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Sulforaphane induces cell type–specific apoptosis in human breast cancer cell lines

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Abstract

Sulforaphane, an isothiocyanate found in cruciferous vegetables, has been shown to induce phase 2 detoxication enzymes and inhibit the growth of chemically induced mammary tumors in rats, although the exact mechanisms of action of sulforaphane are not understood. In this study, we evaluated the effects of sulforaphane on cell growth and death in several human breast cancer cell lines and examined the hypothesis that sulforaphane acts as a histone deacetylase (HDAC) inhibitor in these cell lines. Sulforaphane treatment inhibited cell growth, induced a G_2-M cell cycle block, increased expression of cyclin B1, and induced oligonucleosomal DNA fragmentation in the four human breast cancer cell lines examined, MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells. Activation of apoptosis by sulforaphane in MDA-MB-231 cells seemed to be initiated through induction of Fas ligand, which resulted in activation of caspase-8, caspase-3, and poly(ADP-ribose) polymerase, whereas apoptosis in the other breast cancer cell lines was initiated by decreased Bcl-2 expression, release of cytochrome *c* into the cytosol, activation of caspase-9 and caspase-3, but not caspase-8, and poly(ADP-ribose) polymerase cleavage. Sulforaphane inhibited HDAC activity and decreased the expression of estrogen receptor-α, epidermal growth factor receptor, and human epidermal growth factor receptor-2 in each cell line, although no change in the acetylation of H3 or H4 was seen. These data suggest that sulforaphane inhibits cell growth, activates apoptosis, inhibits HDAC activity, and decreases the expression of key proteins involved in breast cancer proliferation in human breast cancer cells. These results support testing sulforaphane *in vivo* and warrant future studies examining the clinical potential of sulforaphane in human breast cancer. [Mol Cancer Ther 2007;6(3):1013–21]

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Introduction

Data from several epidemiologic studies have suggested that diets rich in cruciferous vegetables, such as broccoli, cabbage, and kale, reduce the risk of developing many common cancers, including breast cancer (1–6). Currently, it is hypothesized that the anticarcinogenic effects of cruciferous vegetables may be due to their relatively high levels of glucosinolates, which, following hydrolysis by the enzyme myrosinase, result in the formation of isothiocyanates (1, 7). The primary glucosinolate in broccoli, glucoraphanin [4-(methylsulfinyl)butyl glucosinolate], is a precursor of the isothiocyanate sulforaphane (8, 9). Both glucoraphanin and sulforaphane have shown significant chemopreventive activity in several animal models, including a recent study showing the ability of sulforaphane to decrease intestinal polyp formation in Apc^{Min} mice (7, 10–15). Several different potential mechanisms of action have been proposed for the role of sulforaphane in chemoprevention, including inhibition of carcinogen-activating enzymes, such as the cytochrome *P*450 isoenzyme 2E1, induction of conjugating enzymes, such as glutathione *S*-transferases, and reduction of the DNA binding ability of nuclear factor-κB (16–20). More recently, the ability of sulforaphane to inhibit histone deacetylase (HDAC) activity *in vitro* and *in vivo* was postulated as another mechanism for its chemopreventive abilities (21–23).

In addition to its chemopreventive effects, sulforaphane has also been shown to exert antiproliferative effects on several cancer cell lines *in vitro* and *in vivo* (24–27). Sulforaphane induced apoptosis in PC-3 human prostate cancer cells, via both caspase-8 and caspase-9 pathways, and inhibited the growth of PC-3 xenografts *in vivo* (28). In quiescent HT29 human colon carcinoma cells, sulforaphane treatment inhibited the reinitiation of growth *in vitro*, whereas in exponentially growing HT29 cells, sulforaphane exposure inhibited cell growth, induced a cell cycle arrest, and activated cell death (26, 29). Sulforaphane-induced apoptosis in HT29 cells was associated with an up-regulation of Bax, release of cytochrome *c* into the cytosol, and proteolytic cleavage of poly(ADP-ribose) polymerase (PARP; ref. 26).

Sulforaphane has also been evaluated in several models of breast cancer. Sulforaphane is able to block 7,12-dimethylbenz(*a*)anthracene–induced mammary tumor development in Sprague-Dawley rats (10). When sulforaphane was given concurrently with 7,12-dimethylbenz(*a*)anthracene, the incidence, multiplicity, latency, and weight of mammary tumors that developed were significantly decreased (10). Sulforaphane treatment of F3II mouse mammary carcinoma cells resulted in a G_2-M cell cycle arrest, elevated $p34^{cdc2}$ (cdc2) kinase activity, and activation of apoptosis (30). BALB/c mice injected s.c. with F3II cells and subsequently injected with sulforaphane

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developed significantly smaller tumor volumes than control groups (30). Finally, in MCF-7 human breast cancer cells, sulforaphane treatment was shown to inhibit cell growth and disrupt tubulin polymerization *in vitro* (31).

Because both epidemiologic and rodent tumor studies suggest activity against mammary carcinogenesis, we analyzed the effects of sulforaphane on key human breast cancer cell lines representative of a wide range of breast tumor phenotypes. Our results show that sulforaphane inhibits cell growth, effects a G_2-M cell cycle block, and induces apoptosis in multiple breast cancer cell lines via two distinct pathways of activation in a cell type–specific manner. Furthermore, sulforaphane exposure inhibited HDAC activity and decreased the expression of critical proteins involved in breast cancer growth, such as estrogen receptor (ER)-α, epidermal growth factor receptor (EGFR), and human EGFR-2 (HER-2). These data provide molecular insight into the mechanism whereby sulforaphane inhibits cell growth in human breast cancer cell lines.

Materials and Methods

Cell Lines, Culture Conditions, and Reagents

MDA-MB-231, MCF-7, and T47D cells were cultured at 37°C, 5% CO_2 in DMEM supplemented with 5% fetal bovine serum (Hyclone, Logan, UT) and 2 μmol/L L-alanyl-L-glutamine (Mediatech, Herndon, VA). MDA-MB-468 cells were cultured at 37°C, 5% CO_2 in improved MEM (BioSource International, Camarillo, CA) supplemented with 10% fetal bovine serum and 2 μmol/L L-alanyl-L-glutamine. D,L-sulforaphane (LKT Laboratories, Minneapolis, MN) was prepared in DMSO and stored at a stock concentration of 10 mmol/L at −20°C.

Cell Growth Assays

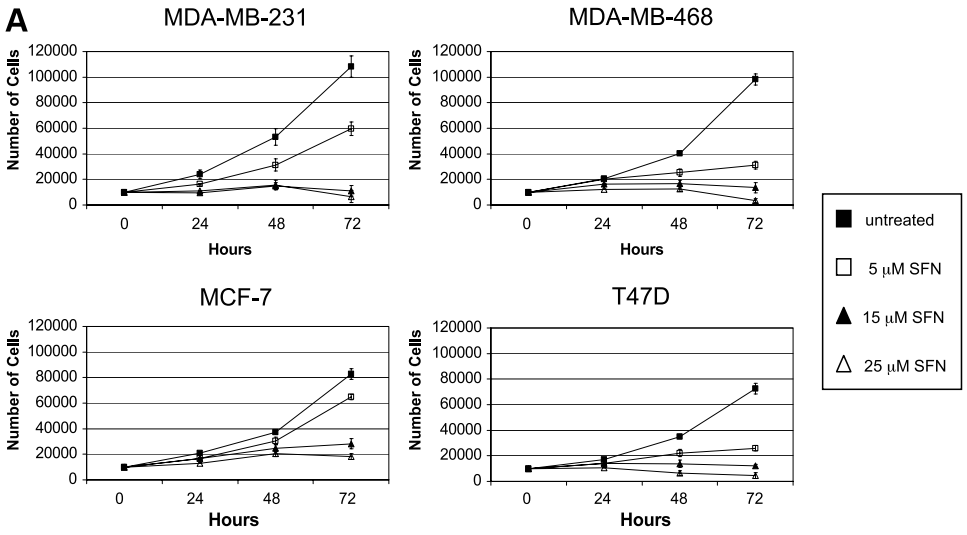
Cells were plated at a cell density of 5,000 cells per well in six-well tissue culture plates. After attachment overnight, the medium was replaced and cells were treated with 0, 5, 15, or 25 μmol/L sulforaphane for 72 h. Every 24 h, cells were detached by trypsinization and counted using a Coulter particle counter (Beckman Coulter, Fullerton, CA). The IC_{50} values were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, which were done as described previously (32). Briefly, following attachment overnight, cells were incubated with increasing concentrations of sulforaphane for 48 h. Immediately following treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done thrice in quadruplicate. All experiments were done thrice in triplicate.

Fluorescence-Activated Cell Sorting

Cells in the exponential growth phase were plated at a cell density of 100,000 cells in 10-cm culture dishes and treated with 0 or 15 μmol/L sulforaphane for up to 72 h. Every 24 h, adherent and nonadherent cells were collected and analyzed by fluorescence-activated cell sorting as described previously (33).

Determination of Internucleosomal DNA Cleavage

After treatment with 0, 5, 15, or 25 μmol/L sulforaphane for 96 h, cells were collected and DNA ladder fragments



B

MTT Assay of SFN	
Cell Line	**IC_{50} (μM)**
MDA-MB-231	8.3 ± 0.4
MDA-MB-468	8.1 ± 0.3
MCF-7	9.2 ± 0.4
T47D	9.5 ± 0.5

Figure 1. Sulforaphane (*SFN*) inhibits cell growth in several human breast cancer cell lines in a dose-dependent manner. **A,** MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells were treated with different concentrations of sulforaphane (0, 5, 15, or 25 μmol/L) for 72 h. Cells were detached by trypsinization and counted every 24 h as described in Materials and Methods. **B,** MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells were exposed to increasing concentrations of sulforaphane for 48 h. The IC_{50} values were determined as described in Materials and Methods. *Points,* mean of three independent experiments done in triplicate; *bars,* SD.

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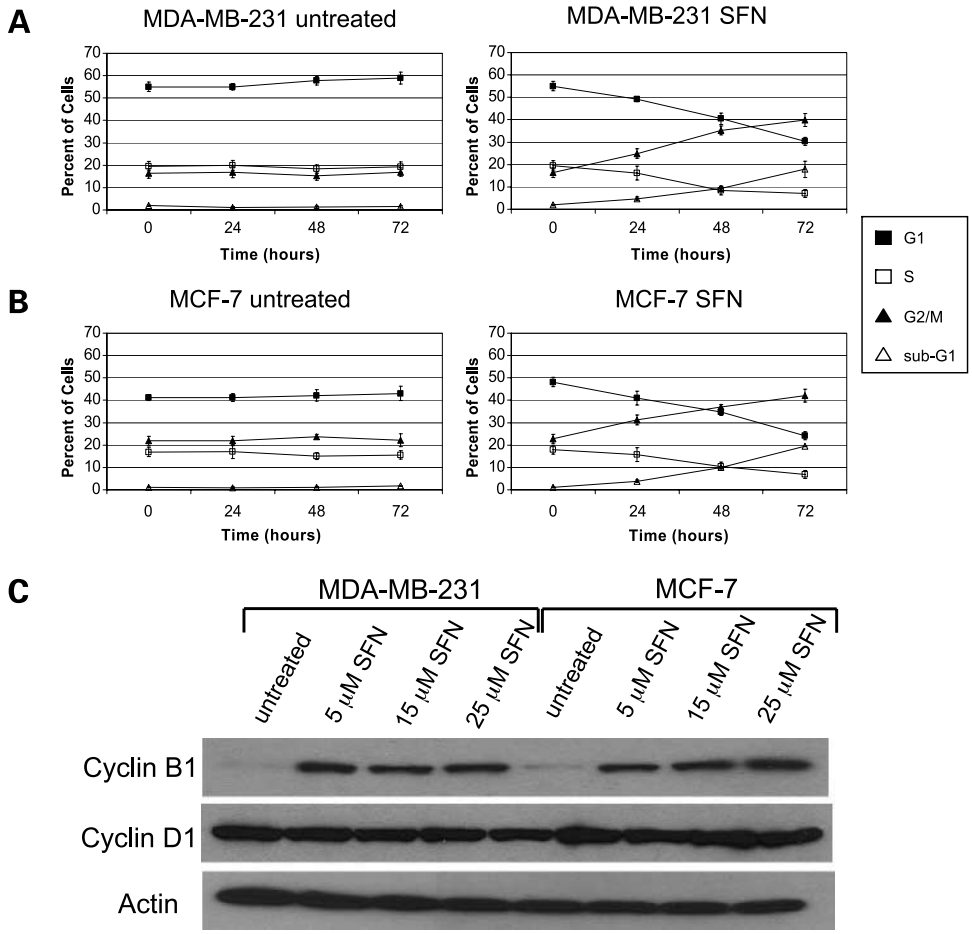

Figure 2. Sulforaphane induces a G_2-M cell cycle block and cyclin B1 protein expression in MDA-MB-231 and MCF-7 cells. MDA-MB-231 (**A**) and MCF-7 cells (**B**) were treated with 15 μmol/L sulforaphane for 72 h. Every 24 h, cells were harvested and analyzed by fluorescence-activated cell sorting as described in Materials and Methods. Percentage of cells in each phase of the cell cycle at each time point. *Points,* mean of three independent experiments done in triplicate; *bars,* SD. **C,** MDA-MB-231 and MCF-7 cells were treated with 0, 5, 15, or 25 μmol/L sulforaphane for 48 h. Equal amounts (100 μg/lane) of whole-cell extracts were fractionated on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with anti–cyclin B1 or anti–cyclin D1 antibodies as described in Materials and Methods. Actin protein was blotted as a loading control. Representative blots from one of the three experiments.

were prepared as described previously (34). DNA samples were analyzed by electrophoresis using a 2% agarose gel containing 0.2 μg/mL ethidium bromide and visualized under UV illumination.

Western Blotting

Cells were treated with 0, 5, 15, or 25 μmol/L sulforaphane for 48 h, harvested by trypsinization, and washed with ice-cold PBS. Whole-cell extracts were isolated using a protein extraction buffer containing 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 5 mmol/L EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS. Equal amounts of protein (100 μg/lane) were fractionated using 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies against caspase-3 (1:1,000), caspase-8 (1:500), caspase-9 (1:500), FasL (1:1,000), Bcl-2 (1:1,000), PARP (1:1,000), ER-α (1:1,000), HER-2 (1:2,000), EGFR (1:2,000), acetylated tubulin (1:1,000), or actin (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were incubated with peroxidase-conjugated goat antimouse or goat anti-rabbit secondary antibody (1:5,000; DAKO Corp., Carpinteria, CA) followed by enhanced chemiluminescence staining (Amersham Biosciences, Piscataway, NJ).

Detection of Cytochrome *c* Release

Selective plasma membrane permeabilization with digitonin was used to examine the release of cytochrome *c* from the mitochondria into the cytosol as described previously (35). Equal amounts of protein (100 μg/lane) were fractionated using 12% SDS-PAGE and analyzed by Western blotting as described above. The membranes were incubated with a primary antibody against cytochrome *c* (1:1,000; Santa Cruz Biotechnology) and actin (1:10,000).

In vitro HDAC Activity Assays

In vitro HDAC activity was determined using the colorimetric HDAC activity assay kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. Briefly, 10 μg of whole-cell extract for each sample were incubated with the HDAC assay buffer and the HDAC colorimetric substrate for 30 min at 37°C. Lysine developer was then added and the samples were incubated at 37°C for another 30 min and then read in an ELISA plate reader at 405 nm.

Histone Isolation and Analysis

Histone proteins from sulforaphane-treated and untreated control cells were isolated according to previously published protocols (36). Thirty micrograms of histone protein from both untreated and sulforaphane-treated

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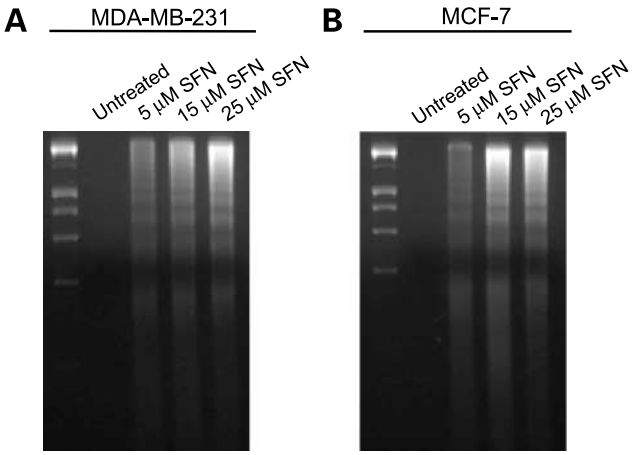

Figure 3. Sulforaphane induces internucleosomal DNA fragmentation. MDA-MB-231 (**A**) and MCF-7 cells (**B**) were treated with 0, 5, 15, or 25 μmol/L sulforaphane for 96 h. Cells were harvested, and fragmented DNA was extracted as described in Materials and Methods. Fragmented DNA was analyzed by agarose gel electrophoresis. Representative gels from one of the three experiments.

cells were analyzed by Western blotting using anti-acetyl H3 and anti-acetyl H4 antibodies (1:1,000; Upstate Biochemical, Lake Placid, NY) and horseradish peroxidase–conjugated goat anti-rabbit secondary antibody (1:5,000).

Results

Sulforaphane Inhibits Cell Growth in Several Human Breast Cancer Cell Lines in a Dose-dependent Manner

Because sulforaphane has been shown to inhibit the growth of several cancer cell lines *in vitro* and *in vivo*, we examined the effects of sulforaphane on the growth of four human breast cancer cell lines, MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells. These cell lines were chosen as they are the representative of a wide range of breast cancer phenotypes. ER, one of the most important targets in breast cancer therapy, is expressed in both MCF-7 and T47D cells, whereas MDA-MB-231 and MDA-MB-468 cells lack expression of ER due to epigenetic silencing. The expression of two other key proteins in breast cancer growth and treatment, EGFR and HER-2, also varies among these cell lines. EGFR is overexpressed in MDA-MB-468 cells, highly expressed in T47D cells, and expressed at lower levels in MDA-MB-231 and MCF-7 cells. HER-2 is expressed at lower levels in the ER-negative cell lines MDA-MB-231 and MDA-MB-468 and expressed at higher levels in the ER-positive cell lines MCF-7 and T47D. Sulforaphane exposure for 72 h inhibited the growth of each cell line in a dose-dependent manner (Fig. 1A). To complement this data, the IC_{50} values for sulforaphane in each cell line were determined and also did not differ significantly between these four cell lines (Fig. 1B). Inhibitory potency did not differ substantially between cell lines.

Sulforaphane Induces a G_2-M Block and Cyclin B1 Protein Expression in MDA-MB-231 and MCF-7 Cells

Fluorescence-activated cell sorting analysis was used to examine the mechanism of cell growth inhibition by sulforaphane. A concentration of 15 μmol/L was chosen because it was growth inhibitory in all cell lines studied (Fig. 1). In both MDA-MB-231 and MCF-7 cells, treatment with sulforaphane increased the percentage of cells in G_2-M and decreased the percentage of cells in G_1 and S within 24 h of treatment (Fig. 2A and B). An increase in cells in sub-G_1 (~20%) was also apparent after 72 h of sulforaphane treatment in each cell line. This G_2-M block was also accompanied by an increase in cyclin B1, but not cyclin D1, protein expression in both MDA-MB-231 and MCF-7 cells (Fig. 2C). Similar findings of a G_2-M block and cyclin B1 protein induction were observed in MDA-MB-468 and T47D cells (data not shown).

Sulforaphane Induces Apoptotic Cell Death

DNA fragmentation assays were done to establish whether the sulforaphane-induced increase in sub-G_1 cells was a result of apoptosis. Sulforaphane treatment for 96 h induced DNA fragmentation in all four cell lines with concentrations as low as 5 μmol/L (Fig. 3). No fragmentation was detected at earlier time points examined (data not shown). Western blotting was used to dissect the apoptotic pathways induced by sulforaphane. MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells were treated with 0, 5, 15, or 25 μmol/L sulforaphane for 48 h and whole-cell protein extracts were prepared as described in Materials and Methods. In MDA-MB-231 cells, treatment with 15 or 25 μmol/L sulforaphane increased the expression of Fas ligand and the cleavage of caspase-3 and caspase-8, but not caspase-9, in a dose-dependent fashion (Fig. 4). PARP cleavage without altered expression of Bcl-2 or cytochrome *c* localization was also seen. The pattern of apoptotic protein expression seen in sulforaphane-treated MDA-MB-231 cells is markedly different from changes induced by sulforaphane in the other three breast cancer cell lines examined. In MDA-MB-468 and T47D cells, sulforaphane treatment activated cleavage of PARP, caspase-3, and caspase-9, but not caspase-8; decreased Bcl-2 expression; and activated the release of cytochrome *c* from the mitochondria to the cytosol. Identical changes were seen in MCF-7 cells except for a lack of expression of caspase-3, which has been previously noted (35).

Sulforaphane Inhibits Global HDAC Activity in Several Human Breast Cancer Cell Lines

Recent studies have shown inhibition of HDAC activity by sulforaphane *in vitro* in a variety of cancer cell lines, including HCT116, LnCaP, and PC-3 cells, and *in vivo* in Apc^{min} mice (21–23). Therefore, we examined the effects of sulforaphane on HDAC activity in human breast cancer cell lines. MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells were treated with 0, 5, 15, or 25 μmol/L sulforaphane for 48 h, and global HDAC activity was measured using an *in vitro* activity assay. Sulforaphane treatment significantly inhibited HDAC activity in all four cell lines, particularly in the ER-negative cell lines, MDA-MB-231 and MDA-MB-468

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cells (Fig. 5A). Despite this significant inhibition in global HDAC activity, no significant changes were observed in the acetylation of H3 or H4 in any cell line following 48 h exposure to 15 μmol/L sulforaphane; a known HDAC inhibitor trichostatin A was used as a positive control (Fig. 5B). Moreover, no changes were seen in the acetylation of H3 or H4 in any of the cell lines using a dose range of 5 to 25 μmol/L sulforaphane with time points of 24 to 96 h (data not shown). Inhibition of HDACs may also result in the accumulation of acetylated tubulin through the inhibition of the major cellular tubulin deacetylase HDAC6 (37, 38). However, no increase in the acetylation of α-tubulin on K40 was seen in MDA-MB-231, MDA-MB-468, MCF-7, or T47D cells treated with 15 μmol/L sulforaphane for 48 h (data not shown).

Sulforaphane Down-regulates ER-α, EGFR, and HER-2 Protein in Multiple Breast Cancer Cell Lines

ER, EGFR, and HER-2 are three of the most critical proteins in breast cancer proliferation and treatment. Because other studies have shown that HDAC inhibition decreases their expression, the effects of sulforaphane exposure on ER, EGFR, and HER-2 protein expression were evaluated by Western blotting. EGFR and HER-2 were down-regulated with 15 and 25 μmol/L sulforaphane in all cell lines examined except that EGFR protein in MCF-7 cells was below the level of detection using this method (Fig. 6). This was accompanied by a decrease in the mRNA expression of both EGFR and HER-2 in all four cell lines using quantitative real-time PCR (data not shown). Exposure of MCF-7 and T47D cells to 15 and

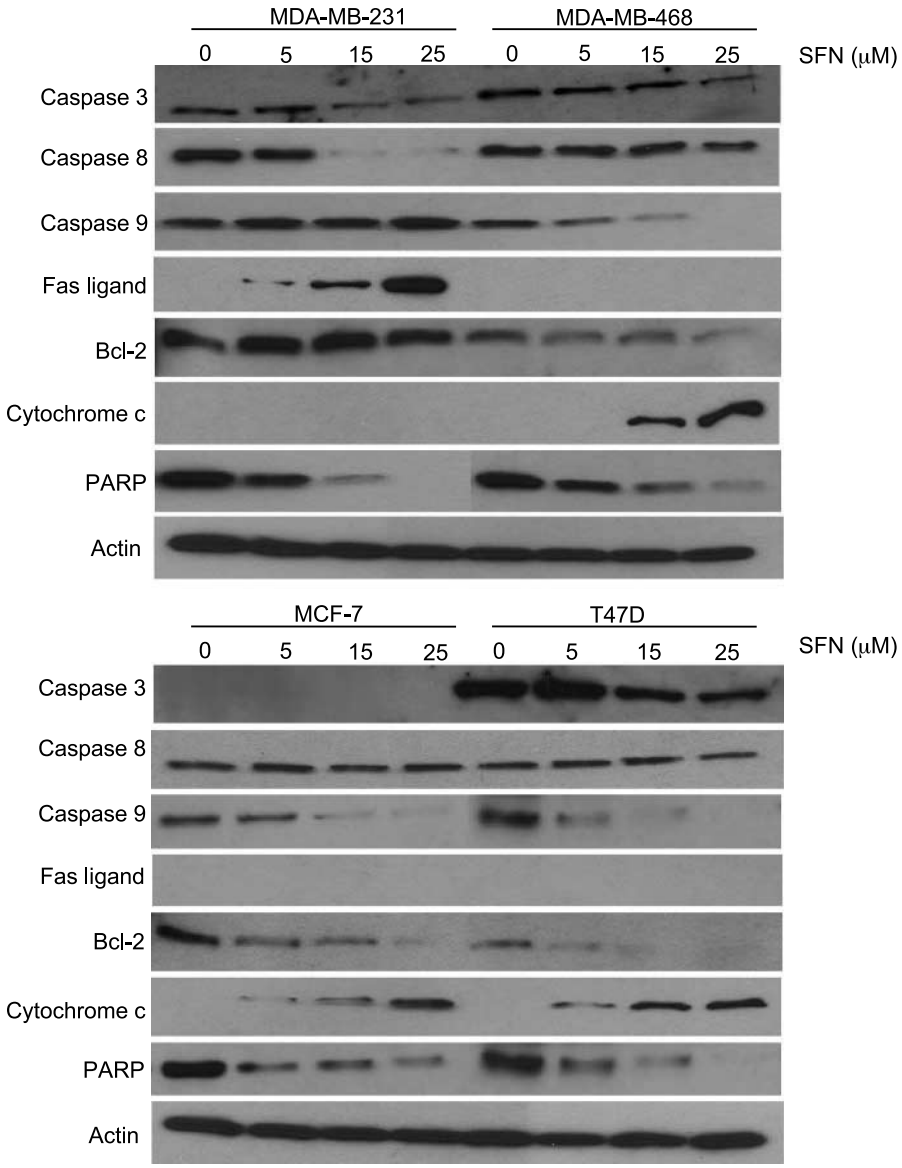

Figure 4. Effects of sulforaphane on apoptotic proteins. MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells were treated with sulforaphane (0, 5, 15, or 25 μmol/L) for 48 h. Equal amounts (100 μg/lane) of whole-cell extracts were fractionated on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies as described in Materials and Methods. Actin protein was blotted as a loading control. Representative blots from one of the three experiments.

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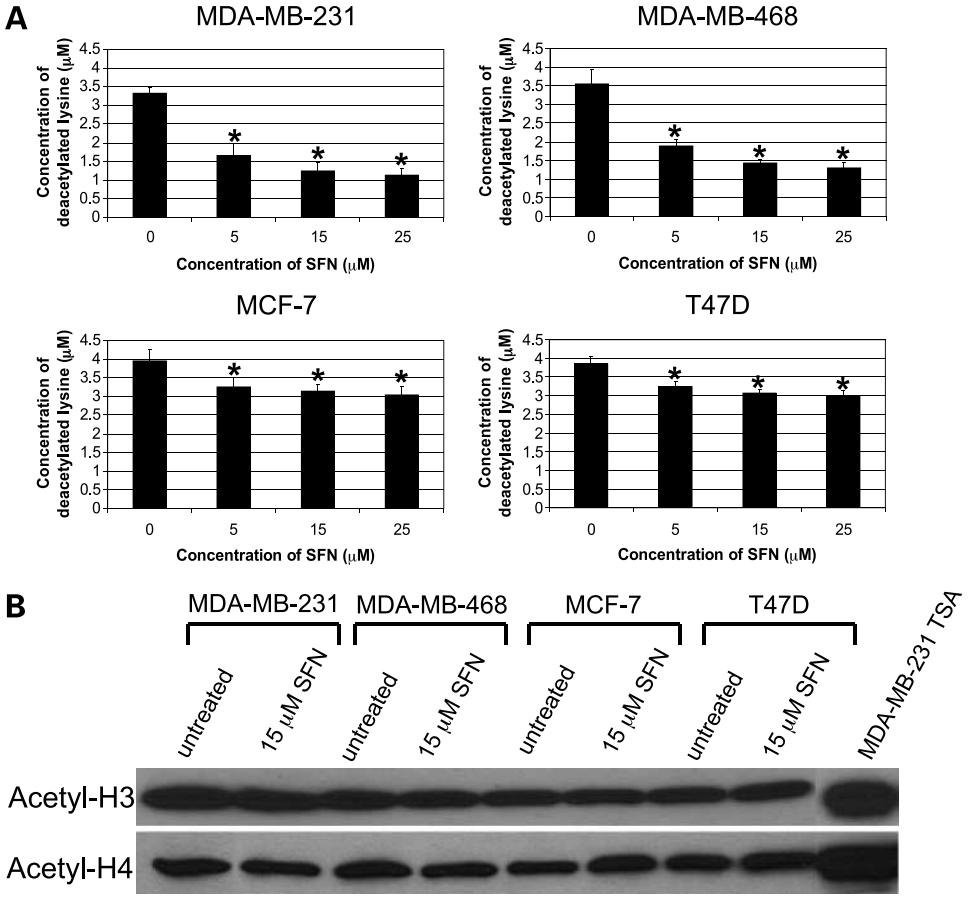

Figure 5. Sulforaphane inhibits global HDAC activity in several human breast cancer cell lines. **A,** MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells were treated with sulforaphane (0, 5, 15, or 25 μmol/L) for 48 h. An *in vitro* HDAC activity assay kit was used to measure HDAC activity as described in Materials and Methods. *Columns,* mean of three independent experiments done in triplicate; *bars,* SD. *, $P < 0.05$, as determined using a Student's *t* test. **B,** MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells were treated with 15 μmol/L sulforaphane for 48 h and histone proteins were isolated as described in Materials and Methods. MDA-MB-231 cells were also treated with 300 nmol/L trichostatin A (*TSA*) for 48 h as a positive control. Equal amounts (30 μg) of histone proteins were fractionated on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies as described in Materials and Methods. Representative blots from one of the three experiments.

25 μmol/L sulforaphane down-regulated ER mRNA and protein but no alteration of ER protein expression in MDA-MB-231 or MDA-MB-468 cells was observed (Fig. 6; data not shown).

Discussion

Human diets that include a consistently high intake of vegetables, fruits, and whole grains have been associated with a markedly reduced risk of cancer in several studies (1–6). Isothiocyanates, which are formed following the hydrolysis of glucosinolates, are currently believed to be responsible for some of the anticancer effect of cruciferous vegetables, such as broccoli, kale, and cauliflower. Sulforaphane is an isothiocyanate that displays significant chemopreventive activity believed to result, in part, from its inhibition of carcinogen-activating enzymes and induction of detoxication enzymes affecting carcinogen metabolism and disposition, as well as inhibition of HDAC activity (9, 16–23). Sulforaphane has been reported to inhibit mouse mammary carcinoma cell growth and disrupt microtubule polymerization (30, 31). Here, we evaluated the effects of sulforaphane on cell growth and death in several human breast cancer cell lines representative of a wide range of breast tumor phenotypes.

Sulforaphane inhibited the growth of the four human breast cancer cell lines examined as determined by direct cell growth assays and IC_{50} values (Fig. 1). The cell growth inhibition we observed by sulforaphane in MCF-7 cells is consistent with the published literature (31). Sulforaphane-induced cell growth inhibition was the result of a G_2-M cell cycle block and was associated with an increase in cyclin B1, but not cyclin D1, levels as well as an increase in the population of cells in sub-G_1 as determined via fluorescence-activated cell sorting analysis (Fig. 2). We hypothesized that the increase in the population of sub-G_1 cells was due to sulforaphane-induced activation of apoptosis; this hypothesis was confirmed by our DNA fragmentation results (Fig. 3). Interestingly, two distinct pathways of apoptosis were induced by sulforaphane in the cell lines evaluated. In MDA-MB-231 cells, apoptosis seems to be initiated with an up-regulation of Fas ligand, which results in activation of caspase-8 and caspase-3, leading to the proteolytic cleavage of PARP. In MDA-MB-468, MCF-7, and T47D cells, apoptosis seems to be initiated through an internal, mitochondrial pathway. In each of these cell lines, sulforaphane down-regulates Bcl-2, causes the release of cytochrome *c* from the mitochondria to the cytosol, and activates caspase-9, caspase-3, and PARP cleavage (Fig. 4). Thus, although sulforaphane inhibits cell growth in a very similar manner in the breast cancer cell lines evaluated, the activation of apoptosis occurs in a cell type–specific manner.

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Other studies have reported the ability of sulforaphane to induce apoptosis in human prostate cancer cells via both caspase-8 and caspase-9 pathways, whereas the induction of apoptosis in two human glioblastoma cell lines, T98G and U87MG, occurs solely through the caspase-9 pathway (28, 39). Indeed, the induction of apoptosis via different pathways has been observed in human breast cancer cell lines with a variety of agents. Ho et al. (40) reported recently the finding that a *Coriolus versicolor* extract induced p53 expression in T47D cells, down-regulated Bcl-2 protein expression in MCF-7 and T47D cells, but had no effect on the expression of either of these proteins in MDA-MB-231 cells. Huang et al. (34) found that exposure to the antitumor polyamine analogue SL-11144 activated apoptosis in MDA-MB-231 cells via the caspase-9 pathway, in MCF-7 cells via up-regulation of Fas ligand, and in MDA-MB-435 cells via both the caspase-8 and caspase-9 pathways. These findings show that the selective activation of two apoptotic pathways in breast cancer cell lines is not unique to sulforaphane but rather is observed with several different classes of antitumor agents. Our data suggest that sulforaphane-induced apoptosis in the human breast cancer cell lines examined occurs via either the caspase-8 or the caspase-9 pathways, but not both, in a cell line–dependent manner. The molecular basis by which sulforaphane selectively activates one or the other of these apoptotic pathways is unresolved.

The differential effects of sulforaphane on human breast cancer cell lines may be an effect of the variation in gene expression patterns between these cell lines. Detailed microarray studies have characterized human breast tumor phenotypes and have identified five subtypes of breast cancer: luminal A, luminal B, basal, ERBB2 overexpressing, and normal like (41). Several microarray studies using breast cancer cell lines have identified a mesenchymal subtype, which has not yet been identified in tumor samples (42, 43). A recent study classified 31 breast cancer cell lines using whole-genome DNA microarrays followed by validation at the protein level via immunohistochemistry (44). In this study, MCF-7 and T47D cells were defined as luminal, whereas MDA-MB-231 cells were classified as mesenchymal; MDA-MB-468 cells were not evaluated in this study. This classification of MDA-MB-231 cells into a different subtype of breast cancers from MCF-7 and T47D cells may explain the differential effects of sulforaphane on apoptosis proteins between these cell lines. Similar changes were seen in apoptotic protein patterns in MCF-7 and T47D cells, whereas sulforaphane induced apoptosis in MDA-MB-231 cells via a different pathway (Fig. 4). Our results suggest that the apoptotic pathways activated by sulforaphane may be determined for certain breast cancer cell lines according to their distinct subtype and gene expression patterns. However, this classification of cell lines may not necessarily be predictive of the response of all types of cell lines to every class of agents.

It has been shown previously that the exposure of PC-3 human prostate cancer cells to sulforaphane results in an increase in the production of reactive oxygen species, which is hypothesized to be the initial signal for sulforaphane-induced apoptosis in this cell line (45). To examine the production of H_2O_2 by sulforaphane in human breast cancer

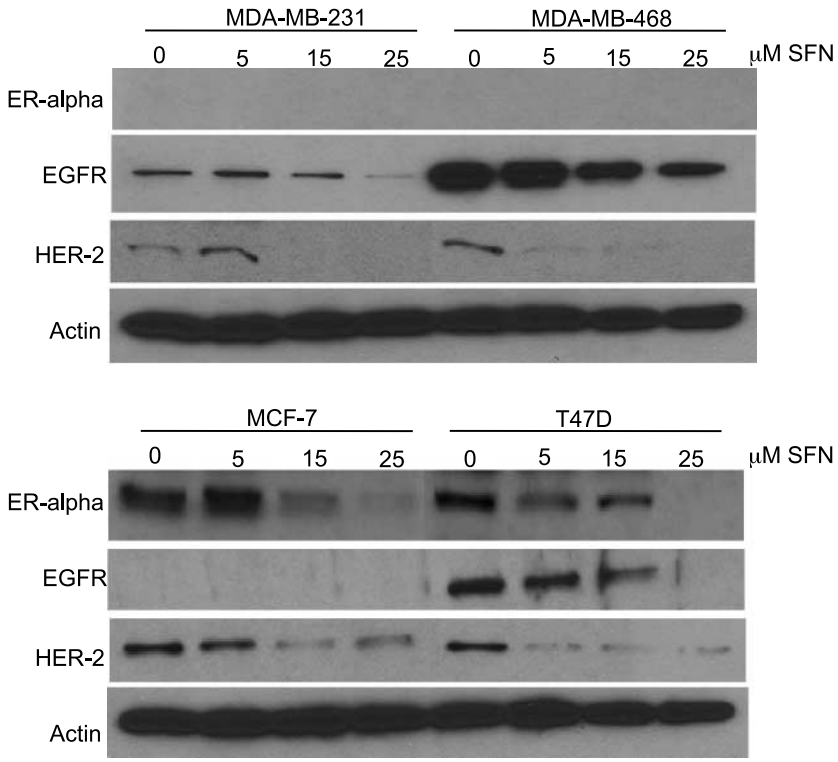

Figure 6. Sulforaphane down-regulates ER-α, EGFR, and HER-2 protein in multiple human breast cancer cell lines. MDA-MB-231, MDA-MB-468, MCF-7, and T47D cells were treated with different concentrations of sulforaphane (0, 5, 15, or 25 μmol/L) for 48 h. Equal amounts (100 μg/lane) of whole-cell extracts were fractionated on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies as described in Materials and Methods. Actin protein was blotted as a loading control. Representative blots from one of the three experiments.

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cell lines, CM-H_2DCFDA, an oxidation-sensitive fluorescent probe, was used to detect H_2O_2 production in sulforaphane-treated MDA-MB-231 and MCF-7 cells. Treatment of MDA-MB-231 and MCF-7 cells for up to 8 h with concentrations between 5 and 40 μmol/L sulforaphane did not produce any significant increase in fluorescence compared with vehicle-treated control cells (data not shown). PC-3 cells treated for 6 h with 40 μmol/L sulforaphane displayed a 6.2-fold increase in fluorescence (45). Higher concentrations of sulforaphane were not evaluated in our study to ensure that the results were relevant to a clinical setting.

Sulforaphane acts as a HDAC inhibitor *in vitro* in several prostate and colon cancer cell lines and *in vivo* in Apc^{min} mice, but until now, its ability to inhibit HDAC activity has not been evaluated in breast cancer (21–23). Results from our studies suggest that sulforaphane can significantly inhibit HDAC activity in all four breast cancer cell lines as measured with an *in vitro* activity assay, although no significant changes in global histone H3, H4, or tubulin acetylation were observed despite detailed time course and dose response analysis. However, reported changes in the acetylation of H3 and H4 by sulforaphane have been slight. In HEK293 cells, treatment with 15 μmol/L sulforaphane for 48 h resulted in a 1.9-fold increase in acetylated H3 and a 1.4-fold increase in acetylated H4, whereas increases between 1.2- and 2-fold were seen in the prostate epithelial cell lines BPH-1, LnCaP, and PC-3 under the same conditions (21, 22). Although we did not detect significant changes in global acetylation, sulforaphane treatment down-regulated the expression of EGFR and HER-2 mRNA and protein in all cell lines and down-regulated ER mRNA and protein in MCF-7 and T47D cells. Future studies will examine the potential of sulforaphane to alter histone acetylation at specific promoters. Collectively, these findings of down-regulation of growth-regulatory proteins suggest that sulforaphane may act as a HDAC inhibitor despite the lack of evidence for accumulation of global histone acetylation. The ability of sulforaphane to act as a HDAC inhibitor and down-regulate these proteins is consistent with previously published data indicating that the HDAC inhibitor trichostatin A down-regulates ER protein and the HDAC inhibitor LAQ824 down-regulates HER-2 protein through attenuation of mRNA levels and increased proteasomal degradation in human breast cancer cell lines (46, 47).

A fundamental question is whether the concentrations of sulforaphane required to activate programmed cell death and affect ER, EGFR, and HER-2 protein expression in this study are achievable in the human. The pharmacokinetic variables for sulforaphane have been examined in humans receiving a single dose of 200 μmol/L broccoli sprout isothiocyanates (predominantly sulforaphane with low concentrations of iberin and erucin). These isothiocyanates were rapidly absorbed, reaching a peak concentration of 0.94 to 2 μmol/L in plasma, serum, and erythrocytes at 1 h after ingestion and declining thereafter with a half-life of ~1.8 h (48). The pharmacokinetic variables for sulforaphane were also evaluated recently in rats that were dosed p.o. with 50 μmol/L sulforaphane. Sulforaphane was detectable in the plasma 1 h after dosing, reached a peak concentration of 20 μmol/L at 4 h after dosing, and declined with a half-life of 2.2 h (49). Previous studies have also suggested that sulforaphane and other isothiocyanates rapidly accumulate to very high levels in several different cell lines, and intracellular concentrations can reach millimolar levels (50). Activation of apoptosis and HDAC inhibition were seen in all four human breast cancer cell lines with 15 μmol/L sulforaphane in our studies. Thus, it is highly likely that the concentrations of sulforaphane used in this study are achievable in humans, although the definitive answer to this question awaits further pharmacokinetic testing in humans.

In summary, sulforaphane significantly inhibits the growth of several human breast cancer cell lines that are representative of a wide range of breast tumor phenotypes. Sulforaphane treatment activates two distinct apoptotic pathways in a breast cancer cell line–specific manner. Sulforaphane also acts as a HDAC inhibitor in breast cancer cell lines and decreases the protein expression of ER, EGFR, and HER-2. These data suggest that sulforaphane may display significant potential in an *in vivo* setting and warrant future studies examining the clinical potential of sulforaphane, alone and in a combination setting, in human breast cancer.

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