

# Dietary Indoles and Isothiocyanates That Are Generated from Cruciferous Vegetables Can Both Stimulate Apoptosis and Confer Protection against DNA Damage in Human Colon Cell Lines<sup>1</sup>

Christine Bonnesen, Ian M. Eggleston, and John D. Hayes<sup>2</sup>

Biomedical Research Centre [C. B., J. D. H.], Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland, United Kingdom; Department of Chemistry [I. M. E.], Park Place, University of Dundee, Dundee DD1 4HN, Scotland, United Kingdom

## ABSTRACT

The natural indoles 3,3'-diindolylmethane (DIM), ascorbigen (ASG), indole-3-carbinol (I3C), and indolo[3,2-*b*]carbazole (ICZ), as well as the natural isothiocyanates sulforaphane (SUL), benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC), all possess cancer chemopreventive properties. It is now shown that DIM, ICZ, SUL, and BITC can each stimulate apoptosis in human colon adenocarcinoma LS-174 and Caco-2 cells. Treatment of LS-174 cells with nontoxic doses of DIM, ASG, I3C, or ICZ affected an increase of up to 21-fold in cytochrome P450 1A1 (CYP1A1). None of these indoles caused an elevation in either aldo-keto reductase 1C1 (AKR1C1) or the  $\gamma$ -glutamylcysteine synthetase heavy subunit (GCS<sub>h</sub>), but DIM, I3C, and ICZ produced a very modest increase in NAD(P)H:quinone oxidoreductase 1 (NQO1). By contrast, nontoxic doses of SUL, BITC, or PEITC failed to induce expression of CYP1A1 in LS-174 cells, but caused an increase of between 11- and 17-fold in the protein levels of AKR1C1, NQO1, and GCS<sub>h</sub>. Treatment of the colon cell line with ICZ or SUL caused increases in the levels of mRNA for CYP1A1, AKR1C1, and NQO1 that were consistent with the enzyme data. Exposure of Caco-2 cells to media containing indoles or isothiocyanates gave similar results to those obtained using LS-174 cells. Evidence is presented that the ability of indoles and isothiocyanates to stimulate either xenobiotic response element- or antioxidant response element-driven gene expression accounts for the two groups of phytochemicals inducing different gene batteries. Pretreatment of LS-174 cells for 24 h with ICZ and SUL before exposure for 24 h to benzo(a)pyrene (BaP) reduced to <20% the number of single-strand DNA breaks produced by the carcinogen. Neither ICZ alone nor SUL alone were able to confer the same degree of protection against DNA damage produced by BaP as they achieved in combination. Similar results were obtained with H<sub>2</sub>O<sub>2</sub> as the genotoxic agent. Together, these phytochemicals may prevent colon tumorigenesis by both stimulating apoptosis and enhancing intracellular defenses against genotoxic agents.

## INTRODUCTION

Epidemiological studies suggest that high dietary intake of fruit and vegetables protects against tumorigenesis in many tissues, including the colon (1), but little is known about the mechanisms by which these types of food serve as cancer chemopreventive agents in humans.

Among vegetables with anticarcinogenic properties, members of the Cruciferae family, particularly those of the *Brassica* genus, such as broccoli, Brussels sprouts, cabbage, and cauliflower, appear to be most effective at reducing the risk of colorectal cancer (1, 2). The ability of cruciferous vegetables to protect against neoplastic disease has been attributed to the fact they contain a relatively high content of glucosinolates. One of the most commonly studied glucosinolates is

glucobrassicin, a 3-indolylmethyl-substituted compound, found in particularly high concentrations in broccoli and Brussels sprouts (2). Glucoraphanin, a 4-methylsulfinylbutyl-containing glucosinolate, is also present in high concentrations in broccoli (3). Gluconasturtin, a phenylethyl-containing compound, and glucotropaeolin, a benzyl-substituted glucosinolate, are found in particularly high concentrations in cress and turnip (4).

Physical damage to cruciferous vegetables results in the hydrolysis of glucosinolates through the actions of myrosinase, an enzyme released from ruptured plant cells. It is probable that the chemopreventive properties of cruciferous vegetables are attributable to these hydrolysis products, rather than the parent glucosinolates. During ingestion, glucobrassicin is converted in the body of the host to various products, including I3C.<sup>3</sup> In turn, I3C may form adducts with ascorbic acid to generate ASG (5), or it can undergo self-condensation reactions resulting in production of DIM and ICZ (Ref. 6; Fig. 1). Similarly, a major degradation product formed from glucoraphanin by the actions of myrosinase is the isothiocyanate, SUL. Gluconasturtin and glucotropaeolin are likewise hydrolyzed to release BITC and PEITC, respectively (Fig. 1).

The fact that, in rodents, the dietary intake of indoles and isothiocyanates increases the levels of drug-metabolizing enzymes in stomach, liver, and small intestine suggests that enhanced detoxification represents at least one mechanism by which these phytochemicals may prevent cancer (2, 7). In rats and mice, I3C has been shown to induce CYP, epoxide hydrolase, GST, and NQO (7–11). By contrast, administration of isothiocyanates to such animals does not appear to alter CYP expression but does induce NQO and GST in the liver and GI tract (11, 12).

Chemopreventive compounds that increase drug-metabolizing enzyme activity are called blocking agents, and indoles and isothiocyanates have been included in this category of anticarcinogen (7). Blocking agents that increase CYP activity as well as GST and NQO activities have been designated bifunctional inducers, whereas those agents that increase only GST and NQO activities have been called monofunctional inducers (13). Prototypic examples of bifunctional and monofunctional inducers are provided by the synthetic compounds  $\beta$ -NF and t-BHQ, respectively (13). The molecular mechanism by which  $\beta$ -NF induces detoxication enzymes involves, first, its binding as a ligand to the AhR, an event that leads to induction of

<sup>3</sup> The abbreviations used are: I3C, indole-3-carbinol; ASG, ascorbigen; DIM, 3,3'-diindolylmethane; ICZ, indolo[3,2-*b*]carbazole; SUL, sulforaphane; BITC, benzyl isothiocyanate; PEITC, phenethyl isothiocyanate; CYP, cytochrome P450; GST, glutathione S-transferase; NQO, NAD(P)H:quinone oxidoreductase; GI, gastrointestinal;  $\beta$ -NF,  $\beta$ -naphthoflavone; t-BHQ, *tert*-butylhydroquinone; AhR, arylhydrocarbon receptor; XRE, xenobiotic responsive element; ARNT, AhR nuclear translocator; Nrf2, NF-E2-related factor 2; ARE, antioxidant responsive element; Maf, transcription factor related to an oncogene isolated from a musculo-aponeurotic fibrosarcoma; GCS<sub>h</sub>,  $\gamma$ -glutamylcysteine synthetase heavy subunit; AP-1, activator protein-1; AKR, aldo-keto reductase; FBS, fetal bovine serum; HCEC, human colon epithelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; CAT, chloramphenicol acetyl transferase; DAPI, diamidino-2-phenylindole; EROD, ethoxyresorufin O-deethylase; DCPIP, dichlorophenol indophenol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BaP, benzo(a)pyrene; CD<sub>CAT</sub>, concentration of inducing agent required to double CAT reporter activity; RT-PCR, reverse transcription-PCR.

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<sup>2</sup> To whom requests for reprints should be addressed, at the Biomedical Research Centre, Ninewells Hospital and Medical School, Level 5, University of Dundee, Dundee DD1 9SY, Scotland, United Kingdom. E-mail: hayes@icrf.icnet.uk.

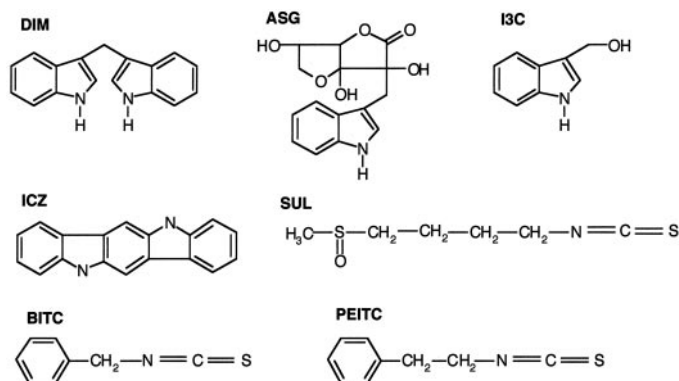


Fig. 1. Structure of phytochemicals used to treat human colon cells.

*CYP1A1* and *CYP1A2* genes through XRE (5'-T<sub>G</sub>NGCGTG<sup>A</sup>/<sub>C</sub><sup>G</sup>/<sub>C</sub>A-3') enhancers in their promoters (14). Transcriptional activation through the XRE is achieved by the AhR binding to the enhancer as a heterodimer with ARNT (14). The resulting increase in *CYP1A1* enzyme activity allows biotransformation of  $\beta$ -NF itself to a thiol-active metabolite that, in turn, transcriptionally activates *NQO* and *GST* genes through ARE (5'-<sup>A</sup>/<sub>G</sub>TGA<sup>C</sup>/<sub>T</sub>NNNGC<sup>A</sup>/<sub>G</sub>-3') enhancers (15–18). In this case, induction of ARE-driven gene expression is mediated by the basic region leucine zipper transcription factor Nrf2 that is recruited to the ARE as a heterodimer with small Maf proteins (19, 20). By contrast with bifunctional inducing agents, t-BHQ does not interact with the AhR but causes activation of ARE-regulated genes without recourse to prior metabolism (13, 15). On the basis of enzyme induction patterns in rodents, it might be concluded that, although indoles and isothiocyanates are both blocking agents, the former phytochemicals possibly act as bifunctional inducers, whereas the latter compounds probably represent monofunctional inducers.

Relatively little is known about regulation of drug-metabolizing enzymes in human intestine by diet. Within the promoters of a number of human genes, potential XRE and ARE enhancers have been identified, and these may be responsive to dietary phytochemicals. For example, the 5'-flanking region of human *CYP1A1* contains multiple XRE enhancers (reviewed in Ref. 14). The promoter region of human *NQO1* contains both a single XRE and an ARE (21), whereas the gene encoding the human GCS<sub>h</sub> subunit lacks an XRE but contains at least two AREs, both of which may also represent AP-1-binding sites (22).

It seems likely that at least some detoxication enzymes are inducible in human intestine by cruciferous vegetables because model inducing agents are active both *in vivo* and in cell lines. Specifically, the levels of mRNA for *NQO1* and GCS<sub>h</sub> have been shown to be increased in the colonic mucosa of patients given the synthetic chemopreventive agent oltipraz (23). One of the dihydrodiol dehydrogenases is also inducible by ethacrynic acid,  $\beta$ -NF, and t-BHQ in human colon HT-29 cells (24), and this has more recently been identified as the AKR that is designated AKR1C1 (25).

In the present paper, the hypothesis that induction of detoxication proteins represents a mechanism by which glucosinolate hydrolysis products prevent colon cancer has been examined.

## MATERIALS AND METHODS

**Cells, Chemicals, and Molecular Biology Reagents.** DMEM, without pyruvate, containing 4500 mg/liter D-glucose; DMEM containing 0.11 g sodium pyruvate/liter; MEM containing Earle's salts; heat-inactivated FBS; heat-inactivated FCS; L-glutamine; penicillin/streptomycin; nonessential amino acids, trypsin, versene, and TRIzol were purchased from Life Technologies, Inc. Ltd (Paisley, Scotland, United Kingdom). Matrigel basement membrane matrix was supplied by Becton Dickinson Labware (Bedford, MA).

Human colon LS-174 and Caco-2 adenocarcinoma cell lines were obtained, respectively, from the American Type Culture Collection (Rockville, MD) and from the European Cell Culture Collection (Salisbury, United Kingdom). Drs. Andrea Pfeifer and Elizabeth Offord (Nestlé Research Center, Lausanne, Switzerland) kindly provided the HCEC line.<sup>4</sup>

MTT, 1-acenaphthenol, I3C (>97% pure), BITC (>97% pure), and PEITC (>97% pure) were bought from Sigma Chemical Co.-Aldrich Company Ltd (Poole, Dorset, United Kingdom). SUL (>98% pure) was from LKT Laboratories (St. Paul, MN). Protein assay reagent was purchased from Bio-Rad Laboratories (Hemel Hempstead, Herts, United Kingdom). Immobilon-P membrane was obtained from Millipore (Watford, Herts, United Kingdom), and nylon membrane was from Amersham Pharmacia Biotech United Kingdom Ltd (Little Chalfont, Bucks, United Kingdom). Reporter lysis buffer and pSV- $\beta$ -galactosidase ( $\beta$ -gal) control vector were from Promega (Madison, WI). Restriction enzymes were from New England Biolabs (UK) Ltd. (Hitchin, Herts, United Kingdom).

Plasmid CAT reporter constructs were used that contained a single copy of the XRE enhancer 5'-TCAGGCATGTTGCGTGCATCCCTGAGGCCAGCC-3' (26), or four copies of the ARE sequence 5'-GTGACAAAGC-3' (4xARE; Ref. 27); multiple AREs were linked with three C or G nucleotides separating each of the enhancers. Constructs that contained these *cis*-acting elements ligated to the rat *GSTA2* minimal promoter driving the bacterial *CAT* gene were kindly provided by Dr. Cecil B. Pickett and Truyen Nguyen (Schering-Plough Research Institute, Kenilworth, NJ).

**Synthesis of ASG, ICZ, and DIM.** ASG, ICZ, and DIM were synthesized by previously published methods (28–34).

**Culture of Human Colon Cells and Treatment with Phytochemicals.** The LS-174, Caco-2, and HCEC cells were all cultured as monolayers at 37°C in 5% CO<sub>2</sub> and 90% relative humidity. The LS-174 cells were maintained in MEM supplemented with 10% FBS and 2 mM L-glutamine. Caco-2 cells were maintained in DMEM containing sodium pyruvate supplemented with 10% FBS, 2 mM L-glutamine, 2.5  $\mu$ g/ml bovine insulin, and 0.01% nonessential amino acids. The HCEC line was grown in DMEM with 4500 mg/liter D-glucose and glutamax without sodium pyruvate, supplemented with 10% FCS. Before seeding and plating HCEC cells, the tissue culture vessels were coated with Matrigel basement membrane matrix. The coating solution was added to the flasks and incubated for 15 min at 37°C, and any excess solution was removed. Cells were cultured in the presence of penicillin (50 IU/ml) and streptomycin (50  $\mu$ g/ml). Experiments were carried out within 20 passages.

Colon cell lines were grown in 60-mm dishes and allowed to reach ~80% confluence before they were exposed to xenobiotic. Various concentrations of the phytochemicals [all dissolved in DMSO to a final concentration of 0.1% (v/v) in the media] were used to treat the cells. Typically, colon cells were exposed to the compounds of interest for 24 h. The highest dose of each xenobiotic used for cytotoxicity testing was as follows: 300  $\mu$ M DIM; 1000  $\mu$ M ASG; 1000  $\mu$ M I3C; 50  $\mu$ M ICZ; 50  $\mu$ M SUL; 50  $\mu$ M BITC; and 50  $\mu$ M PEITC. In the enzyme-induction experiments, care was taken that nontoxic doses of xenobiotic were used. These were chosen on the basis of the results from the MTT assays (see below) and the concentrations that have been used previously by other workers.

**Cytotoxicity Assay and Identification of Apoptotic Cells.** The viability of colon cells after treatment with phytochemicals was assessed using the MTT assay (35). Cells were seeded in 96-well microtiter plates (8  $\times$  10<sup>3</sup> cells per well in 200  $\mu$ l of medium), and left to adhere to the plastic plates overnight before being exposed to indoles or isothiocyanates for 24 h at a concentration range appropriate for determination of IC<sub>50</sub> values. In every experiment, each dose of phytochemical was tested in six separate wells, and the cytotoxicity curve was constructed from at least eight different concentrations of xenobiotic. The phytochemicals were each dissolved in DMSO, and the final concentration of the solvent in all of the wells was 0.1% (v/v). After 24-h exposure to phytochemical, 50  $\mu$ l of a 5-mM MTT solution was added to each well, and the cells were incubated in the dark at 37°C for an additional 4 h. Thereafter, medium was removed, the formazan crystals were dissolved in 200  $\mu$ l of DMSO, and the absorbance was measured at 570 nm in a Microplate reader (Bio-Rad Laboratories).

<sup>4</sup> S. Blum, E. Offord, M. Turini, T. Y. Macé, Y. Tromvoukis, M. Rouvet, and A. M. A. Pfeifer. SV40 immortalized human colon epithelial cells (HCEC): a nontumorigenic *in vitro* model with inducible COX-2 expression, submitted for publication.

Apoptotic cells were identified by visual inspection after DAPI staining. Colon cells were seeded at a concentration of  $6 \times 10^4$  cells/ml media in 4-well chamber slides (Nalge Nunc International Corp., Naperville, IL) and left to adhere overnight at 37°C. Once the cells had attached to the chamber slide, they were treated with various concentrations of phytochemical for 24 h before being washed gently with ice-cold PBS and fixed in a solution of 25% (v/v) acetic acid in methanol for 15 min. The fixed cells were washed briefly with ice-cold PBS and stained for 15 min in a solution of DAPI (0.5 mg/liter in PBS). The staining solution was then removed and the cells allowed to dry for ~15 min before a coverslip was placed over each slide using 5  $\mu$ l of mounting solution (Vector Laboratories Inc., Burlingame, CA). The nuclear morphology of cells was studied by fluorescence microscopy, with apoptotic cells showing condensed or fragmented nuclei.

**Preparation of Cell Extracts and Enzyme Analyses.** Colon cells were scraped off the plate with a rubber policeman and collected as a suspension in a 1-ml solution of PBS. The cells were harvested by centrifugation ( $5,000 \times g$ , 5 min, 4°C) and resuspended in ~200  $\mu$ l of 50 mM Tris-HCl (pH 8.0), containing 150 mM NaCl and 1% (v/v) IPEGAL. Lysates were prepared by subjecting the resuspended cells to three consecutive freeze-thaw cycles; this was achieved by placing the microfuge tube alternately in liquid nitrogen and a 37°C water bath for about 15 s for each step. Finally, cell debris was removed by centrifugation ( $11,000 \times g$ , 4°C, 10 min). The resulting supernatant was collected and stored at -70°C for later analyses. Protein levels in cell extracts were determined by the method of Bradford (36) using BSA to calibrate the assay.

CYP EROD activity was measured in whole cells grown in 96-well microtiter plates. The assay was performed at 37°C in PBS containing 4  $\mu$ M 7-ethoxyresorufin by fluorescence spectroscopy with excitation at 530 nm and measurement of emission at 585 nm (37). Dihydrodiol dehydrogenase activity was measured in a reaction mixture containing 0.2 mM NADP<sup>+</sup> in NaOH-glycine buffer [25 mM glycine and 4.4 mM NaOH (pH 9.0)] and 1 mM 1-acenaphthenol as substrate. The AKR-catalyzed reduction of NADP<sup>+</sup> at 25°C was followed at 340 nm (38). NQO activity was measured at 25°C in the presence and absence of 10  $\mu$ M dicoumarol in a buffer containing 25 mM Tris-HCl (pH 7.4), 0.2 mg/ml BSA, 100  $\mu$ M NADH, and 50  $\mu$ M flavin adenine dinucleotide. The reaction was initiated by the addition of 10  $\mu$ M DCPIP, and enzyme activity was measured as the dicoumarol-sensitive reduction of the phenol assessed by the decrease in absorbance at 600 nm (39). GST activity was measured with 1 mM 1-chloro-2,4-dinitrobenzene as substrate. The assay was carried out in a reaction mixture containing 2 mM GSH in 100 mM sodium phosphate buffer (pH 6.5). The reaction was monitored at 37°C by following the increase in absorbance at 340 nm (40).

**Western Blotting.** This was performed as described previously (11). The membranes were probed with either monoclonal antibodies against CYP (a gift from Dr Helmut Thomas, Ciba Geigy, Basel, Switzerland) or polyclonal antibodies against human AKR isoenzymes (38), human GST subunits (40), rat NQO1 (11), or human GCS<sub>n</sub> (41).

**Northern Blotting.** The RNA was electrophoretically size-fractionated in formaldehyde-agarose gels and transferred to nylon membranes by osmosis (11). The samples were probed with radioactively labeled cDNA for human CYP1A1 (42), AKR1C1 (43), and NQO1 (44). GAPDH (45) was used as a loading control.

**Polymorphic Forms of NQO1 in Human Colon Cell Lines.** Three alleles encoding the reductase have been identified that vary at nucleotides 464 and 609; these are called NQO1\*1 (T464, C609), NQO1\*2 (T464, T609), and NQO1\*3 (C464, C609). The C→T substitution at nucleotide 609, which defines the NQO1\*2 allele, was identified after PCR with 5'-GAGACGCTA-GCTCTGAAGTAT-3' and 5'-ATTTGAATTCGGGCGTCTGCTG-3' using *Hinf*I to recognize the polymorphic site (46). In the assay, restriction of the amplified product from both NQO1\*1 and NQO1\*3 yields 22 and 218 bp *Hinf*I fragments, whereas NQO1\*2 yields 22, 53, and 165 bp fragments (46). The T→C substitution at nucleotide 464, which defines the NQO1\*3 allele, was determined after PCR with 5'-CTGGTCTACCTCAATGATGTC-3' and 5'-CCTGCATCAGTACAGACCACC-3' using *Msp*I to identify the polymorphic site (47). In the assay, restriction of the amplified product from both NQO1\*1 and NQO1\*2 yields 62 and 144 bp *Msp*I fragments, whereas NQO1\*3 yields a 209-bp fragment (47). The products obtained from these two PCR-RFLP analyses were resolved by electrophoresis in 2% (w/v) agarose-gels. Human lymphocyte samples of known genotype were included as positive controls.

**Identification of Transcription Factors in Colon Cell Lines.** The presence of mRNA for AhR, ARNT, Nrf2, and small Maf protein in the various cell lines was detected by RT-PCR using a kit purchased from Qiagen Ltd. (Crawley, West Sussex, United Kingdom); GAPDH was used as a positive control. In each reaction, 0.1  $\mu$ g of total RNA from the colon cells was used as template. Reverse transcription of the message (30 min at 50°C) was used to generate cDNA, and the reaction was terminated by heating at 95°C for 15 min. The "target" sequences within the resulting cDNA products were amplified by PCR using the following reaction conditions: denaturation at 94°C for 1 min, annealing of the primers at 50°C for 1 min, and extension at 72°C for 2 min. This was performed for 35 cycles, with the extension time on the final occasion being increased from 2 min to 10 min. All of the reactions contained forward and reverse primers for GAPDH (0.6  $\mu$ M of each) that were, respectively, 5'-TGAAGGTCGGAGTCAACGGATTGGT-3' and 5'-CATGTGGCCATGAGGTCCACCAC-3' (45), giving a product of 983 bp. The forward and reverse primers for AhR (0.6  $\mu$ M each) were 5'-TCCACAGCAA-GACCCACAAC-3' and 5'-AGGGTCAAAAATGGGCTTGG-3', respectively, and should produce a product of 578 bp (48). The forward and reverse primers for ARNT (0.6  $\mu$ M each) were 5'-CGGAACAAGATGACAGC-CTAC-3' and 5'-ACAGAAAGCCATCTGCTGCC-3', respectively, and are predicted to produce a 225-bp product (49). The forward and reverse primers for Nrf2 (0.6  $\mu$ M each) were 5'-TCACCATCTCAGGGCAG-3' and 5'-CAACATACTGACACTCCAATGC-3', respectively, and ought to generate a product of 755 bp (50). The forward and reverse primers for pan-small Maf (0.6  $\mu$ M each) were 5'-TGTCGGTGGGGAGCTGAA-3' and 5'-CTG-CAGCGCCTCGTACTTG-3', respectively, which will amplify a product of 251 bp (51, 52); these will not distinguish between MafF, MafG, or MafK.

**Transfections and Assay of CAT Reporter Activity.** The XRE-CAT reporter plasmid, the 4xARE-CAT reporter plasmid and the pSV- $\beta$ -galactosidase control plasmid were prepared by cesium chloride-ethidium bromide gradient centrifugation. Transient transfection of Caco-2 cells and measurement of CAT and  $\beta$ -galactosidase activities were performed by standard methods (15).

**Assessment of Genotoxicity.** Measurement of damage to cellular DNA was carried out using modifications to the "comet" method described by Singh *et al.* (53). Briefly, colon cells were seeded in six-well plates at a concentration of  $2 \times 10^5$  cells per well and allowed to adhere overnight at 37°C before being exposed to ICZ, or SUL, or both, for 24 h. After induction with indole and/or isothiocyanate, the cells were subjected to DNA damage using either 25  $\mu$ M BaP (24 h or 48 h at 37°C) or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (5 min on ice). Immediately after treatment with these agents, the cells were scraped from the plates, resuspended in 1 ml of ice-cold PBS, and dis-aggregated by repetitive pipetting. A 10- $\mu$ l aliquot of the resuspended cells was transferred to a microfuge tube containing 70  $\mu$ l of a premelted solution (at 55°C) of 0.75% (w/v) low-melting point agarose in PBS. The cells were mixed rapidly, and the resulting suspension was layered onto a microscope slide that had already been coated, 2–3 h earlier, with 0.75% (w/v) agarose (normal melting point) in PBS. A coverslip was placed over the molten agarose, and the slides were incubated at 4°C in the dark for a minimum of 30 min. Once the top layer of agarose that contained the cells had solidified, the coverslip was removed and the slide was immersed in lysis buffer [10 mM Tris, 2.5 M NaCl, 100 mM EDTA, 1% (w/v) SDS, and adjusted to pH 10.0 with HCl before the addition of Triton X-100 (to 1% by volume) and DMSO (to 10% by volume)]. After lysis of the cells embedded in agarose, the slides were transferred to a Bio-Rad DNA Sub Cell flat-bed electrophoresis tank and immersed in a solution of 300 mM NaOH containing 1 mM EDTA at 4°C for 1 h in the dark. The fragments of DNA released from the lysed cells were resolved into comets by applying a current of 250 mA (60 V) for 20 min. After electrophoresis, the slides were removed from the Sub Cell tank, neutralized with two 5-min washes in a solution of 0.4 M Tris-HCl (pH 7.5), left to dry for 30 min, and fixed in methanol for 10 min. After staining the DNA with DAPI, the comets from individual cells were examined using a fluorescence microscope. For each experiment, 50 comets were scored for their size and shape by computerized image analysis (54).

## RESULTS

### Toxicity of Indoles and Isothiocyanates to Human Colon Cells.

As shown in Table 1, ASG and I3C were tolerated well by the LS-174, Caco-2, and HCEC lines, and there was no evidence of toxicity at

Table 1 Toxicity of indoles and isothiocyanates towards human colon cells

The IC<sub>50</sub> values ( $\mu\text{M}$ ) were determined by MTT assay as described in "Materials and Methods." The LS-174, Caco-2, or HCEC cells were treated for 24 h with a range of concentrations of phytochemical that encompassed the IC<sub>50</sub> for each compound. Each treatment with a specific concentration of a compound was repeated in an entire row of six wells. The data presented are representative of results obtained from three replicate experiments; and on each of the three occasions, the IC<sub>50</sub> calculated lay within 10% of the value presented in the table.

Cell line	Phytochemical, IC <sub>50</sub> ( $\mu\text{M}$ )						
	DIM	ASG	I3C	ICZ	SUL	BITC	PEITC
LS-174	40	>1000	600	25	40	15	20
Caco-2	70	>1000	600	>50 <sup>a</sup>	55	2	12
HCEC	175	>1000	>1000	30	95	22	20

<sup>a</sup> The maximum concentration of ICZ employed in the MTT assay was limited to 50  $\mu\text{M}$  because of solubility problems.

levels considered to be of physiological significance; the IC<sub>50</sub> values were >500  $\mu\text{M}$ . By contrast, DIM and ICZ were substantially more toxic to the human colon cells than were either ASG or I3C. The IC<sub>50</sub> of DIM varied between 40 and 175  $\mu\text{M}$  in the three cell lines studied. The IC<sub>50</sub> of ICZ in the LS-174 and HCEC lines was similar at 25  $\mu\text{M}$  and 30  $\mu\text{M}$ , respectively, but its limited solubility prevented the IC<sub>50</sub> from being measured in Caco-2 cells.

The aliphatic isothiocyanate, SUL, was tolerated by all three of the cell lines better than either of the aromatic isothiocyanates, BITC and PEITC (Table 1). The IC<sub>50</sub> values for SUL were 40, 55, and 95  $\mu\text{M}$  in LS-174, Caco-2, and HCEC, respectively. By contrast, in these cell lines, the IC<sub>50</sub> values for BITC lay between 2 and 22  $\mu\text{M}$ , and the IC<sub>50</sub> value for PEITC lay between 12 and 20  $\mu\text{M}$ . Marked differences in the resistance of the three cell lines to the various isothiocyanates was observed. For example, Caco-2 cells were relatively resistant to SUL, but sensitive to BITC and PEITC. The lowest IC<sub>50</sub> value calculated was obtained with BITC treatment of Caco-2 cells.

When compared with LS-174 and Caco-2 cells, the HCEC line was equally sensitive to ICZ and PEITC. In this study, transformed and nontransformed colon cells did not appear to exhibit major differences in resistance to toxic phytochemicals.

**Differences Exist in Sensitivity to Apoptotic Cell Death in the Transformed and the Nontransformed Colon Cells.** To determine whether indoles and isothiocyanates caused cell death by apoptosis or by necrosis, the cell lines were incubated for 24 h with the concentrations of phytochemical equivalent to one-half of the IC<sub>50</sub>, the IC<sub>50</sub>, and twice the IC<sub>50</sub>, before being DAPI stained. Fluorescence microscopy of the three human colon cell lines treated with DIM revealed that, although apoptosis was observed in both the transformed LS-174 and Caco-2 cells, the abnormal nuclear morphology characteristic of apoptosis was not observed in the nontransformed HCEC line (data not shown). Similarly, exposure of LS-174 cells to ICZ produced the characteristic changes in the structure of the nucleus seen during apoptosis. However, ICZ did not produce the same changes in the HCEC line. The isothiocyanates SUL and BITC were also found to induce apoptotic morphological alterations in LS-174 and Caco-2, but not in the HCEC line. In conclusion, all of the phytochemicals examined appeared to be capable of stimulating apoptosis in LS-174 and Caco-2 cells, but none were able to initiate this process in the HCEC line.

**The Detoxication Enzyme Activity of Human Colon Cells Can Be Enhanced by Phytochemicals.** Experiments to determine whether indoles or isothiocyanates can induce drug-metabolizing enzymes used standardized doses of the phytochemicals that were deemed to be nontoxic by the MTT assay. By these criteria, the LS-174 and HCEC lines were treated with the following amounts of phytochemical: 25  $\mu\text{M}$  DIM, 700  $\mu\text{M}$  ASG, 100  $\mu\text{M}$  I3C, 1  $\mu\text{M}$  ICZ, 5  $\mu\text{M}$  SUL, 5  $\mu\text{M}$  BITC, and 5  $\mu\text{M}$  PEITC. The same concentrations of phytochemical were used to treat Caco-2 cells, with the exception that the amount of BITC was reduced to 0.5  $\mu\text{M}$ , the maximum concentration that permitted greater than 85% survival. In these experiments the bifunctional inducing agent,  $\beta$ -NF, and the monofunctional inducing agent, t-BHQ, were also included as positive controls, both at a concentration of 10  $\mu\text{M}$ .

Under basal conditions [*i.e.*, media containing 0.1% (v/v) DMSO], CYP activity towards 7-ethoxyresorufin was not detected in either LS-174 cells or HCEC, but it was observed in Caco-2 cells (Table 2). Treatment of the colon cells with phytochemicals demonstrated that CYP activity was inducible in the LS-174 and Caco-2 cells, whereas it was not increased in the HCEC line. Exposure of LS-174 cells to the indoles DIM, ASG, I3C, and ICZ, or to  $\beta$ -NF, resulted in a >100-fold increase in CYP activity. By contrast, the isothiocyanates SUL, BITC, and PEITC, or t-BHQ, did not cause an increase in the EROD activity. A broadly similar pattern of CYP enzyme activity was noted in Caco-2 cells that had been exposed to the same xenobiotics as the LS-174 cells. However, the relative elevation in EROD activity in Caco-2 cells was substantially lower than was found in LS-174 cells. This is probably attributable to the relatively high constitutive CYP activity in Caco-2 cells.

Dihydrodiol dehydrogenase activity towards 1-acenaphthenol was observed in the LS-174, Caco-2, and HCEC lines, with greatest basal amounts being found in LS-174 cells. As shown in Table 3, treatment with SUL, BITC, or PEITC achieved a significant induction of up to 4.3-fold in dihydrodiol dehydrogenase activity in both the LS-174 and Caco-2 lines. The model inducing agents  $\beta$ -NF and t-BHQ both increased dehydrogenase activity in LS-174 and Caco-2 cells by up to 3.6-fold. Whereas most of these xenobiotics produced a small decrease in dihydrodiol dehydrogenase activity in the HCEC line, treatment with SUL resulted in a small but significant induction of this enzyme activity.

Table 2 CYP activity towards 7-ethoxyresorufin in human colon cells treated with indoles or isothiocyanates

The LS-174 and HCEC lines were treated for 24 h with chemicals (all in 0.1% DMSO) at the following concentrations: control, DMSO alone; 10  $\mu\text{M}$   $\beta$ -NF; 10  $\mu\text{M}$  t-BHQ; 25  $\mu\text{M}$  DIM; 700  $\mu\text{M}$  ASG; 100  $\mu\text{M}$  I3C; 1  $\mu\text{M}$  ICZ; 5  $\mu\text{M}$  SUL; 5  $\mu\text{M}$  BITC; 5  $\mu\text{M}$  PEITC. The doses of xenobiotic used to treat Caco-2 cells were identical to those used for LS-174 and HCEC with the exception of BITC, which was used at a concentration of 0.5  $\mu\text{M}$ . Enzyme activity was measured using 7-ethoxyresorufin by the fluorometric assay described by Müller *et al.* (37). Each assay was performed in triplicate, and the results are means  $\pm$  SE. The experiment was carried out on two separate occasions.

Cell line	EROD activity ( $\mu\text{mol}$ resorufin formed/min/mg protein) on treatment with									
	Control	$\beta$ -NF	t-BHQ	DIM	ASG	I3C	ICZ	SUL	BITC	PEITC
LS-174	<5	910 $\pm$ 103 <sup>a</sup>	<5	885 $\pm$ 55 <sup>a</sup>	705 $\pm$ 45 <sup>a</sup>	560 $\pm$ 65 <sup>a</sup>	1040 $\pm$ 150 <sup>a</sup>	<5	<5	<5
Caco-2	355 $\pm$ 55	1325 $\pm$ 385 <sup>a</sup>	470 $\pm$ 95	1210 $\pm$ 265 <sup>a</sup>	690 $\pm$ 280	830 $\pm$ 160 <sup>a</sup>	1255 $\pm$ 350 <sup>a</sup>	380 $\pm$ 70	355 $\pm$ 45	400 $\pm$ 30
HCEC	<40	<40	<40	<40	<40	<40	<40	<40	<40	<40

<sup>a</sup> Significantly different from the activity in cells in control medium ( $P < 0.05$ ) using the Student *t* test.

Table 3 Dihydrodiol dehydrogenase activity in colon cells treated with indoles or isothiocyanates

Colon cells were treated with various xenobiotics as described in Table 2. Activity for dihydrodiol dehydrogenase was measured using 1-acenaphthenol (38). Each assay was performed in triplicate, and the results are expressed as means  $\pm$  SE. The experiment was carried out on three separate occasions, and the results are typical of those obtained. The relative enzyme activity when compared with control is shown in parentheses below the specific activity.

Cell line	dihydrodiol dehydrogenase activity (nmol NAD <sup>+</sup> reduced/min/mg protein) on treatment with									
	Control	$\beta$ -NF	t-BHQ	DIM	ASG	I3C	ICZ	SUL	BITC	PEITC
LS-174	1.9 $\pm$ 0.2 (100)	3.3 $\pm$ 0.3 (170) <sup>a</sup>	7.0 $\pm$ 0.1 (360) <sup>a</sup>	1.8 $\pm$ 0.1 (90)	2.1 $\pm$ 0.3 (110)	1.9 $\pm$ 0.2 (100)	1.8 $\pm$ 0.2 (100)	4.0 $\pm$ 0.4 (210) <sup>a</sup>	2.7 $\pm$ 0.1 (140) <sup>a</sup>	2.7 $\pm$ 0.3 (140) <sup>a</sup>
Caco-2	0.5 $\pm$ 0.1 (100)	1.0 $\pm$ 0.3 (210) <sup>a</sup>	1.5 $\pm$ 0.3 (320) <sup>a</sup>	0.6 $\pm$ 0.1 (120)	0.6 $\pm$ 0.1 (120)	0.5 $\pm$ 0.1 (110)	0.6 $\pm$ 0.1 (110)	1.8 $\pm$ 0.2 (370) <sup>a</sup>	0.7 $\pm$ 0.2 (150)	2.1 $\pm$ 0.3 (430) <sup>a</sup>
HCEC	1.1 $\pm$ 0.1 (100)	0.9 $\pm$ 0.1 (90)	0.8 $\pm$ 0.3 (70) <sup>a</sup>	0.9 $\pm$ 0.2 (90)	0.8 $\pm$ 0.1 (80) <sup>a</sup>	0.5 $\pm$ 0.3 (50)	0.8 $\pm$ 0.3 (70)	1.4 $\pm$ 0.2 (130) <sup>a</sup>	0.7 $\pm$ 0.5 (60)	1.1 $\pm$ 0.2 (100)

<sup>a</sup> Significantly different from cells in control medium ( $P < 0.05$ ).

NQO activity was observed in both the LS-174 and HCEC lines, but it was not detected in Caco-2 cells, even after treatment with inducing agents. In LS-174 cells, this activity was marginally increased by indoles. Table 4 shows that NQO activity was increased in these cells by between 1.8- and 2.2-fold after treatment with SUL,  $\beta$ -NF, or t-BHQ. Smaller levels of induction, of about 1.5-fold, were observed in this cell line after treatment with BITC or PEITC. No induction of NQO activity was detected in the HCEC line.

Basal GST activity was observed in all of the cells examined. However, none of the xenobiotics caused significant increases in transferase activity in these cell lines (data not shown).

**Indoles and Isothiocyanates Increase the Level of Drug-metabolizing Proteins in Colon Cells.** Significant increases in the level of CYP1A1 protein were observed after treatment of LS-174 cells with indoles and  $\beta$ -NF (Fig. 2). Under the conditions used, the level of

induction achieved by the indoles was 4-fold for ASG, 11-fold for I3C, 17-fold for DIM, and 21-fold for ICZ. By contrast, no CYP1A1 induction was observed in these cells treated with any of the isothiocyanates or with t-BHQ.

AKR1C1 showed little obvious increase in amount after treatment of LS-174 cells with indoles, but substantial induction was observed after treatment with the isothiocyanates. Densitometry of the blots showed increases in the levels of the reductase of 17-fold with SUL, 16-fold with BITC, and 15-fold with PEITC. The amount of AKR1C1 protein was also increased after treatment with either the bifunctional inducer  $\beta$ -NF (20-fold) or the monofunctional inducer t-BHQ (12-fold). Although the antibody raised against AKR1C1 cross-reacts with AKR1C2, and possibly AKR1C3 (38), it is assumed from the work of Burczynski *et al.* (25) that AKR1C1 is the inducible isoenzyme.

A modest increase of about 2-fold in NQO1 protein occurred after

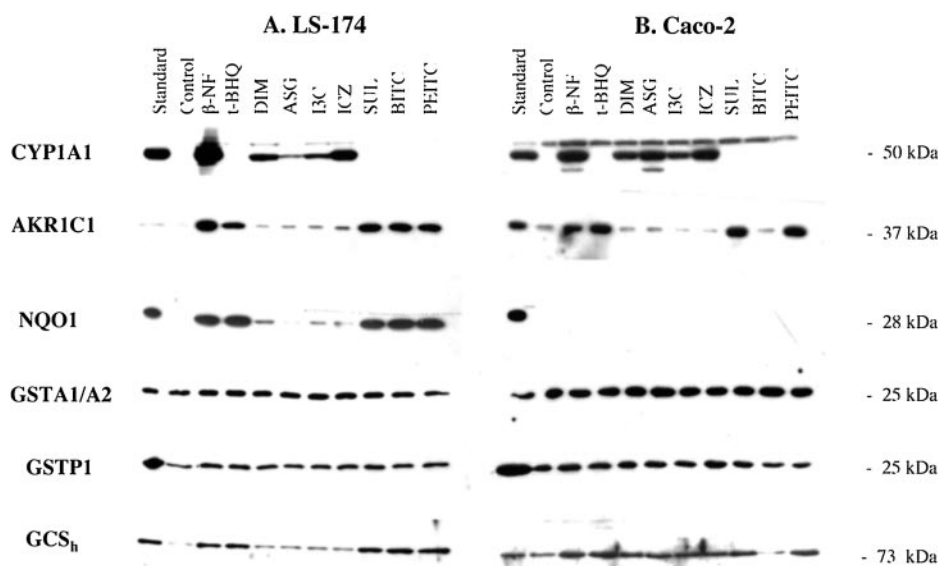
Table 4 NQO activity in colon cells treated with indoles or isothiocyanates

Colon cells were treated as described in Table 2. NQO was monitored by measuring the dicoumarol-sensitive DCPIP reductase activity (39). Each assay was performed in triplicate, and the results are means  $\pm$  SE. The experiment was carried out on three separate occasions, and the results are typical of those obtained. The relative NQO activity when compared with control is shown in parentheses below the specific activity.

Cell line	NQO activity (nmol DCPIP reduced/min/mg protein) on treatment with									
	Control	$\beta$ -NF	t-BHQ	DIM	ASG	I3C	ICZ	SUL	BITC	PEITC
LS-174	540 $\pm$ 12 (100)	990 $\pm$ 45 (180) <sup>a</sup>	1160 $\pm$ 50 (220) <sup>a</sup>	580 $\pm$ 45 (110)	630 $\pm$ 70 (120)	610 $\pm$ 65 (110)	640 $\pm$ 45 (120) <sup>a</sup>	1090 $\pm$ 55 (200) <sup>a</sup>	810 $\pm$ 20 (150) <sup>a</sup>	770 $\pm$ 80 (140) <sup>a</sup>
Caco-2	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
HCEC	175 $\pm$ 10 (100)	175 $\pm$ 10 (100)	130 $\pm$ 20 (70) <sup>a</sup>	175 $\pm$ 10 (100)	180 $\pm$ 10 (100)	150 $\pm$ 20 (90)	170 $\pm$ 15 (100)	190 $\pm$ 25 (110)	165 $\pm$ 25 (100)	165 $\pm$ 20 (100)

<sup>a</sup> Significantly different from cells in control medium ( $P < 0.05$ ).

Fig. 2. Induction of detoxication proteins in LS-174 (A) and Caco-2 (B) cells by indoles and isothiocyanates. LS-174 cells were treated for 24 h as follows: Control, 0.01% DMSO;  $\beta$ -NF, 10  $\mu$ M; t-BHQ, 10  $\mu$ M; DIM, 25  $\mu$ M; ASG, 700  $\mu$ M; I3C, 100  $\mu$ M; ICZ, 1  $\mu$ M; SUL, 5  $\mu$ M; BITC, 5  $\mu$ M; and PEITC, 5  $\mu$ M. The Caco-2 cells were treated in an identical fashion with the exception that the dose of BITC was reduced to 0.5  $\mu$ M. Portions of lysate (10  $\mu$ g of protein) from the cells, along with appropriate protein standards, were subjected to SDS-PAGE and transferred to an Immobilon-P membrane. The bound polypeptides were probed with antibodies against one of the following enzymes: CYP1A1, AKR1C1, NQO1, and GCS<sub>h</sub>, or one of the following GST subunits: A1/A2 and P1 (indicated on the left of the blots). Cross-reacting polypeptides were detected by enhanced chemiluminescence (ECL) and visualized by autoradiography. Horizontal bars on the right side of the blots, position of bands of interest. Blots from replicate experiments have been carried out on at least three occasions, and the results shown are from a typical analysis. *kDa*, *M<sub>r</sub>* in thousands.



treatment of LS-174 cells with the indoles DIM, I3C, and ICZ, whereas substantially greater increases of between 15-fold and 20-fold were observed when the cell line was cultured in the presence of isothiocyanates. Both  $\beta$ -NF and t-BHQ caused induction of the reductase in LS-174 cells of  $\sim$ 18-fold.

The GST subunits A1/A2 and P1 were detectable in LS-174 cells, but they appeared to be uninducible by any of the compounds used in this study. Although GCS<sub>h</sub> was not inducible by indoles, it was induced between 10- and 18-fold by isothiocyanates,  $\beta$ -NF and t-BHQ.

Similar patterns of induction were observed in Caco-2 cells (Fig. 2B) as were seen in LS-174 cells. In common with LS-174 cells, the Caco-2 cells supported induction of CYP1A1 by indoles but not by isothiocyanates. Like LS-174 cells, the level of AKR1C1 could be increased in Caco-2 cells by SUL and PEITC, whereas the indoles had no effect. The inability of Caco-2 cells to tolerate BITC (IC<sub>50</sub>, 2  $\mu$ M) required the dose of the isothiocyanate to be reduced to 0.5  $\mu$ M. As a consequence, the induction of AKR1C1 by BITC in Caco-2 cells is not as obvious as it is in LS-174 cells.

In marked contrast to LS-174 cells, NQO1 was not detected in the Caco-2 cells cultured in control media, nor was it inducible by indoles, isothiocyanates,  $\beta$ -NF, or t-BHQ.

The GCS<sub>h</sub> subunit was modestly induced by isothiocyanates in Caco-2 cells. However, the low basal levels of the protein in this cell line made it difficult to quantify the increase.

Western blots for HCEC cells showed no significant increase in enzyme expression after treatment with indoles. After treatment with SUL, a modest increase in AKR1C1 was, however, observed in the HCEC line (results not shown). This is in accordance with the enzyme activity assays.

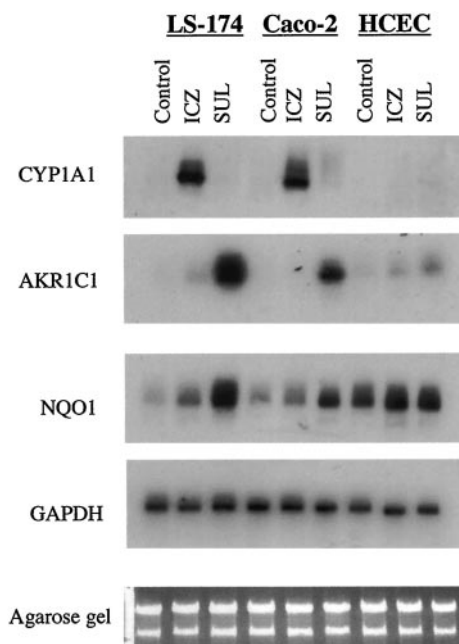


Fig. 3. Increase in mRNA for CYP, AKR, and NQO in human colon cells treated with phytochemicals. The LS-174, Caco-2, and HCEC lines were cultured in either normal media containing DMSO (Control) or media containing either 1  $\mu$ M ICZ or 5  $\mu$ M SUL (both dissolved in DMSO). Total RNA was extracted from the colon cells and quantitated by  $A_{260}/A_{280}$  and by ethidium bromide staining after agarose-gel electrophoresis. Equal portions of RNA from the three cell lines were electrophoretically size-fractionated and transferred to nylon membranes. The immobilized RNA from the control and treated cells were probed with  $^{32}$ P-labeled cDNAs for CYP1A1, AKR1C1, and NQO1 (indicated on the left), and the level of hybridization was visualized by autoradiography. The samples were also probed with GAPDH as a loading control.

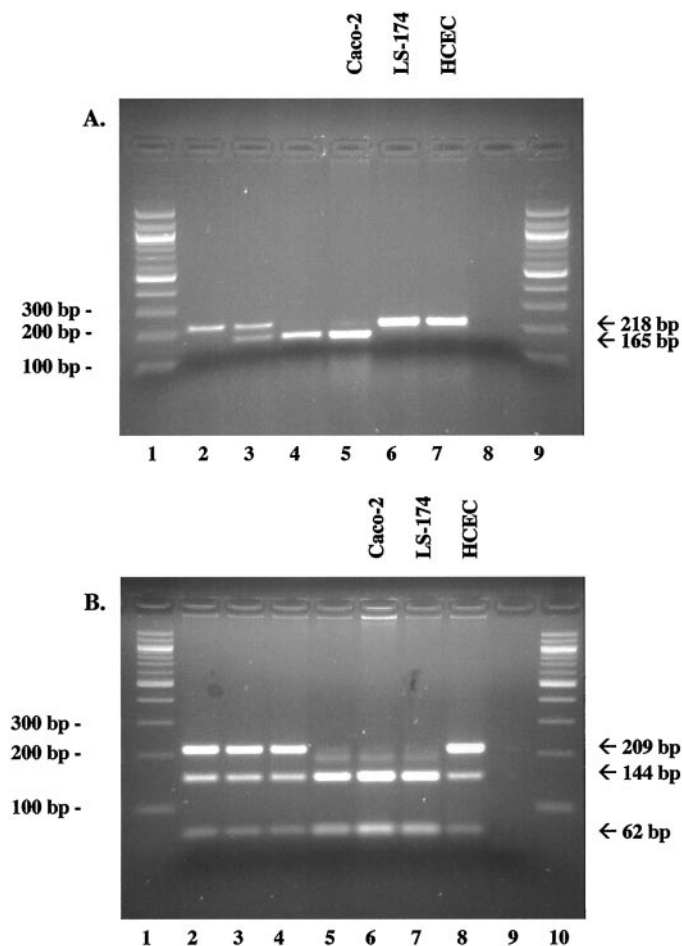
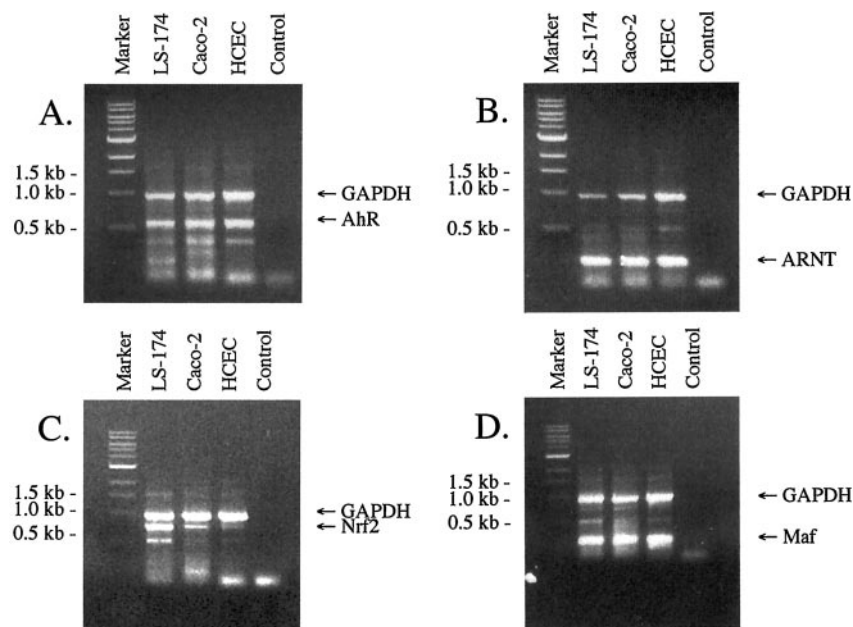


Fig. 4. Caco-2 cells express a different *NQO1* allele from that expressed in the LS-174 and HCEC lines. Regions of the *NQO1* gene encompassing nucleotides 464 and 609 were amplified from DNA isolated from Caco-2, LS-174, and HCEC cells using oligonucleotide primers described in the "Materials and Methods" section. To identify *NQO1*\*2, the amplified product from each cell line was digested with *HinI1*, to yield 22 and 218 bp fragments in the cases of *NQO1*\*1 and *NQO1*\*3 and 22, 53, and 165 bp fragments in the case of *NQO1*\*2. A, agarose-gel electrophoresis of the *HinI1* restriction products from human colon cells, along with size standards and samples from patients that had been genotyped previously. The samples were applied to the gel as follows: Lanes 1 and 9, DNA size standards; Lane 2, homozygous *NQO1*\*1 patient; Lane 3, heterozygous *NQO1*\*1/*NQO1*\*2 patient; Lane 4, homozygous *NQO1*\*2 patient; Lane 5, Caco-2 sample; Lane 6, LS-174 sample; Lane 7, HCEC sample; Lane 8, negative control using PCR off diluent used for other reactions. To identify *NQO1*\*3, the amplified product from each cell line was digested with *MspI*, to yield 62 and 144 bp fragments in the cases of *NQO1*\*1 and *NQO1*\*2, and a 209-bp fragment in the case of *NQO1*\*3. B, agarose-gel electrophoresis of the *MspI* restriction products from human colon cells, along with size standards and samples from patients that had been genotyped previously. The samples were applied to the gel as follows: Lanes 1 and 10, DNA size standards; Lanes 2-4, heterozygous *NQO1*\*3 patients; Lane 5, homozygous *NQO1*\*1 patient; Lane 6, Caco-2 sample; Lane 7, LS-174 sample; Lane 8, HCEC sample; Lane 9, negative control using PCR off diluent used for other PCR amplifications.

**Induction of Detoxication Enzymes by Phytochemicals Involves an Elevation in the Intracellular Levels of Their mRNAs.** Fig. 3 shows that the steady-state level of mRNA for CYP1A1 in LS-174 and Caco-2 cells was increased by ICZ treatment but not by SUL treatment. No increase in CYP1A1 mRNA was seen in the HCEC line. Conversely, the level of AKR1C1 mRNA in LS-174 and Caco-2 cells was increased by SUL treatment but not by ICZ treatment. In the HCEC line, ICZ did not alter CYP1A1 expression, but a relatively small increase in AKR1C1 mRNA was detected after SUL treatment.

mRNA for NQO1 was detected in all three of the cell lines (Fig. 3). A marked increase in NQO1 mRNA was observed in LS-174 cells treated with SUL, with a smaller increase being detectable after ICZ treatment. This was possibly anticipated from the Western blot data.

Fig. 5. Comparison of AhR, ARNT, Nrf2, and Maf expression in human colon cells. Total RNA was extracted from LS-174, Caco-2, and HCEC cells cultured in normal media. Equal portions of RNA from the three cell lines were used in RT-PCR reactions. The presence of mRNA species for AhR, ARNT, Nrf2, and small Maf were detected using primers designed to give PCR products of the following sizes: AhR, 578 bp; ARNT, 225 bp; Nrf2, 755 bp; and small Maf, 251 bp. In these experiments, GAPDH was included as a positive control, with the amplimers yielding a product of 983 bp. A–D, RT-PCR data using primers for AhR, ARNT, Nrf2, and small Maf, respectively, from LS-174, Caco-2, and HCEC cells; the negative control was provided by a RT-PCR mixture which contained all components except colon cell RNA. The experiment was carried out twice, with similar results being obtained on both occasions.



By comparison with the control, a 3-fold increase in the steady-state NQO1 mRNA was detected in Caco-2 cells treated with SUL. These data indicate that the existence of constitutive and inducible mRNA for NQO1 in Caco-2 cells does not result in detectable reductase protein in the cell line (see Fig. 2B). Northern blotting revealed no differences in NQO1 mRNA levels in HCEC cells after treatment with ICZ or SUL.

**Caco-2 Cells Express a Variant Form of NQO1.** Because Northern blotting indicated that the level of NQO1 mRNA in Caco-2 cells can be induced by SUL, but the reductase protein could not be detected by Western blotting, it was considered possible that this cell line might express the nonfunctional *NQO1\*2* allele. The three cell lines were, therefore, genotyped by PCR-RFLP. The Caco-2 cell line gave a *HinfI* digestion pattern (major band, 165 bp) consistent with only T609 being present, which, therefore, suggested it is homozygous for the *NQO1\*2* allele (Fig. 4A). In this assay, both LS-174 and HCEC gave a 218-bp *HinfI* fragment indicating that they possess *NQO1\*1* and/or *NQO1\*3*. When the assay for the *NQO1\*3* was performed, it was found that the HCEC line gave a pattern consistent with it being heterozygous for the variant allele (Fig. 4B). These data suggest Caco-2 is homozygous for *NQO1\*2*, LS-174 is homozygous for *NQO1\*1*, and HCEC is heterozygous for *NQO1\*1* and *NQO1\*3*.

**Human Colon Cells Express AhR, ARNT, Nrf2, and Small Maf.** The possibility that, in colon cells, the induction of CYP1A1 by indoles, and the induction of AKR1C1, NQO1, and GCS<sub>h</sub> by isothiocyanates might be mediated by heterodimers of AhR/ARNT and Nrf2/small Maf was examined by RT-PCR. Fig. 5 shows that transcripts for the AhR and ARNT exist in LS-174 and Caco-2 cells, both of which supported induction of CYP1A1 by indoles. Surprisingly, message for AhR and ARNT was also found in the HCEC line, which is unresponsive to both indoles and  $\beta$ -NF. Transcripts for Nrf2 and small Maf were detected in LS-174 and Caco-2 cells, both of which supported induction of AKR1C1, NQO1, and GCS<sub>h</sub> by isothiocyanates. Interestingly, it was noted that the level of Nrf2 is significantly higher in the former cell line than in the latter. Nrf2 was not detected in the HCEC line, an observation that is consistent with the poor level of AKR1C1 induction caused by SUL in the HCEC line.

**Stimulation of Gene Expression by Dietary Indoles and Isothiocyanates Occurs through Separate Mechanisms.** The patterns of enzyme induction affected by indoles and isothiocyanates indicate

that these two groups of phytochemicals influence gene expression by distinct processes. To analyze the molecular basis for this further, Caco-2 cells were transiently transfected with a *CAT* reporter gene driven by either the XRE or the 4xARE. Results from these experiments showed that the XRE-driven *CAT* activity is inducible by indoles but not by isothiocyanates at the concentrations used in the enzyme induction experiments described above. Conversely, the ARE-driven *CAT* activity was inducible by the isothiocyanates but not by the indoles.

The potencies of the indoles and isothiocyanates as inducing agents were compared by determining the concentration of xenobiotic required to double the reporter gene activity (*i.e.*,  $CD_{CAT}$  value) driven by either the XRE or the ARE. Table 5 shows that the indoles vary in  $CD_{CAT}$  values from 5 nM to 37  $\mu$ M, whereas essentially no effect on the ARE-driven *CAT* activity was observed at the doses used. The isothiocyanates SUL and PEITC were found to have similar potencies as inducers of the ARE-driven *CAT* activity, with  $CD_{CAT}$  values of 1.3 and 1.7  $\mu$ M, respectively. The XRE-driven *CAT* activity was not induced by any of the isothiocyanates at the doses used in the present study.

**Pretreatment of Colon Cells by Both ICZ and SUL Affords Protection against DNA Damage.** To assess whether stimulation of gene expression by glucosinolate breakdown products confers resistance against genotoxic compounds, LS-174 cells were treated with either BaP or H<sub>2</sub>O<sub>2</sub> in the presence or absence of phytochemical.

Table 5 Activation of XRE- and ARE-driven gene expression by indoles and isothiocyanates

Compound	XRE-driven <i>CAT</i> activity <sup>a</sup> $CD_{CAT}^b$ ( $\mu$ M)	ARE-driven <i>CAT</i> activity <sup>a</sup> $CD_{CAT}^b$ ( $\mu$ M)
DIM	1.0	>25
ASG	37.0	>700
I3C	6.0	>100
ICZ	0.005	>25
SUL	>5.0 <sup>c</sup>	1.3
BITC		>0.5 <sup>c</sup>
PEITC	>5.0 <sup>c</sup>	1.7

<sup>a</sup> The data were obtained using XRE-*CAT* and 4xARE-*CAT* reporter constructs that were generated, respectively, by Rushmore and Pickett (26) and Nguyen *et al.* (27).

<sup>b</sup> Concentration of phytochemical required to double the reporter gene activity.

<sup>c</sup> The concentration of these compounds used in the assay could not exceed the given value because of their toxicity.

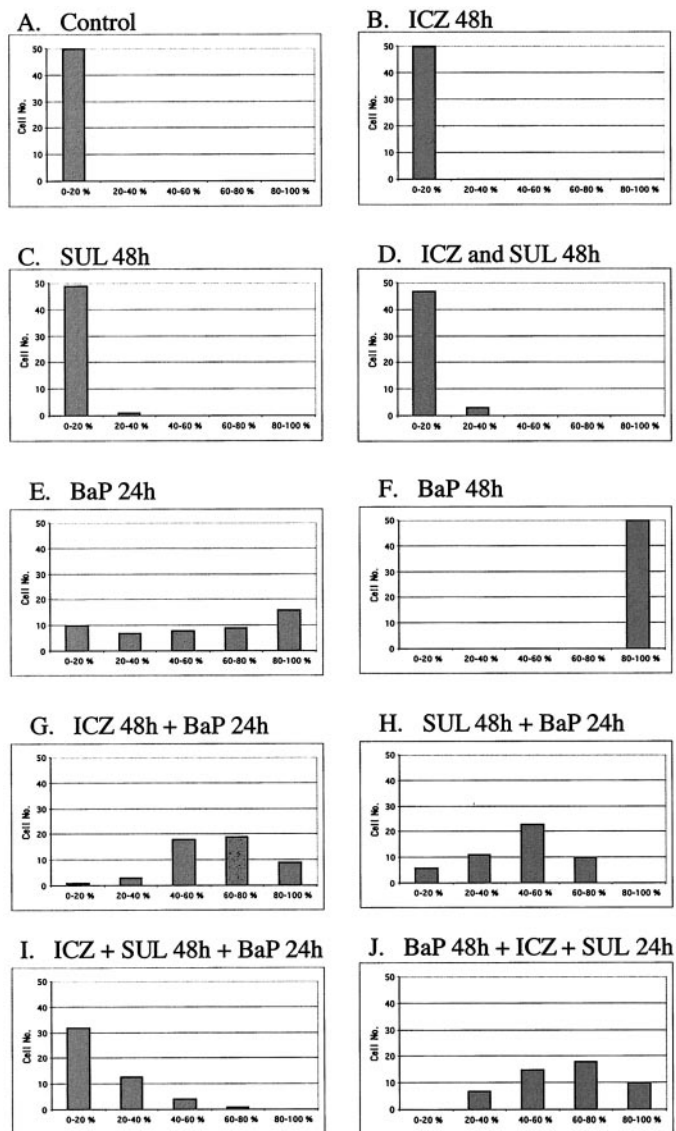


Fig. 6. Phytochemicals protect colon cells against DNA damage caused by exposure to BaP. Single-strand breaks in cellular DNA were detected by the comet assay. The distribution of damage in LS-174 cells after treatment with phytochemicals ( $1 \mu\text{M}$  ICZ or  $5 \mu\text{M}$  SUL) and/or exposure to  $25 \mu\text{M}$  BaP has been determined by calculating the proportion of DNA in the comet tail. A–D, comet data from LS-174 cells cultured for 48 h in either media alone, media containing ICZ, media containing SUL, and media containing ICZ plus SUL, respectively. E and F, distribution of DNA damage resulting, respectively, from culture of cells in media alone for 24 h followed by BaP for 24 h, or culture of cells in media containing BaP for 48 h. G, distribution of DNA damage resulting from culture of cells in media containing ICZ for 24 h followed by BaP plus ICZ for 24 h. H, the extent of DNA damage resulting from culture of cells in media containing SUL for 24 h before exposure to BaP plus SUL for 24 h. I, DNA damage observed in cells pretreated with ICZ and SUL for 24 h, followed by exposure to BaP in the presence of both phytochemicals for 24 h. J, DNA damage observed in cells exposed first to BaP for 24 h, followed by BaP plus ICZ and SUL for an equal period of time. The experiments were performed twice, and essentially identical results were obtained on both occasions.

DNA damage was measured using alkaline single-cell gel electrophoresis, otherwise known as the comet assay (53, 54). At the concentrations used, neither ICZ nor SUL exhibited cytotoxicity (Table 1) or caused significant DNA damage, as assessed by comet assay (Fig. 6). After 48-h exposure to BaP, all of the LS-174 cells possessed extensive DNA damage, with the number of lesions being sufficient to cause between 80 and 100% of total cellular DNA to be recovered as small fragments in the tails of comets. By contrast, after 24-h exposure to BaP, only one-third of the LS-174 cells possessed damage that was sufficiently extensive to result in 80–100% of cellular DNA

migrating during electrophoresis in the tails of comets. Pretreatment of LS-174 cells with  $1 \mu\text{M}$  ICZ for 24 h before exposure to BaP for an equal period of time was found to diminish modestly the amount of DNA damage (Fig. 6G). Similar pretreatment of the LS-174 cells with  $5 \mu\text{M}$  SUL for 24 h before exposure to the genotoxic agent appeared to confer slightly greater protection against DNA damage (Fig. 6H). In this case, treatment of the colon cells with SUL prior to 24-h exposure to BaP completely prevented the formation of comets containing 80–100% of the total DNA. It should be noted however that DNA damage was not eliminated by pretreatment with SUL alone, because electrophoresis revealed that 88% of the cells yielded comets with more than 20% of cellular DNA in the tail. Substantially greater protection against DNA damage could be conferred on LS-174 cells by simultaneous pretreatment with both ICZ and SUL (Fig. 6I). The use of both indole and isothiocyanate in the chemoprevention regimen significantly reduced the ability of BaP to produce DNA damage in LS-174 cells. More than 60% of the colon cells pretreated with both phytochemicals yielded comets containing less than 20% of the total cellular DNA.

In addition to treatment of LS-174 cells with phytochemicals before exposure to BaP, the converse experiment was also performed. Specifically, LS-174 cells were exposed to BaP for 24 h before cotreatment for an additional 24 h with both ICZ and SUL in the presence of the genotoxic compound. In these latter experiments, although the LS174 cells were exposed to BaP for a total of 48 h, the relative amount of DNA damage was comparable with that obtained from 24-h exposure to BaP alone (Fig. 6, E and J). This finding indicates that treatment of colon cells with phytochemicals probably cannot reverse DNA damage once it has occurred. However, it appears that if phytochemicals are coadministered with genotoxic agents they can prevent DNA damage (Fig. 6, F and J).

A series of experiments was also conducted using  $\text{H}_2\text{O}_2$  to determine whether indoles and isothiocyanates can protect against genotoxicity caused by reactive oxygen species. Exposure of LS-174 cells to  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 5 min resulted in damage sufficient to cause comets that contained at least 40% of the total cellular DNA in about 65% of cells. Pretreatment of the cells either with  $1 \mu\text{M}$  ICZ for 24 h or with  $5 \mu\text{M}$  SUL for 24 h before exposure to  $\text{H}_2\text{O}_2$  did relatively little to prevent comet formation (Fig. 7). However, combined pretreatment with ICZ and SUL was found to have a marked inhibitory effect on comet formation (Fig. 7H).

## DISCUSSION

The present investigation was undertaken to determine whether indoles and isothiocyanates, which are formed *in vivo* from glucosinolates, are capable of inducing gene expression in the human, and whether such induction protects against DNA damage.

**Regulation of Enzyme Expression by Indoles and Consequences for Health Risk.** I3C, a major autolysis product of the glucosinolate glucobrassicin, gives rise to a range of metabolites within the GI tract, such as ASG, DIM, and ICZ (Fig. 1). All of these indoles induced CYP1A1 protein in both LS-174 and Caco-2 cells (Fig. 2). Northern blots of LS-174 and Caco-2 cells showed that ICZ treatment causes an increase in the steady-state level of mRNA for CYP1A1 in both cell lines (Fig. 3). Induction of CYP1A1 in human colon cells almost certainly occurs through the XRE. Use of a CAT reporter construct demonstrated that ICZ is a potent inducer of XRE-driven gene expression, giving a  $\text{CD}_{\text{CAT}}$  value of 5 nM. By comparison, DIM, I3C, and ASG are poorer inducing agents, concluded from the fact they gave  $\text{CD}_{\text{CAT}}$  values of 1.0, 6.0, and  $37 \mu\text{M}$ , respectively (Table 5). The relative potency of the different indoles as inducing agents is mirrored by their affinity for the AhR (8).

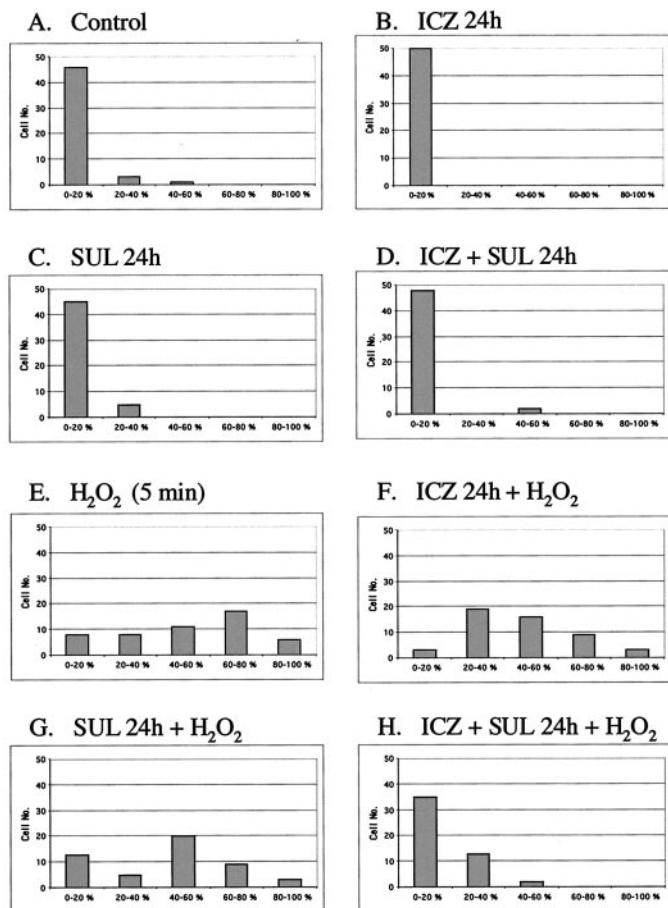


Fig. 7. Phytochemicals protect colon cells against DNA damage caused by exposure to H<sub>2</sub>O<sub>2</sub>. The distribution of damage in LS-174 cells after treatment with phytochemicals (1  $\mu$ M ICZ, or 5  $\mu$ M SUL, or 1  $\mu$ M ICZ and 5  $\mu$ M SUL) and/or exposure to 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> has been determined by calculating the proportion of DNA in the comet tail. A–D, comet data from LS-174 cells cultured for 24 h in media alone, media containing ICZ, media containing SUL, and media containing ICZ plus SUL, respectively. E, distribution of DNA damage resulting from culture of cells in media alone for 24 h followed by H<sub>2</sub>O<sub>2</sub> for 5 min. F, G, and H, the distribution of DNA damage resulting from exposure to H<sub>2</sub>O<sub>2</sub> after treatment for 24 h with ICZ alone, SUL alone, and ICZ plus SUL, respectively.

Indoles caused a small induction of NQO1 in LS-174 and Caco-2 cells but did not induce AKR1C1, GCS<sub>n</sub>, or GST. It was also found that, whereas indoles can stimulate XRE-driven transcription, they were relatively inactive with the ARE-CAT reporter construct. It is important to note that failure to induce ARE-driven gene expression sets DIM, ASG, I3C, and ICZ apart from the classic bifunctional inducer  $\beta$ -NF. Thus, induction of CYP1A1 in colon cells by ICZ, and the presumed accompanying increase in oxidative stress (55), does not necessarily transcriptionally activate genes through the ARE. Furthermore, it can be concluded that none of the indoles studied are metabolized in colon cells to thiol-active compounds, an event that is required for activation of Nrf2 (17, 18). It is apparent that previous work showing that I3C can induce NQO, GST, and aflatoxin B<sub>1</sub> aldehyde reductase in rodent organs (9–11) reflects XRE-driven gene expression and probably not ARE-driven gene expression.

The ability of indoles to induce CYP1A1, coupled with their poor induction of NQO1 and apparent inability to induce GST, raises the interesting question of whether this group of phytochemicals ought to be considered hazardous to mankind. Xenobiotics that induce CYP1A1 are regarded as potentially harmful because of the propensity of the cytochrome to activate carcinogenic polycyclic aromatic hydrocarbons. Agents that induce XRE-driven gene expression without stimulating ARE-driven gene expression are thought to be more

likely to accelerate chemical carcinogenesis rather than retard the process. Consistent with this hypothesis is the observation that exposure of animals to polychlorinated dibenzo-*p*-dioxin, a potent AhR agonist that does not stimulate the ARE (15, 26), causes teratogenesis and tumor promotion (14). Moreover, targeted disruption of the gene encoding the AhR abolishes the teratogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (56). Although these findings indicate that all AhR ligands may promote neoplastic disease, this does not seem to hold true. For example, treatment of rats with I3C has been shown to inhibit both aflatoxin B<sub>1</sub> hepatocarcinogenesis (10) and the formation of DNA adducts by heterocyclic amines in colon carcinogenesis models (57, 58). Although the AhR is itself associated with cell proliferation, recent work suggests that certain AhR ligands act to inhibit cell proliferation (59). The question as to whether indoles inhibit colon cell growth in an AhR-dependent fashion warrants further investigation.

**Enzyme Induction by Isothiocyanates.** SUL, BITC, and PEITC, formed from glucoraphanin, gluconasturtin and glucotropaeolin, respectively, each have similar effects on protein expression. None of these isothiocyanates increased CYP1A1 expression, but all three induced AKR1C1, NQO1, and GCS<sub>n</sub>. The LS-174 and Caco-2 cells that had been treated with SUL demonstrated substantial increases in the steady-state level of mRNA for AKR1C1 and NQO1. It is unlikely that induction of these enzymes by isothiocyanates is mediated by the AhR. Rather, it is most probable that induction by isothiocyanates is mediated by Nrf2 through the ARE. The human *NQO1* gene has been shown to have a functional ARE site in its 5'-flanking region (21). The human gene encoding GCS<sub>n</sub> contains two ARE enhancers in its promoter, both of which embody an AP-1-binding site (22). Presumably, both of these genes are regulated directly by Nrf2 or related basic region leucine zipper proteins. By contrast, no perfect ARE consensus has been identified in the human *AKR1C1* gene promoter, but Burczynski *et al.* (25) have reported that the proximal 819 bp of the 5'-flanking region contains six potential AP-1 like sites. It, therefore, remains to be determined whether induction of AKR1C1 is a direct or indirect effect of Nrf2.

In this study, the use of a CAT reporter assay revealed that SUL and PEITC stimulate ARE-driven gene expression with CD<sub>CAT</sub> values of 1.3 and 1.7  $\mu$ M, respectively. Previous experiments using human liver HepG2 cells to examine the effect of isothiocyanates on ARE-directed reporter gene activity gave a CD<sub>GH</sub> value (the concentration of inducing agent required to double growth hormone reporter activity) for SUL of 0.43  $\mu$ M (60). Our CD<sub>CAT</sub> result for SUL in Caco-2 cells is in reasonable agreement with that obtained in HepG2 cells.

**Polymorphic Expression of NQO1 in Colon Cells and Significance for Cancer Patients.** Our finding that NQO1 protein could not be detected in Caco-2 cells, either before or after treatment with inducing agents was a surprise. The fact that Northern blotting revealed that both LS-174 and Caco-2 cells contained an inducible mRNA species that hybridized to the NQO1 cDNA led us to consider whether the two cell lines might express different allelic variants of the reductase. PCR-based genotyping showed that the Caco-2 cell line is homozygous for the *NQO1*\*2 allele, which translates into an unstable reductase containing Ser187 rather than Pro187. During the original description of a polymorphism in human *NQO1*, it was shown that BE cells express *NQO1*\*2 whereas HT-29 cells express *NQO1*\*1 (61). To our knowledge, this is the first report that Caco-2 cells contain *NQO1*\*2.

**Cell-specific Differences in Enzyme Induction and Apoptosis.** The data presented in this paper about regulation of AKR1C1 and NQO1 in LS-174 and Caco-2 cells, as well as that presented by other workers about these proteins in HT-29 cells (24, 62), suggest that detoxication enzymes are inducible by dietary factors in the epithelial

cells of the human GI tract. The LS-174, Caco-2, and HT-29 cells all represent transformed lines. We have also examined the nontumorigenic HCEC line. Treatment of this latter cell line with SUL produced a modest increase in dihydrodiol dehydrogenase activity (Table 3), which was reflected in the induction of AKR1C1 protein (data not shown) and mRNA (Fig. 3). The relatively limited amount of AKR1C1 induction achieved by SUL in the HCEC line is consistent with the observation that these colon cells express substantially less mRNA for Nrf2 than do LS-174 and Caco-2 cells (Fig. 5).

It is perplexing that the XRE was not activatable in HCEC cells because the RT-PCR experiments indicate that both AhR and ARNT are expressed in the cell line. Possibly HCEC express a polymorphic variant of AhR (49), or maybe this line overexpresses the natural AhR repressor protein (63). Determining the reason for the nonresponsiveness of AhR in HCEC will require further study.

Besides failure to induce CYP1A1, the HCEC line also differed from the LS-174 and Caco-2 cell lines in that apoptosis could not be stimulated by the phytochemicals studied. In view of the fact that SV40-T antigen has been used to immortalize the HCEC line,<sup>4</sup> it can be assumed that p53-dependent apoptosis mechanisms will be inhibited in these cells.

**Phytochemicals Can Protect Human Cells against DNA Damage.** The finding that indoles and isothiocyanates can induce gene expression in colon cells led us to test the hypothesis that the increased expression of detoxication enzymes caused by treatment with phytochemicals confers protection against chemicals that damage DNA. To this end, BaP and H<sub>2</sub>O<sub>2</sub> were used as prototypic genotoxic agents. They were chosen not only because the GI tract is likely to be exposed to these compounds in the normal course of events, but also because these two compounds are detoxified by different pathways. ICZ and SUL were each selected, respectively, as a model indole and isothiocyanate because of their relatively high potencies as inducing agents (Table 5).

Treatment of LS-174 cells with either ICZ or SUL reduced the number of single-strand DNA breaks caused by BaP or H<sub>2</sub>O<sub>2</sub>. However, chemoprevention by ICZ or SUL required the colon cells to be treated with phytochemicals before exposure to the genotoxic compounds. Neither ICZ nor SUL were capable of reducing the amount of DNA damage in colon cells when used after the cells had been exposed to BaP or H<sub>2</sub>O<sub>2</sub>. Pretreatment of LS-174 cells with SUL appeared to confer a greater degree of protection against genotoxic damage by BaP or H<sub>2</sub>O<sub>2</sub> than did ICZ. It was also found that pretreatment with both SUL and ICZ conferred significantly greater protection against DNA damage than did either phytochemical alone. This suggests that combinations of indoles and isothiocyanates will be of greater health benefit than the use of a single chemopreventive monofunctional inducing agent on its own.

**Concluding Comments.** Data are presented showing that indoles and isothiocyanates are capable of inducing detoxication and antioxidant enzymes in human colon cancer cells. The actions of these two groups of phytochemical are distinct. Indoles transcriptionally activate gene expression through an XRE enhancer, whereas isothiocyanates activate genes via an ARE. Treatment of colon cells with both ICZ and SUL together confers protection against genotoxic agents, to a degree that neither compound can achieve alone. It remains to be proven whether protection against DNA damage conferred by ICZ and SUL is determined genetically and, in particular, whether it is dependent on the AhR and Nrf2.

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