Dietary Isothiocyanates Inhibit Caco-2 Cell Proliferation and Induce G₂/M Phase Cell Cycle Arrest, DNA Damage, and G₂/M Checkpoint Activation

James M. Visanji, Susan J. Duthie, Lynn Pirie, David G. Thompson, and Philip J. Padfield

Section of Gastrointestinal Science, University of Manchester, Manchester, UK and *The Rowett Research Institute, Aberdeen, UK

ABSTRACT Benzyl isothiocyanate and phenethyl isothiocyanate, two aromatic phytochemicals present in substantial concentrations in edible vegetables of the genus Brassica, were investigated for their effects on Caco-2 cell proliferation. Benzyl and phenethyl isothiocyanate inhibited DNA synthesis, with 50% inhibitory concentrations of 5.1 and 2.4 μmol/L, respectively, and significantly increased the doubling times of Caco-2 cells from 32 h to 220 and 120 h, respectively. There was no adverse effect of either chemical on cell viability in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, but benzyl isothiocyanate and phenethyl isothiocyanate both caused an accumulation of cells in the G₂/M phase of the cell cycle, which was maintained for at least 48 h in cells synchronized at prometaphase with nocodazole and subsequently treated with 10 μmol/L benzyl isothiocyanate or phenethyl isothiocyanate. Both benzyl and phenethyl isothiocyanate increased DNA strand breakage, increased phosphorylation of the G₂/M checkpoint enforcer Chk2, and induced p21 expression. These results suggest that the antiproliferative effects of benzyl and phenethyl isothiocyanates toward Caco-2 cells are due at least in part to the activation of the G₂/M DNA damage checkpoint, and that sustained G₂/M phase cell cycle arrest in response to benzyl and phenethyl isothiocyanates may be maintained through upregulation of p21. This study indicates that some dietary isothiocyanates may exert an antiproliferative effect through activation of the G₂/M DNA damage checkpoint.


KEY WORDS: isothiocyanates • proliferation • DNA damage • checkpoints

The human diet exerts a profound influence on the risk of cancer, particularly in the gastrointestinal system (1), and there is currently considerable interest in the application of dietary phytochemicals, including isothiocyanates (ITCs), in cancer chemoprevention, that is, the long-term pharmacologic management of cancer risk (2–4).

ITCs are a diverse group of phytochemicals present in substantial quantities in Brassica vegetables (5). They are of particular interest because of their abundance in the human diet, and the observation that Brassica vegetable consumption is associated with reduced overall cancer risk (6), whereas the effect of total fruit and vegetable consumption on cancer risk is less clear (7). Animal studies provide further evidence for a potential cancer chemopreventive effect of ITCs; for example, benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) inhibit lung and esophageal tumorigenesis by tobacco carcinogens in rats and mice (8–10). ITCs modulate activity of the phase I and II detoxifying enzymes, and competitively inhibit cytochromes P₄₅₀ (11); these activities are thought to be responsible at least in part for their chemopreventive effects.

More recently, further possible cellular mechanisms for the chemopreventive effects of ITCs were investigated. Several ITCs induce apoptosis, which is an important event in protection against tumorigenesis in the gastrointestinal system and elsewhere, with a variety of mechanisms involving p53-dependent and -independent pathways (12,13). Furthermore, some ITCs affect the cell cycle and may thereby impede cell proliferation. Indole-3-carbinol disrupts CDK6 transcription to induce G₁ arrest (14), and sulforaphane induces G₂/M arrest, with increased levels of cyclins A and B in HT29 cells (15). Allyl ITC was also shown to inhibit cell proliferation through the induction of G₂/M phase cell cycle arrest, although not all cell lines were equally susceptible to allyl ITC (16).

Despite the growing body of information on the cell cycle effects of some ITCs, nothing is known about the induction of cell cycle checkpoint mechanisms by ITCs. Cell cycle checkpoints are important growth arrest mechanisms that ensure the orderly progression of cell-cycle events and prevent aberrant mitosis in response to a range of events, including DNA damage. The aim of this study was to investigate the antiproliferative effects of BITC and PEITC, two common dietary ITCs, and to establish whether the G₂/M phase DNA damage checkpoint was involved in inhibition of proliferation by BITC and PEITC.
MATERIALS AND METHODS

Materials. Nocodazole, aprotinin, peptatin, and leupeptin were purchased from CalBiochem. Antibodies to Chk2 were purchased from Santa Cruz Biotechnology. Antibodies to p21 and the Thr68 phosphorylated form of Chk2 were purchased from Cell Signaling Technology. Horseradish peroxidase conjugated secondary antibodies were purchased from Bio-Rad. Tissue culture media were purchased from Invitrogen. All other reagents were purchased from Sigma unless stated otherwise.

Cell culture, proliferation and cytotoxicity assays. Caco-2 cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mmol/L l-glutamine, 50 kU/L penicillin G, 50 mg/L streptomycin and 1X nonessential amino acids (Invitrogen) in a tissue-culture incubator at 37°C, 5% CO2 and subcultured every 7–10 d. For DNA synthesis assays, 12-well tissue culture plates were grown to confluence for 24 h before exposure to 10 μCi [3H]thymidine incorporation (Invitrogen) in culture medium. For the final 3 h, 1 μCi [3H]thymidine (Amersham Pharmacia Biotech) was added to each well; then the cells were washed with PBS and 5% trichloroacetic acid. Each treatment was performed in triplicate and data shown derive from 3 or more independent experiments. For cell proliferation assays, 25-cm² tissue culture flasks were inoculated with Caco-2 cells and incubated for 24 h before exposure to 10 μmol/L BITC or PEITC. Cells were harvested from treated and untreated flasks every 24 h and cell density and optical density measured. For all experiments, BITC and PEITC were added to culture media as 50 mmol/L solutions in dimethyl sulfoxide and controls were matched for dimethyl sulfoxide concentration.

Western blotting. Western blotting was performed as described previously (17). Flow cytometric determination of cell cycle phase. Cells were treated for up to 48 h with 10 μmol/L BITC or PEITC, harvested using trypsin, and fixed in 1 mL ice-cold 70% ethanol. Fixed cells were resuspended in 0.5 mL PBS containing 35 mg/L propidium iodide and 35 mg/L RNase A. Samples were analyzed on a Beckton Dickinson FACSVantage flow sorter measuring forward and side scatter, peak width, and area of fluorescence at 488 nm. Events were gated for peak width and area to exclude subcellular debris and aggregates. A minimum of 5000 gated events were recorded for each sample, and a frequency histogram of peak area was generated and analyzed using Modfit LT software (Verity).

Where appropriate, cells were synchronized at prometaphase by treatment with 0.2 μmol/L nocodazole for 24 h before exposure to BITC or PEITC. After nocodazole treatment, cells were washed twice with PBS to release the nocodazole-induced G2/M arrest, and subsequently treated with BITC or PEITC.

Single-cell alkaline gel electrophoresis. Caco-2 cells were treated for 24 h with 10 μmol/L BITC or PEITC before assessment of DNA strand breakage by single-cell alkaline gel electrophoresis (SCGE) as described previously (18). Etoposide, a potent inducer of DNA strand breakage, was used as a positive control. Cells were scored by fluorescence microscopy by a trained and experienced observer who was unaware of the treatment. A minimum of 100 cells from each sample were analyzed visually on the basis of comet tail intensity, and placed in 1 of 5 classes reflecting the proportion of DNA in the comet tail relative to the head. Visual scoring shows a clear relation to the percentage DNA in the tail measured by computer image analysis (19). The mean comet score (normalized to 100 cells) was calculated for each treatment and control sample from class (0, 1, 2, 3, or 4) of a minimum of 100 cells/sample from each of ≥3 separate experiments.

Statistical analysis. Results of [3H]thymidine incorporation assays, cell cycle studies, and SCGE were analyzed by Student’s t test. Doubling times for cell populations were calculated using a linear least-squares best fit of log-transformed data and differences between control and treated cell doubling times analyzed using Student’s t test. Results of MTT assays were analyzed using 1-way ANOVA. All tests were 1-tailed and P = 0.05 was taken as the limit of significance. Data are presented as means ± SE.

RESULTS

ITCs inhibit Caco-2 cell proliferation. To establish whether BITC or PEITC (Fig. 1 A) inhibited Caco-2 cell proliferation, the influence of both ITCs on [3H]thymidine incorporation and cell doubling was determined. Both BITC and PEITC produced a concentration-dependent decrease in thymidine incorporation (Fig 1B). The 50% inhibitory concentrations were 5.1 μmol/L for BITC and 2.4 μmol/L for PEITC. Exposure to BITC or PEITC (10 μmol/L) also significantly increased cell doubling time. For control cells, the doubling time was 32 h. This increased to 220 h (P < 0.0001) and 120 h (P < 0.0001) for cells exposed to 10 μmol/L BITC and PEITC, respectively.

To demonstrate that the ITCs were not directly inhibiting DNA synthesis, cells were treated acutely for 3 h with 10 μmol/L BITC or PEITC, and the [3H]thymidine incorporated during this shorter period of exposure was determined. [3H]thymidine incorporation among cells treated with BITC and PEITC was 92 ± 15 and 86 ± 16% of that among control cells (P = 0.3 and 0.2, respectively), whereas treatment with 6 μmol/L of the DNA polymerase α inhibitor aphidicolin reduced [3H]thymidine incorporation to 2.8 ± 0.2% (P < 0.0005) of the control value. Finally, treatment with either ITC (10 μmol/L) for up to 48 h had no detectable influence on the viability of Caco-2 cells (data not shown).

ITCs induce cell cycle arrest after prometaphase. Next, we determined whether either ITC arrested cells in a specific phase of the cell cycle.
phase of the cell cycle. BITC and PEITC both caused a gradual accumulation of cells in G2/M phase at the expense of G0/G1 phase (Fig. 2). After 48 h, the percentage of cells in G2/M phase was increased from 12.4 ± 0.5% among controls to 48.3 ± 4.1% (P < 0.001) and 36.0 ± 2.5% (P = 0.001) for cells treated with 10 μmol/L BITC or PEITC, respectively. The slight accumulation of cells at S phase after 24 h of treatment with BITC was not significant.

We then investigated the effect of BITC and PEITC on cells synchronized in prometaphase by exposure to nocodazole. Approximately 90% of cells were arrested in G2/M at the start of the experiment; 48 h after removal of nocodazole, the proportion of cells at G2/M fell to 26.9 ± 6.3%, with a corresponding increase in the proportion of cells at G0/G1 and S phase (Fig. 3). However, when cells were treated with 10 μmol/L BITC or PEITC, the G2/M arrest was maintained for at least 48 h after release of the nocodazole-enforced arrest.

**ITCs induce DNA damage and the G2/M DNA damage checkpoint.** Because a common mechanism for the induction and maintenance of G2/M cell cycle arrest is via the activation of the DNA damage checkpoint, the next step was to determine whether either ITC induced DNA damage (Fig. 4A). Treatment with the ITCs (10 μmol/L) increased the comet score from 40.2 ± 11.4 (out of a theoretical maximum of 400) among controls to 140.3 ± 25.3 (P = 0.01) and 158.8 ± 9.3 (P < 0.001) among cells treated with 10 μmol/L BITC and PEITC, respectively. As a positive control, cells were incubated for 24 h with 5 μmol/L etoposide, which increased the comet score to 149.0 ± 17.2 (P < 0.005). The distribution of comet scores by class for each treatment is shown in Figure 4B. Representative examples of Caco-2 cells classified according to the degree of strand breakage are shown in Figure 4A.

Having ascertained that both ITCs induced DNA damage, we next examined whether this triggered activation of the G2/M DNA damage checkpoint by determining whether ITC exposure activated Chk2 and induced p21 expression (Fig. 5A and B). Both responses are key elements in the checkpoint pathway, and p21 is involved in the sustained maintenance of G2/M phase cell cycle arrest in response to DNA damage (20–22). According to [3H]thymidine incorporation experiments, the onset of the antiproliferative effect of BITC and PEITC occurred between 3 and 21 h after the initiation of treatment. We therefore sought to determine the earliest in-
intermediate time point at which the activation of DNA damage checkpoint controls could be observed. Chk2 activation was gauged by determining the relative levels of the Thr68 phosphorylated active form of Chk2. The phosphorylation of Chk2 was dramatically and reproducibly increased in response to treatment with BITC or PEITC for 8–12 h (Fig. 5A), whereas the minor variation in Chk2 protein levels seen among control cells in Figure 5A was not consistently found among several experiments. p21 levels were consistently elevated in response to an 8- to 12-h exposure to BITC or PEITC (Fig. 5B). Although p21 levels were slightly reduced after 12 h of treatment with PEITC compared with 8 h of treatment, p21 levels remained higher than controls after 24 h of exposure to both BITC and PEITC (data not shown).

DISCUSSION

Although it has been known for over 30 years that some ITCs affect cell proliferation or viability (23), there have been relatively few studies since then of the mechanisms by which ITCs inhibit cell proliferation. Most work on the cellular effects of ITCs relates to their influence on detoxifying enzymes and these studies, together with the epidemiologic evidence for a protective effect of Brassica vegetable consumption against cancer, and animal studies using experimental chemical carcinogens, led to the prevailing view that ingestion of ITCs reduces cancer risk by promoting the detoxification of chemical carcinogens. This study showed that BITC and PEITC (10 μmol/L) profoundly inhibit Caco-2 cell proliferation, but have no detectable influence on cell viability, even after prolonged (48 h) exposure. Furthermore, this study demonstrated that BITC and PEITC cause cells to accumulate in late G2/M phase (after prometaphase). The induction of cell cycle arrest in response to several other ITCs was reported previously. For example, indole-3-carbinol induces cell cycle
arrest at G1 phase (14), whereas allyl ITC and sulforaphane, like BITC and PEITC, cause arrest in G2/M phase (15,16). However, no previous studies showed an antiproliferative effect of an ITC in association with DNA damage and checkpoint activation.

The DNA damage checkpoint mediated by the ataxiatelangiectasia mutated and/or ataxia-telangiectasia mutated and rad3-related kinases and the resultant activation of Chk1 and/or Chk2 likely represent the most common mechanism for the induction of G2/M arrest. Our findings that BITC and PEITC cause both DNA damage and phosphorylation (activation) of Chk2 strongly imply that both BITC and PEITC induce arrest via activation of the DNA damage checkpoint enforced by Chk2. To date, only one other dietary phytochemical, genistein, was shown to cause induction of the G2/M checkpoint and cell cycle arrest through Chk2 (24,25).

We also observed increased protein levels of p21 in response to treatment with BITC and PEITC. Because Caco-2 cells are p53 negative, the ITC-induced expression of p21 would appear to be independent of the p53 pathway. p21 is a broad-specificity CDK inhibitor that acts to prevent cell cycle progression in response to a wide range of stimuli, including DNA damage and other forms of cell stress. Its classical function is to prevent cell cycle progression at G1 phase; however, it now has established functions at other phases of the cell cycle. In particular, p21 is required for sustained cell cycle arrest at G1/M phase (20–22) and inhibition of apoptosis (26–28). The upregulation of p21 levels by BITC and PEITC (Fig. 5B) is therefore consistent with the failure of BITC and PEITC to affect cell viability adversely in the MTT assay. Also consistent with the established cell cycle effects of p21 upregulation, we found that cell cycle arrest induced by BITC and PEITC in cells previously synchronized at prometaphase with the microtubule inhibitor nocodazole was sustained for 48 h (Fig. 3). Enforcement of the G2/M DNA damage checkpoint by p21 involves inhibition of a stimulatory phosphorylation (at Thr161) of cdc2, the cyclin B1 kinase partner (20). Because cyclin B1/cdc2 activity is required for passage through mitosis until anaphase, the observation that G2/M arrest is sustained after prometaphase in response to BITC and PEITC is consistent with the involvement of p21 in the cell cycle arrest induced by BITC and PEITC in these experiments.

DNA strand breakage is undoubtedly a procarcinogenic, rather than an anticarcinogenic phenomenon; thus, the induction of DNA strand breakage by BITC and PEITC seems inconsistent with the epidemiologic evidence, which indicates a reduction of cancer risk with increasing consumption of ITC-rich Brassica vegetables. Nevertheless, previous reports also suggested that ITCs may induce DNA strand breakage or have mutagenic effects (29,30). Most recently, glucoraphanin, the precursor of the ITC sulforaphane, was found to increase activity of phase I (procarcinogen-activating) enzymes, increase oxidative stress, and damage DNA (31). Substances that appear capable of exerting both “pro-” and “anti-” mutagenic and carcinogenic effects have been termed “Janus mutagens” (32), and the data presented here, taken together with results from other laboratories (29,30), strongly suggest that BITC and PEITC at least, and possibly a wider range of structurally related ITCs, should be considered potential Janus mutagens. Animal models provided further evidence for the possible carcinogenic effects of ITCs, in particular suggesting that ITCs may in fact be cocarcinogenic with common experimental chemical carcinogens, rather than simply opposing their action as suggested in previous studies (9); in fact, some studies showed that ITCs may promote urinary bladder carcinogenesis in rodents (33,34).

In conclusion, these results demonstrate that BITC and PEITC are potent antiproliferative agents toward Caco-2 cells in vitro, and that this antiproliferative effect may be mediated through the G2/M DNA damage checkpoint. To the best of our knowledge, the induction of this checkpoint by any dietary phytochemical is, with the exception of genistein, unprecedented (24,25). Both agents induced sustained G2/M cell cycle arrest, which was associated with increased phosphorylation of Chk2, and upregulation of p21 levels. Both BITC and PEITC also induced DNA strand breakage, suggesting that their antiproliferative effects result from G2/M cell cycle arrest enforced by at least 2 distinct checkpoint mechanisms, namely, upregulation of Chk2 activity and upregulation of p21 protein levels. In addition to demonstrating novel effects of these ITCs, and novel mechanisms of cell cycle arrest-induction by these ITCs, these observations indicate the need for further toxicological studies of these and other ITCs, before they are employed in doses in excess of normal dietary levels as part of a long-term pharmacologic approach to the management of cancer risk (35).

ACKNOWLEDGMENT

We thank Michael Jackson for excellent technical assistance.

LITERATURE CITED


