Dietary Organic Isothiocyanates Are Cytotoxic in Human Breast Cancer MCF-7 and Mammary Epithelial MCF-12A Cell Lines

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Organic isothiocyanates (ITCs) are dietary components present in cruciferous vegetables. The purpose of this investigation was to examine the cytotoxicity of 1-naphthyl isothiocyanate (NITC), benzyl isothiocyanate (BITC), β-phenethyl isothiocyanate (PEITC), and sulforaphane in human breast cancer MCF-7 and human mammary epithelium MCF-12A cell lines, as well as in a second human epithelial cell line, human kidney HK-2 cells. The cytotoxicity of NITC, BITC, PEITC, and sulforaphane, as well as the cytotoxicity of the chemotherapeutic agents daunomycin (DNM) and vinblastine (VBL), were examined in MCF-7/sensitive (wt), MCF-7/Adr (which overexpresses P-glycoprotein), MCF-12A, and HK-2 cells. Cell growth was determined by a sulforhodamine B assay. The IC_{50} values for DNM and VBL in MCF-7/Adr cells were 7.12 ± 0.42 μM and 0.106 ± 0.004 μM (mean ± SE) following a 48-hr exposure; IC_{50} values for BITC, PEITC, NITC, and sulforaphane were 5.95 ± 0.10, 7.32 ± 0.25, 77.9 ± 8.03, and 13.7 ± 0.82 μM, respectively, with similar values obtained in MCF-7/wt cells. Corresponding values for BITC, PEITC, NITC, and sulforaphane in MCF-12A cells were 8.07 ± 0.29, 7.71 ± 0.07, 33.6 ± 1.69, and 40.5 ± 1.25 μM, respectively. BITC and PEITC can inhibit the growth of human breast cancer cells as well as human mammary epithelium cells at concentrations similar to those of the chemotherapeutic drug DNM. Sulforaphane and NITC exhibited higher IC_{50} values. The effect of these ITCs on cell growth may contribute to the cancer chemopreventive properties of ITCs by suppressing the growth of preclinical tumors, and may indicate a potential use of these compounds as chemotherapeutic agents in cancer treatment.

Exp Biol Med 229:835–842, 2004.

Key words: phenethyl isothiocyanate; benzyl isothiocyanate; naphthyl isothiocyanate; sulforaphane; cytotoxicity

Organic isothiocyanates (ITCs) are plant-derived dietary compounds commonly known as mustard oils. Present in the *Brassica* genus of the Cruciferae family, they are found in vegetables such as cabbage, cauliflower, broccoli, and brussels sprouts, in the form of glucosinolates, the biosynthetic precursors of ITCs in plants. When vegetables are chewed, the glucosinolates are cleaved by the enzyme myrosinase (1) to form ITCs. Glucosinolate levels have been estimated to be as high as 180 mg/g in some vegetables (2). More than 20 natural and synthetic ITCs have demonstrated cancer-preventive properties in animals treated with chemical carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines (3–6). There is substantial evidence that inhibition of tumorigenesis is partly due to the direct inhibition or down-regulation (or both) of the cytochrome P-450 enzymes responsible for carcinogen activation, resulting in decreased amounts of ultimate carcinogens formed (7). In addition, ITCs have also demonstrated the ability to induce certain phase II enzymes, which are responsible for the detoxification of electrophilic intermediates formed during phase I metabolism (7). For example, ITCs can inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK)-induced carcinogenesis by inhibiting the microsomal metabolism of NNK to reactive species that form methyl and pyridyloxobutyl adducts in DNA (8). ITCs may also be able to suppress tumor cell growth. Recent studies have indicated that certain ITCs can affect the cell cycle; for example, sulforaphane induces cell cycle arrest and apoptosis in HT29 human colon cancer cells (9), PEITC also induces apoptosis *in vitro* (10, 11), and allyl ITC can induce apoptosis in dimethylhydrazine-induced rat colon cancer (12).

This research was supported in part by U.S. Army Contract DAMD17-00-1-0376 and by a grant from the Kapoor Charitable Foundation, SUNY. E.A.S.-R. was supported in part by a Gateway Research Scholarship from the American Foundation for Pharmaceutical Education.

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Received March 7, 2004.
Accepted May 4, 2004.

1535-3702/04/2298-0835 $15.00

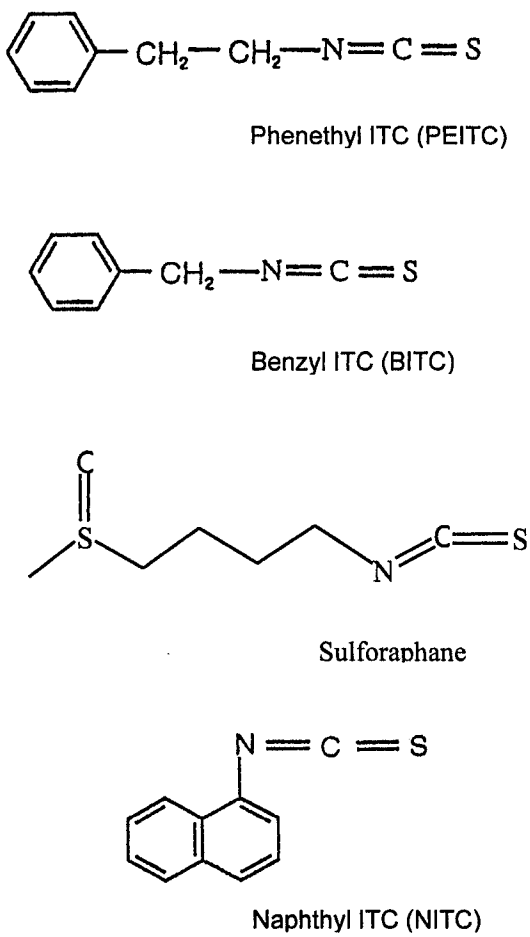

Figure 1. Chemical structures of phenethyl isothiocyanate, benzyl isothiocyanate, sulforaphane, and 1-naphthyl isothiocyanate.

The main objective of this investigation was to examine the cytotoxic effects of benzyl isothiocyanate (BITC), β-phenethyl isothiocyanate (PEITC), sulforaphane, and α-naphthyl isothiocyanate (NITC; Fig. 1) in a human breast cancer cell line (MCF-7) and a human mammary epithelium cell line (MCF-12A), and to compare the results with the cytotoxicity observed for two chemotherapeutic agents, daunomycin (DNM) and vinblastine (VBL). Cytotoxicities were also compared with those obtained using another human epithelial cell line, human kidney HK-2 cells.

Materials and Methods

Materials. BITC, PEITC, NITC, daunorubicin HCl, and vinblastine sulfate were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sulfuraphane was purchased from LKT Laboratories (St. Paul, MN). Cell culture reagents were supplied by Gibco BRL (Buffalo, NY). Cell culture flasks and 96-well plates were purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ). The MCF-7/Adr cell line was obtained from the National Cancer Institute. The MCF-12A and HK-2 cell lines were purchased from American Type Culture Collection (Manassas, VA). Epidermal growth factor (20 ng/ml) was obtained from BD Biosciences (Bedford, MA). Cholera toxin (100 ng/ml), insulin (10 μg/ml), and hydrocortisone (500 ng/ml) were purchased from Sigma Chemical.

Table 1. IC_{50} Values of DNM and ITCs in MCF-7 Cells[a]

Compound	Time (hours)	MCF-7/Adr IC_{50} Mean ± SE, μM	MCF-7/wt IC_{50} Mean ± SE, μM
DNM	2	40.1 ± 1.74***	
	4	34.2 ± 1.32***	—
	6	21.9 ± 2.47***	
	48	7.12 ± 0.42	
VBL	2	9.99 ± 0.803*	
	4	5.87 ± 0.469	—
	6	6.99 ± 0.787	
	48	0.106 ± 0.004	
BITC	1	14.5 ± 0.28***	17.0 ± 1.53***
	2	10.4 ± 0.31***	14.1 ± 1.46***
	3	7.37 ± 0.16***	10.3 ± 1.62***
	6	5.55 ± 0.19***	6.94 ± 0.46*
	48	5.95 ± 0.10	4.11 ± 0.26
PEITC	1	20.4 ± 0.78***	30.3 ± 0.98***
	2	14.0 ± 0.54***	20.8 ± 1.95***
	3	12.8 ± 0.65***	15.2 ± 0.10*
	6	7.56 ± 0.31***	22.6± 3.54*
	48	7.32 ± 0.25	6.51 ± 0.86
NITC	3	—	89.2 ± 10.8
	48	77.9 ± 8.03	78.4 ± 5.26
Sulforaphane	3	167 ± 3.61***	144 ± 26.1***
	6	64.1 ± 3.37	119 ± 12.4***
	48	13.7 ± 0.82	27.9 ± 5.30

[a]Significantly different from 48-hr values: $^*P < 0.01$, $^{**}P < 0.05$, $^{***}P < 0.001$; n = 2–8.

Cell Culture. MCF-7/wt and MCF-7/Adr cells (passages 16–24) were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (10 units/ml), and streptomycin (10 μg/ml). Cells were incubated at 37°C supplemented with 5% CO_2/95% air. Cells were subcultured two to three times a week using 0.05% trypsin-0.53 mM EDTA. Cells were grown in 75-cm^2 plastic culture flasks and seeded in 96-well plates.

MCF-12A cells, used between passages 56–58, were grown in a 1:1 mixture of Dulbecco modified Eagle medium and Ham's F12 medium supplemented (95%) with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 500 ng/ml hydrocortisone and 5% horse serum. Cells were incubated at 37°C supplemented with 5% CO_2/95% air. Cells were subcultured using 0.25% trypsin-0.03% EDTA in phosphate-buffered saline (PBS). Cells were grown in 75-cm^2 plastic culture flasks and seeded in 96-well plates.

HK-2 cells, used between passages 15–25, were grown in Keratinocyte-SFM containing 5 ng/ml recombinant epidermal growth factor and 0.04 mg/ml bovine pituitary extract. Cells were incubated at 37°C supplemented with 5% CO_2/95% air. Cells were subcultured once a week using

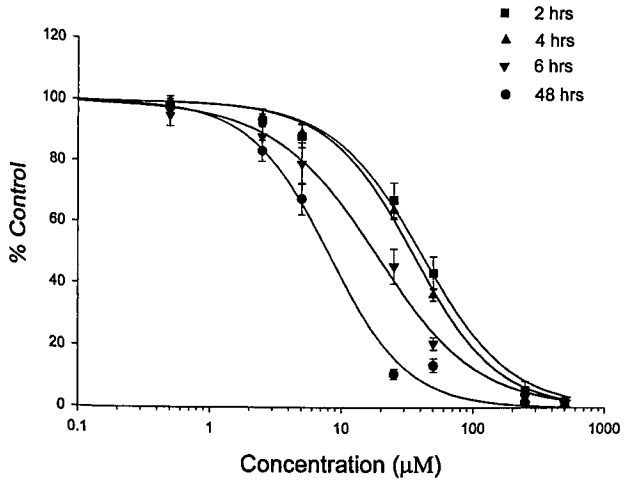

Figure 2. DNM cytotoxicity in MCF-7/Adr cells. The effect of varying concentrations of DNM on cell growth of MCF-7/Adr cells following exposure times of (■) 2 hrs, (▲) 4 hrs, (▼) 6 hrs, and (●) 48 hrs. Each data point represents the mean ± SE from four wells in one representative study. The study was repeated five to eight times.

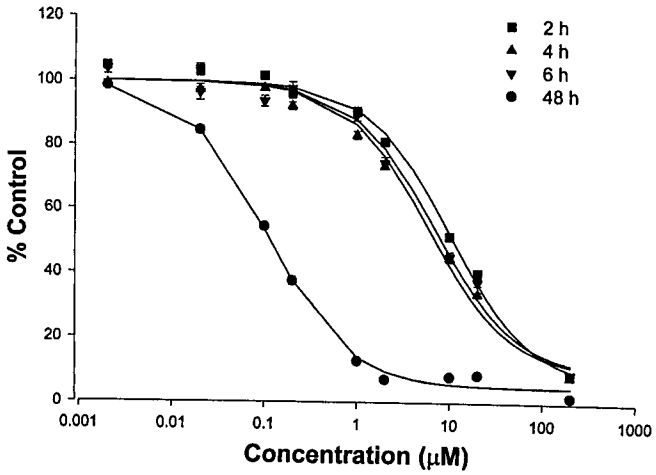

Figure 3. VBL cytotoxicity in MCF-7/Adr cells. The effect of varying concentrations of VBL on cell growth of MCF-7/Adr cells following exposure times of (■) 2 hrs, (▲) 4 hrs, (▼) 6 hrs, and (●) 48 hrs. Each data point represents the mean ± SE from four wells in one representative study. The study was repeated twice.

0.05% trypsin-0.53 m*M* EDTA. Cells were grown in 75-cm^2 plastic culture flasks and seeded in 96-well plates.

Cytotoxicity Assay. Cells were plated at a density of 5,000 cells/well for 48 hrs prior to drug exposure. The doubling rate for MCF-7 cells is ~28 hrs; therefore, plating cells for 48 hrs makes certain that cells are in their exponential phase when exposed to drugs. Cells were then exposed to 200 μl of growth media (containing 0.5% dimethyl sulfoxide; DMSO) in the absence or presence of increasing concentrations of NITC, BITC, PEITC, sulforaphane, DNM, or VBL for time intervals up to 48 hrs. At the end of the incubation period, the cells were rinsed twice with 1× PBS, and fresh media was added. Control wells were incubated with the vehicle DMSO; the percentage of DMSO in media was kept constant (0.5%) and demonstrated no cytotoxicity in any cell line. At 48 hrs, media was removed and a sulforhodamine B (SRB) assay was performed (13). Briefly, cells were fixed to the plate with 100 μl of 10% trichloroacetic acid and stored at 4°C for 1 hr. Cells were then rinsed five times with water and allowed to dry for 5–10 mins. A 100-μl aliquot of SRB 0.4% dye was added to each well for 15 mins and the wells were rinsed 3–4 times with 1% acetic acid. Plates were allowed to air dry for another 5–10 mins before the addition of 10 m*M* Tris-base for 5 mins. Absorbance was read at 570 nm, with a Spectra Rainbow plate reader (Tecan US; SLT Lab Instruments, Research Triangle Park, NC). The absorbance values (optical density (O.D.) 570) from the SRB assay indicate the cell number in each well of the 96-well plates.

Data Analysis. Data were fitted to a sigmoidal E_{max} equation using WinNonlin pharmacokinetic software (Pharsight Corp., Mountain View, CA):

$$E = E_0 - \left(\frac{E_{max} \times C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}}\right)$$

where E represents the effect, E_0 is the initial effect, E_{max} is the maximal effect, C is the concentration of substrate, IC_{50} is the concentration that inhibits cell growth by 50%, and γ represents a sigmoidicity factor. Statistical analysis was determined by a one-way ANOVA followed by a Dunnett posthoc test using the analysis software GraphPad Prism (GraphPad Inc., San Diego, CA). Differences were considered to be significant when $P < 0.05$.

Results

Cytotoxicity as a Function of Time Exposure. The general pattern observed in the MCF-7 cell lines was a decrease in IC_{50} values with longer exposure times to the test drugs (Table 1). The IC_{50} values for DNM and VBL decreased significantly over the 48-hr exposure period, with the lowest values obtained following a 48-hr exposure.

Cytotoxicity Studies with MCF-7 Cells. The IC_{50} value of DNM in MCF-7/Adr cells determined following a 48-hr exposure was 7.12 ± 0.42 μ*M*, while that of VBL was 0.106 ± 0.004 μ*M*. The general potency, determined after a 48-hr exposure, was VBL > BITC ≈ PEITC ≈ DNM > sulforaphane > NITC (Table 1; Figs. 2–4). In MCF-7/Adr cells, IC_{50} values for the ITCs were generally similar to those in MCF-7/wt and MCF-12A cells (except for NITC in MCF-12A cells). PEITC and BITC were more cytotoxic than DNM after an exposure of 6 hrs and comparable after a 48-hr exposure.

Cytotoxicity Studies with MCF-12A Cells. The IC_{50} value of DNM was not able to be determined using concentrations up to 100 μ*M* following a 48-hr exposure time in MCF-12A cells. When BITC, NITC, PEITC, and sulforaphane were incubated with MCF-12A cells *in vitro*, there was a dose-dependent inhibition of cell growth (Table 2; Fig. 5). BITC and PEITC inhibited the growth of MCF-12A cells with similar potency; IC_{50} values ranged from

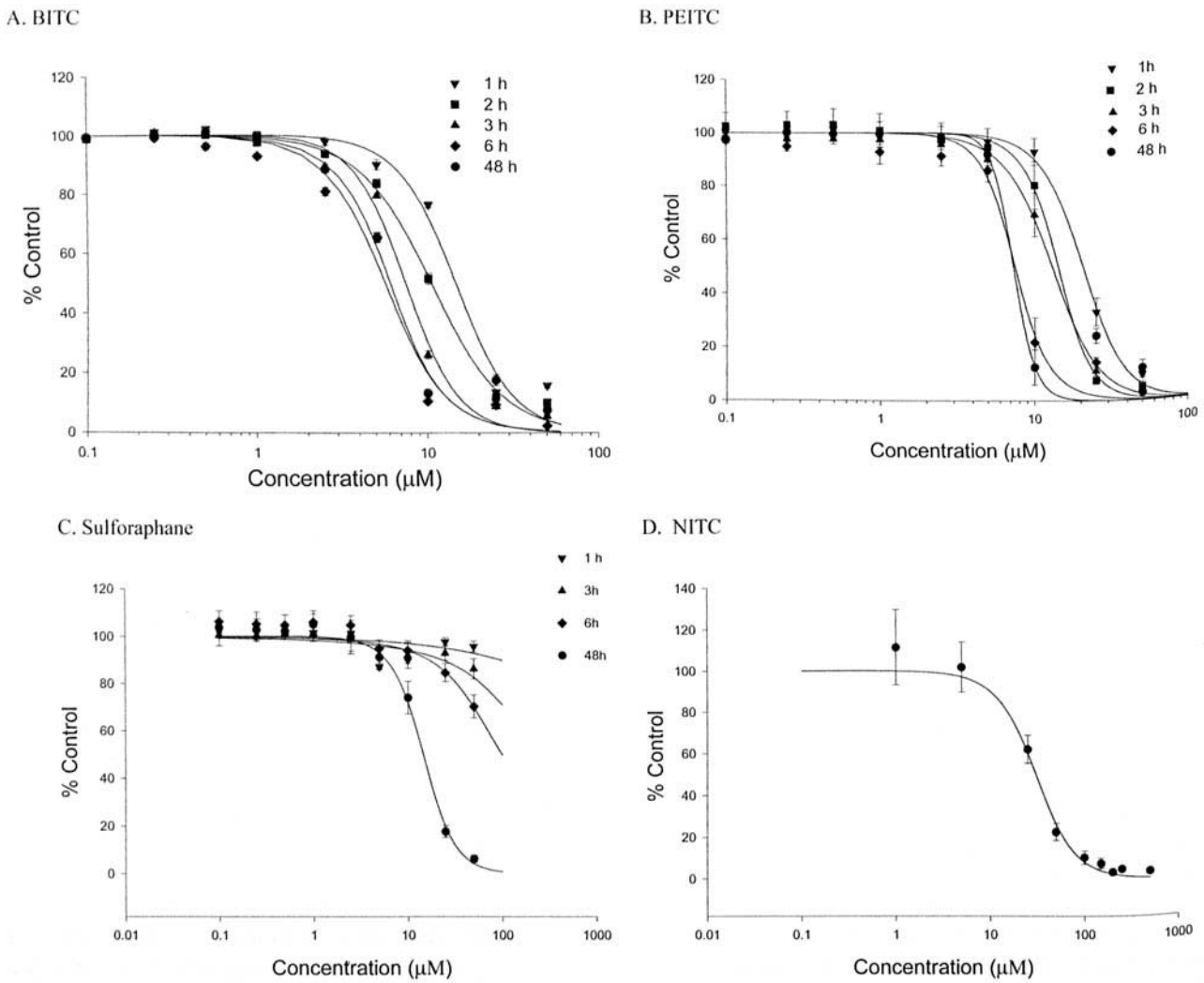

Figure 4. ITC cytotoxicity in MCF-7/Adr cells. The effect of varying concentrations of (A) BITC, (B) PEITC, (C) sulforaphane, and (D) NITC on cell growth of MCF-7/Adr cells following exposure times of (▾) 1 hr, (■) 2 hrs, (▲) 3 hrs, (♦) 6 hrs, and (●) 48 hrs. Each data point represents the mean ± SE from four wells in one representative study. The study was repeated two to four times.

7.71 to 8.07 μ*M* for a 48-hr exposure. Sulforaphane inhibited cell growth with a similar IC_{50} value as that in MCF-7 cell lines. NITC exhibited less toxicity than BITC and PEITC, although the IC_{50} value was about one-half that of the MCF-7 cell lines.

Cytotoxicity Studies with HK-2 cells. DNM was observed to have a mean IC_{50} of 0.77 μ*M* following a 48-hr exposure in HK-2 cells. When BITC, PEITC, and NITC were incubated with HK-2 cells *in vitro,* all produced inhibition of cell growth (Table 3). BITC and PEITC inhibited the growth of HK-2 cells with similar potency: IC_{50} values ranged from 2.12 to 2.62 μ*M* for a 2-hr exposure and from 2.97 to 3.27 μ*M* for a 48-hr exposure. However, NITC exhibited less toxicity than BITC and PEITC. The IC_{50} values for NITC averaged 10.73 μ*M* for a 2-hr exposure and 7.26 μ*M* for a 48-hr exposure.

Discussion

The capacity for organic ITCs to block chemical carcinogenesis was first recognized more than 30 years ago with NITC. ITCs have been demonstrated to be potent chemopreventive agents in numerous animal models of cancer (5, 14–16). The inhibition of carcinogen metabolic activation via inhibition of cytochrome P-450 enzymes (CYPs) and increased detoxification of carcinogens through induction of glutathione S-transferases (GSTs) and UDP-glucuronyl transferases (UGT) or NADPH quinone oxidoreductase (NQO1) are recognized as important mechanisms (17). Human and rodent studies have demonstrated that PEITC blocks metabolic activation of NNK and benzo(a)pyrene, major lung carcinogens in tobacco smoke, via CYPs, resulting in increased urinary excretion of detoxified metabolites, and suggesting inhibitory effects on CYP1A1, CYP1A2, and CYP2B1 (8, 18, 19). In humans, watercress ingestion resulted in a reduction in the levels of oxidative

Table 2. IC_{50} Values of ITCs in MCF-12A Cells

Compound	Time (hours)	IC_{50} Mean ± SE μM (n = 4)
BITC	48	8.07 ± 0.29
PEITC	48	7.71 ± 0.07
NITC	48	33.6 ± 1.69
Sulforaphane	48	40.5 ± 1.25

metabolites of acetaminophen, which was attributed to inhibition of oxidative metabolism by CYP2E1 (20), and enhancement in the area under the plasma concentration–time curve of chlorzoxazone, a clinical probe for CYP2E1 (21). ITCs are able to increase the expression or activity (or both) of phase II enzymes in rat organs (7) and in cell culture (22). Watercress extract increases the activities of UGT, GST, and NQO1 (23). PEITC treatment of LS-174 human colon cells produced an increased protein expression of NQO1 and γ-glutamylcysteine synthetase (24), and PEITC, administered to rats by gavage or via diet, induced hepatic NQO1, GST, and UGT (18, 25).

In this investigation we found that BITC and PEITC inhibit cell growth in breast cancer cells at similar concentrations as those observed for DNM. These concentrations are 4- to 6-fold lower than the IC_{50} for the isoflavonoid genistein, a compound that has also been studied in MCF-7 cells. Genistein has been reported to have the lowest IC_{50} value among the dietary flavonoids tested in MCF-7 cells (26). Although the plasma concentrations of ITCs following dietary consumption in humans is unknown, it has been estimated that the consumption of 100 g of broccoli could release 40 μmoles of the ITC sulforaphane, which would result in low micromolar concentrations in plasma (27). PEITC is a naturally occurring ITC, being found as its glucosinolate conjugate gluconasturiin in vegetables, including watercress. Consumption of about 30 g of watercress releases about 46.5 μmoles of PEITC (28). Another factor that will highly influence plasma ITC levels is the polymorphic expression of the major metabolizing enzymes for many ITCs, GSTM1 and GSTT1 (29). Polymorphisms in the GSTM1 and GSTT1 genes are caused by a complete deletion of the gene, which results in loss of function. The incidence of homozygous null deletion is approximately 50% for GSTM1 in white subjects in the United States, as well as in Japanese and Chinese subjects; for GSTT1 the incidence is 12%–16% in subjects from Germany and England, and 60–64% in subjects from China and Korea (30). Moreover, the protective effect of broccoli on the prevalence of colorectal adenomas was observed only among subjects with the GSTM1-null genotype (31). Studies examining the correlation of ITC intake obtained through vegetable consumption and GSTM1 and GSTT1 genotypes among various populations have also suggested that the reduction in lung cancer risk was stronger among persons genetically deficient in GSTM1, GSTT1, or both, although GSTM1 deficiency appears to be more important (32–34). In those subjects with detectable levels of ITCs in their urine and a GSTM1 deficiency, there was a 64% decrease in the risk for developing lung cancer (32). These studies suggest the importance of the polymorphic expression of GSTs in determining the systemic concentrations and efficacy of ITCs.

The mechanism underlying the cytotoxicity of ITCs is unknown, but apoptosis may play an important role. PEITC is capable of inducing apoptosis in a dose-dependant manner in HeLa cervical cancer cells, human leukemia HL-60 cells, PC-3 prostate cancer cells, and in human HT-29 colon carcinoma cells (11). In HT-29 cells, PEITC induces apoptosis via the mitochondria caspase cascade and the activation of c-Jun N-terminal kinase (JNK) is critical in this process. This leads to cytochrome *c* release, activation of caspase-9 and caspase-3, followed by nuclear condensation and DNA fragmentation (11). The tumor suppressor protein p53 is involved in ITC-mediated apoptosis but is not essential, because apoptosis can also occur in p53-null cells. Additionally, the PEITC-induced apoptosis in p53-deficient PC-3 prostate cancer cells is mediated by extracellular-regulated kinase and not JNK, suggesting cell line dependence. Thornalley (22) has suggested that depletion of glutathione due to the formation and subsequent expulsion of glutathione conjugates from cells may result in protein thiocarbamoylation by the ITC. Protein thiocarbamoylation may lead to the activation of JNK, with the subsequent induction of apoptosis (22). BITC and PEITC can deplete intracellular glutathione (GSH) concentrations in cell lines, whereas NITC does not (35). While there are no reported cellular toxicities of NITC, there have been reported cases of hepatotoxicity by this compound. Primary rat hepatocyte cultures incubated for 18 hrs with NITC (0–100 μM) produced cytotoxicity at concentrations >25 μM (36). Therefore, the mechanisms involved in the cytotoxicity of NITC may differ from that of BITC and PEITC.

There are a number of possible reasons for the differences in IC_{50} values observed in this study in MCF-7/Adr, MCF-12A, and HK-2 cells, besides differences in intrinsic sensitivity to the ITCs. The human breast cancer cell line MCF-7/Adr has pronounced expression of P-glycoprotein, which may result in lower intracellular concentrations of the ITCs in MCF-7/Adr cells if the ITCs are substrates for P-glycoprotein. However, we have shown that PEITC is not a substrate for P-glycoprotein in MCF-7/Adr cells, and that intracellular concentrations are similar in sensitive and resistant MCF-7 cells (35). Additionally, in this study, the IC_{50} values of the ITCs were similar in MCF-7/wt and MCF-7/Adr cells, indicating that the presence of P-glycoprotein did not influence efficacy. Differences in intracellular concentrations in cell lines may also occur due to differences in protein binding of the ITC in the cell media. In a study by Xu and Thornalley (37), it was found that in the absence of FBS, the IC_{50} of PEITC in HL-60 cells was lower compared with the increased IC_{50} values in

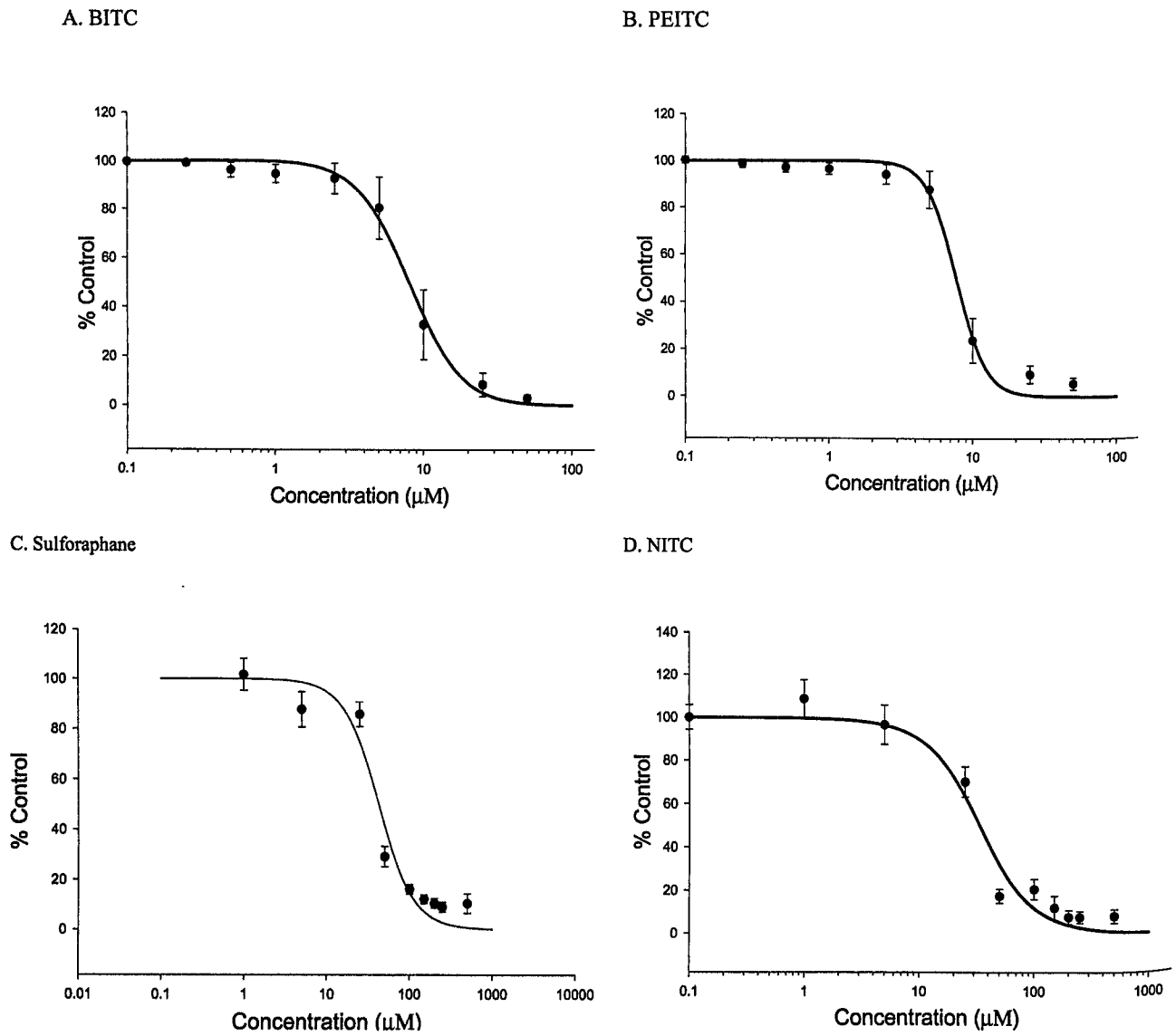

Figure 5. ITC cytotoxicity in MCF-12A cells. The effect of varying concentrations of (A) BITC, (B) PEITC, (C) sulforaphane, and (D) NITC on cell growth of MCF-12A cells following an exposure time of (•) 48 hrs. Each data point represents the mean ± SE from four wells in one representative study. The study was repeated 4 to 10 times.

the presence of 10% and 25% FBS in the media. The MCF-7/Adr media used in this investigation contained 10% FBS and the MCF-12A media contained 5% horse serum, whereas the HK-2 media did not contain protein. If the ITCs are protein-bound, then lower free concentrations would be present in protein-containing media, and would therefore be available to diffuse across cell membranes in MCF-7 cells. Preliminary studies in our laboratory have indicated extensive protein binding of PEITC in rat serum (Y. Kuo and M. E. Morris, unpublished results). The lack of protein binding in HK-2 cell media may be responsible for the lower IC_{50} values for ITCs observed in the HK-2 cell studies. Intracellular levels of ITCs will also be dependent on drug metabolism within these cells and on stability of the ITC in the culture medium, which may differ substantially. The intracellular accumulation of some ITCs has been shown to be highly dependent on intracellular glutathione concentrations; differences in GSH and GST activity in the two cell lines could result in different degrees of accumulation at the same media ITC concentration (38, 39).

The IC_{50} values for the ITCs remained the same in both MCF-7/Adr and MCF-12A cells following 6- and 48-hr exposure times. This contrasted with our findings with DNM and VBL, in which the IC_{50} values were significantly lower following a 48-hr exposure. Although the reason for this difference is unknown, this may be due to the stability and metabolism of ITCs at 37°C in these cells lines. We have found that the half-life for PEITC in buffer at pH 7.4 at room temperature is about 58 hrs, while that of BITC is about 40 hrs; NITC is less stable in buffer, with a half-life of approximately 20–25 hrs (Y. Kuo and M. E. Morris, unpublished). Metabolism in these cell lines for any of the

Table 3. IC_{50} Values of DNM and ITCs in Human Kidney Proximal Tubule HK-2 Cells[a]

Compound	Time (hours)	IC_{50} Mean ± SE (μM)
DNM	48	0.768 ± 0.060
BITC	2	2.19 ± 0.04
	6	1.47 ± 0.03***
	48	2.97 ± 0.05
PEITC	2	2.62 ± 0.07***
	6	2.35 ± 0.06***
	48	3.27 ± 0.04
NITC	2	10.7 ± 0.41***
	6	5.69 ± 0.29**
	48	7.26 ± 0.09

[a]Significantly different from 48-hr values: $^*P < 0.01$, $^{**}P < 0.05$, $^{***}P < 0.001$; n = 4–8.

tested compounds has not been examined. As well, adsorption to plastic wells for these very lipophilic compounds may represent a source of loss of the ITCs (35).

In conclusion, we have shown that the ITCs BITC, PEITC, NITC, and sulforaphane are able to exert cytotoxic effects in a dose- and time-dependent manner. This cytotoxic effect, which is comparable for BITC and PEITC with that of DNM, may represent another mechanism that is important for the chemopreventive effect of the ITCs, or it may indicate the potential use of BITC and PEITC as anticancer agents. Elucidation of the mechanisms underlying the cancer-protective effects of ITCs is of crucial importance, not only for the use of these compounds as chemopreventive agents, but also for the identification or design of more effective chemopreventive agents.

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