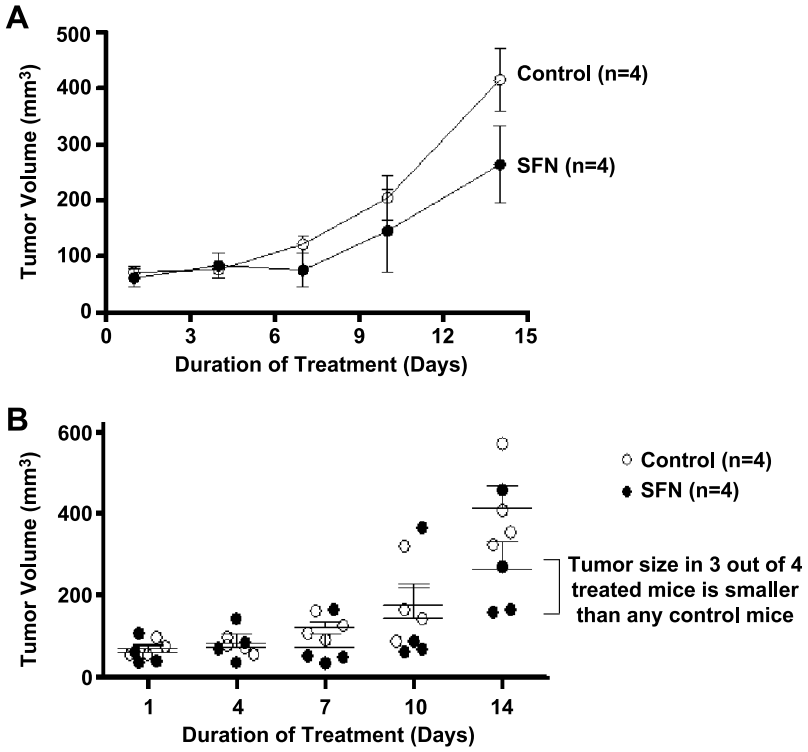

Figure 6. *In vivo* efficacy of SFN in BEAC. SCID mice were inoculated subcutaneously in the interscapular area with FLO-1 (BEAC) cells, and after the appearance of tumors, mice were treated intraperitoneally with PBS alone or SFN 25 mg/kg (5 times per week). Average tumor sizes in all treated and control mice (A) and tumor sizes in individual mice (B) are shown.

of other antiproliferative agents, at least in part, by increasing intracellular drug concentration.

Exposure of BEAC cells to SFN was associated with both the cell cycle arrest and apoptosis, and it is consistent with the observations made in other cancer cell types including, osteosarcoma, ovarian carcinoma, colon, prostate, and lung cancers [1,14,19,32,41,42]. As shown in Figure 4, the treatment with SFN led to the accumulation of FLO-1 cells at the G_1 phase of the cell cycle. Although a G_2/M cell cycle arrest is more commonly seen after exposure of cancer cells to SFN [15,18,43], arrest at G_1 has been observed for human colon cancer (HT-29) cells [44]. Consistent with our observations in BEAC cells, the G_1 arrest in colon HT-29 cells was also associated with induction of p21.

Several mechanisms have been proposed for induction of apoptosis after treatment of cancer cells with SFN. SFN-induced apoptosis in colon cancer (HT-29) cells is attributed to up-regulation of Bax and release of cytochrome *C* from mitochondria [16]. In both the prostate and colon cancer cells, SFN treatment was associated with inhibition of histone deacetylase, leading to increased histone acetylation and apoptosis [42,45]. SFN has also been reported to induce apoptosis through activation of AP-1 [46], activation of MAPK pathways (ERK, JNK, p38) [44,47], and down-regulation of nuclear factor κB [48]. In our study, the apoptosis in BEAC cells could be attributed, at least in part, to the induction of caspase 8 and p21 and suppression of hsp90. Exposure of BEAC cells to SFN led to a marked induction of caspase 8, an initiator caspase implicated in death receptor–mediated apoptosis [45]. Consistent with this, the transcript levels of caspase 8 and several death receptors were also elevated in SFN-treated BEAC cells (not shown). SFN, paclitaxel, and the combination of both led to 9-fold, 5-fold, and 57-fold induction of p21 in FLO-1 cells, respectively. These data indicate that there could be a remarkable effect of adding SFN with paclitaxel in treating BEAC.

In summary, these studies demonstrate that SFN inhibits the proliferation of BEAC cells at a nontoxic concentration, reduces tumor growth *in vivo*, and significantly enhances the anticancer activity of other chemotherapeutic and antiproliferative agents in BEAC. These data therefore indicate that a natural product with antioxidant properties from broccoli has a specific activity against BEAC, making it an ideal compound for therapy and possible chemoprevention of this disease.

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