DOI:10.1158/1535-7163.MCT-05-0476

Molecular Cancer Therapeutics

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Mol Cancer Ther 2006;5:935-944. Published online April 28, 2006.

DOI:10.1158/1535-7163.MCT-05-0476

Potent activation of mitochondria-mediated apoptosis and arrest in S and M phases of cancer cells by a broccoli sprout extract

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Abstract

We have previously shown that broccoli sprouts are a rich source of chemopreventive isothiocyanates, which potently induce carcinogen-detoxifying enzymes and inhibit the development of mammary and skin tumors in rodents. However, the principal isothiocyanate present in broccoli sprout extracts, sulforaphane, not only induces carcinogen-detoxifying enzymes but also activates apoptosis and blocks cell cycle progression. In this article, we show that an aqueous extract of broccoli sprouts potently inhibits the growth of human bladder carcinoma cells in culture and that this inhibition is almost exclusively due to the isothiocyanates. Isothiocyanates are present in broccoli sprouts as their glucosinolate precursors and blocking their conversion to isothiocyanates abolishes the antiproliferative activity of the extract. Moreover, the potency of isothiocyanates in the extract in inhibiting cancer cell growth was almost identical to that of synthetic sulforaphane, as judged by their IC_{50} values (6.6 versus 6.8 μmol/L), suggesting that other isothiocyanates in the extract may be biologically similar to sulforaphane and that nonisothiocyanate substances in the extract may not interfere with the antiproliferative activity of the isothiocyanates. Further study showed that the isothiocyanate extract of broccoli sprouts activated the mitochondria-mediated apoptosis pathway and halted cells in S and M phases. Cell cycle arrest was associated with down-regulation of Cdc25C and disruption of mitotic spindles. These data show that broccoli sprout isothiocyanate extract is a highly promising substance for cancer prevention/treatment and that its antiproliferative activity is exclusively derived from isothiocyanates. [Mol Cancer Ther 2006;5(4):935–44]

Introduction

We have previously shown that broccoli sprouts are an exceptionally rich source of sulforaphane [1-isothiocyanato-4-(methylsulfinyl)butane], a well-known cancer chemopreventive isothiocyanate (1). The chemopreventive activity of sulforaphane was first brought to light in the early 1990s when we found it to be the principal ingredient of mature broccoli extracts exhibiting potent induction of carcinogen-detoxifying phase 2 enzymes (2) and to inhibit mammary tumor induction by 7,12-dimethylbenz(*a*)anthracene in rats (3). Sulforaphane has since been rigorously and extensively studied in many laboratories (see ref. 4 for detailed review), and additional chemopreventive mechanisms have been discovered, among which the induction of apoptosis and arrest of cell cycle progression have been shown to occur without cell and tissue specificity. More recently, sulforaphane was shown to inhibit the growth of prostate cancer xenografts in nude mice (5), first indicating its cancer therapeutic potential. In this context, several recent studies have shown that sulforaphane targets cellular molecules that are critical for cancer cell survival and proliferation, including inhibition of histone deacetylase (6) and inhibition of tubulin polymerization (7). Although broccoli sprout extracts were previously shown to induce phase 2 enzymes and to inhibit 7,12-dimethylbenz(*a*)anthracene–induced mammary tumor development in rats (1), and very recently to inhibit UV-induced skin tumor development in mice (8), the antiproliferative activity of broccoli sprout extracts has not yet been evaluated.

In addition to sulforaphane, broccoli sprout extracts also contain in relatively lower quantities two isothiocyanates that are structurally similar to sulforaphane, iberin and erucin (see Fig. 1 for their chemical structures; ref. 9). Several studies have shown that iberin and erucin possess chemopreventive properties similar to sulforaphane (2, 10, 11). The isothiocyanates in broccoli sprouts are generated only when the plants are damaged, through myrosinase-catalyzed hydrolysis of glucosinolates (β-thioglucoside

Received 11/17/05; revised 12/22/05; accepted 2/21/06.

Grant support: USPHS grants CA100623 and CA93780, and The Lewis B. and Dorothy Cullman Foundation.

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Note: Jed W. Fahey and The Johns Hopkins University own stock in Brassica Protection Products LLC, which sells broccoli sprouts. He is a founder of and an unpaid scientific consultant to Brassica Protection Products, and his stock is subject to certain restrictions under University policy. The terms of this arrangement are being managed by The Johns Hopkins University in accordance with its conflict of interest policies.

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doi:10.1158/1535-7163.MCT-05-0476

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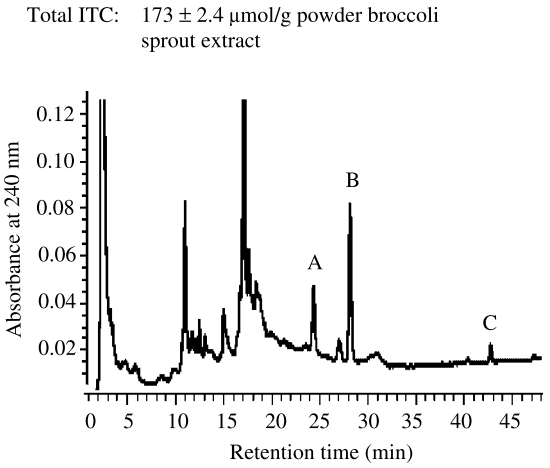

Figure 1. Isothiocyanate content in broccoli sprout extract. Myrosinase-treated broccoli sprout extracts (lyophilized powder) were dissolved in water. Total isothiocyanate content was determined by the cyclocondensation assay (reaction with 1,2-benzenedithiol; ref. 18), whereas the levels of iberin [*A;* $CH_3S(O)(CH_2)_3NCS$, 25%], sulforaphane [*B;* $CH_3S(O)(CH_2)_4NCS$, 70%], and erucin [*C;* $CH_3S(CH_2)_4NCS$, 5%] were measured with reverse-phase high-performance liquid chromatography, as described in Materials and Methods. Representative of at least three runs.

N-hydroxysulfates; refs. 1, 9). However, recent studies have revealed that formation of isothiocyanates in broccoli sprouts is highly temperature dependent, not only because of its dependence upon myrosinase, but also due to the presence of epithiospecifier protein. Epithiospecifier protein binds to and converts the intermediates of glucosinolate hydrolysis (thiohydroximate-*O*-sulfonates) to nitriles or cyanoepithioalkanes at the expense of isothiocyanates (12). Epithiospecifier protein is highly temperature sensitive, and Matusheski et al. (13) showed that heating broccoli sprouts to 60°C to 70°C for only 5 to 10 minutes destroyed virtually all epithiospecifier protein and increased sulforaphane yield by 3- to 7-fold, although further increase in temperature caused a decline in sulforaphane formation, presumably due to myrosinase inactivation. Thus, the method of preparation of broccoli sprouts for human consumption is an important issue, but the common practice of eating raw broccoli sprouts may lead to significant loss of sulforaphane and perhaps other isothiocyanates. It is noteworthy, however, that glucosinolates that escape myrosinase of plant origin can also be (at least partially) converted to isothiocyanates, following ingestion, by myrosinase from intestinal microflora (14–16); myrosinase is not known to exist in mammalian cells.

In the present study, we have evaluated the antiproliferative activity of broccoli sprout extracts and explored the underlying mechanism of action using human bladder carcinoma UM-UC-3 cells. We previously showed that several isothiocyanates, including sulforaphane, induced apoptosis and cell cycle arrest in this cell line (17). Our current results show that the broccoli sprout extracts impart potent antiproliferative activity and that such activity depends almost entirely on the enzymatic conversion of glucosinolates to isothiocyanates. The antiproliferative activity of broccoli sprout extracts is associated with induction of apoptosis and cell cycle arrest. We further show that broccoli sprout extracts preferentially activate the mitochondria-mediated apoptosis pathway and arrest cells in S and M phases. The latter is associated with down-regulation of cell division cycle 25C (Cdc25C) and disruption of the mitotic spindle assembly.

Materials and Methods

Chemicals

Sulforaphane and glucoraphanin were purchased from LKT Laboratories (St. Paul, MN) and Royal Veterinary and Agricultural University (KVL, Denmark), respectively. The antibodies specific for caspases and poly(ADP-ribose)polymerase were purchased from Cell Signaling Technology (Beverly, MA). The antibodies specific for cell cycle regulators and glyceraldehyde-3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon (Temecula, CA), respectively. Rhodamine 123 was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture

Human bladder cancer UM-UC-3 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in McCoy's 5A medium with L-glutamine, supplemented with 10% fetal bovine serum. The medium and fetal bovine serum were purchased from Cellgro (Herndon, VA) and Biosource International (Camarillo, CA), respectively. Cells were maintained in 75 cm^2 flasks in a humidified incubator at 37°C with 5% CO_2.

Preparation of Broccoli Sprout Extracts

Broccoli sprout extracts were prepared from 3-day-old fresh sprouts, which were grown by Sprouters Northwest (Kent, WA) using seeds purchased from Caudill Seed Co., Inc. (Louisville, KY). Briefly, fresh 3-day-old broccoli sprouts were added to boiling water at a ratio of 100 kg sprouts per 300 liters of water, in a steam-jacketed kettle with stirring. The sprouts were added slowly to ensure that water temperature never dropped below 95°C. The sprouts were boiled for 30 minutes and were then filtered out by passing the mixture through a conical stainless mesh screen (1 mm mesh) after the mixture was cooled to ~38°C. This process inactivates endogenous epithiospecifier protein and myrosinase and simultaneously extracts glucosinolates. The filtrates were kept in stainless-steel drums, and the conversion of glucosinolates to isothiocyanates in the filtrates was initiated by adding a homogenized daikon sprout preparation containing myrosinase. Fresh daikon sprouts (also produced at Sprouters Northwest) contain low amounts of glucosinolates but a very high amount of active myrosinase, and unlike broccoli sprouts, are not known to contain epithiospecifier protein. Approximately 3.2 kg daikon sprouts (myrosinase activity: ~6 units/g fresh sprouts) were homogenized in a small volume of the

DOI:10.1158/1535-7163.MCT-05-0476

above-described broccoli sprout filtrates and then immediately mixed with the remaining filtrates (final volume: ~325 liters). This mixture was kept in the original kettle, incubated at 32°C to 38°C for 3.5 hours with occasional stirring, and passed through a stainless mesh screen as described above. The filtrates were transferred to trays and freeze-dried to yield 2.8 kg powder (from 100 kg broccoli sprouts). The freeze-dried extract was stored at −70°C until used.

When comparing the antiproliferative activity of broccoli sprout extracts with and without conversion of glucosinolates to isothiocyanates, we prepared additional extracts. Briefly, 20 g broccoli sprouts were added to 100 mL water at either 100°C or 70°C for 3 minutes. As already described, myrosinase is rapidly inactivated at 100°C, resulting in no isothiocyanate formation, whereas at 70°C myrosinase is still active but epithiospecifier protein is totally inactivated, thus leading to the formation of a maximal amount of isothiocyanates. After heat treatment and cooling to room temperature, evaporated water was replaced and the mixture was homogenized in a Waring blender for 10 minutes. The homogenates were centrifuged at 500 × *g* for 10 minutes to remove insoluble materials and the supernatants were stored at −70°C until use.

Measurement of Isothiocyanates in Broccoli Sprout Extracts

Total isothiocyanate content in each broccoli sprout extract was determined by the high-performance liquid chromatography–based cyclocondensation assay (18). For the freeze-dried broccoli sprout isothiocyanate extracts, the proportion of each of the three isothiocyanates that have been previously shown to exist in broccoli sprout extracts, including sulforaphane, iberin, and erucin (9), was determined by reverse phase high-performance liquid chromatography. Briefly, the extract was dissolved in water and the insoluble material was removed by filtration. The sample was then applied to an analytic C18 column (Partisil 10, ODS-2, 4.6 × 250 nm, 10 μm, Whatman, Clifton, NJ), which was equilibrated with 1% acetonitrile/99% water and eluted at 1 mL/min. A linear gradient run from 1% acetonitrile/99% water to 100% acetonitrile in 60 minutes was initiated 2 minutes after sample injection. Elution was monitored at 240 nm (the approximate λ_{max} of all three isothiocyanates being evaluated). The retention time of each isothiocyanate and its content in the sample was determined by comparison with authentic standards purchased from LKT Laboratories. The extract was also examined for presence of the corresponding glucosinolate (e.g., glucoraphanin, glucoiberin, and glucoerucin) by hydrophilic interaction liquid chromatograph as reported by Troyer et al. (19).

Cell Proliferation Assay

Cells were seeded in 96-well plates (5×10^3 cells per well with 150 μL medium) and grown for 24 hours. The broccoli sprout extracts, sulforaphane, and glucoraphanin were dissolved in cell culture medium, acetonitrile, and water, respectively. The final concentration of total isothiocyanate of extracts prepared with glucosinolate hydrolysis, sulforaphane, glucoraphanin, and total glucosinolate of extracts prepared without glucosinolate hydrolysis in each well ranged from 1.56 to 100 μmol/L (isothiocyanate extracts and sulforaphane) and 1.56 to 1,000 μmol/L (glucoraphanin and glucosinolate extracts). The final concentration of acetonitrile in sulforaphane-containing medium was ≤0.1% (v/v). Cells were incubated with the sprout extracts, sulforaphane, or glucoraphanin for 72 hours and cell density in each well was measured by 3-(4,6-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (20). The growth curve of extract-treated cells was plotted to determine the IC_{50}.

Western Blot Analysis

A total of 1.5×10^6 cells were grown in each 10 cm dish with 10 mL medium for 24 hours and then were treated with the extracts at a desired isothiocyanate concentration for 24, 48, and 72 hours, respectively. At the end of treatment, cells in each dish were harvested by trypsinization and centrifugation at 500 × *g* for 5 minutes at 4°C. The cell pellet from each dish was washed once with 10 mL ice-cold PBS and was centrifuged again. Each cell pellet was then suspended in 200 μL cell lysis buffer from Cell Signaling Technology, which was supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, and was sonicated using a Branson's Model 450 sonifier. The lysates were centrifuged at 10,000 × *g* for 5 minutes at 4°C, and the supernatant was used for analysis. Protein concentration in each sample was measured using a bicinchoninic acid assay kit (Pierce, Rockford, IL). Each sample (50 μg protein) was resolved by SDS-PAGE (8-15%) and blotted to polyvinylidene difluoride membranes, which were probed by specific antibodies and visualized using an ECL chemiluminescence system from Amersham Biosciences (Piscataway, NJ).

Measurement of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$)

Cells were grown in 10 cm dishes (1.5×10^6 per dish with 10 mL medium) for 24 hours and then treated with the broccoli sprout extract at an isothiocyanate concentration of 0, 15, or 30 μmol/L in the culture medium for 48 hours. At the end of treatment, cells were harvested and washed with fresh medium. A total of 1×10^6 cells were suspended in 1 mL fresh medium and incubated with 10 μg rhodamine 123 for 30 minutes at 37°C. Cells were then washed twice with fresh medium and immediately analyzed by flow cytometry to determine fluorescence intensity. Rhodamine 123 is readily and selectively sequestered by normal mitochondria (cells showing strong fluorescence) but is removed from the latter when $\Delta\Psi_m$ is lost (cells showing diminished fluorescence). Ten thousand cells were examined in each sample using a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA).

Analysis of Apoptosis

The cell death detection ELISA^{PLUS} kit from Roche Applied Science (Indianapolis, IN), which detects histone-associated DNA fragments (mononucleosomes and oligonucleosomes) in cytoplasmic fractions of cells, was used to assess apoptosis in cells treated by the broccoli sprout extract. Ten thousand cells were placed in each well of 96-well plates with 150 μL

DOI:10.1158/1535-7163.MCT-05-0476

medium and grown for 24 hours and then incubated with the extract at a total isothiocyanate concentrations of 0, 15, or 30 μmol/L in fresh medium for 48 hours. Apoptosis was measured according to the instructions of the manufacturer.

Analysis of Cell Cycle Arrest

Cells were treated with the broccoli sprout extract and then harvested as described in the Western blot analysis. For each sample, 1×10^6 cells were suspended in 1 mL modified Krishan buffer containing 1 g/L sodium citrate, 20 mg/L RNase, 0.3% NP40, and 50 mg/L propidium iodide (21), and incubated on ice in the dark for at least 1 hour. Cell cycle distribution was then determined by flow cytometry, and 10,000 cells per sample were analyzed.

Immunofluorescence Staining for Microtubules

Cells were grown on 12 mm glass cover slides placed in six-well plates (0.3×10^6 cells per slide with 2 mL medium in each well) for 24 hours, and then exposed to the broccoli sprout extract at a total isothiocyanate concentration of 0 or 15 μmol/L for 24 hours. After washing once with ice-cold PBS, cells were fixed with 3.7% formaldehyde in PBS for 10 minutes at room temperature. Following three washes with PBS, cells were permeabilized with wash buffer (0.1% Triton X-100 and 1% bovine serum albumin in PBS) for 10 minutes, and treated with 10% mouse serum in PBS for another 10 minutes. Following three more rinses with wash buffer, cells were incubated for 1 hour at 37°C with a monoclonal antibody against α-tubulin raised in mice (Sigma). Following another three washes through wash buffer, cells were incubated with a FITC-conjugated goat anti-mouse secondary antibody from Santa Cruz Biotechnology for 30 minutes in a dark chamber. Finally, cells were washed again with wash buffer and mounted on microscope slides with mounting medium (Molecular Probes), which contained 4′,6-diamidino-2-phenylindole to counterstain DNA in cells. Fluorescence microscopy was used to analyze samples.

Wright-Giemsa Staining and Quantification of Mitotic Figures

Cells were seeded in 10 cm dishes (1.5×10^6 per dish with 10 mL medium) and grown for 24 hours, treated with broccoli sprout extracts, and harvested as described above. After washing them once with PBS, cells were pelleted by low-speed centrifugation, resuspended in PBS, and transferred to microscope slides using a Shandon Cytospin 4 from Thermo Electron Corp. (Pittsburgh, PA). Cells on the slides were dried at room temperature and stained with Wright-Giemsa solution (Sigma). After rinsing and air drying, cover slides were affixed and slides were analyzed by light microscopy. The percentage of mitotic figures was determined by analyzing a minimum of 150 cells in at least 20 fields per slide.

Statistics

Results were expressed as mean ± SD (at least three determinations). Data were analyzed by one-way ANOVA, followed by Dunnett's t test for separate comparisons with the control group. When the comparison involved only two groups, the data were analyzed by Student's t test. Differences were considered significant at $P < 0.05$.

Results

Isothiocyanate Contents in Broccoli Sprout Extracts

Our previous studies have shown that only three isothiocyanates, sulforaphane, iberin, and erucin, are present in aqueous broccoli sprout extracts, and they are derived from glucoraphanin, glucoiberin, and glucoerucin, respectively (1, 9). Because our preliminary study showed that two glucosinolates, including sinigrin (the precursor of allyl isothiocyanate) and gluconasturtinn (the precursor of phenethyl isothiocyanate), had no antiproliferative activity against human bladder cancer cells (results not shown), whereas both allyl isothiocyanate and phenethyl isothiocyanate displayed potent activity (17), we focused in the current study on broccoli sprout extracts in which all glucosinolate had been converted to isothiocyanates. In fact, as will be described later, both glucoraphanin and a broccoli sprout extract in which the glucosinolate were not hydrolyzed due to heat-induced myrosinase inactivation showed little antiproliferative activity. The freeze-dried isothiocyanate-rich extract of broccoli sprouts contained 173 μmol total isothiocyanate per gram of extract (dry powder), of which 70%, 25%, and 5% were sulforaphane, iberin, and erucin, respectively (Fig. 1). These results are consistent with our previous findings (1, 9). The glucosinolates in the extract were fully hydrolyzed by myrosinase, as they were undetectable in the isothiocyanate extract (results not shown).

Stability of Isothiocyanates in Lyophilized Broccoli Sprout Extracts

The lyophilized isothiocyanate-rich extract had been stored for nearly 1 year at the time this report was prepared for publication. We were interested in the stability of the isothiocyanates in the extract stored under different conditions. The isothiocyanates were quite unstable when the extract was stored at room temperature, as only 48% and 24% isothiocyanates remained after 5 and 11 months, respectively (Fig. 2). The degradation rates of the isothiocyanates in the extract when stored at −20°C and −70°C were identical and were considerably slower than at room temperature, as 91% and 84% isothiocyanates remained in the extract after 5 and 11 months of storage under these conditions, respectively (Fig. 2). These results show that freezing is required to significantly retard the rate of degradation of the isothiocyanates in the extracts.

Growth Inhibition of Human Bladder Cancer Cells by the Isothiocyanate Extracts of Broccoli Sprouts

We next asked three questions: (*a*) does the broccoli sprout isothiocyanate extract display antiproliferative activity; (*b*) if so, is the antiproliferative activity of the extract accounted for by the isothiocyanates; and (*c*) if the isothiocyanates are responsible for the antiproliferative activity of the extract, is such an activity comparable with that of sulforaphane, because sulforaphane comprises 70% of the total isothiocyanates in the extract and the remaining two isothiocyanates are structurally similar to sulforaphane. The results are shown in Fig. 3 and can be summarized as follows: First, incubation of UM-UC-3

DOI:10.1158/1535-7163.MCT-05-0476

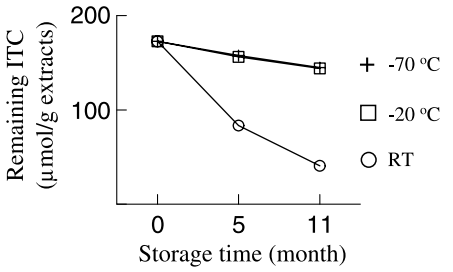

Figure 2. Stability of isothiocyanate (*ITC*) in a dry broccoli sprout extract. Myrosinase-treated broccoli sprout extracts (lyophilized powder) were either stored at room temperature (*RT*), −20°C, or −70°C, and at the indicated times the total isothiocyanate content in the extracts were determined by the cyclocondensation assay. *Points,* mean (n = 3); *bars,* SD. Note that the two lines showing the degradation rates of the isothiocyanates at −20°C and −70°C overlap one another.

cells with isothiocyanate-rich sprout extracts led to inhibition of cell growth, with an IC_{50} value of 6.8 μmol/L isothiocyanate (Fig. 3A). Second, the conversion of glucosinolates to isothiocyanates was essential for broccoli sprout extracts to exhibit antiproliferative activity, as neither glucoraphanin (Fig. 3A) nor broccoli sprout extracts in which myrosinase was heat-inactivated (thus blocking conversion of glucosinolates to isothiocyanates; Fig. 3B), could significantly retard the growth of UM-UC-3 cells. Third, the potency of the isothiocyanates in broccoli sprout extracts in inhibiting the growth of UM-UC-3 cells is almost identical to that of sulforaphane, as the IC_{50} value of the latter is 6.6 μmol/L (Fig. 3A). This suggests that the antiproliferative activities of iberin and erucin may be similar to that of sulforaphane and that other substances in broccoli sprout extracts do not interfere with the antiproliferative activity of the isothiocyanates.

In this context, it is noteworthy that our previous study (1) has shown that broccoli sprouts contain very low levels of indole glucosinolates (~3% of isothiocyanate-forming glucosinolates). Hydrolysis of indole glucosinolates by myrosinase may give rise to indole-3-carbinol, which has been shown to display antiproliferative activity in cultured cells at relatively high concentrations (≥50 μmol/L; ref. 22). However, the level of indole-3-carbinol in the broccoli sprout extracts, if any, is probably far below what is needed to have an effect on the antiproliferative effects of the isothiocyanates.

Induction of Apoptosis by Broccoli Sprout Isothiocyanate Extract in Human Bladder Cancer Cells

Sulforaphane was previously shown to induce apoptosis of UM-UC-3 cells at 7.5 to 30 μmol/L, which was associated with mitochondrial damage, cytoplasmic relocation of cytochrome *c*, cleavage/activation of caspase-9 and caspase-3, as well as cleavage of poly(ADP-ribose)-polymerase (17, 23). The isothiocyanates in broccoli sprout extracts behaved similarly. When cells were treated by the extract at the total isothiocyanate concentrations of 7.5, 15, and 30 μmol/L for 24 to 72 hours, there was a dose- and time-dependent increase in the cleavage of caspase-9, caspase-3, and poly(ADP-ribose)polymerase. Similar to sulforaphane (17), the extract had no detectable effect on caspase-8 (Fig. 4A). Moreover, the isothiocyanate extract also caused mitochondrial damage, as cells with loss of $\Delta\Psi_m$ increased 2.4- and 3.2-fold after incubation with the extract for 48 hours at the total isothiocyanate concentrations of 15 and 30 μmol/L (Fig. 4B). Apoptosis induction by the extract was further documented by increased cytoplasmic accumulation of histone-associated DNA fragments. After treatment of cells with the extract at the total isothiocyanate concentrations of 15 and 30 μmol/L for 48 hours, cytoplasmic levels of histone-associated DNA fragments increased 4.4- and 5.2-fold, respectively (Fig. 4C). Taken together, these results show that, much like sulforaphane, broccoli sprout isothiocyanate extracts stimulate the mitochondria-mediated intrinsic apoptosis pathway.

Arrest of Cell Cycle Progression by Broccoli Sprout Isothiocyanate Extract in Human Bladder Cancer Cells

In addition to inducing apoptosis, sulforaphane is also known to cause cell cycle arrest in various cell lines. It arrested UM-UC-3 cells in both S and G_2-M phases at 7.5 to 30 μmol/L (17). Likewise, incubation of UM-UC-3 cells

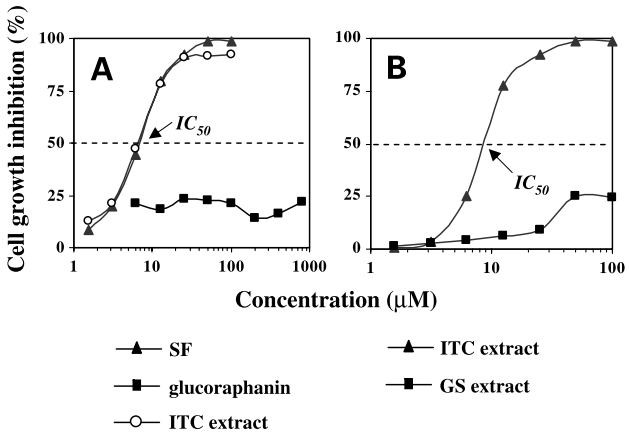

Figure 3. Effect of sulforaphane (*SF*), glucoraphanin, and broccoli sprout extracts on cancer cell growth. Human bladder cancer UM-UC-3 cells were grown in 96-well plates (5×10^3 per well) for 24 h and then exposed to the test agent at indicated concentrations for 72 h. At the end of treatment, cell density in each well was determined using 3-(4,6-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (20). *Points,* mean (n = 6–8); the SD values are too small to be visible. The isothiocyanate extract used in **A** was the freeze-dried broccoli sprout extract in which all glucosinolates were converted to isothiocyanates, and the contents of isothiocyanates in the extract are shown in Fig. 1. The extracts used in **B** were prepared without lyophilization as described in Materials and Methods. Briefly, broccoli sprouts were mixed with water either at 70°C [maximal conversion of glucosinolate to isothiocyanate (*ITC extract*) by endogenous myrosinase after epithiospecifier protein inactivation] or 100°C [minimal conversion of glucosinolates to isothiocyanates (*GS extract*) due to myrosinase inactivation] for 3 min, followed by homogenization in a Waring blender. The homogenates were centrifuged at low speed to remove insoluble materials before use for bioassay. The total isothiocyanate content was 1.06 ± 0.03 μmol/mL isothiocyanate extract and 0.02 ± 0 μmol/mL glucosinolate extract, as measured by the cyclocondensation assay.

DOI:10.1158/1535-7163.MCT-05-0476

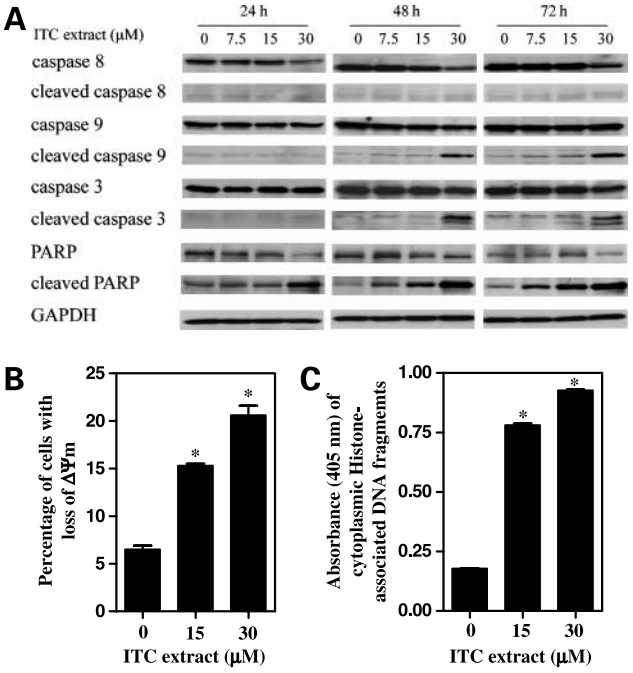

Figure 4. Activation of mitochondria apoptosis pathway by broccoli sprout isothiocyanate extract. UM-UC-3 cells (1.5×10^6 per plate) were grown in 10 cm plates with 10 mL medium for 24 h and were then treated with freeze-dried broccoli sprout isothiocyanate extract at the indicated isothiocyanate concentrations for 24, 48, or 72 h. At the end of treatment, cells were harvested for various analyses. **A,** detection of cleavage of caspase-3, caspase-8, caspase-9, and poly(ADP-ribose)-polymerase by Western blot, using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a loading control. **B,** measurement of $\Delta\Psi_m$ by flow cytometry, using rhodamine 123 as a mitotracker. Cells were treated with the extract for 48 h, and 10,000 cells per sample were analyzed. **C,** detection of accumulation of cytoplasmic histone-associated DNA fragments by an ELISA kit. Cells were treated with broccoli sprout isothiocyanate extract for 48 h before analysis. *Columns,* mean (n = 3); *bars,* SD.

with broccoli sprout extract at the isothiocyanate concentrations of 7.5, 15, and 30 μmol/L generally led to time- and dose-dependent cell cycle arrest in G_2-M phase and to a lesser extent in S phase (Table 1). The number of cells in G_2-M and S phases increased 6.4 and 4.5 times, respectively, after treatment with the extract at the highest isothiocyanate concentration for 72 hours. Interestingly, prolonged culture of UM-UC-3 cells led to accumulation of control cells in G_1 phase, as there were 79.0% and 83.8% control cells in G_1 phases after growing for 48 and 72, hours, respectively, compared with 53.3% cells in G_1 phase at the 24 hours time point (Table 1). The reason for this phenomenon is not entirely clear, but may result from an increasingly unfavorable growth environment, e.g., increased cell density/increased cell contact and diminished availability of nutrients in the culture medium. Moreover, treatment of UM-UC-3 cells with the extract also caused the formation of both subdiploid cells (sub-G_1) and cells that were more than tetraploids (>4n DNA; Table 1). However, formation of sub-G_1 cells occurred maximally at 15 μmol/L isothiocyanate and was not correlated with caspase-mediated apoptosis, which was the strongest at 30 μmol/L isothiocyanate (Fig. 3). Formation of cells containing >4n DNA is consistent with arrest of mitosis as described later, but was detected only at 15 μmol/L isothiocyanate.

We next examined the effect of the broccoli sprout isothiocyanate extract on cyclin B1/cdc2 (important for G_2-M phase transition) and cyclin A/cdk2 (important for S-phase transition) in UM-UC-3 cells, but no down-regulation of any of these proteins was detected (Fig. 5). Instead, there were slight increases in the levels of cdc2 and cdk2 after treatment with the extract, which might result from increased cell numbers in these phases due to arrest. This contrasts with our previous finding that sulforaphane down-regulates cyclin B1 (17). It is possible that certain chemicals in the broccoli sprout extracts may interfere with the effects of sulforaphane on cyclin B1. Cdc25B and Cdc25C are nuclear phosphatases and are involved in both G_2-M and S phase regulation by modulating the activity of cyclin-dependent kinases. Sulforaphane has previously been shown to down-regulate both Cdc25B and Cdc25C in human prostate cancer cells (24). The broccoli sprout isothiocyanate extract had no effect on Cdc25B but caused down-regulation of Cdc25C at 30 μmol isothiocyanate per liter in UM-UC-3 cells (Fig. 5), suggesting that these proteins either are not involved or play a limited role in mediating cell cycle arrest by the isothiocyanate extract. Because S-phase arrest was more prominent at 30 μmol isothiocyanate per liter, it is possible that down-regulation of Cdc25C may contribute more to S-phase arrest than to G_2-M phase arrest. Previous studies have shown that down-regulation of Cdc25C by chemopreventive agents may be associated with cell cycle arrest in G_2-M, S and G_2-M, or S phase (25–27).

Disruption of Mitotic Spindle and Arrest of Mitosis by Broccoli Sprout Isothiocyanate Extract

Sulforaphane and erucin, both of which are present in the broccoli sprout isothiocyanate extract, were previously shown to disrupt mitotic spindles and cause M-phase arrest in mammary carcinoma cells, presumably by inhibiting tubulin polymerization (7). We reasoned that broccoli sprout isothiocyanate extract might affect mitotic spindles and cause M-phase arrest in bladder cells as well. Thus, mitotic spindles were visualized under fluorescence microscopy after immunostaining of α-tubulin with an FITC-tagged antibody. As shown in Fig. 6, control cells showed characteristic two-polar mitotic spindles with aligned DNA (Fig. 6A and B), mitotic figures ranging from early metaphase to telophase were scattered throughout the whole cell population. However, after treatment with the isothiocyanate extract at 15 μmol isothiocyanate per liter for 24 hours, cells were devoid of well-organized mitotic spindles, microtubules were scrambled together, and cells showed single or multipolar structures without equatorial metaphase alignment or separation of DNA (Fig. 6C and D). The mitotic spindle disruption was associated with cell arrest in mitosis as documented by Wright-Giemsa staining

DOI:10.1158/1535-7163.MCT-05-0476

Table 1. Effect of broccoli sprout isothiocyanate extract on cell cycle progression of UM-UC-3 cells

Isothiocyanate extract (μmol/L)	Cell cycle distribution (%)				
	Sub-G_1	G_1	S	G_2-M	Cells with >4n DNA
24-h treatment					
0	2.1 ± 0.4	53.3 ± 1.7	32.9 ± 1.6	10.9 ± 0.4	0.8 ± 0.2
7.5	2.2 ± 0.5	44.1 ± 0.7	37.6 ± 0.8*	14.4 ± 0.5*	1.6 ± 0.2
15	3.4 ± 0.8	12.1 ± 0.4	42.5 ± 2.4*	38.7 ± 0.9*	3.4 ± 1.4*
30	3.3 ± 1.0	47.0 ± 0.1	31.3 ± 1.2	17.0 ± 0.7*	1.4 ± 0.1
48-h treatment					
0	2.1 ± 0.2	79.0 ± 0.8	11.8 ± 0.5	6.0 ± 0.5	1.1 ± 0.2
7.5	5.7 ± 0.7*	61.2 ± 1.2	22.7 ± 0.7*	8.9 ± 1.0*	1.6 ± 0.8
15	5.6 ± 1.2*	33.4 ± 0.3	15.5 ± 0.5*	30.7 ± 0.9*	14.8 ± 1.0*
30	4.7 ± 1.0*	44.8 ± 2.1	25.9 ± 0.8*	23.0 ± 0.7*	1.6 ± 0.6
72-h treatment					
0	5.1 ± 1.2	83.8 ± 2.9	6.3 ± 0.4	3.8 ± 1.8	1.0 ± 0.4
7.5	10.0 ± 1.2*	71.7 ± 1.5	13.0 ± 0.5*	3.8 ± 0.9	1.5 ± 0.1
15	14.4 ± 1.5*	36.8 ± 4.3	14.6 ± 0.1*	20.7 ± 2.1*	13.5 ± 3.1*
30	5.7 ± 0.7	39.7 ± 2.7	28.6 ± 1.6*	24.2 ± 1.7*	1.8 ± 0.1

NOTE: Cells were grown in 10 cm plates (1.5×10^6 per plate with 10 mL medium) for 24 hours and then exposed to isothiocyanate extract at desired concentrations for 24, 48, or 72 hours, respectively. Cells were harvested and analyzed by flow cytometry. Values are mean ± SD, $n = 3$.
*$P < 0.05$, different from the control.

of nuclei. The number of mitotic cells after treatment with the isothiocyanate extract at 15 μmol isothiocyanate per liter for 24 hours increased from 7.6% in the control to 30.6% in extract-treated cells, a net increase of 23% (Fig. 7A and B). Significantly, examination of cells by flow cytometry showed that G_2-M phase cells increased from 10.9% in the control to 38.7% after treatment with the isothiocyanate extract at 15 μmol/L for 24 hours (Table 1), a net increase of 27.8% (very close to the 23% mitotic cells detected by Wright-Giemsa staining, as described above). These findings therefore revealed that UM-UC-3 cells were arrested by the isothiocyanate extract primarily in M phase rather than in G_2 phase.

Moreover, when UM-UC-3 cells were grown for 72 hours in the cultured medium initially dosed with the isothiocyanate extract at 15 μmol isothiocyanate per liter, there was an abundance of giant cells with large nuclei (sign of failed nuclear division) or multiple micronuclei (sign of mitotic catastrophe; Fig. 7C). Cell cycle distribution measured by flow cytometry–based DNA analysis showed that there were 13.5% cells with >4n DNA after treatment with the isothiocyanate extract, compared with 1% in the control group (Fig. 7D). These results suggest that nuclear division in UM-UC-3 cells may be persistently inhibited by the isothiocyanate extract, whereas DNA synthesis may continue and the cells may be subsequently killed by mitotic catastrophe.

Discussion

Our results show that isothiocyanate-rich broccoli sprout extracts are potent inhibitors of cancer cell growth, and that neither glucoraphanin nor broccoli sprout extracts in which glucosinolates were not hydrolyzed (due to heat-induced myrosinase inactivation) showed growth-inhibitory activity against UM-UC-3 cells (Fig. 3). To the best of our knowledge, this is the first study to show that the antiproliferative activity of broccoli sprout extracts is exclusively derived from the isothiocyanates. In light of these results, it is worth noting that we have previously shown that induction of carcinogen-detoxifying phase 2 enzymes by broccoli sprout extracts also depends on isothiocyanate formation (1). Moreover, the antiproliferative potency of the isothiocyanates in the crude broccoli sprout extract is almost identical to that of sulforaphane, suggesting that such an extract may be a replacement for sulforaphane for potential clinical test and use. This is significant, not only because chemical synthesis of sulforaphane is complex and costly, but also because when considered in the context of a

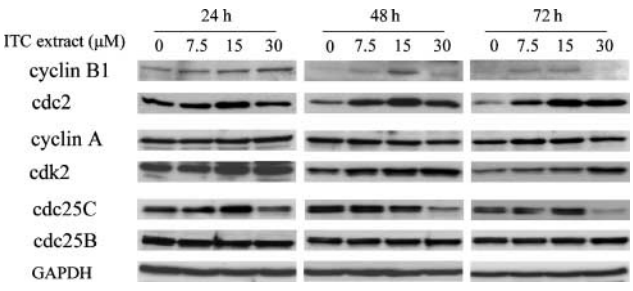

Figure 5. Effect of broccoli sprout isothiocyanate (*ITC*) extract on select cell cycle regulators. UM-UC-3 cells (1.5×10^6 per plate) were grown in 10 cm plates with 10 mL medium for 24 h and were then treated with freeze-dried broccoli sprout isothiocyanate extract at indicated isothiocyanate concentrations for 24, 48, or 72 h. At the end of treatment, cells were harvested for analysis for various cell cycle regulators by Western blot. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control.

DOI:10.1158/1535-7163.MCT-05-0476

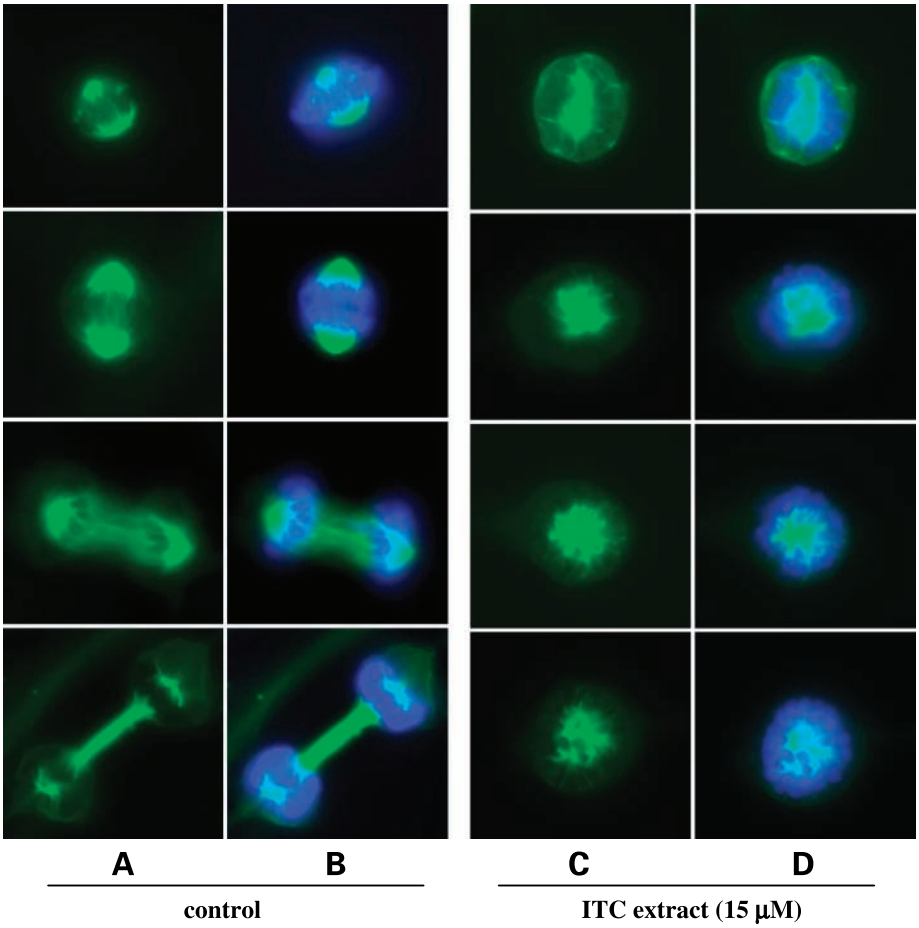

Figure 6. Effect of broccoli sprout isothiocyanate (*ITC*) extract on mitotic spindles and nuclear division. UM-UC-3 cells were grown in 12-mm glass cover slides for 24 h and then treated with either vehicle or freeze-dried broccoli sprout isothiocyanate extract at 15 μmol isothiocyanate per liter for 24 h. The cells were then fixed in formaldehyde, permeabilized with Triton X-100, and probed with an anti-α-tubulin primary antibody and an FITC-conjugated secondary antibody (*green*). Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (*blue*). The fluorescence micrographs (magnification, ×1,000) are representative of cells in the control (**A,** tubulin; **B,** tubulin and DNA) and extract-treated groups (**C,** tubulin; **D,** tubulin and DNA).

disease prevention strategy, plant extracts may be an important alternative to pharmaceutical agents. Broccoli sprout isothiocyanate extracts are also more attractive than fresh broccoli sprouts, as eating the latter likely causes significant loss of isothiocyanates due to the presence of epithiospecifier protein, as discussed in the Introduction. However, further development to increase the stability of isothiocyanates in such broccoli sprout extracts is warranted, as the isothiocyanates in our lyophilized extracts degraded at a considerable rate, particularly at room temperature.

Treatment of UM-UC-3 cells with broccoli sprout isothiocyanate extracts led to activation of mitochondria-mediated apoptosis pathway, including mitochondrial damage, cleavage of caspase-3/9 and poly(ADP-ribose)-polymerase, cytoplasmic accumulation of histone-associated DNA fragments, and absence of cleavage of caspase-8 (Fig. 4). Similar changes were previously observed in these cells after sulforaphane treatment (17, 23), indicating that the broccoli sprout isothiocyanate extract and sulforaphane elicit the same apoptosis-inducing mechanism. Both the broccoli sprout isothiocyanate extract in the current study and sulforaphane in our previous study (17) also caused S and G_2-M phase arrest in UM-UC-3 cells. However, whether the isothiocyanate extract and sulforaphane share the same mechanisms of cell cycle arrest requires further investigation. Whereas sulforaphane was previously shown to decrease the level of cyclin B1 in these cells, the isothiocyanate extract had no effect on cyclin B1. Rather, the isothiocyanate extract down-regulated Cdc25C, which may account for S-phase arrest. The effect of sulforaphane on Cdc25C has not been examined in UM-UC-3 cells, although it was previously shown to down-regulate Cdc25C in prostate cancer cells through checkpoint kinase 2–mediated phosphorylation of the protein (24). More importantly, in addition to causing S-phase arrest, our results show that the isothiocyanate extract potently arrests UM-UC-3 cells in M phase while exerting little effect on G_2 phase. This raises the question whether individual isothiocyanates, including sulforaphane, selectively target M phase but not G_2 phase, as isothiocyanates have been widely reported to arrest cells in G_2-M phase but there have been no studies to discern the effect of the compounds on G_2 and M phases separately. Our studies clearly show that the isothiocyanate extract potently disrupts spindle assembly and blocks mitosis. Disruption of spindle assembly most likely resulted from inhibition of tubulin polymerization, because sulforaphane was

DOI:10.1158/1535-7163.MCT-05-0476

previously shown to inhibit tubulin polymerization in an *in vitro* assay (7). Hence, both sulforaphane and broccoli sprout isothiocyanate extract seem to be microtubule-destabilizing agents, resembling *Vinca* alkaloids and some other antimitotic agents that are currently in clinical use for cancer treatment.

The broccoli sprout extracts show potent anticancer activity at 7.5 to 30 μmol/L isothiocyanate concentrations. These concentrations seem to be achievable *in vivo*. Hu et al. (28) recently reported that after a single p.o. dose of 50 μmol sulforaphane (the major isothiocyanate in broccoli sprout extracts) to each rat weighing 120 to 150 g, plasma sulforaphane concentrations peaked at ~20 μmol/L. Isothiocyanates are principally metabolized through the mercapturic acid pathway *in vivo*, giving rise to dithiocarbamates, which are biologically similar to their parent isothiocyanates (29, 30). Although plasma concentrations of isothiocyanates plus dithiocarbamates peaked at 0.9 to 2.3 μmol/L, when human volunteers were each fed a single dose of broccoli sprout extracts containing ~200 μmol total isothiocyanate, the total urinary concentrations of isothiocyanates plus dithiocarbamates on average were estimated to be >100 μmol/L during the first 8-hour period after isothiocyanate dosing (31, 32). Hence, the bladder epithelium where the majority of bladder cancers occur is exposed to high concentrations of isothiocyanates plus dithiocarbamates after dietary consumption of small amounts of broccoli sprout extracts.

In summary, broccoli sprouts are a rich source of sulforaphane and related isothiocyanates and potently inhibit the growth of human bladder cancer cells. The latter is associated with induction of apoptosis and cell cycle arrest. Our results show that isothiocyanate-rich broccoli sprout extracts activate the intrinsic apoptosis pathway and arrest cells in both S and M phases. Arrest of cell cycle progression by the extracts was associated with down-regulation of Cdc25C and disruption of mitotic spindles. Although the current study has involved only a single bladder cell line, it is tempting to predict that the broccoli sprout isothiocyanate extract will exert similar effects in other cancer cells. This prediction is based on the finding that the isothiocyanate extract and sulforaphane show very similar biological activity in UM-UC-3 cells, and the antiproliferative activity of sulforaphane has been reported in cell lines derived from various human cancers.

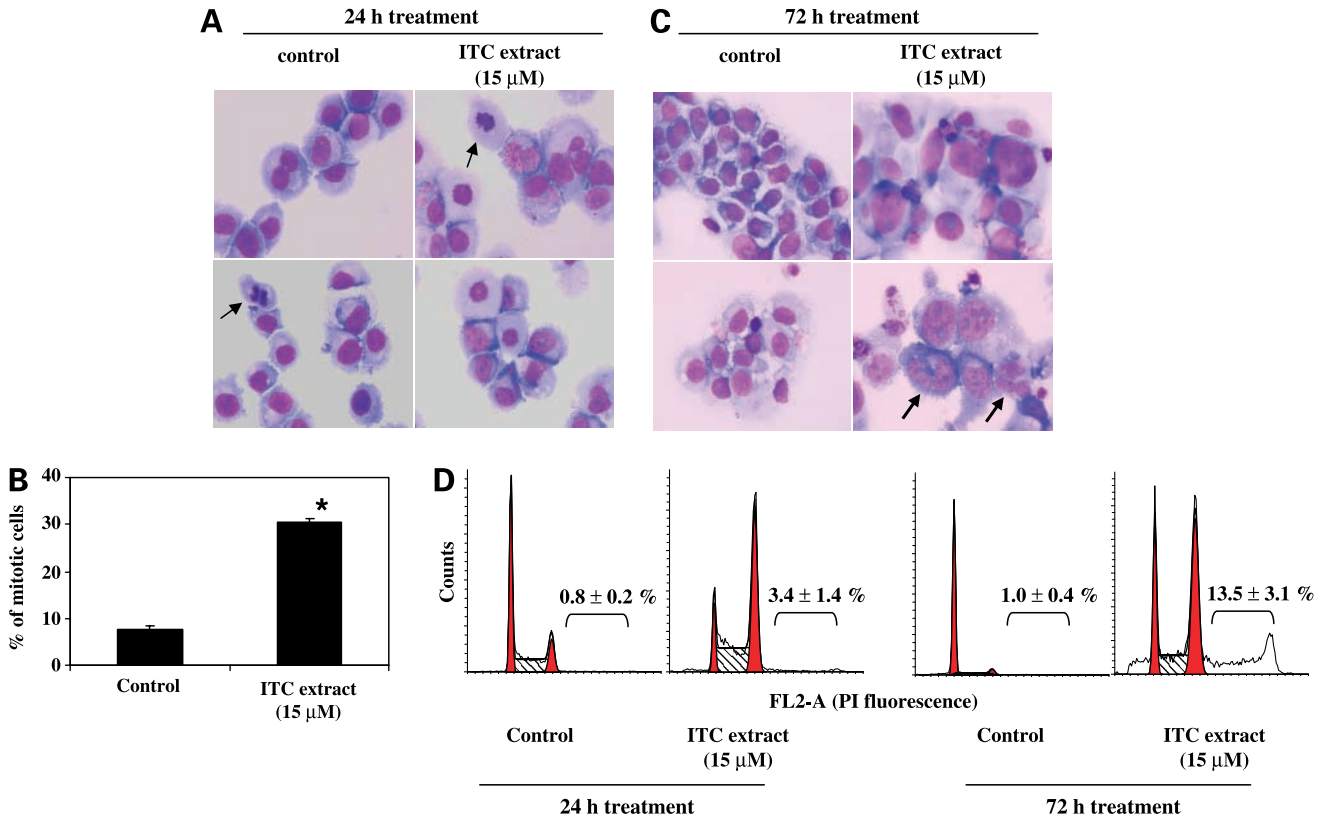

Figure 7. Arrest in mitosis by broccoli sprout isothiocyanate (*ITC*) extracts. UM-UC-3 cells were seeded in 10 cm plates and grown for 24 h before exposure to either vehicle or freeze-dried broccoli sprout isothiocyanate extract at 15 μmol isothiocyanate per liter for 24 or 72 h. **A** and **C,** cells were stained with a Wright-Giemsa solution and cell morphologies were examined under a light microscope (magnification, ×1,000). *Arrows,* mitotic cell (**A**) or a giant cell with a large nucleus or multiple nuclei (**C**). **B,** the percentage of mitotic figures was determined by analyzing a minimum of 150 cells in at least 20 fields per slide; only cells that were treated with the extract or vehicle for 24 h were analyzed. **D,** analysis of cell cycle distribution by flow cytometry (10,000 cells per sample). *PI,* propidium iodide.

DOI:10.1158/1535-7163.MCT-05-0476

Acknowledgments

We thank James R. Marshall at Roswell Park Cancer Institute for providing access to instruments for image analysis.

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