Nutrition and Cancer

Mechanisms of Action and Antiproliferative Properties of *Brassica oleracea* Juice in Human Breast Cancer Cell Lines[1,2]

Giorgio Brandi,*†[3] Giuditta F. Schiavano,* Nadia Zaffaroni,‡ Cinzia De Marco,‡ Mirko Paiardini,** Barbara Cervasi,** and Mauro Magnani†**

*Institute of Hygiene, †Center of Biotechnology, **Institute of Biochemistry "G. Fornaini" University of Urbino, 61029 Urbino, Italy and ‡Department of Experimental Oncology, Unit 10, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy

ABSTRACT Cruciferous vegetables are an important source of compounds that may be useful for chemoprevention. In this study, we evaluated the antiproliferative activity of juice obtained from leaves of several varieties of *Brassica oleracea* on both estrogen receptor (ER)-positive (ER+; MCF-7 and BT474) and ER-negative (ER−; MDA-MB-231 and BT20) human breast cancer cell lines. The effect of juice on cell proliferation was evaluated on DNA synthesis and on cell cycle–related proteins. Juice markedly reduced DNA synthesis, evaluated by [^{3}H]thymidine incorporation, starting from low concentrations (final concentration 5–15 mL/L), and this activity was independent of ER. All cauliflower varieties tested suppressed cell proliferation in a dose-dependent manner. Cell growth inhibition was accompanied by significant cell death at the higher juice concentrations, although no evidence of apoptosis was found. Interestingly, the juice displayed a preferential activity against breast cancer cells compared with other mammalian cell lines investigated (ECV304, VERO, Hep2, 3T3, and MCF-10A) ($P < 0.01$). At the molecular level, the inhibition of proliferation was associated with significantly reduced CDK6 expression and an increased level of p27 in ER+ cells but not in ER− cells, whereas a common feature in all cell lines was significantly decreased retinoblastoma protein phosphorylation. These results suggest that the edible part of *Brassica oleracea* contains substances that can markedly inhibit the growth of both ER+ and ER− human breast cancer cells, although through different mechanisms. These results suggest that the widely available cruciferous vegetables are potential chemopreventive agents. J. Nutr. 135: 1503–1509, 2005.

KEY WORDS: • *breast cancer* • *Brassica juice* • *CDK6* • *p27* • *chemoprevention*

In recent years, chemoprevention has attracted considerable attention as a means of blocking malignant transformation in its early stages and disease progression in later stages. The presence of a large number of potentially anticarcinogenic compounds in fruits and vegetables was suggested, particularly in those belonging to the genus *Brassica* (e.g., cauliflower and broccoli) (1–3). These cruciferous plants contain high quantities of glucosinolates (GLS),[4] whose hydrolytic products were shown to prevent, delay, or reverse carcinogenesis induced by various chemical compounds (4–6). In fresh plants, GLS are present in a stable form, compartmentalized in different sites from the endogenous enzyme myrosinase; when tissues are destroyed, however, myrosinase comes into contact with and hydrolyzes GLS into a series of breakdown products such as isothiocyanates (ITCs; isothiocyanates sulforaphane, benzylisothiocyanate and prophylisothiocyanate, phenethyl isothiocyanates), and indoles (3,3′-diindolylmethane, ascorbagen, indole-3-carbinol, indolo-[3,2-b]carbazole) (7).

Although the mechanism(s) by which cruciferous vegetables exert their cancer chemopreventive action is largely unclear, several hypotheses were proposed; first among these is that some agents contained in these vegetables exert a protective effect by modulating the activity of Phase II and Phase I drug metabolism enzymes. In particular, ITCs and indole-3-carbinol were shown to induce the so-called phase-II enzymes, (i.e., glutathione *S*-transferase, quinone reductase, glucoronosyl transferase), which play an important role in the detoxification of toxins and carcinogens (4,8–11) and inhibit phase I enzymes, which are responsible for metabolic activation of most chemical carcinogens (10,12).

Recently, several other mechanisms were proposed to explain the protective effect associated with *Brassica* consumption in breast cancer, such as interactions between agents contained in this vegetable and estrogen metabolism (or signal transduction pathways) (13,14), the induction of apoptotic cell death (15–18), and interference with cell cycle regulatory proteins (19–22). In a previous study (22) we demonstrated that a cyclic tetrameric derivative (tetramer) of indole-3-

[1] Presented in part at "40° Congresso Nazionale SItI," September 2002, Cernobbio, Italy [Brandi, G., Cervasi, B., Amagliani, G., Schiavano, G. F., Sisti, M. & Paiardini, M. (2001) Valutazione dell'attività antiproliferativa di succo di Brassica Oleracea in cellule di tumore mammario umano. Panorama della Sanitá. P07–P09 (abs.)].

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[3] To whom correspondence should be addressed. E-mail: brandi@uniurb.it.

[4] Abbreviation used: CDK, cyclin-dependent kinase; DMSO, dimethyl sulfoxide; ER, estrogen receptor; FCS, fetal calf serum; GLS, glucosinolates; I3C, indole-3-carbinol; IC_{50}, 50% inhibitory concentration; ITC, isothiocyanate; pRb, retinoblastoma protein.

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carbinol (I3C), a naturally occurring compound found in Brassica vegetables, is able to inhibit the growth of breast cancer cells. It does so by inducing their arrest in the G1 phase of the cell cycle as a consequence of inhibition of CDK6 expression and activity, an increase in the p27 expression level, and a decrease in retinoblastoma protein (pRb) expression and phosphorylation.

Most of the evidence concerning the anticarcinogenic effects of the products of hydrolysis of GLS comes from animal experiments, in which indoles and ITCs reduced the incidence and the multiplicity of experimentally induced tumors (4,6,23–26). On the contrary, in humans, the role of cruciferous consumption in preventing cancer is still controversial (6). Many epidemiologic studies indicated a reduction in the incidence of various types of human cancer in subjects consuming large amount of vegetables rich in GLS, such as those of the cruciferous family (2,3,27–31); the degree of protection that resulted was proportional to the level of cruciferous vegetable consumption (6). In other cohort studies, weaker or no associations were found between total vegetable or fruit intake and colorectal cancer (32–34). However, the effects of a diet rich in *Brassica* vegetables in cancer prevention may differ depending on the cancer type and sites (24,35). In addition, the association between *Brassica* consumption and breast cancer in humans remains unclear because some studies showed significant protective effects associated with the consumption of *Brassica* vegetables, whereas others revealed no relation whatsoever (31,36–40). In some cases, these discrepancies may depend on the different methods utilized in these studies or on the end points tested (41). Thus, the role of naturally occurring compounds found in *Brassicaceae* as cancer chemopreventive agents warrants further investigation.

Although a great number of studies dealing with the anticarcinogenic activity of indoles or ITCs or other single components have been conducted, very few studies were performed using whole *Brassica* vegetables. It cannot be excluded that the cancer chemopreventive properties of whole *Brassica* vegetables may be more effective than those of a single compound due to possible interactions among compounds in the whole vegetable.

In this study, we investigated the effect and the mechanism of action of whole fresh cauliflower juice on the growth and viability of human breast cancer cells.

MATERIALS AND METHODS

Materials. DMEM, insulin, Hoechst 33342, and ethidium bromide were obtained from Sigma Chemical. Fetal calf serum (FCS) was obtained from Mascia Brunelli. [^{3}H]Thymidine was purchased from NEN Life Science Products.

Preparation of cauliflower juice. Fresh cauliflower leaves were obtained from local producers during the winter and spring months. Whole juice was prepared by mechanical breaking of the leaves followed by homogenization. The homogenate was centrifuged at 8000 × *g* for 10 min at 4°C, the supernatant decanted, sterilized by filtration on 0.22-μm filters, and used immediately for treatments.

Preparation of dried extracts from cauliflower juice. Fresh cauliflower leaves were washed with a solution of 0.4 mg/L of chlorine, rinsed with sterile water, and dehydrated at 40°C under air ventilation. Leaves were chopped and processed for ethanolic or aqueous extractions. The dried extracts contained ≤5% water. The extract was prepared by processing the juice obtained from the leaves by spray drying (Model B-191-Buchi, Milan) at 180°C inlet temperature and 90°C outlet temperature.

Cell culture. The human breast adenocarcinoma ER+ (MCF-7 and BT474) and ER− (MDA-MB-231 and BT 20) cell lines were obtained from the Centro di Biotecnologie Avanzate. Cells were cultured in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, 10 g/L nonessential amino acids, 50 mg/L streptomycin, 1000 U/L penicillin, with (MCF-7) or without 10 mg/L insulin.

VERO African green monkey fibroblastoid kidney cells, permanent ECV304 cells, Hep2 human epithelial cells, 3T3 mouse embryo fibroblast cells, and nontumorigenic human breast MCF-10A cells were cultured in their appropriate growth media supplemented with 10% FCS, 50 mg/L streptomycin, and 1000 U/L penicillin. All cell lines were grown as monolayers in a humidified atmosphere at 37°C with 5% CO_2. The experiments were performed with cells in the logarithmic phase of growth.

[^{3}H]thymidine incorporation. Cells were seeded at a density of 30,000/well in 24-well tissue culture dishes and allowed to attach overnight. Duplicate samples of growing cells were treated for 48 h (ECV304, VERO, Hep2, and 3T3) or 72 h (MCF-7, MDA-MB-231, and MCF-10A) with increasing concentrations of juice (from 0.5 to 60 mL/L). During the last 4 h of treatment, cells were pulsed with 111 MBq/L of [^{3}H]Thymidine (962 Bq/mmol) and processed as reported by Brandi et al. (22). The results were expressed as the mean dpm value in juice-treated samples compared with control samples.

Assay for cell viability. MCF-7 cells were seeded at the density of 6 × 10^5 cells/60-mm dish and allowed to attach overnight. Cells were treated with increasing concentrations of cauliflower juice; at 72 h from the beginning of treatment, total cells (floating and attached cells harvested by trypsinization) were stained with 0.4% trypan blue and counted using a hematocytometer.

Evaluation of apoptotic cell morphology. Cells were harvested at different intervals after juice treatment; floating and adherent cells were collected separately and apoptosis was assessed by staining the cells with Hoechst 33342 (2 mg/L; Sigma) for 15 min at 37°C and costained with ethidium bromide (5 mg/L) added for 5 min just before observation. After staining, the slides were observed under fluorescence microscopy. The percentage of cells with an apoptotic nuclear morphology was determined by scoring at least 300 cells for each sample.

Flow cytometric analyses of DNA content. Cells were seeded onto 75-cm^2 plastic flasks and treated with increasing concentrations of juice. At different intervals (24, 48, and 72 h) from the beginning of juice treatment, cells were harvested and samples of 1 × 10^6 cells were fixed in 70% ethanol, washed in PBS, and stained with a solution containing 50 mg/L propidium iodide, 50 g/L RNAse, and 0.05% NP40 for 30 min at 4°C and then analyzed with a FACScan flow cytometer (Becton Dickinson). The cell cycle distribution was evaluated on DNA plots by CellFit software according to the SOBR model (Becton Dickinson).

Caspase activity measurement. Caspase catalytic activity was determined on cells exposed to 10 mL/L of cauliflower juice for 72 h by means of the Caspase-9/Mch6 Fluorometric Protease Assay Kit (MBL) and the Caspase-3 Assay Kit (BD Biosciences, Pharmingen), respectively. Total protein and the specific fluorogenic substrate (leu-glu-his-asp-7-amino-4-trifluoromethylcoumarin, LEHD-AFC, for caspase-9 and *N*-acetyl-Asp-Glu-Val-Asp-aldehyde-7-amino-4-methylcoumarin, Ac-DEVD-AMC, for caspase-3) were mixed for 1 h at 37°C. In the assay for caspase-3 activity, a negative control was obtained by incubating each sample in the presence of the inhibitor Ac-DEVD-CHO. The hydrolysis of substrates for caspase-9 and caspase-3 was monitored by spectrofluorometry at 505 and 440 nm, respectively.

Western blot analysis. CDK6, $p27^{kip1}$, and pRb protein expression was assessed by Western blot. Untreated and juice-treated cells were lysed for 20 min on ice as described by Brandi et al. (22). From the total protein extracted, 25 μg were fractionated on SDS-PAGE, electrically transferred to nitrocellulose membranes, and incubated with anti-CDK6 (1:200), anti-$p27^{kip1}$ (1:500), and anti-pRb (1:1000) antibodies (Santa Cruz Biotechnology). Proteins detected with enhanced chemiluminescence reagents were quantitated by ChemiDoc System (Bio-Rad). Actin was used to confirm equal protein loading.

RNA isolation, hybridization, and macroarray data analysis. Gene expression was analyzed using a commercially available nucleic acid array, the Panorama™ Human Apoptosis Gene Array (Sigma Genosys), consisting of 198 different human apoptosis-related genes. Total RNA was isolated by Trizol LS Reagent from cauliflower juice treated or untreated MCF-7 cells untreated. Complex probes were then prepared by reverse transcription in presence of 740 kBq[α-^{32}P]

dCTP, Unincorporated nucleotides were removed by Sephadex G-25 spin column.

The cDNA generated was incubated in hybridization solution and denatured at 95°C for 10 min. The membrane was hybridized overnight and finally exposed to a phosphor-imager screen. The results were normalized by dividing the intensity of the signal gene by the mean intensity of the signals of the 5 housekeeping genes that had a relatively constant expression level among the different samples.

Statistical analysis. The results are presented as means ± SEM of at least 3 separate experiments, each performed in duplicate. Data were analyzed using 1- or 2-way ANOVA as appropriate followed by Tukey's post hoc test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of cauliflower juice on cell proliferation. Cauliflower juice markedly reduced DNA synthesis in MCF-7 cells (**Fig. 1**A). The inhibition was dose dependent, with an inhibition of cell proliferation of ~50% at a concentration of 3.5 mL/L and >90% at 25 mL/L. Juice administered for 72 h at the same concentration as for MCF-7 cells dose dependently inhibited the growth of ER− cells (Fig. 1*B*). To confirm these data, we repeated the juice treatment on other ER+ (BT20) and ER− (BT474) human breast cancer cell lines. The results obtained (not shown) were similar to those in Figure 1, and the ranking of sensitivity to juice-induced inhibition of [^{3}H]thymidine incorporation, evaluated as the 50% inhibitory concentration (IC_{50}), was as follows: BT474 > MCF-7 = MDA-MB-231 > BT20 (BT474 vs. MCF-7, $P < 0.05$; MCF-7 vs. BT20, $P < 0.05$).

Although a strong dose-dependent inhibition of DNA synthesis was evident in MCF-7 breast cancer cells, only a modest reduction in [^{3}H]thymidine incorporation was observed in other mammalian cell lines ($P < 0.01$) (**Fig. 2**). In fact, the IC_{50} for MCF-7 cells was 5.1 mL/L, whereas in other cell lines, it was 44.3 (VERO), 19.8 (3T3), 29.6 (Hep2), and 25.5 (ECV304) mL/L. Exposure of nontumorigenic human breast MCF-10A cells to 15 μL juice for 72 h did not affect [^{3}H]thymidine incorporation (89 ± 4.5% of control), whereas 30 μL juice inhibited incorporation ($P < 0.05$) (38.8 ± 3.4% of control). However, at this concentration, [^{3}H]thymidine incorporation into breast cancer cell lines, MCF-7 and MDA-MB-231, was almost completely abrogated (Fig. 1). Thus, these results indicate that the juice of *Brassica oleracea* is a potent inhibitor of DNA synthesis in human breast cancer cells. We also demonstrated that these antiproliferative properties are preserved, although to different extents, in all varieties *of Brassica oleracea* (**Fig. 3**)

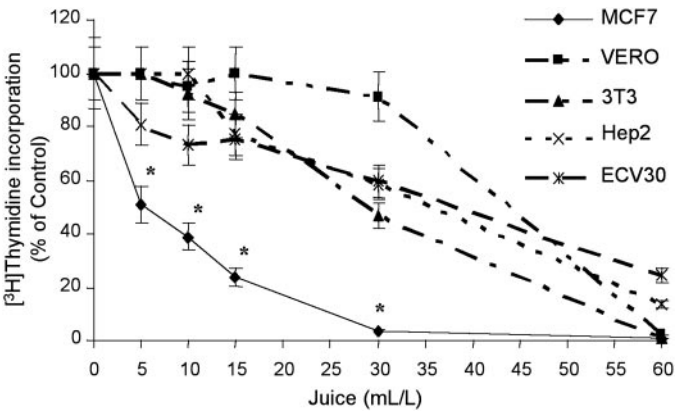

FIGURE 2 Effect of cauliflower juice on DNA synthesis in various mammalian cell lines. Cells were treated with the indicated concentrations of juice for 48 h and processed as reported in the legend of Figure 1. The IC_{50} for MCF-7 cells was ~5 mL/L, whereas it was >20 mL/L for the other cell lines. Values are means ± SEM, $n = 3$. *Different from the all other cell lines, $P < 0.01$.

Cauliflower juice treatments. Dry extracts of cauliflower juice (aqueous or hydroalcholic) obtained by freeze-drying markedly affected ($P < 0.05$) DNA synthesis in MCF-7 cells, and the effects were concentration dependent (**Fig. 4**A). The activity of the aqueous extract stored in a cool place was stable over a 1-mo period (Fig. 4*B*). Conversely, no antiproliferative activity was observed in cells exposed to relatively high con-

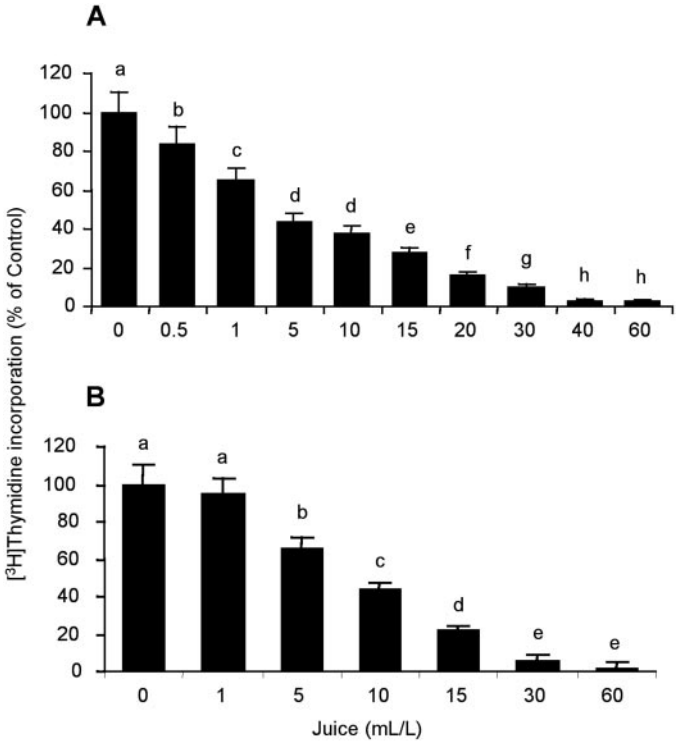

FIGURE 1 DNA synthesis in MCF-7 (*A*) and MDA-MB-231 (*B*) breast cancer cells treated with the indicated concentrations of juice for 72 h. During the last 4 h of treatment, cells were pulsed with [^{3}H]thymidine and the incorporation into DNA was determined. Values are means ± SEM, $n = 5$. Values without a common letter differ, $P < 0.05$.

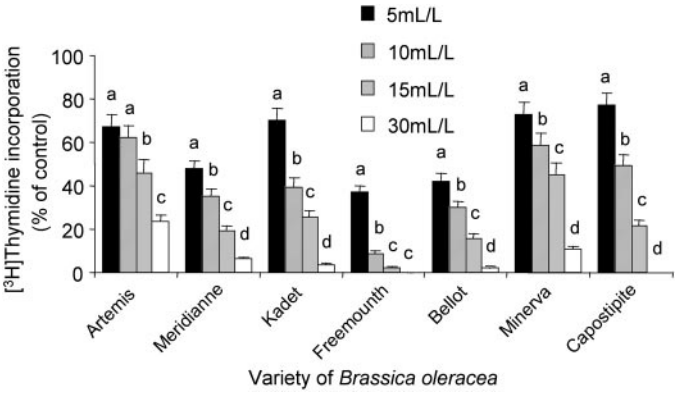

FIGURE 3 DNA synthesis in MCF-7 cells treated with various concentrations of juices obtained from different varieties of *Brassica oleracea* for 72 h. Values are means ± SEM, $n = 3$. Values for each variety without a common letter differ, $P < 0.05$.

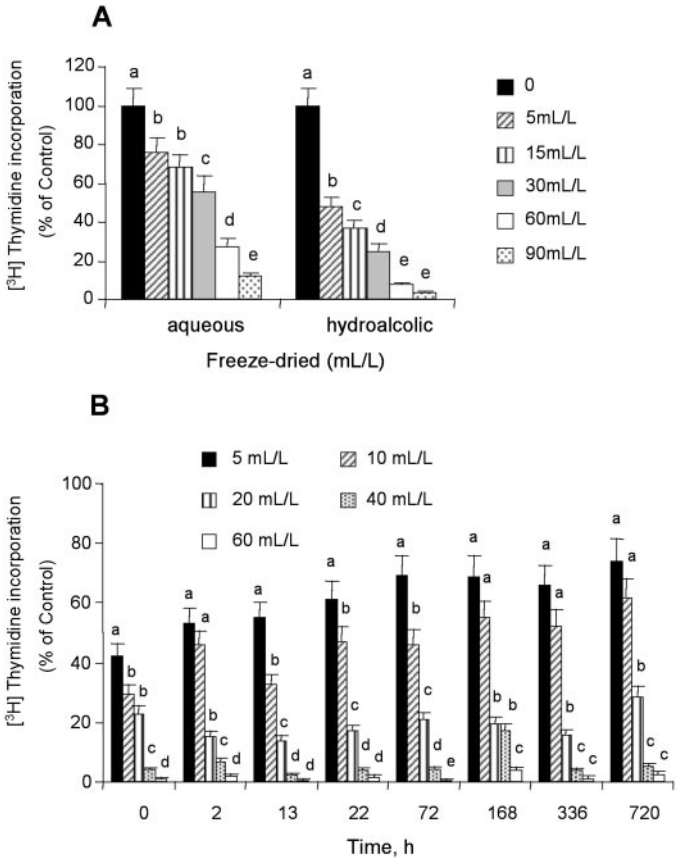

FIGURE 4 DNA synthesis in MCF-7 cells treated for 72 h with increasing concentrations of (*A*) freeze-dried extract immediately after preparation and (*B*) freeze-dried extract (aqueous) after preservation at a cool temperature for different period of time. Values are the means ± SEM; $n = 3$. Values for an extract (*A*) or at a time (*B*) without a common letter differ, $P < 0.05$.

centrations of extract obtained by spray-drying, suggesting that the active substances are heat labile.

Cell cycle effects. To assess the effects of the cauliflower juice on the cell cycle, we used concentrations ranging from 2.5 to 10 mL/L, levels that are able to inhibit [^{3}H]thymidine incorporation into DNA 10–71% and 22–91% in MCF-7 and MDA-MB-231 cells, respectively. Flow cytometry profiles of nuclear DNA content in the ER+ and ER− cells revealed that the juice did not interfere with the cell cycle of MCF-7 cells, whereas only a slight accumulation of MDA-MB-231 cells in the S compartment was noted after treatment with the higher concentration (10 mL/L) of juice (**Table 1**). These results suggest that, at least at low concentrations, the juice suppressed cell growth without inducing a specific block of the cell cycle. However, when the ER+ and ER− cells were exposed to higher concentrations of juice (>20 mL/L), an appreciable number of cells accumulated in the S and G_2/M phases after 48 h of treatment. This was paralleled by a decrease in cell number in the G_0/G_1 phase and an increase in the percentage of debris (data not shown).

Cytostatic and cytotoxic effects. To evaluate whether the reduced [^{3}H]thymidine incorporation observed in juice-treated cells was due to a cytotoxic or a cytostatic effect, we evaluated the cell viability of MCF-7 cells after juice treatment over the range of linear dose-response obtained in the antiproliferative assay (Fig. 1). Juice treatment caused a dose-dependent inhibition of cell proliferation ($P < 0.05$) without loss of cell

TABLE 1

Effects of cauliflower juice on the cell cycles of ER-positive (MCF-7) and ER-negative (MDA-MB-231) cell lines[1,2]

	48 h				72 h			
Juice, μL	Debris	$G_{0/1}$	S	G_2M	Debris	$G_{0/1}$	S	G_2M
				%				
MCF-7								
0	5	57	34	9	8	59	32	9
2.5	18	58	32	10	19	59	29	12
5.0	32	59	31	10	25	63	25	13
10.0	42	60	29	11	38	54	25	11
MDA-MB-231								
0	7	50	31	19	12	55	30	15
2.5	12	51	29	20	15	58	28	14
5.0	17	45	33	22	20	52	29	19
10.0	30	35	45	20	34	38	40	22

[1] Data are expressed as the percentage of total cells and represent the means of duplicate experiments that did not differ by >10%.
[2] The pre-$G_{0/1}$ apoptotic cell peak was undetectable in all samples.

viability at the lower concentrations ($\leq IC_{50}$), whereas a proportional increase in the percentage of necrotic cells (up to 25% at 20 mL/L of juice, $P < 0.05$), as shown by loss of viability and increase of debris, occurred even at concentrations above the IC_{50} (**Fig. 5**). Because flow-cytometric analyses conducted in MCF-7 and in MDA-MB-231 cells after treatment with concentrations of juice that were able to reduce cell proliferation (≤10 mL/L) did not indicate the presence of a detectable pre-G_1 apoptotic cell population (Table 1), we analyzed apoptosis; we also evaluated nuclear chromatin condensation and nuclei fragmentation, both common features in apoptotic cells. After 48 and 72 h of treatment, the analyses of fluorescent nuclei revealed that only a very small number (<3%) of juice-treated cells displayed the typical apoptotic features. Moreover, at the molecular level, there was no evidence of treatment-induced apoptosis. In fact, exposure of MDA-MB-231 cells to 10 mL/L of cauliflower juice for 72 h did not increase the caspase-9 and caspase-3 catalytic activities compared with untreated samples (not shown). Similarly, caspase-9 activity was not enhanced by juice treatment in

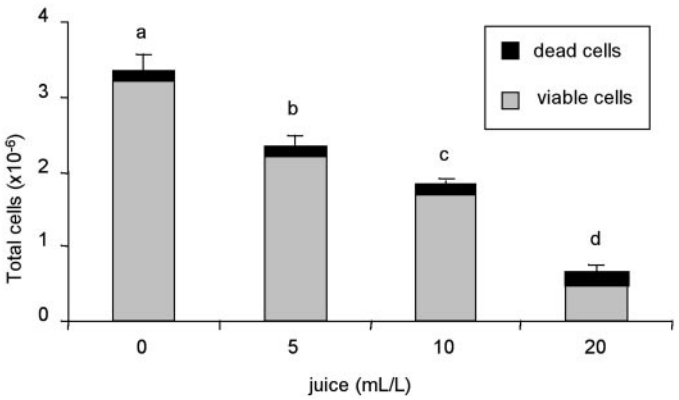

FIGURE 5 Cell growth inhibition induced by cauliflower juice in MCF-7 cells treated with different concentrations of juice; after 72 h of culture, the number of viable and dead cells was evaluated. Values are means ± SEM, $n = 3$. Values without a common letter differ, $P < 0.05$.

MCF-7 cells (not shown). In these cells the analysis was limited to caspase-9 because they do not express caspase-3 (42). These results indicate that growth arrest in juice-treated cells is associated with a cytostatic mechanism and with necrotic cell death that occurs at the higher concentrations utilized.

Effect on cell cycle-dependent proteins. Western blot analysis carried out in ER+ cells revealed that juice markedly increased the levels of the p27 CDK inhibitor in both MCF-7 ($P < 0.01$) (**Fig. 6**) and BT474 ($P < 0.01$, not shown) cells and selectively decreased, especially in MCF-7 cells, the level of CDK6 protein ($P < 0.01$). In MCF-7 cells, the levels of p27 protein increased in a dose-dependent manner up to 200% of the baseline level with 10 μL of juice (Fig. 6). In the same cells, treatment for 72 h with 5 and 10 μL of juice reduced the baseline level of CDK6 to 20 and 15%, respectively. Conversely, the intracellular level of CDK4 was not modified by treatment with juice (not shown). We also analyzed the expression of $p27^{kip1}$ and CDK6 in ER− cells (MDA-MB-231 and BT20). Interestingly, in these cells, the expressions of p27 and CDK6 were not affected by the same concentrations of juice (**Fig. 7**A).

One of the key substrates for the G_1 CDKs is the retinoblastoma protein (pRb). The antiproliferative activity of Rb is regulated by post-translational modification; in particular, phosphorylation of pRb is critical for progression through the cell cycle (43). The amount of total and hyperphosphorylated pRB was markedly lower in juice-treated ER+ cells ($P < 0.01$) (Fig. 6A, B) and, surprisingly, also in ER− cells ($P < 0.01$) (Fig. 7B). Thus, different mechanisms are most likely responsible for the reduction in Rb phosphorylation in ER+ and ER− cells and consequently for cell proliferation arrest.

Expression of cell cycle and apoptosis-related genes. In the treated cells, the expression of several apoptosis-related genes was upregulated (data not shown). Among genes whose products have antiapoptotic functions, we found overexpression of *Survivin* (150% increase). Among genes whose products have a proapoptotic function, we found overexpression of tumor necrosis factor receptor associated death domain (*TRADD,* 180% increase), tumor necrosis factor receptor associated factor 2 (*TRAF 2,* 120% increase), and *BID* (170% increase), and a decrease in the expression of Fas-associated death domain (*FAAD,* 50% decrease).

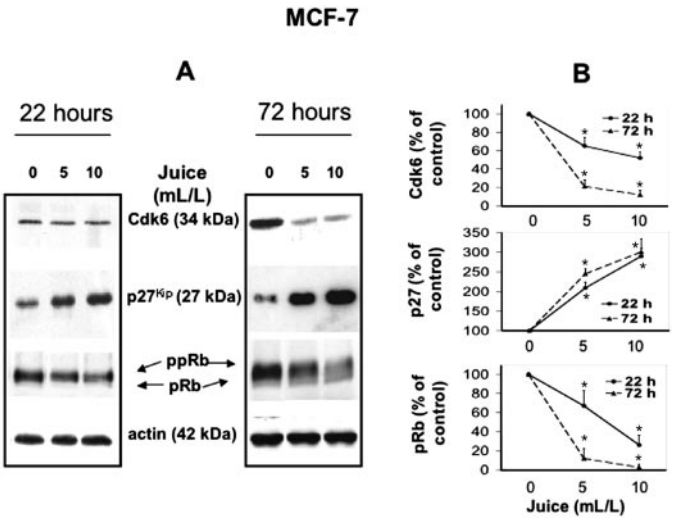

FIGURE 6 G_1 cell cycle-dependent protein expression and the level of endogenous phosphorylation of Rb protein in MCF-7 cells treated with the juice for 22 and 72 h. (*A*) Total cell extracts were analyzed for Cdk6, $p27^{kip1}$, pRb, and actin proteins by Western blot. Hyperphosphorylated Rb (ppRb) exhibits a characteristic mobility shift compared with the hypophosphorylated (pRb) alone. (*B*) The relative level of Cdk6, $p27^{kip1}$, and ppRb proteins in MCF-7 treated with different concentrations of the juice was quantitated by ChemiDoc System, normalized to actin, and expressed as a percentage of untreated value (which was set at 100). Values are means ± SEM, $n = 3$. *Different from the untreated cells, $P < 0.01$.

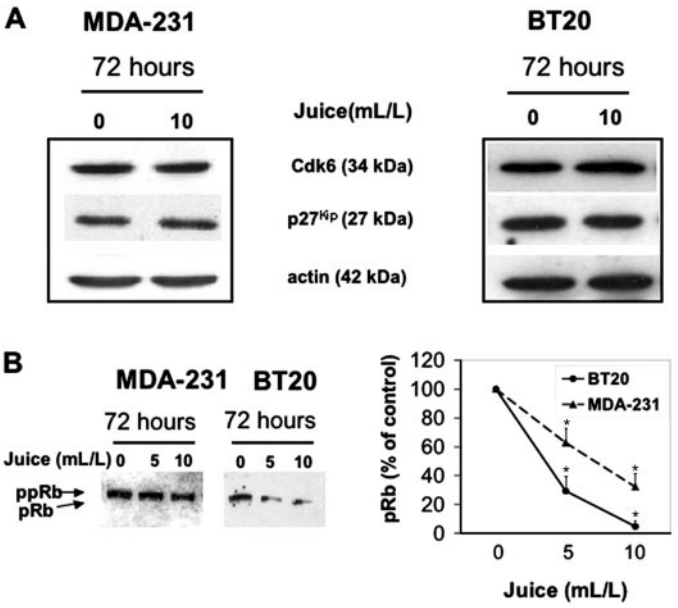

FIGURE 7 G_1 cell cycle–dependent protein expression and the level of endogenous phosphorylation of Rb protein in ER− cells. Cells were treated and processed as reported in the legend of Fig. 7. (*A*) Western blot analysis of Cdk6, $p27^{kip1}$, and actin proteins in MDA-MB-231 (*left*) and BT20 (*right*) cells treated with 10 mL/L juice. (*B*) Representative Western blot analysis of pRb and ppRb (*left*) and levels of ppRB (*right*) in MDA-MB-231 and BT20 cells treated for 72 h with different concentrations of juice. The levels of proteins were normalized to actin. Values are means ± SEM, $n = 3$. *Different from the untreated cells, $P < 0.01$.

DISCUSSION

During the past decade, breast cancer has become the most common cause of cancer in women in developed countries. Surgical tumor removal and chemotherapy represent the principal means to combat this neoplasm. Unfortunately, surgery is often ineffective in the metastatic phase, and a considerable number of patients are intrinsically resistant to antiestrogen compounds or become resistant to conventional therapies as a consequence of prolonged treatments. In recent years, many vegetables have been studied for their possible efficacy as chemopreventive compounds; among these, cruciferous vegetables are indicated in many epidemiologic studies as having protective effects against various kinds of cancer in vivo, including breast carcinoma (3,31,44). It was suggested that the antitumorigenic activity of some agents contained in these vegetables is associated with the induction of detoxification enzymes, the inhibition of activation of precancerogens, antioxidant effects, and the binding of carcinogens by fibers in the digestive tract (5). In this study, we showed that raw cauliflower juice is a potent growth inhibitor of human breast cancer cells in vitro and that its antiproliferative properties are a common feature of various cultivars of *Brassica oleracea*, although varying in efficacy. These differences in efficacy may

depend on genotype, growth condition, time of harvest, and climate (45,46) and warrant further studies.

Our data demonstrate that the juice was able to block the proliferation of both ER+ and ER− breast cancer cells and suggest that distinct growth-inhibitory pathways may be involved, with a unique common target represented by the marked decrease in endogenous Rb phosphorylation.

In fact, at the molecular level, juice selectively reduces the levels of CDK6 and increases the level of the CDK6 inhibitor p27 in ER+ cells. Because the endogenous substrate for G_1 CDK-6 is the Rb, the reduced level of CDK-6 decreases the amount of Rb phosphorylation; as a consequence, Rb in the hypophosphorylate form binds and inactivates E2F transcription, thus arresting the cell cycle. In ER− cells, CDK6 and p27 remain unchanged after treatment, suggesting that other proteins and/or kinases may be involved in reducing the level of Rb phosphorylation and consequently the block of cell proliferation. Hong et al. (47) reported that 3,3′-diindolylmethane, an indole derived from *Brassica*, induced G_1 cell cycle arrest in breast cancer cells through a mechanism involving p21 and that the induction of p21 expression is independent of the estrogen receptor.

A mechanism of growth arrest in breast cancer cells involving the G_1 cell cycle proteins p27 and CDK6 was reported by Cover et al. (19) for I3C and by us for the I3C tetrameric derivative (22). These compounds induced the overexpression of p27 and also reduced the levels of CDK6 in ER− cells. Furthermore, cell proliferation arrest was associated with a block of cells in the G_1 phase of the cell cycle, a situation that was not observed in juice-treated cells. It is important to mention, however, that in our previous study, we also observed that a pRb-negative breast cancer cell line (BT-549) was highly sensitive to the tetramer even in the absence of any G_1 phase cell accumulation (22). Therefore, in agreement with the data obtained with the tetramer in BT-549 cells, it is unlikely that CDK6 is the only important molecular target for the activity of *Brassica* juice.

The antiproliferative activity exerted by juice appears to be due to both cytostatic and cytotoxic mechanisms, depending on juice concentrations. At lower concentrations ($\leq IC_{50}$), juice induced an appreciable inhibition of proliferation without any significant loss of cell viability, suggesting growth arrest via a cytostatic mechanism, whereas at the highest concentrations ($>IC_{50}$), juice induced significant cell death. In this study, we also utilized concentrations of juice $> IC_{90}$ and found that an appreciable fraction of cells accumulated in the S and G_2/M phases. Cell death at the highest concentrations, however, may also be due to the specific toxic effect of juice, as reported for ITCs (48). Several studies indicated that some compounds naturally present in cauliflower, such as indoles, ITC, and sulforaphane, induce apoptosis in various types of cancer cells (15,17,48–53) including breast cancer cells (15,17,50). In our study, we demonstrated that raw cauliflower juice is unable to induce apoptosis in ER+ and ER− breast cancer cells, at least during the observation period used in this work, and that cell death in our experimental conditions is caused by necrotic events. The lack of apoptosis induction observed after cauliflower juice treatment is also compatible with data obtained from macroarray analysis in MCF-7 cells, indicating an increased expression of both anti- and pro-apoptotic genes.

Taken together these results suggest that several mechanisms may be responsible in inducing cell growth inhibition in breast cancer cells exposed to cauliflower juice, and the anticarcinogenic activity of juice differs at least in part from that suggested for I3C and its tetramer (19,22).

An important finding in this study is that cauliflower juice displays a preferential activity against breast cancer cells. Indeed, comparing the IC_{50} for MCF-7 cells with those obtained in the other mammalian cell lines tested, breast cells were from 3.7 to 8.7 times more susceptible to juice action. Because we used cell lines with a high replicative rate, this observation strongly suggests that, at the concentration that blocks the growth of breast cancer cells, it is unlikely that the juice could damage normal cells in vivo. However, because different culture media were use to grow different cell lines, we cannot exclude the possibility that an interaction between extract and component(s) of specific media can contribute at least in part to the different sensitivity observed among cell lines. The evidence that a minor antiproliferative effect was observed in the nontumorigenic human breast cell line only at a high extract concentration, one that is not physiologically relevant, corroborates the selective activity of cauliflower juice for breast cancer cells.

Many cruciferous vegetables, including cauliflower, are cooked before consumption. However, cooking destroys myrosinase, thus reducing bioavailability of ITC and other potential chemopreventive compounds. To avoid this problem, we processed cauliflower leaves to obtain a dry extract and demonstrated that the dry powder obtained with a procedure conducted at a low temperature, preserves the antiproliferative activity, which remains stable over an extended time period. Thus, this extract could be utilized by the nutraceutical industry for chemoprevention. In contrast, the spray-drying technique destroyed this activity. We do not know which substance is destroyed by that treatment. However, several compounds, including those responsible for antioxidant activity, were lost (not shown).

This study, to our knowledge, reports for the first time that cauliflower acts as a potent inhibitor of both ER+ and ER− breast cancer cell growth. Although the mechanisms by which growth inhibition occurs must be investigated further, results suggest that cauliflower contains compounds having anticarcinogenic properties that are likely to act through both ER-independent and ER-dependent pathways. Whole juice rather than a single purified compound was used in this study to take advantage of all of the active compounds present in cauliflower. The possibility of using this edible plant, widely present in nature, for the chemoprevention of some types of cancer in subjects who are considered to be at high risk or as a chemotherapeutic agent is of great interest and must be examined further.

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