Autophagy Inhibition Enhances Sulforaphane-induced Apoptosis in Human Breast Cancer Cells

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Abstract. *Aim: Sulforaphane (SFN), which is present in cruciferous vegetables, induces growth arrest and/or cell death in cancer of various organs. The involvement of autophagy in the SFN-induced apoptotic death of human breast cancer cells was investigated. Materials and Methods: Cell proliferation and viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue exclusion assay. Flow cytometry, immunofluorescence, electron microscopy, and Western blot analysis were used for detection of apoptosis and autophagy, and the role of autophagy was assessed using autophagy inhibitors. Results: SFN dose- and time-dependently retarded the growth and induced cell death in MCF-7 and MDA-MB-231 human breast cancer cells. In MDA-MB-231 cells, 30 μM SFN caused S and G_2/M cell-cycle arrest associated with increased $p21^{WAF1}$ and $p27^{KIP1}$ levels and decreased cyclin A, cyclin B1 and CDC2 levels. Cell death was due to apoptosis with increased caspase-3 and lowered BCL-2 levels. In addition, the SFN-treated cells exhibited autophagy, as characterized by the appearance of autophagic vacuoles by electron microscopy, the accumulation of acidic vesicular organelles by flow cytometry, and the punctuate patterns of microtubule-associated protein 1 light chain 3 (LC3) by fluorescein microscopy. The levels of LC3-I and -II proteins (processed forms of LC3-I) and LC3 mRNA were increased. Treatment with autophagy inhibitor bafilomycin A1 (but not 3-methyladenine) with SFN significantly enhanced apoptosis, which was accompanied by increases in the level of BAX and the cleavage of caspase-3 and poly(ADP-ribose)polymerase (PARP)-1 and decreases in the mitochondrial membrane potential ($\Delta\Psi m$). Conclusion: These results indicate a cytoprotective role of autophagy against SFN-induced apoptosis and that the combination of SFN treatment with autophagy inhibition may be a promising strategy for breast cancer control.*

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Breast cancer is the most prevalent type of cancer among women worldwide, however, there are geographic variations in its prevalence. The incidence and mortality of breast cancer is 5-times higher in Western countries than in some Asian countries, and Asian migrants to the United States eventually acquire the breast cancer incidence of their host country (1). This observation suggests the importance of environmental and lifestyle-related factors; among such factors, studies suggest that dietary factors play a key role in breast cancer etiology (2). Phytochemicals affect breast cancer, and some phytochemicals suppress the occurrence and progression of the disease (3).

Epidemiological studies have suggested the benefits of the consumption of cruciferous vegetables and the reduction in the risk of cancer at various sites (4). Sulforaphane (SFN; 1-isothiocyanato-4-(methylsulfinyl)-butane), which is found in cruciferous vegetables such as broccoli and Brussels sprouts, is a potent dietary phytochemical effective in cancer chemoprevention (5-7). Laboratory investigations with rodents and cell culture studies have shown that SFN has a potent activity against breast cancer. The administration of SFN (75 or 150 μmol) by gavage for 5 days around the time of carcinogen exposure significantly reduces the incidence and multiplicity and prolongs the latency of 7,12-dimethylbenz[α]anthracene-induced mammary cancer in rats (8). Daily intravenous injections of 15 nmol SFN for 13 days reduce the growth of F3II mouse mammary carcinoma cells subcutaneously transplanted in BALB/c mice (9). In F3II cells in culture, 15 μM SFN causes G_2/M cell-cycle arrest and induces apoptosis (9). In cultures of human breast cancer cell lines (MCF-7, T47D, MDA-MB-231, and MDA-MB-468) the IC_{50} for 72 h ranged from 8.1 to 9.5 μM (10), and 15 μM SFN induced G_2/M cell-cycle arrest through elevated cyclin B1 expression (10, 11). SFN induces apoptosis in human breast cancer cells through the activation of poly(ADP-ribose)polymerase (PARP) and caspase family

proteins (10). Moreover, SFN can more effectively inhibit the growth of MCF-7 human breast cancer cells (IC_{50} for 48 h is 27.9 μM) compared to MCF-12A normal human breast epithelial cells (IC_{50} for 48 h is 40.5 μM) (12). Thus, SFN can cause cell-cycle arrest and apoptosis induction in various human breast cancer cell lines.

Autophagy is an evolutionarily conserved catabolic process of degrading damaged proteins and/or organelles and recycling the materials to maintain the quality of cellular components (13). During autophagy, autophagosomes are formed by the elongation of double membrane-bound vesicles, and they sequester cytoplasmic constituents. Subsequently, autophagosomes fuse with lysosomes to form autolysosomes in which the incorporated organelles are degraded. Recent studies have found that autophagy plays a role in human diseases including cancer (14). Emerging evidence indicates that chemotherapeutic agents induce autophagy in various types of cancer cells (15). On one hand, autophagy exerts a cell protective role, which allows cancer cells to survive against cytotoxic agents; on the other hand, autophagy results in the cell death termed autophagic cell death or type II cell death (16, 17). Recently, SFN was shown to induce autophagy that functions as a cell protective mechanism in human prostate cancer cells (18) and in human colon cancer cells (19). However, the ability of SFN to induce autophagy in human breast cancer cells and its precise role are largely unknown. In the present study, we examined the cytotoxic effects of SFN in human breast cancer cells and the possible induction of autophagy by SFN.

Materials and Methods

Cell lines and chemicals. The human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained as described previously (20). D,L-SFN was obtained from LKT Laboratories (St. Paul, MN, USA). A stock solution of SFN (10 mM) was prepared by using dimethyl sulfoxide (DMSO) as the solvent. The autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin A1 (Baf) were obtained from Sigma (St. Louis, MO, USA) and Wako Pure Chemicals (Osaka, Japan), respectively.

Cell proliferation and viability assay. Cells were seeded at 5×10^3 cells/well on 96-well plates in growth medium supplemented with 10% fetal bovine serum. The cells were treated either with DMSO or the indicated concentrations of SFN for up to 72 h. Cell proliferation was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (20). The cell viability was assessed by trypan blue exclusion assay. After treatment with either DMSO or 30 μM SFN, the cells were collected by trypsin-EDTA and the viable cells and dead cells were counted.

Cell-cycle analysis. Cells were plated at 5×10^5 cells/100-mm dish. The cells were treated with 30 μM SFN for 72 h. At 24, 48 and 72 h after the addition of SFN, cells were collected by trypsinization and fixed in 70% ethanol. The cells were collected by centrifugation at 600×*g* for 5 min at 4˚C and washed twice with PBS. The cells were then resuspended in PBS containing 250 U/ml RNase for 20 min at room temperature and stained with 100 μg/ml propidium iodide (PI). The DNA content was examined with FACScalibar (Becton Dickinson, San Jose, CA, USA). Cell-cycle distribution was analyzed using CellQuest (Becton Dickinson) and ModiFit LT (Verity, Topsham, ME, USA).

Western blotting. Cell lysates were prepared by homogenization in RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40 and 0.1% sodium dodecyl sulfate [SDS]) supplemented with a protease inhibitor cocktail (Pierce, Rockford, IL, USA). The homogenates were incubated on ice for 30 min and then centrifuged at 15,000×*g* for 15 min at 4˚C. The protein concentration of the supernatants was measured by using the DC protein assay kit (BioRad, Hercules, CA, USA). Aliquots of lysates equivalent to 20 μg of protein were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to Hybond-P PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 5% non-fat powdered milk before incubation with the primary antibody. The blots were then washed and incubated with the appropriate secondary antibody coupled to horseradish peroxidase (HRP). The antigen-antibody complexes were detected using ECL plus reagent (Amersham Biosciences). The following primary antibodies were used: anti-p21, -p27, -cyclin B1, -CDC2, -caspase-3, -BAX, -BCL-2 and -BCL-xL antibody (Cell Signaling Technology, Beverly, MA, USA), anti-cyclin A antibody (Novocastra, Newcastle Upon Tyne, UK), anti-LC3 antibody (Abgene, San Diego, CA, USA), and HRP-conjugated anti-actin antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Electron microscopy. The ultrastructure of MDA-MB-231 cells after SFN treatment was determined by electron microscopy. After treatment with DMSO or 30 μM SFN for 72 h, the cells were collected by centrifugation, washed with PBS, fixed in ice-cold Karnovsky's solution (4% paraformaldehyde, 5% glutaraldehyde and 30 mM cacodylate buffer) at 4˚C for 1 h, washed with 0.1 M cacodylate buffer several times, and incubated at 4˚C overnight. The cells were then post-fixed in 2% osmium tetroxide at 4˚C for 1 h, resuspended in 1% sodium alginate, collected by centrifugation, and gelated by adding 1 M calcium chloride. The gelated cells were dehydrated through a graded series of ethanol (50%-100%) and propylene oxide and then processed for epon embedding. Semi-thin sections were stained with toluidine blue, and representative areas were chosen for ultrathin sectioning. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a JEM-1011 electron microscope (JEOL, Tokyo, Japan).

Quantification of acidic vesicular organelles (AVOs) with acridine orange. Autophagy is characterized by the formation of AVOs. To detect AVOs, vital staining with acridine orange was performed (21). In brief, cells were seeded at 5×10^5 cells on a 50-mm culture dish and allowed to attach. After treatment with DMSO or 30 μM SFN for 72 h, cells were stained with 1 μg/ml acridine orange for 15 min, collected by trypsinization, and resuspended in phenol red-free growth medium. Green (510-530 nm) and red (650 nm) fluorescence emission from 1×10^4 cells illuminated with blue (488 nm) excitation light was measured with FACSCalibur (Becton Dickinson) and CellQuest software (Becton Dickinson).

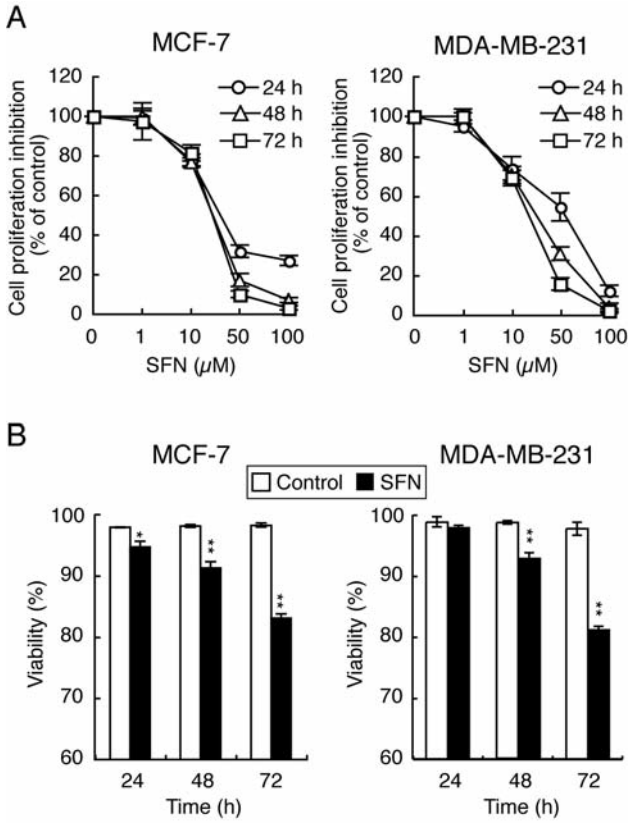
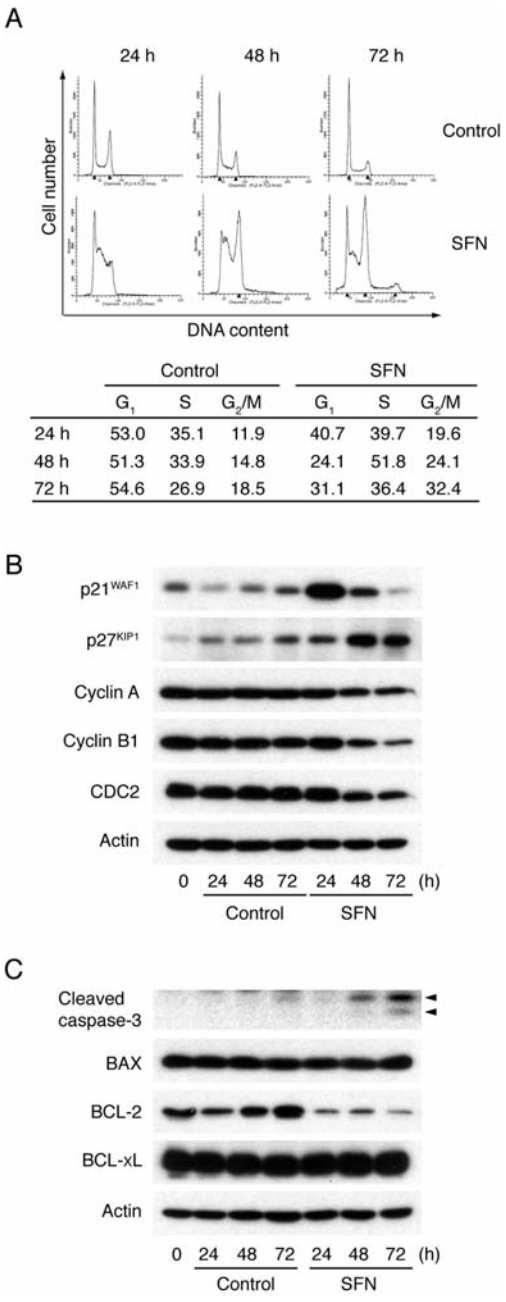


Figure 1. *Dose- and time-dependent effects of sulforaphane (SFN) on cell proliferation and cell viability in human breast cancer cells. A: For the cell proliferation assay, DMSO control and SFN-treated MCF-7 and MDA-MB-231 cells were plated on 96-well plates at 5×10^3 cells/well. Cell proliferation after the indicated amount of time was determined by MTT assay. Data are the means±SD of 3 independent experiments. B: For the cell viability assay, cells were plated on 6-well plates at 5×10^4 cells/well and treated with DMSO control or 30 μM SFN for the indicated period of time. Cell viability was counted by trypan blue exclusion assay. The data are the means±SD. *p<0.05 and **p<0.01 compared with respective controls.*



	Control			SFN		
	G_1	S	G_2/M	G_1	S	G_2/M
24 h	53.0	35.1	11.9	40.7	39.7	19.6
48 h	51.3	33.9	14.8	24.1	51.8	24.1
72 h	54.6	26.9	18.5	31.1	36.4	32.4

Figure 2. *Effects of sulforaphane (SFN) on cell-cycle progression and altered expression of cell-cycle regulatory and apoptosis-related molecules in MDA-MB-231 cells. A: Cells were treated with 30 μM SFN for the indicated period of time, and the cell-cycle distribution was analyzed by flow cytometry with propidium iodide (PI). Data are representative of at least two independent experiments. B: Whole-cell lysates from DMSO control and 30 μM SFN-treated MDA-MB-231 cells at indicated time points were subjected to SDS-PAGE, and the expression level of cell cycle-related molecules was analyzed by immunoblotting. Actin was used as a loading control. C: Whole-cell lysates from DMSO control and 30 μM SFN-treated MDA-MB-231 cells at the indicated time points were subjected to SDS-PAGE, and the expression level of apoptosis-related molecules was analyzed by immunoblotting. Actin was used as a loading control.*

Immunofluorescence localization of microtubule-associated protein 1 light chain 3 (LC3). LC3 is recruited to the autophagosome membrane and is a specific marker of autophagy (22). Immunocytochemistry for LC3 localization was performed essentially as described previously (20). In brief, MDA-MB-231 cells were grown on a 35-mm glass-bottom dish (Matsunami Glass, Osaka, Japan), treated with DMSO or 30 μM SFN for 72 h, washed with PBS, and fixed in 10% neutral buffered formalin for 30 min at room temperature. The cells were washed with Tris-buffered saline (TBS) and blocked in TBS containing 5% bovine serum albumin at room temperature for 30 min. Cells were subsequently incubated for 1 h with the anti-LC3 antibody (Abgene), washed multiple times and then incubated for 1 h with the secondary antibody (Alexa Fluor 488 anti-rabbit IgG; Molecular Probes, Eugene, OR, USA). The cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan) for 5 min. A laser

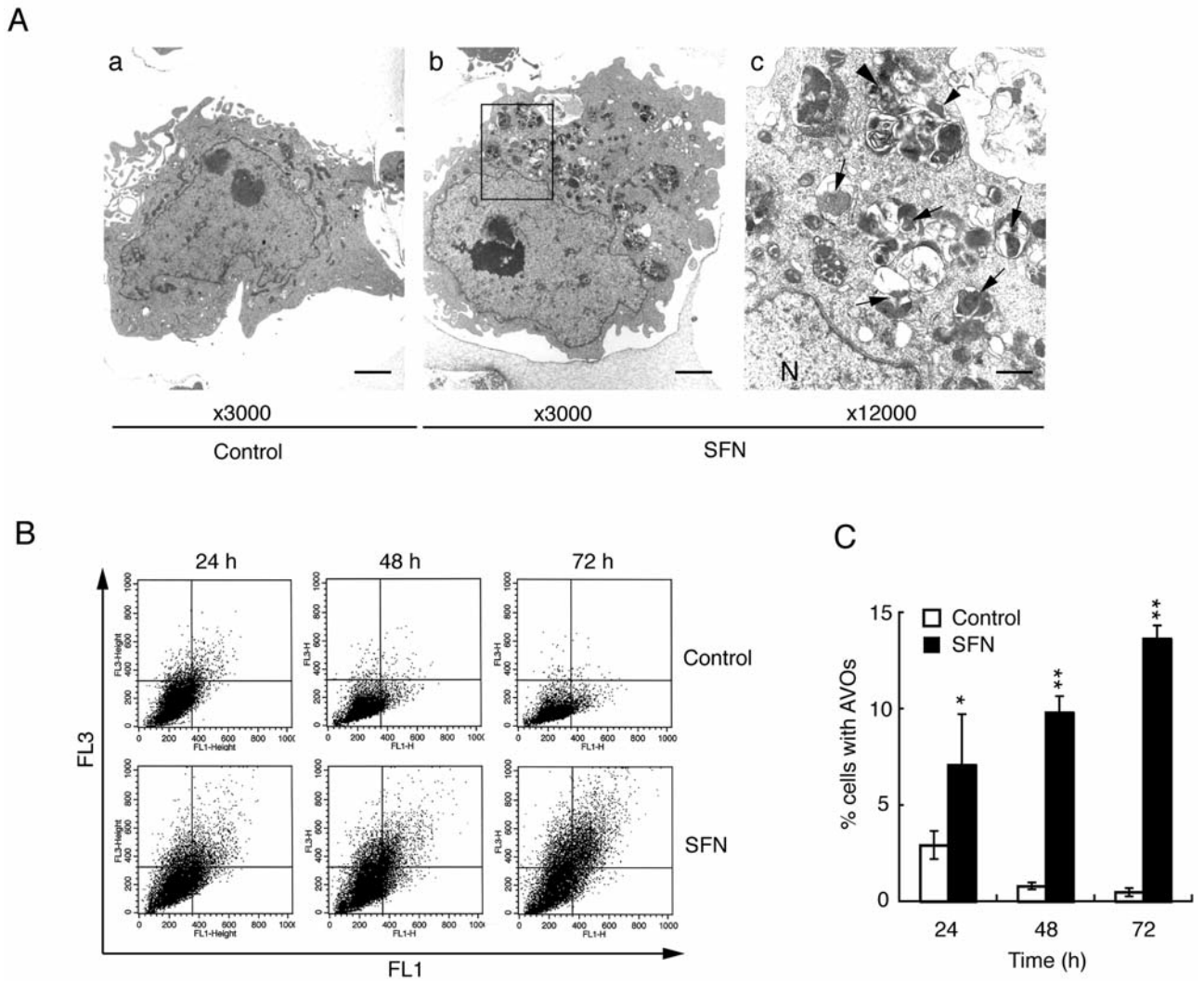


Figure 3. *Sulforaphane (SFN) induces autophagy in MDA-MB-231 cells. A: Cells were treated with 30 μM SFN for 72 h, and ultrastructural features were analyzed with electron microscopy. Panel a: DMSO control; panel b and c: 30 μM SFN treatment. Numerous autophagosome (arrow heads) and autolysosome (arrows) were observed in SFN-treated cells. B: Quantification of acridine orange-positive cells using flow cytometry. C: Representative histograms of acridine orange staining of cells treated with 30 μM SFN for 24, 48 and 72 h are shown. The data are means±SD of 3 independent experiments. *p<0.05 and **p<0.01, compared with controls.*

scanning microscope (LSM510-META, Zeiss, Jena, Germany) was used to collect images of the cells.

Quantitative analysis of LC3 protein and mRNA expression. LC3-II, the processed form of LC3-I, is associated with the autophagosome membrane (23) and is used as a molecular marker of autophagosomes. The protein levels of LC3-I (18 kDa) and LC3-II (16 kDa) were examined by Western blot. *LC3* mRNA expression was analyzed by performing quantitative real-time PCR with iCycler iQ Optical System Software version 3.0A (Bio-Rad). Total RNA from MDA-MB-231 cells was prepared by using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The cDNA was then reverse-transcribed from 1.0 μg of RNA using a SuperScript III first-strand cDNA Synthesis kit (Invitrogen, Grand Island, NY, USA). The following primers used for the quantitative real-time PCR analysis: *LC3*: 5'-ATGCCGTCGGAGAAGACCTT-3' (forward); 5'-TTAC ACTGACAATTTCATCCCG-3' (reverse) and *β-actin*: 5'-AGAAA ATCTGGCACCACACC-3' (forward); 5'-AGAGGCGTACAGGGA TAGCA-3' (reverse). PCR amplification was performed in triplicate using iQ SYBR Green Supermix (Bio-Rad). The PCR conditions were as follows: denaturing at 94˚C for 2 min, 40 cycles of 15 s at 95˚C, 15 s at 60˚C and 15 s at 60˚C. The expression was calculated using the comparative threshold cycle (CT) method (24).

Autophagy inhibition by autophagy inhibitors. To investigate the role of SFN-induced autophagy, two kinds of specific autophagy inhibitors,

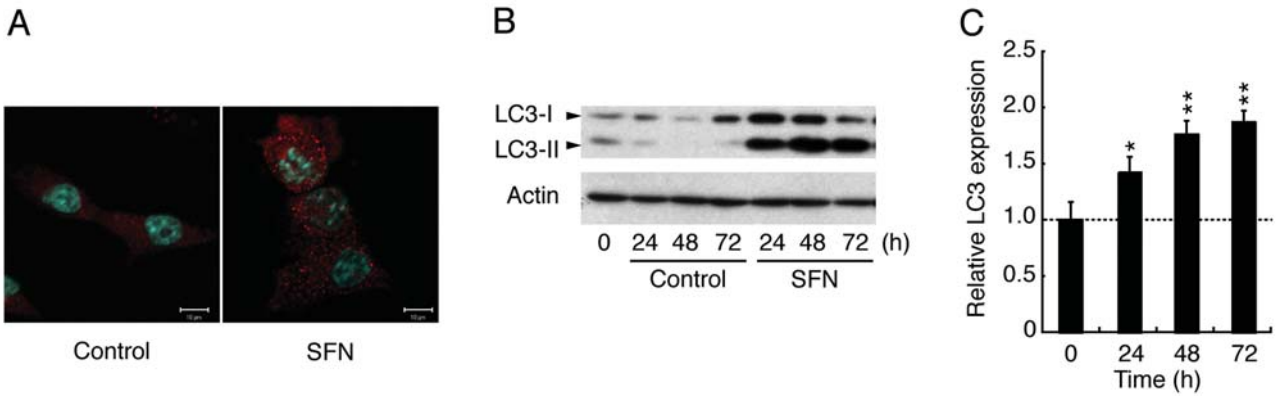


Figure 4. *Effect of sulforaphane (SFN) on LC3 expression in MDA-MB-231 cells. A: Detection of punctate pattern of LC3 by confocal laser microscopy. Immunofluorescence images of DMSO control (left) and SFN-treated (right) MDA-MB-231 cells. Note that SFN-treated cells exhibit the punctate pattern of LC3 (red). Cells were counterstained with 4',6-diamidino-2-phenylindole (green). Bar, 20 μm. B: Cell lysates from DMSO control and SFN-treated cells at indicated time points were subjected to SDS-PAGE and immunoblotted with anti-LC3 antibody. Actin was used as a loading control. C: Quantitative real-time PCR of LC3 mRNA. The expression was normalized to β-actin, and the expression level is shown relative to the value for DMSO control (designated as 1) for comparison. Data represent the means±SD of triplicate PCR assays. *p<0.05 and **p<0.01, compared with control.*

3-MA and Baf, were tested. 3-MA, a specific inhibitor of class III phosphatidylinositol-3 kinase (PI3K), inhibits autophagy at an early stage by inhibiting autophagic sequestration (25). Baf, a specific inhibitor of vacuolar-type H^+-ATPase, inhibits autophagy at a late stage by inhibiting the fusion between autophagosome and lysosome (25). MDA-MB-231 cells were treated with 30 μM SFN and 5 μM of 3-MA, or 1 nM of Baf, or left untreated. The cells were incubated for 24, 48, and 72 h, and then the AVO-positive cells were quantified.

Mitochondrial membrane potential and cell membrane integrity. Apoptosis induction in SFN-treated cells was assessed based on the mitochondrial membrane potential ($\Delta\Psi m$) and membrane integrity by double staining with rhodamine 123 (Rh123; Sigma) and PI (Sigma) (26). In brief, cells were seeded at 5×10^5 cells per 50-mm culture dish and allowed to attach. After treatment with DMSO or 30 μM SFN for up to 72 h, Rh123 (100 nM) was added 15 min prior to the trypsinization, cells were washed twice with PBS, and PI (10 μM) was added 10 min before analysis. Fluorescence intensity was measured with a FACScalibar (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson). Cells that were Rh123 (high) and PI (low) were considered as viable cells. Cells that were Rh123 (low) and PI (low) were considered as early apoptotic cells, and cells that were Rh123 (low) and PI (high) were considered as late apoptotic cells.

Statistical analysis. All discrete values, expressed as mean±SD, were analyzed using Student's *t*-test. *P*-values less than 0.05 and 0.01 were considered as significant and highly significant, respectively.

Results

SFN inhibits cell proliferation and induces cell death in MCF-7 and MDA-MB-231 human breast cancer cells. To evaluate the effect of SFN on cell proliferation, MCF-7 and MDA-MB-231 cells were treated with several concentrations (1-100 μM) of SFN for up to 72 h. The MTT assay revealed that SFN induced growth inhibition in a dose- and time-dependent manner (Figure 1A). The half maximal inhibitory concentration (IC_{50}) against MCF-7 and MDA-MB-231 cells after a 72-h treatment was 33.8±0.1 μM and 31.5±0.1 μM, respectively. To ascertain the efficacy of SFN on cell death induction, cell viability was determined by trypan blue exclusion assay (Figure 1B). SFN-treated cells exhibited a significant decrease in cell viability (MCF-7 cells, 91% at 48 h and 83% at 72 h; MDA-MB-231 cells, 92% at 48 h and 80% at 72 h) as compared with the respective control cells ($p<0.01$). In both cell lines, the viability of cells after treatment with SFN at each time point (24, 48 and 72 h) was lower than for the control cells; the reduction in cell viability indicates that cell death was induced. SFN induced growth inhibition and cell death equally in both human breast cancer cell lines. However, MDA-MB-231 cells were selected for the subsequent *in vitro* experiments because these cells are thought to be more malignant than MCF-7 cells, as judged by estrogen receptor (ER) status and intrinsic subtype classification, and are refractory in various therapeutic trials.

Effect of SFN on cell-cycle progression and the expression of cell-cycle regulatory and apoptosis-related proteins in MDA-MB-231 cells. To examine the mechanism by which SFN inhibits cell growth, the cell-cycle distribution was assessed by fluorescence-activated cell sorting (FACS) analysis. A concentration of 30 μM SFN was chosen for all cell culture experiments. A 72-h treatment with SFN

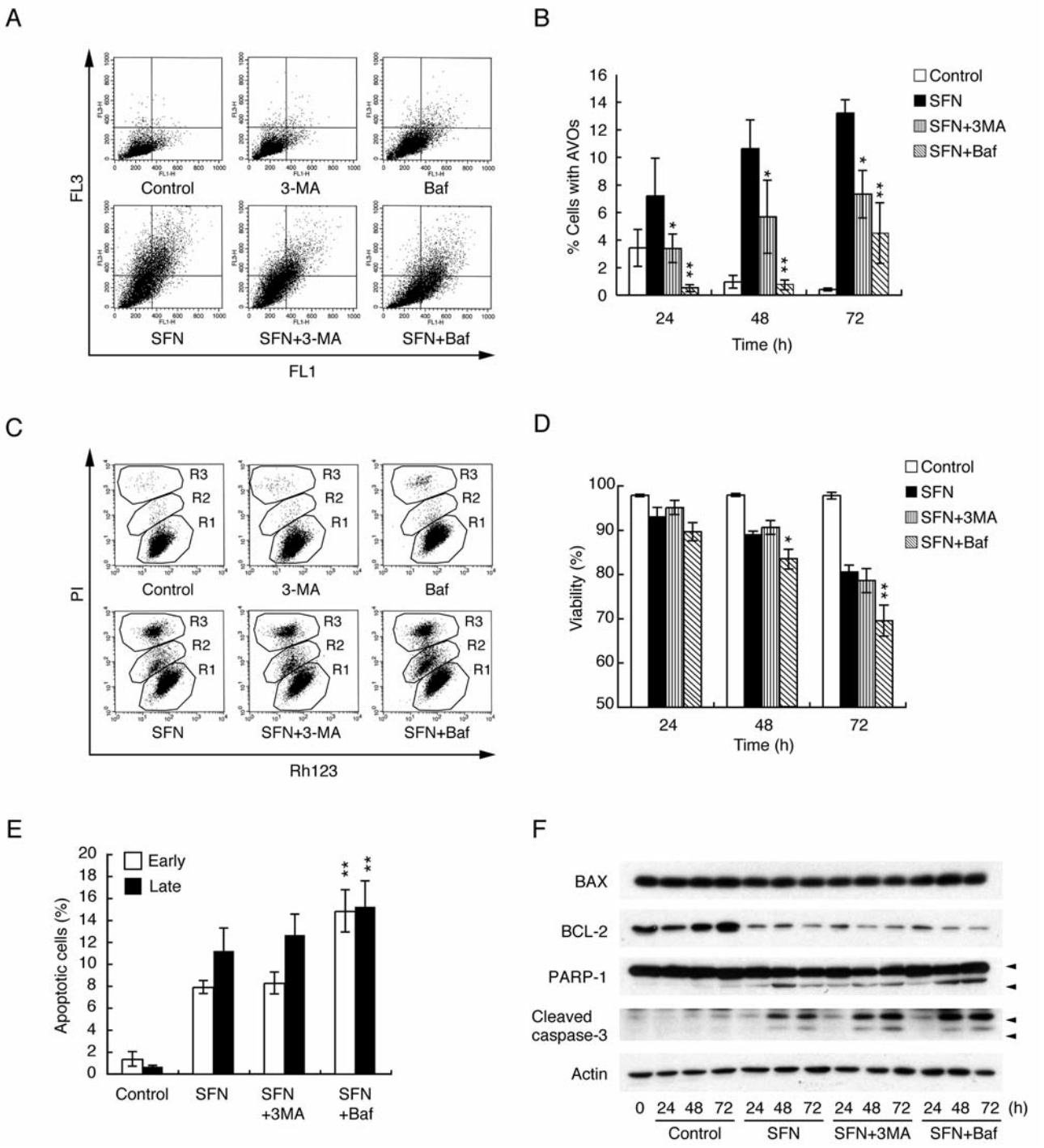


Figure 5. *Effect of 3-methyladenine (3-MA) and bafilomycin A1 (Baf) on sulforaphane (SFN)-induced autophagy in MDA-MB-231 cells. A and B: Cells were treated with 30 μM SFN and 5 μM of 3-MA or 1 nM Baf for the indicated period of time, and acridine orange–positive cells were quantified by using flow cytometry. A: Representative histograms of acridine orange staining of cells treated with SFN and 3-MA or Baf for 72 h are shown. B: The data are means±SD of 3 independent experiments. *p<0.05 and **p<0.01, as compared with SFN-treated cells at each time point. C, D and E: Apoptosis assays were performed by using flow cytometry following staining with propidium iodide (PI) and rhodamine 123 (Rh123). C: Representative histograms of PI and Rh123 staining of cells treated with 30 μM SFN and 3-MA or 1 nM Baf for 72 h are shown. R1, viable cells; R2, early apoptotic cells; R3, late apoptotic cells. D and E: The data are the means±SD. *p<0.05 and **p<0.01, compared with SFN-treated cells. F: Whole-cell lysates from DMSO control and SFN-treated MDA-MB-231 cells with 3-MA or Baf treatment at indicated time points were subjected to SDS-PAGE, and the expression level of apoptosis-related molecules was analyzed by immunoblotting.*

increased the percentages of cells in the S and G_2/M phases and decreased the percentage of cells in the G_1 phase, as compared with control cells (Figure 2A). The accumulation of cells in the S and G_2/M phases was apparent at 48 and 72 h after SFN treatment. We next examined the expression of cell-cycle regulatory molecules by performing Western blots (Figure 2B). SFN treatment induced an increase in the $p21^{WAF1}$ level that peaked at 24 h, and $p27^{KIP1}$ accumulated at 48 and 72 h after SFN treatment. On the other hand, time-dependent decreases in the levels of cyclin A, cyclin B1 and CDC2 proteins occurred after SFN treatment. We then examined the changes in the protein levels of apoptosis-related molecules, such as capase-3, BAX, BCL-2 and BCL-xL, after SFN treatment. Cells treated with SFN exhibit an increase in the cleavage of caspase-3 and a decrease in BCL-2 levels, whereas BAX and BCL-xL protein levels were unchanged (Figure 2C). The increase in cleaved caspase-3 and the decrease in the BCL-2 level were well correlated to the number of dead cells. Thus, SFN-induced apoptosis may be mediated by caspase-3 activation through mitochondrial signaling.

SFN induces autophagy in MDA-MB-231 cells. We examined the SFN-treated cells by electron microscopy (Figure 3A). Numerous membranous vacuoles, autophagosomes and autolysosomes containing residual digested materials appeared in the cytoplasm of SFN-treated cells, while there were relatively few such structures in the cytoplasm of control cells. FACS analysis was performed to assess the development of AVOs. The representative histograms for acridine orange staining after 24, 48, and 72 h treatment with or without SFN are shown in Figure 3B and summarized in Figure 3C. In contrast to control cells, SFN-treated cells accumulated a significant number of AVOs in a time-dependent manner ($p<0.05$ at 24 h, and $p<0.01$ at 48 and 72 h, compared with controls).

To ascertain the expression of LC3 in SFN-treated cells, we examined the localization of LC3 by immuno-fluorescence analysis. SFN-treated cells exhibited punctuate patterns of LC3 fluorescence signals (Figure 4A). Next, we examined the changes in protein level and mRNA expression of LC3. The cells treated with SFN exhibited an increase in both LC3-I and LC3-II protein levels as compared with the control cells (Figure 4B), and the level of *LC3* mRNA expression was significantly increased after 24, 48, and 72 h treatments with SFN ($p<0.05$ at 24 h, $p<0.01$ at 48 and 72 h; ~1.8-fold increase at 72 h) when compared with the respective controls (Figure 4C). These results suggest that SFN treatment induces LC3-associated autophagosome formation and modulates LC-3 expression through mRNA and protein levels. Taken together, these results indicate that SFN induces autophagy in MDA-MB-231 cells.

Autophagy inhibition enhances SFN-induced apoptosis in MDA-MB-231 cells. To investigate the role of SFN-induced autophagy in MDA-MB-231 cells, two kinds of specific autophagy inhibitors, 3-MA and Baf, were tested. The representative histograms for acridine orange staining after a 72-h treatment with SFN in the presence of 3-MA or Baf are shown in Figure 5A. Acridine orange staining after 72 h of SFN treatment showed significant accumulation of AVOs (13.2±1.0%) as compared with control cells (0.4±0.1%); this accumulation was partially inhibited by 3-MA (7.3±1.7%; $p<0.05$) and Baf (4.5±2.1%; $p<0.01$) (Figure 5B). The inhibitory effect of 3-MA and Baf on AVO formation peaked at 24 h and decreased gradually (~72 h), and the inhibitory effect of Baf against SFN-induced AVO formation was greater than that of 3-MA. Next, the effect of 3-MA and Baf on cell viability and the induction of apoptosis was determined by FACS analysis following staining with PI and Rh123. The representative histograms for PI and Rh123 staining after a 72-h treatment with SFN in the presence of 3-MA, and Baf are shown in Figure 5C and summarized in Figure 5D. The cells treated with SFN for 48 and 72 h exhibited a significant decrease in cell viability (89.0±0.8% and 80.5±1.6%, respectively), as compared with control cells ($p<0.01$, respectively). Interestingly, SFN treatment in the presence of Baf for 72 h significantly reduced cell viability as compared to SFN treatment alone (69.5±3.5% *vs*. 80.5±1.6%; $p<0.01$), whereas SFN treatment in the presence of 3-MA had no significant effect (Figure 5D). To further examine whether decreased cell viability induced by Baf is due to apoptosis, the percent of apoptotic cells was determined (Figure 5E). The cells treated with SFN in the presence of Baf exhibited a significant increase in the percentage of both early and late apoptotic cells, as compared to cells treated with SFN alone ($p<0.01$, respectively). 3-MA did not show any adverse effect on the induction of early or late apoptosis. We then examined the alterations in the protein levels of BAX, BCL-2, PARP-1 and caspase-3 after SFN treatment in the presence of 3-MA, and Baf (Figure 5F). The BAX level and cleavage of PARP-1 and caspase-3 after SFN treatment was enhanced in the presence of Baf. SFN treatment with 3-MA resulted in a slight increase in the cleavage of caspase-3, but there was little effect on BAX, BCL-2 and PARP-1 levels. These results indicate that the inhibition of autophagy by 3-MA (at an early stage) had little, if any, effect on cell viability, whereas the inhibition of autophagy by Baf (at a late stage) significantly enhanced SFN-induced cytotoxicity by accelerating apoptosis. Alone 3-MA and Baf had no significant effect on AVO formation, cell viability, or the protein levels of apoptosis-related molecules (data not shown).

Discussion

SFN inhibited cell proliferation and induced cell death in MCF-7 and MDA-MB-231 human breast cancer cells in a dose- and time-dependent manner. When MDA-MB-231 cells were exposed to 30 μM of SFN, cell growth was inhibited by cell-cycle arrest in the S and G_2/M phases and apoptosis was induced. Human breast carcinomas have been divided into five subtypes, luminal A, luminal B, basal, ERBB2-overexpressing, and normal-like, which have different clinical courses and different responses to therapeutic agents (27). The ER-positive MCF-7 cell line is a luminal subtype, and the ER-negative MDA-MB-231 cell line is a mesenchymal subtype, which has not been identified in cancer samples but is similar to the basal subtype (28). Although SFN was equally effective in the two human breast cancer cell lines, MDA-MB-231 cells were selected for detailed study because the basal subtype exerts a more malignant potential than the luminal subtype, and is unresponsive to endocrine and/or anti-HER2 therapy. The question arises as to whether treatment with 30 μM SFN is relevant to humans. SFN was detectable in the plasma of rats 1 h after the administration of an oral dose of 50 μM SFN (29). The peak plasma level of SFN was 20 μM at 4 h, and the level of SFN declined with a half-life of 2.2 h (29). Although further pharmacokinetic studies in humans are necessary, the SFN concentration used in the present *in vitro* study is highly achievable *in vivo*.

Treatment with 15 μM SFN for 72 h induces G_2/M phase arrest in MCF-7 cells and MDA-MB-231 cells (10), and broccoli sprout extract rich in SFN arrests UM-UC-3 human bladder cancer cells in the S and G_2/M phases (30). Cyclin A/CDK2 and cyclin B1/CDC2 are important molecules for the transition of the S phase and the G_2/M phase, respectively. SFN treatment of MDA-MB-231 cells resulted in decreased levels of cyclin A, cyclin B1 and CDC2; increased levels of p21 and p27; and cell-cycle arrest in the S and G_2/M phases. In PC-3 human prostate cancer cells (31) and in T98G and U87MG human glioblastoma cells (32), SFN caused a decrease in the BCL-2 level, an increase in the BAX level, and activation of caspase-3 and PARP activity. In MCF-7, T47D, MDA-MB-231, and MDA-MB-468 human breast cancer cell lines (10), the molecules involved in SFN-induced apoptosis were the increased level of caspase-3 and PARP-1 cleavage and the decreased level of BCL-2; the BAX level was unchanged. The proteins of the BCL-2 family play a central role in regulating apoptosis (33). Although the BAX levels were unchanged, the BAX/BCL-2 ratio may be more important (32). The different concentration or duration of SFN exposure on different types of cells may modulate the apoptosis signaling pathway. However, our results suggest the importance of an increased BAX/BCL-2 ratio, followed by increased caspase-3 cleavage, for SFN-induced apoptosis in MDA-MB-231 cells.

Several chemotherapeutic agents induce autophagy in human breast cancer cells (34, 35). We found that SFN induced autophagy in MDA-MB-231 cells, which was characterized by the formation of autophagosomes and autolysosomes, the accumulation of AVOs, a punctuate pattern of LC3 immunostaining, and an increased level of LC3-II. Autophagy exhibits controversial functions in cell survival or cell death after various anticancer therapies (16, 17). The regulation of autophagy signaling may be a promising therapeutic target for cancer treatment (16, 36).

Autophagy inhibitors 3-MA and Baf enhance the cytotoxicity of anthocyanin to PLC/PRF/5 human hepatocellular carcinoma cells (37) and the cytotoxicity of arsenic trioxide to U373-MG human glioblastoma cells (21), respectively. Both 3-MA and Baf increase the cytotoxicity of resveratrol to U251 human glioblastoma cells (38) and the cytotoxicity of trastuzumab to trastuzumab-refractory SKBR human breast cancer cells (35). However, the cytotoxicity of temozolomide to U373-MG (39) and imatinib to U373-MG and U87-MG human glioblastoma cells (40) is enhanced by Baf, but suppressed by 3-MA (40). The cytotoxicity of tamoxifen to MCF-7 cells is suppressed by 3-MA (34). Effects of autophagy inhibition may be influenced by factors including the differences in autophagy inducer, type of target cells, and/or degree of damage on target cells. Recently, SFN was shown to induce autophagy in PC-3 and LNCaP human prostate cancer cells and in WiDr human colon cancer cells, and the inhibition of autophagy by 3-MA enhanced cytotoxicity such that the SFN-induced apoptosis was potentiated (18, 19). In MDA-MB-231 cells, although both 3-MA and Baf inhibited AVO formation, the inhibitory effect of Baf was greater than that of 3-MA. Inhibition of autophagy has been proposed to induce apoptosis (16, 41). In agreement with previous studies of prostate and colon cancer cells (18, 19), we found that the inhibition of autophagy significantly enhanced apoptosis, which was characterized by increased BAX expression, increased cleavage of caspase-3 and PARP-1, and decreased mitochondrial membrane potential ($\Delta\Psi$m). However, although autophagy inhibition by Baf effectively enhanced apoptosis, 3-MA treatment did not show significant changes in SFN-induced apoptosis; other human breast cancer cell lines should be further studied.

In conclusion, the present findings provide novel evidence that SFN induces autophagy in MDA-MB-231 human breast cancer cells and that autophagy inhibition by Baf (but not by 3-MA) enhances SFN-induced cytotoxicity through the modulation of BAX, BCL-2, caspase-3, PARP-1, and mitochondrial membrane potential ($\Delta\Psi$m). Although the molecular mechanisms of the different responses against 3-MA and Baf in SFN-induced autophagy need to be clarified, and the effects of autophagy inhibition *in vivo* need to be confirmed, SFN in combination with autophagy inhibitor

may be an attractive treatment strategy for breast cancer refractory to tailored therapies such as endocrine therapy and/or anti-HER2 therapy.

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