JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2005, 53, 3895−3901 3895

Effects of Brussels Sprout Juice on the Cell Cycle and Adhesion of Human Colorectal Carcinoma Cells (HT29) in Vitro

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Consumption of *Brassica* vegetables is associated with a reduced risk of cancer of the alimentary tract in animal models and human populations. We used raw juice extracted from Brussels sprouts rich in the glucosinolate sinigrin to explore the effect of naturally occurring glucosinolate breakdown products on cell cycle progression and apoptosis in human colorectal carcinoma cells (HT29). Juice was prepared from sprout tissue immediately before use, and the glucosinolate breakdown products were determined by gas chromatography mass spectrometry and liquid chromatography mass spectrometry. The cell cycle was analyzed by flow cytometry on detached and adherent cells, and apoptosis was measured in the detached population by annexin V staining. Twenty-four hours after challenge with juice (10 μL/mL), 7−13% of adherent cells had detached from the substratum but the majority (82%) of these cells had not entered apoptosis, whereas only 33% of detached control cells were not apoptotic ($p < 0.05$). The main glucosinolate breakdown products were as follows: the sinigrin breakdown product, 1-cyano-2,3-epithiopropane (ca. 38 mM); the gluconapin hydrolysis product, 3-butenyl isothiocyanate (ca. 2.2.mM); the glucobrassicin metabolite, ascorbigen (ca. 8 mM); and low concentrations of other indole glucosinolate-derived hydrolysis products such as neoascorbigen and 3,3′-diindolylmethane. A variety of biologically active glucosinolate breakdown products are released by mechanical disruption of raw Brussels sprout tissue, but contrary to previous assumptions, allyl isothiocyanate is not the main compound responsible for the inhibition of cell proliferation.

KEYWORDS: Brussels sprouts; glucosinolate metabolites; isothiocyanates; cell cycle; cyano-epithioalkanes; adhesion; apoptosis

INTRODUCTION

There is increasing evidence for a protective effect of vegetables against epithelial cancers and particularly those of the alimentary tract (*1*). However, there is little evidence that these benefits can be duplicated by long-term supplementation with antioxidant vitamins (*2*); hence, there is growing interest in the anticarcinogenic effects of other biologically active constituents of edible plants. Diets rich in *Brassica* vegetables are associated with reductions in the relative risks of colorectal carcinoma (*3*), lung cancer (*4*), and possibly prostate cancer (*5*). The leaves and other tissues of edible crucifers are good sources of many potentially protective dietary factors, but one group of sulfur compounds, the glucosinolates, is found only in these vegetables and related *Capparales* species (*6*). Disruption of the raw plant tissue by cutting or chewing results in the hydrolysis of glucosinolates by the endogenous enzyme myrosinase, yielding an unstable intermediate that can give rise to various hydrolysis products (*7*). Depending on the structure of the parent glucosinolate and factors such as the presence of metal ions and specific proteins such as the epithiospecifier protein (ESP), these hydrolysis products can include isothiocyanates (ITCs), nitriles, cyano-epithioalkanes, and thiocyanates (*8*). Glucosinolate hydrolysis products are formed in the presence of active plant myrosinase in the upper gastrointestinal tract (*9*) and by exposure to bacterial myrosinase under anaerobic conditions leading to the formation of ITCs and nitriles in the colon (*10*). Human beings appear to absorb and metabolize a proportion of the ITCs derived from this route, but their biological significance has not been thoroughly investigated (*9*).

ITCs have previously been reported to induce apoptosis in cancer cells in vitro (*11*−*13*), and we have shown that allyl ITC (AITC), which is a derivative of the glucosinolate sinigrin, inhibits proliferation of human colorectal carcinoma cells (HT29) by blocking mitosis (*14*). Furthermore, oral administration of the pure glucosinolate sinigrin (*15*) or raw Brussels sprouts (*Brassica oleracea* var. *gemmifera*) (*16*), which is a particularly rich source of sinigrin, leads to an amplification of apoptosis in the colorectal crypts of rats, 48 h after exposure to the chemical carcinogen 1,2-dimethylhydrazine (DMH). A high level of crypt cell apoptosis is protective against colorectal

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10.1021/jf048025v CCC: $30.25 © 2005 American Chemical Society
Published on Web 04/21/2005

neoplasia in both animal models (*17*) and humans (*18*). The aim of the present study was to investigate the effects of *Brassica* vegetable constituents on cell cycle progression and apoptosis in HT29, using a freshly prepared *Brassica* juice (*B. oleracea* var. *gemmifera*, cultivar Ajax), rich in glucosinolate breakdown products.

MATERIALS AND METHODS

Cell Culture. HT29 colorectal cancer cells obtained from ECACC at passage 135 were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma -D5671) supplemented with L-glutamine (2 mM), penicillin (50 μg/mL), streptomycin (50 μg/mL), and 10% fetal calf serum. Cultures were incubated in 5% CO_2 at 37 °C. Cells were split 1:8 every 7 days, and media were changed every 3−4 days.

Preparation of Brussels Sprout Juice. Brussels sprouts (*B. oleracea* var. *gemmifera* cv. Ajax) previously shown to contain a high concentration of sinigrin (approximately 1 μmol/g wet weight of tissue) were obtained directly from a local farm, stored at 4 °C, and used within 72 h. Juice was prepared immediately before use by passing raw sprouts through a domestic centrifugal juice extractor (Kenwood JE500, Kenwood, United Kingdom). The liquid obtained was filtered through a double layer of muslin and centrifuged (6000 rpm) for 15 min. The supernatant was then filtered through a 0.22 μm single use filter (ICN Flow Ltd., Rickmansworth, United Kingdom) prior to challenge of cells.

Liquid Chromatography/Mass Spectrometry (LC/MS) of Glucosinolates and Phenolics in Sprouts and Hydrolysis Products in Sprout Juices. Sprout juices and subsamples of uncooked, freeze-dried Brussels sprout tissues were analyzed by LC/MS, using previously described methods for intact glucosinolates, phenolics, and flavonoids (*19*, *20*). Briefly, all mass spectra were obtained using a Micromass Quattro II triple quadropole mass spectrometer (Micromass, Manchester, United Kingdom) coupled to a Jasco PU-1585 triple pump high-performance liquid chromatography (HPLC) equipped with an AS-1559 cooled autoinjector, a CO-1560 column oven, and a UV-1575 UV detector (Jasco, Great Dunmow, United Kingdom). Phenomenex (Macclesfield, United Kingdom) Luna C_{18} (2) 250 mm × 4.6 mm, 5 μm colums with Securityguard precolumns were used for all analyses. The mass spectra, obtained in both negative ion and positive ion full-scan electrospray modes using a Micromass Z-spray ion source, were used to analyze the samples, primarily for nonvolatile glucosinolate hydrolysis products (indoles) and other phytochemicals (*20*).

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis of Volatile Glucosinolate-Derived Hydrolysis Products in the Brussels Sprout Juices. Subsamples of the sprout juice (3 × 1 mL) from two separate preparations were placed in 2 mL screw top tubes, and 50 μL of benzyl ITC (BITC; Aldrich, United Kingdom) made up to1 mg/mL in dichloromethane (extraction efficiency standard) plus a further 500 μL of dichloromethane were added. The samples were vortex-mixed for 3 min and centrifuged to partition the samples (17000*g*, 10 °C, 10 min). The samples formed three phases: an upper aqueous phase, a solid interphase "sheet" (insoluble material), and a lower pale green dichloromethane phase. The lower phases were carefully removed using a pipet and transferred to HPLC vials containing 5 mg of anhydrous $MgSO_4$ to absorb any residual water in the organic phase. The samples were mixed and taken up in a syringe and filtered through a 0.2 μm solvent filter into sample vials. For each sample, 3 × 1 μL was injected into a Waters AS2000 GC with a DB-5MS column (J and W Scientific, $L = 30$ m, internal diameter = 0.25 mm, film thickness = 0.25 μm), a column start temperature of 30 °C, increasing to 300 °C at 6 °C min^{-1}. MS conditions: splitless mode; electron impact ionization; source temperature, 200 °C; interface temperature, 280 °C; mass range, 50−500 amu; data collected between 3 and 50 min. Samples of BITC (50 μL of 1 mg/mL added to 500 μL of dichloromethane), 1 mg/mL AITC, and 1 mg/mL 1-cyanoepithiopropane (CEP) in dichloromethane were also run. CEP was prepared from a precursor kindly provided by Dr. John Rossiter (Department of Biology, Imperial College at Wye, University of London). A calibration curve of CEP against the BITC standard was also prepared. GC/MS was performed using previously described methods (*21*).

Enumeration of Attached and Detached Cells. Cells were harvested from log phase cultures and reseeded in 3 mL of warm DMEM medium at 1×10^6 cells per 25 cm^2 flask. Flasks were then incubated at 37 °C for 72 h to achieve logarithmic growth. Two hours prior to challenge with test compounds, the medium was removed, replaced with 3 mL of fresh medium, and returned to the incubator. After the addition of the test compounds (10 μL juice/mL medium), flasks were sealed and returned to the incubator for either 7 or 24 h. At these time points, cells that had detached from the flask surface (detached cells) and adherent cells (adherents) were harvested separately. The number of detached cells in the incubating media was determined after resuspending the cell pellet in a known volume of phosphate-buffered saline (PBS, Sigma, United Kingdom). The adherent population was removed by trypsinisation [0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS, pH 7.4; Sigma] and counted after resuspending the cell pellet in a known volume of PBS. Cell numbers were determined using a hemocytometer.

Cell Cycle Analysis. The cellular DNA content was assessed using the method of Clarke et al. (*22*). Cell cycle analysis by flow cytometry was performed on cells 24 h after challenge with Brussels sprout juice (20 μL/mL) as described above. After the cells were washed twice in PBS containing 1 mM EDTA, samples of cells were resuspended in 80% v/v ice-cold ethanol at a density of 5×10^5 cell per mL and fixed on ice for 30 min. Cells were then washed twice in PBS before being resuspended in 1 mL of staining solution, consisting of PBS containing 30 μg/mL ribonuclease A, 0.1% v/v Triton X-100, and 50 μg/mL propidium iodide (all Sigma). Cells were stained for 30 min at room temperature and analyzed by flow cytometry. Data were analyzed on logarithmic scales for forward angle and right angle light scatter and on a linear scale for red fluorescence. Noncellular material was excluded from the analysis by gating on the forward-angle and right-angle light scattering characteristics of either adherent or detached cells. Red fluorescence intensity was used for the determination of position within the cell cycle, using Phoenix Multicycle for Windows cell cycle analysis software (Coulter Electronics Ltd., Luton, United Kingdom). Cells with a DNA content of less than $2n$ were designated as apoptotic, while those ranging from $2n$ to $4n$ were designated as being in G1, S, or G2/M phases of the cell cycle. Adherent cells were analyzed in triplicate, and nonadherent cells were pooled for analysis.

Annexin V−FITC Labeling of Externalized Phosphatidylserine. Externalization of phosphotidyl serine (PS) and the assessment of membrane integrity in the detached population of cells were assessed using the ApoDetect V−FITC kit (Zymed Laboratories Inc., San Francisco, CA) designed for the quantification of these apoptotic biomarkers. Cells were seeded and challenged as described above for cell cycle analysis. Twenty-four hours after challenge, detached cells were harvested by first removing the medium from all flasks to universal tubes. To ensure complete removal of detached cells, the remaining adherent cells were washed with 3 mL of PBS, which was then added to the previously removed medium. Any nonadherent cells present in the medium together with the PBS washings were spun down in a centrifuge at 1000 rpm for 5 min. The resulting cell pellet was washed in 1 mL of PBS prior to staining, subsamples of 5×10^5 cells were resuspended in 190 μL of binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4), and 10 μL of Annexin V−FITC (10 μg/mL) was added. After the cells were allowed to stain in the dark, at room temperature, for 10 min, 800 μL of binding buffer was added to each sample. Cells were then pelleted and resuspended in 500 μL of the binding buffer, and 20 μL of propidium iodide (20 μg/mL) solution was added. Cells were then analyzed for red and green fluorescence by flow cytometry. Both red and green fluorescence, as well as forward-angle and right-angle scatter, were analyzed on a logarithmic scale. Live cells were defined as those showing no fluorescence and apoptotic cells as those showing only green fluorescence. Cells exhibiting both green and red fluorescence were designated late apoptotic/necrotic, and red cells were regarded as damaged.

Statistical Analysis. Unless stated otherwise, experiments were run in triplicate and results were compared using analysis of variance. The Tukey posthoc test was applied to determine significant differences between groups within the Minitab Statistical Package, release 13.1 (State College, PA).

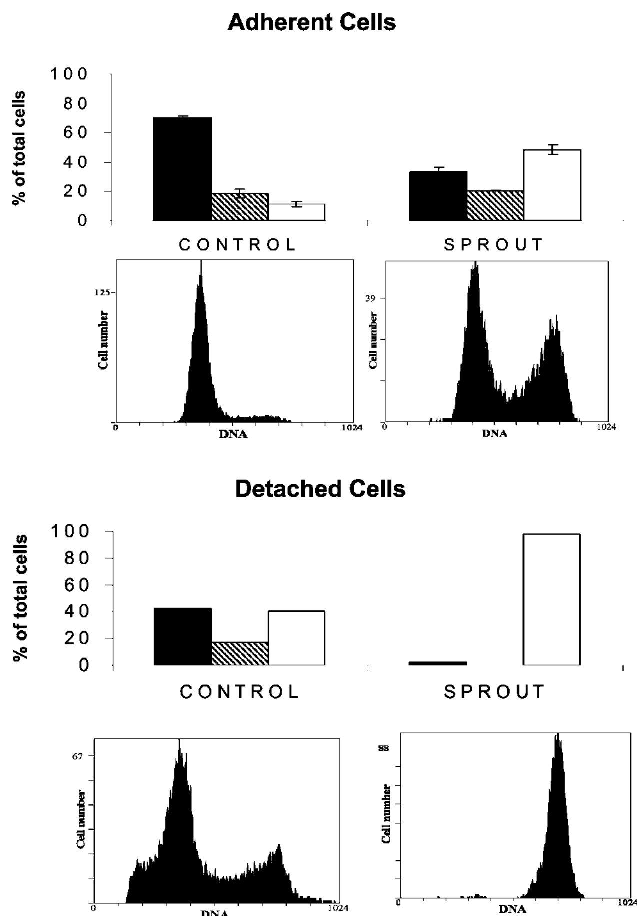

Figure 1. Cell cycle analysis of both adherent and detached populations of the HT29 cell line 24 h after challenge with raw sprout juice (20 μL per mL media). The histograms show the percentage of cells in G0/G1, S, and G2/M expressed as mean and standard error ($n = 3$). The panels show single representative flow cytometer runs.

RESULTS

Determination of Cell Number. Challenging HT29 cells with raw sprout juice (10 μL/mL) resulted in the shedding of cells from the surface of the flask into the surrounding medium 24 h after treatment. Although no cell detachment was detectable 7 h after challenge, inspection under the inverted microscope revealed that many of the adherent cells treated with raw sprout juice were "rounded-up" in appearance, a morphological feature normally seen in cells undergoing mitosis. Twenty-four hours after challenge with raw sprout juice, 7−13% of adherent cells had detached from the substratum and the majority of the cells still attached had rounded-up.

Flow Cytometric Analysis of Cell Cycle. Cell cycle analysis was undertaken 24 h after challenge with raw sprout juice (20 μL/mL media). In all cases, there was a shift in the cell cycle phase distribution. In the adherent population of cells treated with raw sprout juice, a decrease in the number of cells in the G1 phase of the cell cycle was accompanied by a significant ($p < 0.05$) increase in the number of cells in G2/M (**Figure 1**). Practically all cells in the nonadherent population were in the G2/M phase of the cell cycle.

Annexin V−FITC Labeling of Externalized Phosphatidylserine. The majority (82%) of nonadherent cells treated with raw sprout juice remained unstained, suggesting that they were intact, with an undamaged membrane and no PS translocation (**Figure 2**). In contrast only 33% of control cells remained unstained. This difference was statistically significant ($p < 0.05$).

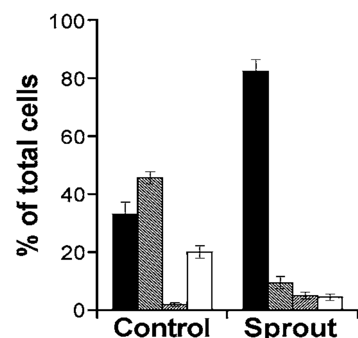

Figure 2. Annexin V–FITC staining of the detached population of HT29 cells 24 h after challenge with raw sprout juice (20 μL per mL media). The graph shows the percentage of cells designated as healthy (PI^-/AnV^-), early apoptotic (PI^-/AnV^+), late apoptotic/necrotic (PI^+/AnV^+), and necrotic (PI^+/AnV^-).

Analyses of Glucosinolates, Glucosinolate Hydrolysis Products, and Phenolics in Brussels Sprouts and Sprout Juices. A typical LC/MS trace for the analysis of the uncooked Brussels sprout phytochemicals is shown in **Figure 3**. The sprouts had a typical profile for both glucosinolate and phenolics content. The concentrations of all the glucosinolates detected in uncooked sprout tissue were well within the ranges previously reported by Rosa et al. (*23*), and as expected for this particular cultivar (Ajax), the level of sinigrin was comparable to the highest levels previously observed. The combined results of the GC/MS and LC/MS analyses of both the uncooked sprout samples and the sprout juices are presented in **Table 1**. No intact glucosinolates were detected in the juice. The major glucosinolate hydrolysis products detected in the freshly prepared Brussels sprout juices were the sinigrin breakdown product CEP (ca. 38 mM); the gluconapin hydrolysis product, 3-butenyl ITC (ca. 2.2.mM); and the glucobrassicin metabolite, ascorbigen (ca. 8 mM). The final concentration of CEP in the medium after the addition of sprout juice, when diluted 1:100, was therefore calculated to be 380 μM, the concentration of 3-butenyl-ITC was 20 μM, and the concentration of ascorbigen was 80 μM. No AITC was detected in any of the samples. The full scan MS data were searched for the presence of other known hydrolysis products of the specific glucosinolates in the Brussels sprouts used in this study, including AITC, nitriles, more complex indole condensation products [homologues of 3,3′-diindolylmethane (DIM) and mixed indole analogues], and conjugates of ITCs with endogenous glutathione and cysteine. No other hydrolysis products or hydrolysis product metabolites were detected in the juices. Although various phenolics and flavonoid glycosides were detected at very low concentrations in the Brussels sprouts, they were not found in the juice, possibly due to the combined action of glycosidases and oxidases leading to breakdown of the compounds and subsequent oxidation producing quinones that reacted with proteins and became insoluble.

DISCUSSION

Sinigrin, which is the major glucosinolate of Brussels sprouts is hydrolyzed by plant myrosinase and by bacterial myrosinases present in the human colon giving rise to AITC and to other compounds such as nitriles and amines (*7*, *24*). We recently established that AITC induces cell cycle block at G2/M and loss of cell adhesion in HT29 cells (*14*), and in the present study, we set out to test the hypothesis that exposure to raw sprout juice would induce cell cycle arrest in HT29 cells, due to the presence of AITC derived from sinigrin. As expected, raw sprout

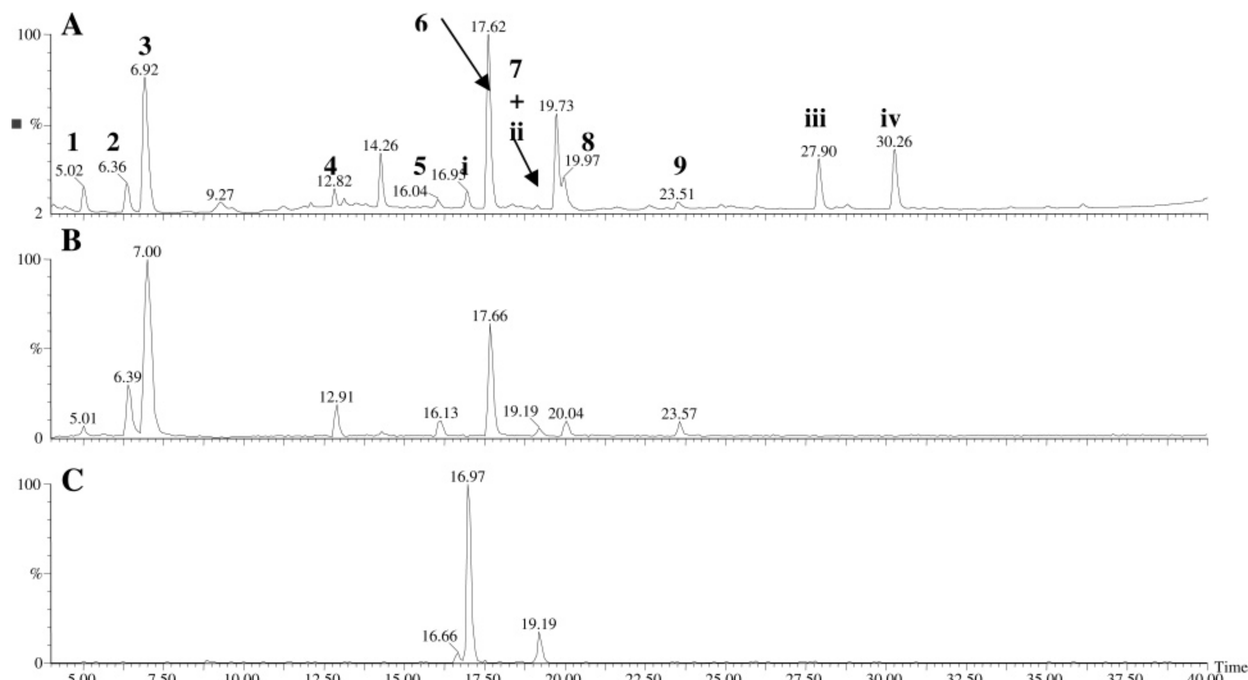

Figure 3. LC/MS analysis of the glucosinolates and phenolics in Ajax Brussels sprouts: A = analogue (227 nm), B = m/z = 96 + 97 (characteristic fragment ions of glucosinolates), and C = m/z = 355 (chlorogenic acids). Only traces of flavonoids were detected, and only negative ion data are shown. Peak ID: Numerically labelled peaks are intact glucosinolates: 1 = 3-methylsulfinylpropyl (glucoiberin), 2 = (*R*)-2-hydroxy-3-butenyl (progoitrin), 3 = 2-propenyl- (sinigrin), 4 = 3-butenyl- (gluconapin), 5 = benzyl- (glucotropaeolin), 6 = 3-indolylmethyl- (glucobrassicin), 7 = 2-phenylethyl- (gluconasturtiin), 8 = 4-methoxy-3-indolylmethyl (4-methoxyglucobrassicin), and 9 = *N*-methoxy-3-indolylmethyl- (neoglucobrassicin). Roman numeral labeled peaks are phenolics: i and ii = chlorogenic acids, iii = 6-O-β-glucopyranosyl-β-D-(1-O-sinapoyl,2-O-sinapoyl)glucopyranose, and iv = 6-O-β-D-(2′-sinapoyl)-glucopyransoyl-β-D-(1-O-sinapoyl,2-O-sinapoyl)glucopyranose.

juice caused HT29 cells to become rounded-up within 7 h of exposure and subsequently to become detached from the surface of the flask. Analysis of the cell cycle established that exposure to sprout juice caused a block at G2/M, prior to loss of adhesion. However, the detached cells were apparently resistant to detachment-induced apoptosis (anoikis).

The behavior of HT29 cells exposed to uncooked Brussels sprout juice was very similar to that seen previously for cells treated with pure AITC, but the main glucosinolate breakdown products present in the raw Brussels sprout juice used in this study were CEP and 3-butenylisothiocyanate, which would have been derived from the glucosinolates, sinigrin and gluconapin (3-butenylglucosinolate), respectively. The formation of CEP rather than AITC in our sprout juice preparation is probably due to the activity of ESP, which is known to regulate the formation of CEP in disrupted *Brassica* tissue (*8*, *25*). CEP was the most abundant of the glucosinolate breakdown products detected in the sprout juice used in the present study. In contrast to the ITCs, the nitriles derived from glucosinolate degradation in *Brassica* vegetables have received relatively little attention. There is no published evidence to confirm that CEP is biologically active against mammalian cells, but another nitrile, crambene (1-cyano-2-hydroxy-3-butene), which is a breakdown product of the glucosinolate progoitrin, has been shown to induce hepatic quinone reductase activity (*26*) and cause apoptosis in pancreatic acinar cells (*27*) in the rat in vivo, albeit at concentrations at least 1 order of magnitude greater than present in these studies.

Several previous studies have shown that a number of ITCs other than AITC are able to modify cell proliferation and induce apoptosis in mammalian cells (*12*, *13*, *28*). Ascorbigen has also previously been investigated in relation to its cytotoxicity and proapoptotic activity (*29*), but it was found to have an IC_{50} of >1000 μM. The presence of ascorbigen is therefore unlikely to be of importance under the conditions of the present study, where the concentration in the medium was only in the region of 80 μM, although synergistic effects with other compounds cannot be excluded. Similarly, the concentration of DIM in the juice (0.8 μM) was well below the concentration shown previously to be biologically active (70 μM) when tested in isolation (*29*). Although synergistic effects of combinations of glucosinolate breakdown products have been reported previously (*12*), it seems probable that the blockade of the cell cycle in G2/M and loss of cell adhesion induced by sprout juice is predominantly attributable to the presence of 3-butenylisothiocyanate, which was present in our incubation media at concentrations similar to those used previously for isolated ITCs. There is little information available on the biological activity of 3-butenylisothiocyanate in mammalian systems, although high doses have been reported to retard the growth of rat embryos (*30*).

In the intact rat, oral administration of a virtually identical sprout juice by gavage had no detectable effect on the colorectal mucosa of otherwise normal rats but led to an amplification of the apoptotic response in rats treated with the colorectal carcinogen DMH (*16*). In the present study, analysis of the detached population of cells, 24 h after challenge with raw Brussels sprout juice, revealed that nearly all cells were in the G2/M phase of the cell cycle with only a very small percentage of apoptotic cells present. However, if we make a conservative estimate of the numbers of apoptotic cells in the culture flask based only on the numbers in the detached population and express this value as a percentage of all cells in both the adherent and the detached populations, we can estimate that less than 0.5% of cells were apoptotic under control conditions, whereas this value increases to 2−4% for cells treated with sprout juice.

Table 1. Glucosinolate Content of Brussels Sprouts (*B. oleracea* Var. *gemmifera* cv. Ajax) and Glucosinolate Hydrolysis Products in Juice Freshly Extracted from Sprout Tissue

intact glucosinolates in Brussels sprout tissue	content in sprouts (μmol/100 g fresh weight)		hydrolysis products derived from specific glucosinolate in Brussels sprouts juices	content (mmol/L) juice	
	sample 1	sample 2		sample 1	sample 2
3-methylsulfinylpropyl (glucoiberin)	16.8 ± 0.4	16.1 ± 0.2	3-methylsulfinylpropylnitrile	ND	ND
			3-methylsulfinylpropylisothiocyanate	ND	ND
			3-methylsulfinylpropyl-DTC	ND	ND
2-propenyl (sinigrin)	225 ± 1.4	305 ± 0.9	allylnitrile	ND	ND
			AITC	ND	ND
			allyl-DTC	ND	ND
			CEP	38.8 ± 3.1	37.4 ± 3.4
3-butenyl (gluconapin)	19.3 ± 0.4	23.5 ± 0.5	3-butenylnitrile	ND	ND
			3-butenylisothiocyanate	1.86 ± 0.06	2.59 ± 0.097
			3-butenyl-DTC	ND	ND
			1-cyano-2,4-epithiobutane	ND	ND
(*R*)-2-hydroxy-3-butenyl (progoitrin)	47.6 ± 1.1	60.3 ± 0.6	(*R*)-2-hydroxy-3-butenylnitrile	ND	ND
			(*R*)-2-hydroxy-3-butenylisothiocyanate	ND	ND
			(*R*)-2-hydroxy-3-butenyl-DTC	ND	ND
			5-vinyloxazolidine-2-thione (goitrin)	trace	trace
benzyl (glucotropaeolin)	10.2 ± 0.3	11.7 ± 0.2	benzylnitrile	ND	ND
			BITC	ND	ND
			benzyl-DTC	ND	ND
2-phenyethyl (gluconasturtiin)	3.5 ± 0.1	4.5 ± 0.2	2-phenylethylnitrile	ND	ND
			2-phenylethylisothiocyanate (PEITC)	ND	ND
			2-phenylethyl-DTC	ND	ND
3-indolylmethyl (glucobrassicin)	57.4 ± 0.1	73.1 ± 0.1	indole-3-acetonitrile	ND	ND
			ascorbigen	8.05 ± 0.01	7.94 ± 0.01
4-methoxy-3-Indolylmethyl (4-methoxyglucobrassicin)	15.9 ± 0.1	20.1 ± 0.1	4-methoxy-indole-3-acetonitrile	ND	ND
			4-methoxy-ascorbigen (expressed as neoascorbigen equivalent)	1.61 ± 0.11	1.58 ± 0.15
N-methoxy-3-indolylmethyl (neoglucobrassicin)	3.1 ± 0.3	4.3 ± 0.2	*N*-methoxy-indole-3-acetonitrile	ND	ND
			neoascorbigen	0.38 ± 0.006	0.45 ± 0.009
			3,3′-di-indolylmethane (DIM)	0.08 ± 0.003	0.09 ± 0.002
			3,3′,3′′-tri-indolylmethane (TIM)	ND	ND
			linear trimer (LTr)	ND	ND
			other di- and tri-indolylmethanes[a]	ND	ND
			phenolics		
chlorogenic acids	low	low	neither chlorogenic acids nor free acids were detected (possibly due to losses through hydrolysis followed by oxidation)	ND	ND
5-caffeoylquinic acid					
3-caffeoylquinic acid					
flavonoid glycosides[b]	trace	trace	neither flavonoid glycosides nor flavonoid aglycones were detected (possibly due to losses through hydrolysis followed by oxidation)	ND	ND

[a] X-DTC = ITC dithiocarbamates formed from reactions with endogenous L-cysteine and/or glutathione. [b] Flavonoid glycosides were present at very low concentrations in this Brussels sprouts cultivar.

The proapoptotic effect may also be enhanced in vivo as compared to in a cell culture system as a result of interactions with neighboring cells, particularly interstitial lymphocytes, in response to cell cycle block (*31*).

Although plants from the *Brassica* family, and related *Capparales*, are unique in containing glucosinolates, these plants also contain other compounds such as a range of polyphenolic compounds, which have been shown to be biologically active. However, analysis of the juice revealed only negligible levels of chlorogenic acids, flavonoid glycosides, and flavonoid aglycones, so that it is unlikely that any biological effect can be attributed to this class of compounds. Alternatively, the juice will have also contained chlorophyll and its water soluble derivative chlorophyllin, which have also been shown to have anticarcinogenic and proapoptotic effects (*32*). However, although chlorophyll was not measured in the present study, we can estimate from published data that the concentration of chlorophyll in our media was approximately 14 μM (*33*), whereas the relevant biological activity of chlorophyllin requires concentrations of at least 10 times this value (*32*).

There is a growing consensus that *Brassica* vegetables protect against cancers of the lung and gastrointestinal tract through modulation of both pre- and postinitiation events. The most likely mechanism is that these effects are mediated by glucosinolate breakdown products, generated during food preparation, during digestion, or in the large intestine by bacteria. Brussels sprouts are usually cooked prior to consumption, but other *Brassicas*, including cabbage varieties, many of which have a glucosinolate profile similar to sprouts (*34*), are often eaten raw. In preliminary studies, we observed that juice from cooked sprouts had no effect on HT29 cell survival. This is consistent with cooking having denatured the endogenous myrosinase activity (*35*). Any metabolites formed during preparation would be leached into the cooking water, volatile compounds would

be lost on heating, and very little catabolism of glucosinolates would occur on breakdown of the cell wall structure during mastication (*7*). Glucosinolate metabolites could, however, be formed in the colon as a result of bacterial fermentation (*10*). When *Brassica* vegetables are cooked briefly at relatively low temperatures, ESP is denatured whereas myrosinase is not, and there is an increased yield of ITC upon consumption (*36*). Thus, biologically active glucosinolate breakdown products can be derived from both raw and lightly cooked *Brassica* vegetables.

Whatever their origin, glucosinolate breakdown products are absorbed from the gut lumen and metabolized by the intestinal epithelial cells. Some induce biologically significant increases in the activities of phase II xenobiotic-metabolizing enzymes, and some can selectively modify the proliferation of precancerous cells. Our previous assumption that the major biologically active breakdown product from glucosinolate degradation in uncooked Brussels sprouts would be AITC has been shown here to be incorrect. However, the present study demonstrates that raw sprout juice yields biologically active glucosinolate products, including 3-butenylisothiocyanate and possibly CEP, which inhibit the proliferation of HT29 cells by blocking the cell cycle. We propose that these phenomena may well account for the biological activity of uncooked sprout tissue observed in vivo (*16*).

ACKNOWLEDGMENT

We acknowledge John Eagles (IFR) for help with the GC/MS analyses and Dr. John Rossiter (Imperial College at Wye) for providing a sample of CEP.

NOTE ADDED AFTER ASAP PUBLICATION

Several corrections have been made after the original ASAP posting of April 21, 2005: in the first paragraph of the Introduction, the word “enzymes” has been changed to “proteins”; in the last paragraph under Results, “3-butyl” has twice been corrected to “3-butenyl”; in the second paragraph under Discussion, “quinine” has been corrected to “quinone”; and in the next to the last paragraph under Discussion, “flavanoid” has been corrected to “flavonoid”. The posting of April 21, 2005 is now correct.

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Received for review November 24, 2004. Revised manuscript received March 24, 2005. Accepted March 29, 2005.

JF048025V