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Studies on the Mechanism of the Inhibition of Human Leukaemia Cell Growth by Dietary Isothiocyanates and Their Cysteine Adducts *In Vitro*

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ABSTRACT. The dietary isothiocyanates and cancer chemopreventive agents phenethyl isothiocyanate and allyl isothiocyanate and their cysteine conjugates inhibited the growth and induced apoptosis of human leukaemia HL60 (p53−) and human myeloblastic leukaemia-1 cells (p53+) *in vitro*. The median growth inhibitory concentration (GC_{50}) values were in the range 1.49–3.22 μM in cultures with 10% serum. Isothiocyanates and cysteine conjugates had increased potency against HL60 cells in serum-free medium, with GC_{50} values of 0.8–0.9 μM. The potency of the compounds decreased with increased serum content of the medium, but that of the cysteine conjugates decreased more markedly. Growth inhibition and toxicity was characterised by either a rapid interaction of the isothiocyanate with the cells in the first hour of culture or exposure to isothiocyanate liberated from the cysteine conjugate in the initial 3 hr of culture, inhibition of macromolecule synthesis, and a commitment to apoptosis which developed in the initial 24 hr. Activities of caspase-3 and caspase-8 were increased during isothiocyanate-induced apoptosis, but caspase-1 activity was not. The general caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone and the specific caspase-8 inhibitor *N*-benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone inhibited apoptosis, but specific caspase-1 and caspase-3 inhibitors did not. The antiproliferative activities were limited by hydrolysis of the isothiocyanate. This suggests that caspase-8 has a critical role, and caspase-3 a supporting role, in isothiocyanate-induced apoptosis in which p53 is not an obligatory participant. Isothiocyanate-induced apoptosis may suppress the growth of preclinical tumours and contribute to the well-established decreased cancer incidence associated with a vegetable-rich diet. BIOCHEM PHARMACOL **60**;2:221–231, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. phenethyl isothiocyanate; allyl isothiocyanate; human leukaemia; lymphocyte; apoptosis; caspases

Glucosinolates are naturally occurring thioglucosides present in cruciferous vegetables, broccoli, cabbage, cauliflower, turnip, radish, and watercress [1]. They are degraded non-enzymatically and enzymatically by myrosinases (thioglucoside glucohydrolase, EC 3.2.3.1) during food preparation, cooking, and chewing to isothiocyanates, thiocyanates, nitriles, and epithiocyanoalkanes. Two of the most prevalent glucosinolates are sinigrin and gluconasturtin, degraded to form AITC† and PEITC, respectively. Dietary isothiocyanates, synthetic analogues, and their cysteine conjugates have recently been of intense interest for their anticarcinogenic activities and potential use in the chemoprevention of cancer [2, 3]. The cysteine conjugates are prodrugs: they are expected to fragment to form the corresponding isothiocyanate and have decreased toxicity compared to the isothiocyanate [4]. Chemopreventive activity is thought to be associated with inhibition of the metabolic activation of carcinogens by cytochrome P450 isozymes [5] and increased excretion of carcinogens by the induction of increased activities of glutathione *S*-transferases (EC 2.5.1.18) involved in elimination of carcinogens by the mercapturic acid pathway [4, 6]. A further feature of the pharmacological activity of dietary isothiocyanates and related *S*-(*N*-thiocarbamoyl)cysteine derivatives was their anticancer activity *in vitro* [7], which has also recently been demonstrated *in vivo* [8] and which may contribute to chemopreventive activity by inhibiting the growth of tumours in preclinical development.

PEITC inhibited the growth of HL60 cells *in vitro* and induced apoptosis. The mercapturic acid pathway metabolites of PEITC had similar antitumour activities. There was decreased toxicity to corresponding differentiated cells [7].

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† *Abbreviations:* AITC, allyl isothiocyanate; HL60, human leukaemia 60; GC_{50}, median growth inhibitory concentration value; JNK1, c-Jun N-terminal kinase 1; MALDI, matrix-assisted laser desorption mass spectrometry; ML-1, human myeloblastic leukaemia-1; PEITC, phenethyl isothiocyanate; ATC-Cys, *S*-(*N*-allylthiocarbamoyl)cysteine; and PETC-Cys, *S*-(*N*-phenethylthiocarbamoyl)cysteine.

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HL60 cells have deletions in the *p53* gene such that there is neither p53 protein nor mRNA in these cells [9]. AITC inhibited the growth and induced the cytotoxicity of human colonic carcinoma HT29 cells *in vitro*; the GC_{50} value was 13.9 μM. It was less toxic to differentiated HT29 cells. These studies suggest that dietary isothiocyanates may have selective toxicity to the malignant phenotype [10]. PEITC, AITC, and benzyl isothiocyanate inhibited the growth of HeLa cells *in vitro*, where the GC_{50} values were *ca.* 3, 10, and 2 μM, respectively. There was an accumulation of cells in the G_2/M phase of the cell cycle associated with growth inhibition [11]. Similar induction of growth arrest and cytotoxicity by these isothiocyanates was found in cultures of simian virus 40-transformed Indian muntjac cells and Chinese hamster ovary cells. Isothiocyanates were *ca.* 1000-fold more potent than the glucosinolate precursors [12, 13].

PEITC was recently found to induce a sustained activation of JNK1 in serum-deprived HeLa cells [14]. This was associated with apoptosis, as mutation of the JNK1 or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 inhibited PEITC-induced apoptosis. An inhibitor of caspase-1 blocked PEITC-induced apoptosis without change in the increased activity of JNK1 in Jurkat cells, suggesting that JNK1 signalling was upstream of caspase-1 activation in the mechanism of PEITC-induced apoptosis [15]. In HeLa cells, however, PEITC induced an increase in the activity of caspase-3, and a caspase-3 inhibitor, but not a caspase-1 inhibitor, attenuated PEITC-induced apoptosis [16]. This suggested that the activation of caspases is important in the mechanism of PEITC-induced apoptosis, but that the importance of individual caspases may vary in different cell types. Overexpression of Bcl-2 and Bcl-x_L suppressed both PEITC-induced activation of JNK1 and apoptosis, suggesting that Bcl-2 and Bcl-x_L could intervene upstream of JNK1 activation in PEITC-induced apoptosis [15]. 2-Mercaptoethanol (10 mM) and *N*-acetylcysteine (20 mM) inhibited PEITC-induced apoptosis, which was interpreted as evidence for the involvement of oxidative stress in PEITC-induced apoptosis by the modification of cellular GSH by PEITC [15].

In this report, we describe the characteristics of apoptosis induced by PEITC, AITC, and their cysteine adducts in the human leukaemia cells HL60 and ML-1 *in vitro*. We conclude that caspase-8 has a critical role, and caspase-3 a supporting role, in apoptosis induced by dietary isothiocyanates and their cysteine conjugates in these cells, but that p53 is not an obligatory participant. Isothiocyanates, free or liberated from fragmentation of corresponding *S*-(*N*-thiocarbamoyl)cysteine adducts, interacted with leukaemia cells between 1 and 3 hr of culture to inhibit macromolecule synthesis and committed cells to apoptosis, wherein isothiocyanate hydrolysis and interaction with serum limited the antitumour effect.

MATERIALS AND METHODS

Materials

PEITC, AITC, and d_6-dimethylsulphoxide were purchased from Aldrich. L-Cysteine, *N*-acetyl-L-cysteine, trypan blue, human insulin, human holotransferrin, β-glycerophosphate, phenylmethylsulphonyl fluoride, dithiothreitol, digitonin, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate, and dimethylsulphoxide were purchased from Sigma. Tissue culture medium RPMI-1640 and foetal bovine serum were purchased from GIBCO Europe Ltd. The caspase-1 substrate *N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD-AMC), the caspase-3-substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC), the caspase-8-substrate *N*-benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-7-amino-4-(trifluoromethyl)coumarin (Z-IETD-AFC), the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-fmk), the caspase-1 inhibitor *N*-acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO), and the caspase-3 inhibitor *N*-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) were purchased from Calbiochem. The caspase-8 inhibitor *N*-benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (Z-IETD-fmk) was purchased from Enzyme Systems Products. PETC-Cys and ATC-Cys were prepared by reaction of PEITC and AITC with cysteine in ethanolic sodium phosphate buffer, pH 6.6 and 20° [17]. Briefly, cysteine (5 mmol) was dissolved in 5 mL of sodium phosphate buffer, pH 7.4. The isothiocyanate (4 mmol), PEITC or AITC, was dissolved in 4.4 mL of ethanol and added drop-wise with stirring to the cysteine solution. An immediate precipitate was formed; after addition of the isothiocyanate was complete, the pH was *ca.* 6.6. The precipitate was collected by filtration and washed with water, ethanol, and diethyl ether, and dried under vacuum. The product was recrystallised from aqueous ethanol. The cysteine conjugate products were characterised by ^1H and ^{13}C NMR spectroscopy and MALDI mass spectrometry. PETC-Cys had molecular characteristics identical to those previously described [5, 17]. ATC-Cys gave: ^1H NMR δH ppm ($J_{X,Y}$ Hz) in d_6-DMSO cysteinyl 2-H 3.53 ($J_{2,3A}$ = 4.4, $J_{2,3B}$ = 6.0), 3-H_A 3.65 ($J_{2,3A}$ = 4.4, $J_{3A,3B}$ = −14.5), 3-H_B 3.31 ($J_{2,3B}$ = 6.0, $J_{3A,3B}$ = −14.5), thiocarbamoyl NH 8.46, allyl 1-H(2H) 4.21 ($J_{1,2}$ = 5.6), 2-H 5.86 ($J_{1,2}$ = 5.6), 3-HA 5.20 ($J_{2,3A}$ = 1.6, $J_{3A,3B}$ = −18.8), 3-HB 5.12 ($J_{2,3B}$ = 1.2, $J_{3A,3B}$ = −18.2); ^{13}C NMR δc ppm cysteinyl C_1 168.4, C_2 53.4, C_3 35.5, thiocarbamoyl 194.7, allyl C_1 48.2, C_2 132.4, C_3 116.4; and molecular ion (M + 1/z) = 221.

Cell Culture

HL60 (p53−) cells and ML-1 (p53+) cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, U.K.) and cultured in RPMI-1640 media containing 10% foetal bovine serum under an atmosphere

of 5% CO_2 in air, 100% humidity, and 37° [7, 18]. Cells were seeded at an initial density of 5×10^4/mL and incubated with 100 nM–50 μM PEITC, AITC, PETC-Cys, or ATC-Cys for 48 hr at 37°. Stock solutions of the compounds (100 mM) were prepared in dimethylsulphoxide and diluted into the growth medium such that the final concentration of dimethylsulphoxide did not exceed 0.05% (v/v), a concentration that did not induce differentiation or toxicity in HL60 or ML-1 cells.

The effect of serum on the inhibition of HL60 cell growth by PEITC and PETC-Cys was investigated by also studying the effect of PEITC and PETC-Cys on the growth of HL60 cells in RPMI-1640 with 25% foetal bovine serum and in serum-free medium. For serum-free medium cultures, HL60 cells grown in RPMI-1640 with 10% foetal bovine serum were washed with serum-free medium (RMPI-1640 with 5 μg/mL of insulin and 5 μg/mL of holotransferrin [19]) and seeded at 5×10^4 cells/mL in serum-free medium. The cells were incubated for 24 hr, 0–5 μM PEITC or PETC-Cys added, incubated for a further 48 hr, and the cell number and viability then determined. The effect of dose period of PEITC and PETC-Cys on the growth of HL60 and ML-1 cells was investigated by incubating HL60 or ML-1 cells with PEITC or PEITC-Cys for 1–48 hr, replacing the medium without compound, and continuing the incubation such that the combined incubation period with and without PEITC or PEITC-Cys was 48 hr.

Human peripheral lymphocytes were isolated from venous blood (50 mL) of healthy human donors under aseptic conditions. Mononuclear cells were separated by density gradient centrifugation using Histopaque 1077 [20]. Monocytes were removed by adherence to walls of plastic 12-well plates by incubating mononuclear cells (2×10^6/mL) in RPMI-1640 for 1 hr at 37°. Residual lymphocytes were removed, counted, and incubated (1×10^5/mL) with 5 μg/mL of concanavalin A in RPMI-1640 containing 25% autologous plasma for 24 hr at 37°. PEITC or PETC-Cys was then added and the incubation continued for a further 48 hr. The cell viability was then determined. Cell viability was judged by the ability of cells to exclude trypan blue.

Macromolecule Synthesis

The effect of isothiocyanates and cysteine conjugates on macromolecule synthesis was assessed in the second to fourth hour of exposure of the cells to the compounds. HL60 and ML-1 cells (5×10^4/mL; 4 mL) were incubated in RPMI-1640 with 10% foetal bovine serum with 0–50 μM compound for 1 h. [methyl-^{3}H]Thymidine (2.5 μCi) for measurement of DNA synthesis, [5-^{3}H]uridine (2.5 μCi) for measurement of RNA synthesis or [^{3}H]leucine (2.5 μCi) for measurement of protein synthesis were then added and the incubation continued for a further 3 hr. DNA, RNA, and protein extracts, as well as cell cytosol fraction (as a measure of the cellular thymidine, uridine, and leucine pools, respectively) were then prepared and counted as described [21].

Flow Cytometric Analysis

HL60 cells (5×10^4/mL; 20 mL) were incubated with PEITC or PETC-Cys (5 μM) for 6 hr, sedimented by centrifugation (200 *g*, 5 min), washed with PBS (10 mL), and the cell pellet fixed by addition of 1 mL of 70% ethanol. The fixed cells were stored in the dark at 4° until required for further analysis (less than 1 week). The cell pellet was re-suspended in 1 mL of 140 mM phosphate buffer, pH 7.4, with 1 mg/mL of ribonuclease A for 30 min at 37°. The cells were then collected by centrifugation and re-suspended in 1 mL of 100 μM propidium iodide in 0.1% sodium citrate with 0.1% Triton X-100 and transferred to a flow cytometric analysis tube. Flow cytometric analysis was performed to determine the percentage of cells undergoing apoptosis [22]. The dependence of apoptosis on PEITC and PETC-Cys concentration and the effect of the caspase inhibitors were investigated. Flow cytometric analysis was performed using a Becton Dickinson FACSCalibur fluorescence-activated cell sorter. Forward light scatter and fluorescence (488 nm excitation, 585 nm emission) were determined for 10,000 cells. Data presented are normalised events (cell number) against specific fluorescence (linear scale). The percentage of cells with specific fluorescence lower than the G_0-G_1, S, and G_2-M phase envelopes was deduced as apoptotic cells.

Activity of Caspase-1, Caspase-3, and Caspase-8

HL60 cells (5×10^4/mL) were incubated with and without 5 μM PETC-Cys for 3–24 hr. After treatment, cells were washed twice with ice-cold PBS and lysed in buffer (50 mM Tris/HCl [pH 7.4], 50 mM β-glycerophosphate, 15 mM $MgCl_2$, 15 mM EDTA, 100 μM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, and 150 μg/mL of digitonin). The lysate was homogenised by passing it through a 23-gauge needle three times and allowed to stay on ice for 30 min. The homogenate was centrifuged at $12{,}500 \times g$ for 20 min at 4°. Caspase activities in the supernatant were determined by a fluorogenic assay. For caspase-1 and caspase-3, 10 μg of total protein, as determined by the Bradford method, was incubated with fluorogenic peptide substrate (200 μM Ac-YVAD-AMC for caspase-1, 200 μM Ac-DEVD-AMC for caspase-3) in 50 μL of protease assay buffer containing 100 mM HEPES (pH 7.5), 10% sucrose, 10 mM dithiothreitol, and 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate. Following incubation at 37° for 2 hr, the release of 7-amino-4-methylcoumarin was measured by fluorescence spectrophotometry with excitation at 360 nm and emission at 460 nm. One unit of enzymatic caspase activity was defined as the release of 1 pmol of 7-amino-4-methylcoumarin per min at 37° under assay conditions [16]. Caspase-8 activity was assayed similarly with minor modifications: 20 μg of total protein, 160 μM Z-IETD-AFC substrate, and incubation for 1 hr at 30°, with the release of 7-amino-4-(trifluoromethyl)coumarin measured by fluorescence spectrophotometry with exci-

tation at 400 nm and emission at 505 nm. One unit of enzymatic caspase-8 activity was defined as the release of 1 pmol of 7-amino-4-(trifluoromethyl)coumarin per min at 30° under assay conditions [23].

Kinetics of Hydrolysis of PEITC and Fragmentation of PETC-Cys

PEITC (50 μM) or PETC-Cys (50 μM) was incubated for 0–200 min in PBS at 37° in a thermostatted autosampler. At the times indicated, aliquots (150 μL) were withdrawn and analysed by reversed-phase HPLC. HPLC was performed with a Waters HPLC system (717plus autosampler, 2 × 510 pumps with gradient controller, and Lambda Max 481 LC spectrophotometer). The column was a NOVAPAK octadecylsilyl (ODS) 4-μm (0.8 cm × 10 cm) cartridge fitted with a precolumn in a 8-cm × 10-cm radial compression unit at 37°. The mobile phase was 20 mM acetic acid in 50% acetonitrile with a linear gradient of 50–100% acetonitrile over 30 min; the eluate absorbance was monitored at 240 nm. The peak areas were calibrated by injection of 1.5–7.5 nmol of analyte in dimethylsulphoxide. The retention times of PETC-Cys and PEITC were 2.41 ± 0.07 min and 12.89 ± 0.13 min, and the limits of detection were 0.36 and 0.47 nmol, respectively. The concentrations of hydrolysis products were deduced as $[PEITC]_0 - [PEITC]_t$, or $[PETC\text{-}Cys]_0 - ([PETC\text{-}Cys]_t + [PEITC]_t)$, as appropriate. Products of the hydrolysis of PEITC were characterised from chloroform extracts of PEITC incubations by MALDI mass spectrometry. The formation of cysteine concomitant with the fragmentation of PETC-Cys was confirmed by withdrawal of aliquots of the reaction mixture (100 μL) and incubation with 500 μM 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) in PBS.

Data Analysis

Viable cell number as a percentage of control (V) and compound concentration ([D]) data from concentration–response studies were fitted to the logistic equation $V = 100 \times GC_{50}^n/(GC_{50}^n + [D]^n)$, where GC_{50} is the median growth inhibitory concentration value and n is the logistic regression coefficient determined from N data points. When $n > 1$, positive cooperativity can be inferred in the mechanism of the biological response and when $n < 1$, negative cooperativity can be inferred in the mechanism of the biological response. Similar logistic regression of data from the study of the effect of compounds on cell viability gave the median toxic concentration TC_{50} value. The GC_{50} value is influenced by a combination of growth arrest and cytotoxic effects of the compounds; the TC_{50} value is influenced by cytotoxicity only. Data from the study of compounds on macromolecule synthesis were analysed similarly to deduce the median inhibitory IC_{50} values for macromolecule synthesis. The data were fitted by non-linear regression using the ENZFITTER program (Biosoft).

PEITC concentration data from studies of the hydrolysis of PEITC were fitted to an exponential decay to determine the first-order rate constant. PETC-Cys and PEITC concentration data were fitted to a kinetic model of PETC-Cys fragmentation and hydrolysis,

$$\text{PETC-Cys} \underset{+\text{Cys}}{\overset{-\text{Cys}}{\rightleftharpoons}} \text{PEITC} \rightarrow \text{hydrolysis products.}$$

Experimental data were fitted to kinetic models by the GIT program, a Gear iterator which integrates complex sets of differential equations by the Gear method, analyses the deviations of these calculations from sets of observations, and then optimises the model's rate constants by minimising the deviation between calculated lines and the observed points (du Pont de Nemours & Co.).

RESULTS

Effect of Dietary Isothiocyanates and Their Cysteine Conjugates on the Growth, Viability, and Macromolecule Synthesis of Human Leukaemia Cells In Vitro

When PEITC, AITC, and their cysteine conjugates PETC-Cys and ATC-Cys were incubated with HL60 cells *in vitro*, there was a dose-dependent inhibition of cell growth and cytotoxicity. All compounds inhibited the growth of HL60 cells with similar potency: the GC_{50} values were in the range 1.49–3.22 μM. AITC and ATC-Cys, however, were less toxic than PEITC and PETC-Cys to HL60 cells (Table 1). PEITC, AITC, and their cysteine conjugates had a remarkably similar effect on the growth and viability of ML-1 cells, exhibiting GC_{50} values in the range 2.41–3.22 μM. Again, AITC and ATC-Cys were less toxic than PEITC and PETC-Cys to ML-1 cells. Under these conditions, macromolecule synthesis was inhibited early in the exposure period. DNA synthesis, RNA synthesis, and protein synthesis were inhibited by all of these compounds in both HL60 and ML-1 cells in the second to fourth hour of culture. Median inhibitory concentration IC_{50} values were similar to or 2- to 3-fold higher than the corresponding GC_{50} values (Table 2). Hence, inhibition of macromolecule synthesis was an early feature of isothiocyanate- and cysteine conjugate-induced growth arrest and toxicity to HL60 and ML-1 cells *in vitro*.

Effect of Dietary Isothiocyanates and Their Cysteine Conjugates on the Viability of Proliferating Human Lymphocytes

PEITC and PETC-Cys (1–500 μM) were incubated with concanavalin A-stimulated peripheral human lymphocytes *in vitro* and the effect on cell viability investigated. The median toxic concentration TC_{50} values were: PEITC 53.1 ± 4.3 μM (N = 15) and PETC-Cys 90.8 ± 2.7 μM (N = 12). PEITC and PETC-Cys were, therefore, *ca.* 10- to 20-fold less toxic to peripheral human lymphocytes than to

TABLE 1. Inhibition of growth of human leukaemia HL60 and ML-1 cells and cytotoxicity induced by dietary isothiocyanates and their cysteine adducts *in vitro*

Compound	Inhibition of growth		Cytotoxicity		N
	GC_{50} (μM) Mean ± SD	n Mean ± SD	TC_{50} (μM) Mean ± SD	n Mean ± SD	
Human leukaemia 60 cells					
Allyl isothiocyanate	2.56 ± 0.11	1.52 ± 0.09	11.04 ± 1.01	1.57 ± 0.26	18
S-(*N*-Allylthiocarbamoyl) cysteine	3.22 ± 0.10	1.40 ± 0.06	11.62 ± 0.02	1.57 ± 0.01	49
Phenethyl isothiocyanate	1.49 ± 0.01	1.97 ± 0.01	4.95 ± 0.28	2.86 ± 0.53	8
S-(*N*-Phenethylthiocarbamoyl) cysteine	2.48 ± 0.09	2.44 ± 0.22	4.76 ± 0.07	2.88 ± 0.13	18
Human leukaemia ML-1 cells					
Allyl isothiocyanate	2.59 ± 0.12	2.48 ± 0.23	7.74 ± 0.39	1.69 ± 0.15	18
S-(*N*-Allylthiocarbamoyl) cysteine	3.22 ± 0.13	2.73 ± 0.29	10.98 ± 0.75	1.33 ± 0.14	18
Phenethyl isothiocyanate	2.67 ± 0.06	4.20 ± 0.35	3.32 ± 0.07	4.40 ± 0.30	18
S-(*N*-Phenethylthiocarbamoyl) cysteine	2.41 ± 0.06	3.58 ± 0.38	3.55 ± 0.01	5.59 ± 0.01	18

Regression equations: cell growth (% of control) = $100 \times GC_{50}^n/(GC_{50}^n + [D]^n)$, where GC_{50} is the median growth inhibitory concentration value, n the logistic regression coefficient, and N the number of determinations. Cytotoxicity (% viable cells) = $100 \times TC_{50}^n/(TC_{50}^n + [D]^n)$, where TC_{50} is the median toxic concentration value, n the logistic regression coefficient, and N the number of determinations.

human leukaemia cells, with the cysteine conjugate having the lowest toxicity and highest selective toxicity for HL60 cells.

Effect of Serum on the Inhibition of HL60 Cell Growth by PEITC and PETC-Cys

In cultures of HL60 cells in serum-free medium and medium containing 10% and 25% foetal bovine serum, increasing the percentage of serum in the medium was found to decrease the antiproliferative effects of both PEITC and PETC-Cys (Fig. 1). Curiously, PEITC and PETC-Cys had similar potencies in serum-free medium, and the effect of serum on the antiproliferative activity of PETC-Cys was more marked than for PEITC. The GC_{50} values for serum-free medium (+5 μg/mL of insulin and 5 μg/mL of holotransferrin), 10% serum, and 25% serum were, respectively: PEITC: 0.84 ± 0.01 μM, 1.49 ± 0.01 μM and 4.57 ± 0.21 μM (N = 18). PETC-Cys: 0.96 ± 0.02 μM, 2.48 ± 0.09 μM, and 8.05 ± 0.20 μM (N = 18). Preincubation of PEITC and PETC-Cys in serum-free medium with 5 mg/mL of BSA, serum-free medium only, or PBS for 3 hr led to a complete loss of antiproliferative activity (Fig. 2). The loss of antiproliferative activity of isothiocyanates and cysteine conjugates is due to spontaneous degradation under physiological conditions. This was, therefore, characterised.

Hydrolysis of Isothiocyanate and Fragmentation of the Cysteine Conjugate

When PEITC (50 μM) was incubated in PBS, pH 7.4 and 37°, and analysed by HPLC there was an exponential decrease in PEITC concentration with time (Fig. 3a).

TABLE 2. Effect of dietary isothiocyanates and their cysteine adducts on DNA, RNA, and protein synthesis of human leukaemia HL60 and ML-1 cells *in vitro*

Compound	DNA Synthesis		RNA Synthesis		Protein Synthesis		N
	IC_{50} (μM) Mean ± SD	n Mean ± SD	IC_{50} (μM) Mean ± SD	n Mean ± SD	IC_{50} (μM) Mean ± SD	n Mean ± SD	
Human leukaemia 60 cells							
Allyl isothiocyanate	7.30 ± 0.76	1.24 ± 0.24	5.41 ± 0.14	1.29 ± 0.05	5.17 ± 0.41	0.93 ± 0.09	6
S-(*N*-Allylthiocarbamoyl)cysteine	15.60 ± 0.94	2.86 ± 0.56	8.97 ± 0.21	1.70 ± 0.07	8.06 ± 2.53	0.78 ± 0.29	6
Phenethyl isothiocyanate	2.24 ± 0.01	1.78 ± 0.01	2.84 ± 0.28	2.09 ± 0.41	2.52 ± 0.14	2.60 ± 0.30	6
S-(*N*-Phenethylthiocarbamoyl)cysteine	5.15 ± 0.20	2.03 ± 0.18	4.03 ± 0.32	1.34 ± 0.18	3.17 ± 0.13	2.46 ± 0.24	6
Human leukaemia ML-1 cells							
Allyl isothiocyanate	3.27 ± 0.01	2.82 ± 0.01	6.16 ± 1.45	0.99 ± 0.34	3.36 ± 0.29	1.46 ± 0.19	6
S-(*N*-Allylthiocarbamoyl)cysteine	4.31 ± 0.01	2.12 ± 0.01	4.73 ± 0.54	1.69 ± 0.30	4.93 ± 0.01	2.44 ± 0.01	6
Phenethyl isothiocyanate	2.42 ± 0.01	4.21 ± 0.02	2.17 ± 0.14	2.93 ± 0.61	2.17 ± 0.05	2.67 ± 0.19	6
S-(*N*-Phenethylthiocarbamoyl)cysteine	4.44 ± 0.08	3.83 ± 0.22	2.15 ± 0.08	2.63 ± 0.24	2.39 ± 0.01	3.68 ± 0.03	6

Regression equations: macromolecule synthesis (% of control) = $100 \times IC_{50}^n/(IC_{50}^n + [D]^n)$, where IC_{50} is the median inhibitory concentration value, n the logistic regression coefficient, and N the number of determinations.

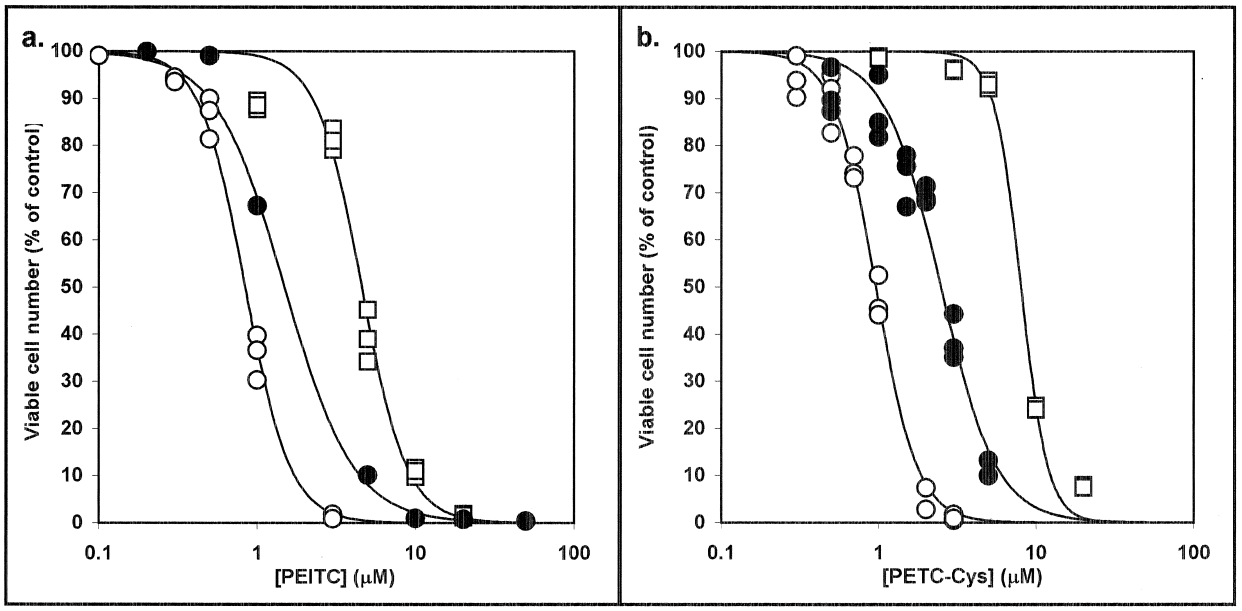

FIG. 1. Effect of serum on the inhibition of HL60 cell growth by (a) PEITC and (b) PETC-Cys. HL60 cells were incubated with 0.1–50 μM compound in RPMI-1640 with serum content: (○) 0% (+5 μg/mL of insulin and 5 μg/mL of holotransferrin), (●) 10% serum, and (□) 25% serum.

MALDI MS analysis of the incubation after 6 hr gave molecular ions of 122 and 285, indicative of the formation of phenethylamine and *N,N′*-diphenethylthiourea by isothiocyanate hydrolysis. The decrease in PEITC concentration followed first-order kinetics with a rate constant $k_{Hydrolysis}$ of $(4.92 \pm 0.23) \times 10^{-3}$ min^{-1} and a half-life $t_{1/2}$ of 141 ± 7 min.

When PETC-Cys was incubated similarly in phosphate-buffered saline, pH 7.4 and 37°, and analysed by HPLC there was a rapid decrease in the concentration of PETC-Cys and a concomitant formation of PEITC; the concentration of PEITC maximised after *ca.* 50 min and then decreased (Fig. 3b). There was a concomitant formation of cysteine, as judged by reactivity with Ellman's reagent. Data were fitted to a kinetic model for the reversible fragmentation of PETC-Cys to PEITC and cysteine, and hydrolysis of PEITC (with $k_{Hydrolysis}$ of 4.92 min^{-1}). The estimate of the rate constant for the fragmentation of PETC-Cys to PEITC and cysteine, $k_{PETC\text{-}Cys}$, was 0.0426 ± 0.0023 min^{-1}, and the estimate the reformation of PETC-Cys from PEITC, $k_{PEITC,Cys}$, was 31.1 ± 0.1 M^{-1} min^{-1}.

After *ca.* 3 hr of incubation in phosphate-buffered saline, pH 7.4 and 37°, the concentrations of PEITC in both incubations of PEITC and PETC-Cys had declined to *ca.* 40% and 60% of the initial PEITC and PETC-Cys concentrations, respectively. Preincubation of PEITC and PETC-Cys in PBS for 3 hr and subsequent addition to HL60 cells in serum-free medium resulted in a loss of growth inhibitory effect (Fig. 2). At the GC_{50} concentration, exposure of HL60 cells to PEITC for 1 hr was the minimum time sufficient to lead to a subsequent 50% inhibition of cell growth (Fig. 3c). With the GC_{50} concentration of PETC-

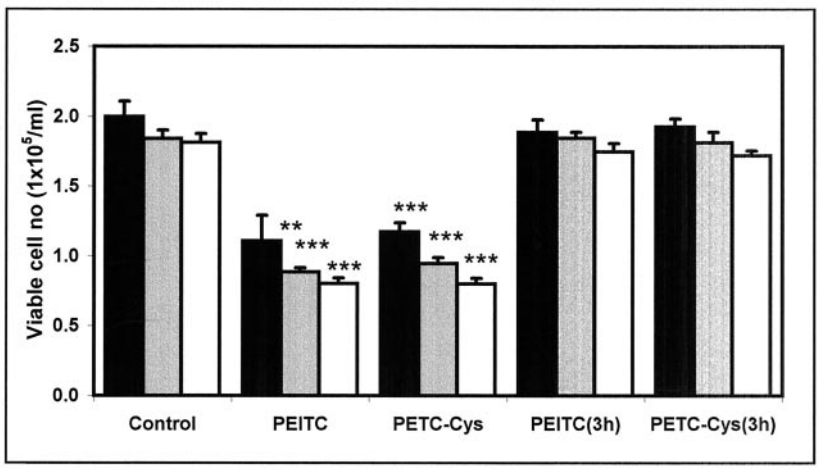

FIG. 2. Effect of 3-hr preincubation of PEITC and PETC-Cys on the inhibition of HL60 cell growth. Preincubations: PEITC and PETC-Cys (3 μM) were incubated in serum-free medium (RPMI-1640 + 5 μg/mL of insulin and 5 μg/mL of holotransferrin) or serum-free medium + 5 mg/mL of BSA at 37° for 3 hr. PEITC and PETC-Cys (30 μM) were incubated in PBS at 37° for 3 hr and then diluted 10-fold in serum-free medium. HL60 cells (5×10^4/mL) were incubated in these media for 45 hr. Bar filling key: (■) serum-free medium, (▨) serum-free medium + 5 mg/mL of albumin, and (□) PBS/serum-free medium. Bar label key: Control, PEITC (3 μM), PETC-Cys (3 μM)–no preincubation; PEITC (3 hr) and PETC-Cys (3 hr)–preincubation for 3 hr. Data are means ± SD of 3 determinations. ***$P < 0.001$ with respect to zero PEITC or PETC-Cys control.

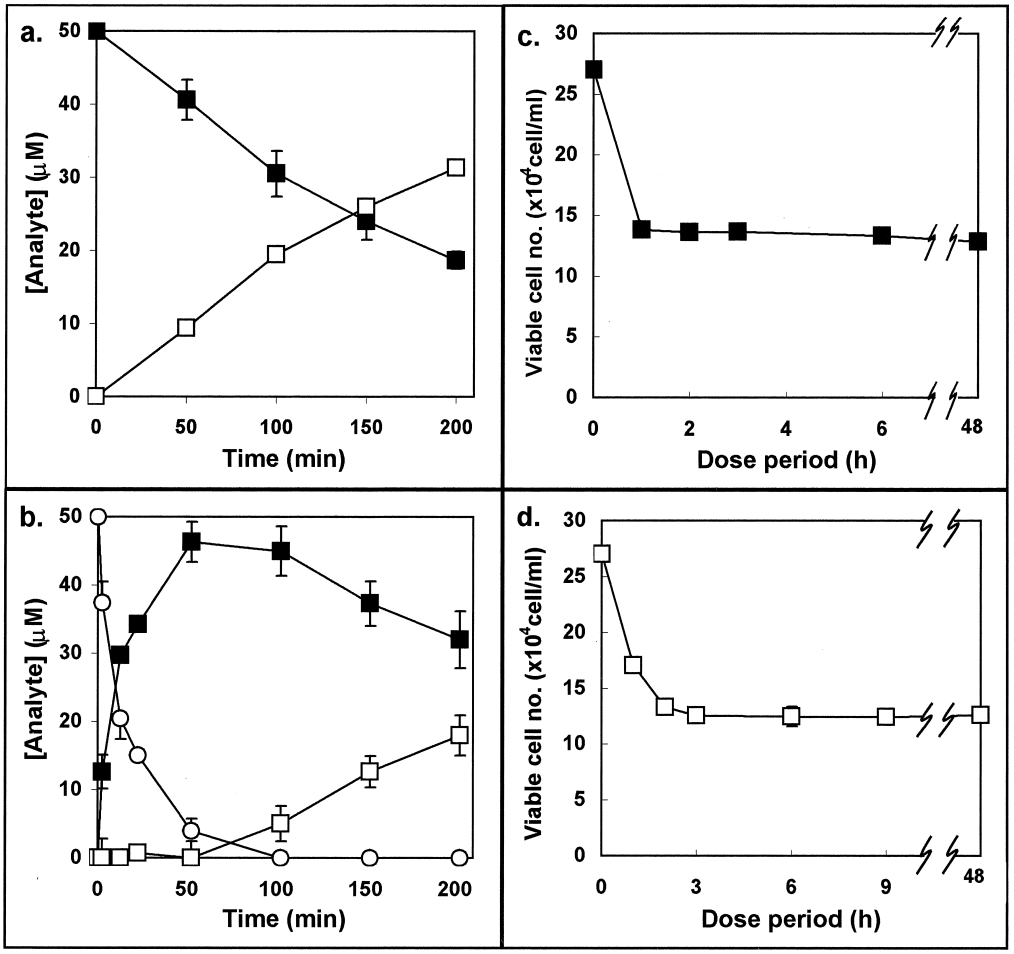

FIG. 3. Hydrolysis of PEITC and fragmentation and hydrolysis of PETC-Cys in phosphate-buffered saline, pH 7.4 and 37° (a and b) and dependence of the antiproliferative effect on the period of exposure of HL60 cells to PEITC, PETC-Cys, and metabolites (c and d). Hydrolysis of PEITC and fragmentation and hydrolysis of PETC-Cys: (a) 50 μM PEITC, (b) 50 μM PETC-Cys. Key: (■) PEITC, (○) PETC-Cys, and (□) PEITC degradation products. Dependence of the antiproliferative effect on the period of exposure of HL60 cells to PEITC, PETC-Cys, and metabolites: HL60 cells (5×10^4/mL) were incubated for the times indicated with 2.5 μM PEITC (c) or PETC-Cys (d) and the cells washed and incubated with fresh medium, RPMI-1640 with 10% serum, for a total of 48 hr. Data are means ± SD of 3 determinations.

Cys, however, exposure of HL60 cells to the compound and metabolites for 3 hr was necessary to produce a 50% inhibition of growth after 48 hr (Fig. 3d). During this period, PETC-Cys fragments to form cysteine and PEITC. Similar minimum exposure periods were needed for 50% inhibition of growth of ML-1 cells by PEITC and PETC-Cys (data not shown).

Induction of Apoptosis by Dietary Isothiocyanates and their Cysteine Conjugates

Caspases are common mediators of apoptosis. We investigated the effect of 5 μM PETC-Cys or PEITC on the activities of caspase-1, caspase-3, and caspase-8 in HL60 cells. Caspase-1 activity was little changed during PETC-Cys-induced apoptosis and tended to decrease slightly after 9 and 24 hr; at these times, apoptosis was well developed (Fig. 4a). Caspase-3 activity increased 4- to 6-fold in HL60 cells after 3–24 hr of incubation with 5 μM PETC-Cys (Fig. 4b). We then studied the effect of specific inhibitors of caspase-1 and caspase-3 on the induction of apoptosis of HL60 cells by PETC-Cys, as judged by flow cytometry. The specific inhibitor of caspase-1, Ac-YVAD-CHO (25 μM), did not prevent the induction of apoptosis of HL60 cells by 5 μM PETC-Cys to a significant degree (Fig. 4c). The specific caspase-3-inhibitor Ac-DEVD-CHO inhibited the increase in caspase-3 activity in HL60 cells during PETC-Cys-induced apoptosis (Fig. 4d), but did not prevent the induction of apoptosis of HL60 cells by 5 μM PETC-Cys significantly (Fig. 4c). The broad spectrum caspase inhibitor Z-VAD-fmk (50 μM), however, inhibited PETC-Cys-induced apoptosis completely (Fig. 4c). This suggested that other caspases may be involved in apoptosis, in particular caspase-8, which is a major processor of procaspase-3 leading to caspase-3 activation.

We investigated the effect of 5 μM PEITC on the

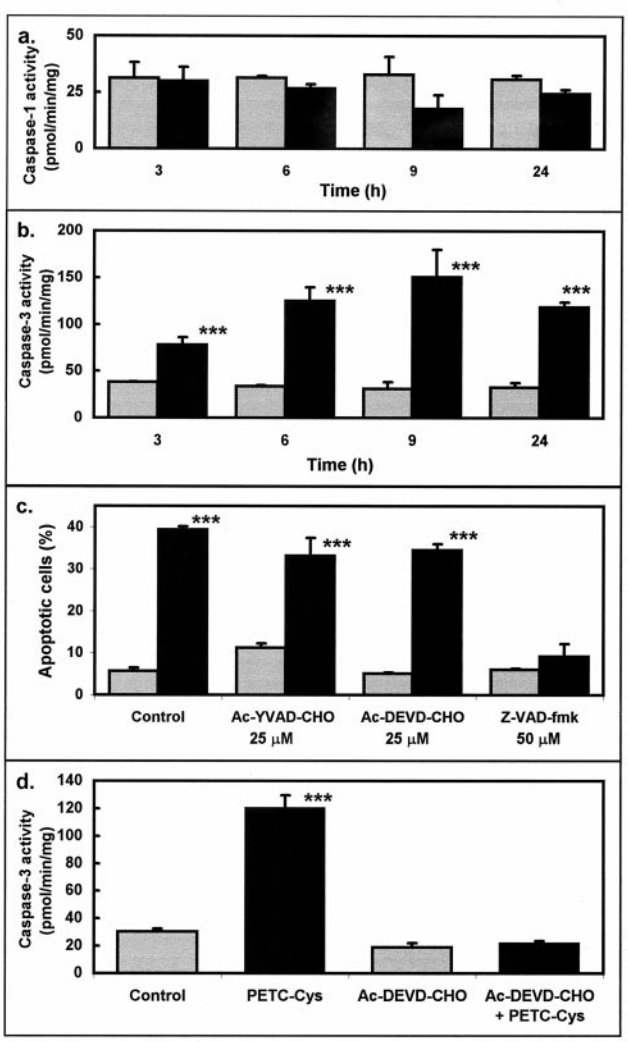

FIG. 4. Activities of caspase-1 and caspase-3 and the effect of caspase inhibitors on apoptosis induced by PETC-Cys. (a) Caspase-1 activity. (b) Caspase-3 activity. (c) Effect of caspase inhibitors on apoptosis. (d) Effect of caspase-3 inhibitor on caspase-3 activity during PETC-Cys-induced apoptosis. HL60 cells (5×10^4/mL) were incubated in RPMI-1640 with 10% foetal bovine serum with and without 5 μM PETC-Cys (a–c) and also with and without 25 μM Ac-DEVD-CHO (d). Incubations were for the times indicated (a. and b.) or 6 hr only (c and d). Caspase activities or the percentage of apoptotic cells (by flow cytometry) were then determined. Key: ▨ control (-PETC-Cys), ■ + 5 μM PETC-Cys. Data are means ± SD of 3 determinations. ***$P < 0.001$ with respect to zero PETC-Cys control.

activity of caspase-8 in HL60 cells. Caspase-8 activity increased rapidly in the initial 3 hr and remained increased *ca.* 100% above control levels over 3–9 hr. After 24 hr, it had declined and returned to control levels (Fig. 5a). We then studied the effect of a specific caspase-8 inhibitor, Z-IETD-fmk, which inhibited PEITC-induced apoptosis completely (Fig. 5b). Flow cytometric analysis indicated that the development of apoptotic bodies with low specific fluorescence, characteristic of PEITC-induced apoptosis, was inhibited in the presence of 180 μM Z-IETD-fmk.

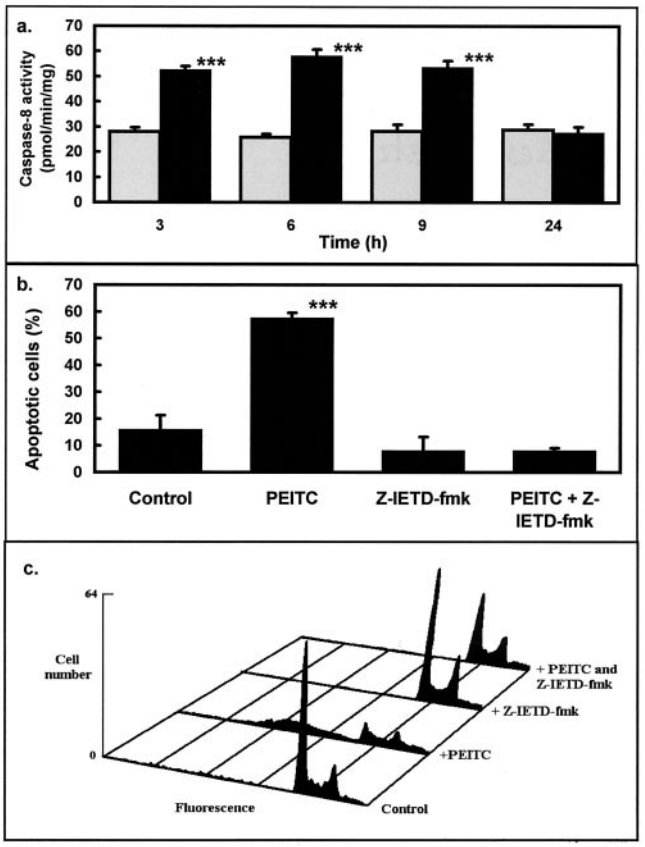

FIG. 5. Caspase-8 activity and the effect of caspase-8 inhibitor on apoptosis induced by PEITC. (a) Caspase-8 activity. (b) Effect of caspase-8 inhibitor on apoptosis. (c) Specimen flow cytometry data. HL60 cells (5×10^4/mL) were incubated in RPMI-1640 with 10% foetal bovine serum with and without 5 μM PEITC (a–c) and also with and without 180 μM Z-IETD-fmk (b). Incubations were for the times indicated (a) or 6 hr (b and c). Caspase activities or the percentage of apoptotic cells (by flow cytometry) were then determined. Key: ▨ control (-PEITC), ■ + 5 μM PEITC. Data are means ± SD of 3 determinations. ***$P < 0.001$ with respect to zero PEITC control.

DISCUSSION

We reported previously that the dietary isothiocyanate PEITC and related mercapturic acid pathway metabolites—including PETC-Cys—induced growth arrest and apoptosis in HL60 cells *in vitro*. DNA synthesis was inhibited early in the development of apoptosis, and the antiproliferative effect of these compounds was lost by preincubation in culture medium for 3 hr [7]. In this study, we found similar effects of AITC and its cysteine conjugate, ATC-Cys. Further characteristics of the mechanism of apoptosis induced by these agents were identified.

Growth arrest and toxicity in human leukaemia HL60 and ML-1 cells *in vitro* induced by PEITC, AITC, and their cysteine conjugates was characterised by either a rapid interaction of the isothiocyanate with the cells in the first hour of culture or exposure to isothiocyanate liberated from the cysteine conjugate in the initial 3 hr of culture. This was demonstrated by investigation of the minimum time of

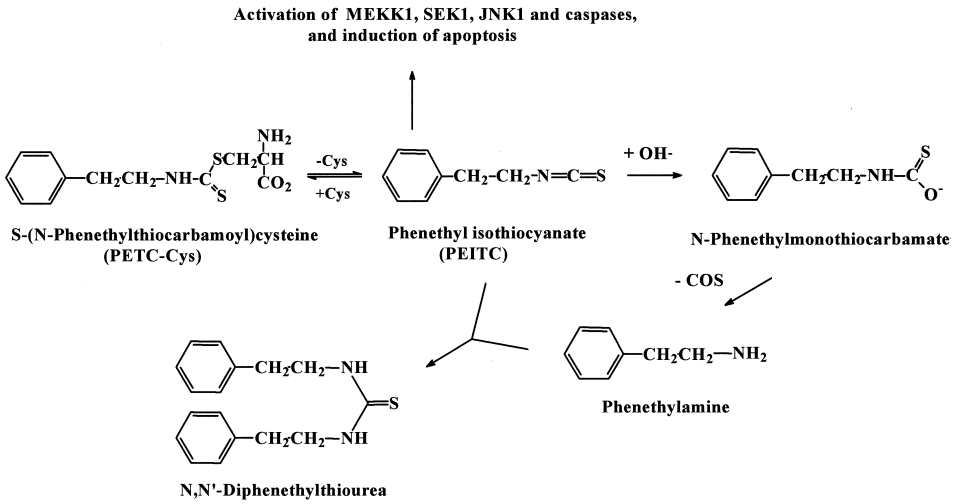

FIG. 6. The *S*-(*N*-phenethylthiocarbamoyl)cysteine/phenethyl isothiocyanate and cysteine equilibrium: spontaneous reactions and induction of apoptosis. Abbreviation: SEK1, stress-activated protein kinase/extracellular signal-regulated kinase kinase.

exposure of tumour cells to PEITC and PETC-Cys for maximum limiting growth inhibitory effect. This interaction induced commitment to apoptosis which developed over the subsequent 3–21 hr. Inhibition of macromolecule synthesis, DNA synthesis, RNA synthesis, and protein synthesis was also an early feature of the commitment of HL60 and ML-1 cells to cell death. The clone of HL60 cells used in this study did not express the tumour suppressor protein 53 [9], whereas human myeloblastic ML-1 cells did express p53 and rapidly increased the expression in response to DNA-modifying agents [24]. DNA modification by isothiocyanates may be implicated in their antitumour activity since they are known to be clastogenic [12]. The induction of apoptosis in HL60 cells, however, suggests that p53 is not always a critical mediator of dietary isothiocyanate-induced apoptosis. This is contrary to a recent report [25] where an essential role of p53 was found in the induction of apoptosis by PEITC in a mouse epidermal cell line. The mediators of isothiocyanate-induced apoptosis may vary, therefore, depending on cell type. Similar toxicity of PEITC, AITC, and their cysteine conjugates was found for p53(+) ML-1 cells. Expression of wild-type p53 in ML-1 cells, therefore, also did not protect against dietary isothiocyanate and related cysteine conjugate growth inhibition and toxicity.

We report here for the first time the kinetics of hydrolysis of PEITC. The hydrolysis of AITC has been studied under non-physiological conditions where allylamine was formed by hydrolysis of AITC to allylmonothiocarbamate and loss of carbonyl sulphide [26]. In our study, MALDI-MS analysis suggested that phenethylamine and *N*,*N*′-diphenethylthiourea were formed from the hydrolysis of PEITC at pharmacologically active concentrations (Fig. 6). Hydrolysis of PEITC for 3 hr prior to addition to HL60 cells led to a loss of antiproliferative activity, suggesting that phenethylamine and *N*,*N*′-diphenethylthiourea did not mediate the cytotoxic effect. Hydrolysis of PEITC and AITC under physiological conditions limits their antiproliferative effects. This should be noted in the preparation of aqueous solutions of isothiocyanates for biological evaluation, although the half-life of PEITC in PBS at 37° was 141 min. PEITC was also stable at room temperature during a working day in dimethylsulphoxide. Hydrolysis of dietary isothiocyanates during food preparation may also limit their chemopreventive activity. This requires further investigation.

We previously reported that PETC-Cys and similar cysteine conjugates lost their antiproliferative activity on exposure to culture medium [7]. This was confirmed in this study and extended to demonstrate that this also occurred in PBS. The cysteine adduct of PEITC, PETC-Cys, was show herein to be in equilibrium with PEITC and cysteine under physiological conditions: PETC-Cys $\rightleftharpoons$ PEITC + cysteine. The reversible binding of AITC and benzyl isothiocyanate to cysteine and glutathione has been reported previously [27]. Isothiocyanates are much more reactive with cysteinyl thiol groups than with lysyl side chain or α-amino groups. This is consistent with the formation and isolation of *S*-thiocarbamoyl derivatives of cysteine, glutathione, and other physiological thiol-containing metabolites with little evidence of corresponding thiourea derivatives. The equilibrium constant for the formation of the cysteine conjugate $K_{\text{PETC-Cys}} = k_{\text{PEITC,Cys}}/k_{\text{PETC-Cys}}$ was 730 M^{-1}. Assuming a similar equilibrium is established in blood plasma with cysteinyl thiols of albumin of *ca.* 500 μM, the ratio $[\text{PETC-Cys}]_{\text{Albumin}}/[\text{PEITC}]_{\text{free}}$ is estimated to be *ca.* 0.37, or *ca.* 27% of PEITC would be bound to albumin. Cellular thiol concentrations are much higher, i.e. in the range 5–10 mM, and the ratio $[\text{PETC-Cys}]_{\text{Cytosol}}/[\text{PEITC}]_{\text{free}}$ is estimated to be up to *ca.* 7, or *ca.* 88% of PEITC would be bound to cell thiols. Thiol conjugates of isothiocyanates, dithiocarbamoyl derivatives, are therefore depot storage sites for isothiocyanates. For 5 μM PEITC, the predicted equilibrium relaxation time τ $(=1/(k_{\text{PEITC,Cys}}[\text{PEITC}]_{\text{equilibrium}} + k_{\text{PEITC,Cys}}[\text{SH}]_{\text{equilibrium}} + k_{\text{PETC-Cys}})) \approx 17$ min for interaction with plasma thiols, and for interaction with cellular thiols, $\tau \approx 3$ min. Interactions of isothiocyanates with cellular thiols are

therefore reactions that are kinetically competent to influence and/or mediate the effects occurring in the initial 1–3 hr of exposure to PEITC and PETC-Cys that induce commitment to apoptosis. The cysteine conjugates of isothiocyanates are expected to have lower toxicity to non-malignant cells, however, since the release of isothiocyanate produced a lower maximum concentration of isothiocyanate than with an equivalent concentration of free isothiocyanate, and cellular cysteine metabolism may be concurrently stimulated. The toxicity of PETC-Cys to proliferating lymphocytes was indeed lower than that of PEITC. Decreased toxicity of PETC-Cys relative to PEITC has been reported in cancer chemoprevention studies in rats [4].

The induction of apoptosis in HL60 cells by PEITC and PETC-Cys was characterised by activation of caspase-3 and caspase-8. Inhibition of caspase-8, but not caspase-3, by specific cell-permeable inhibitors prevented apoptosis. The general caspase inhibitor Z-VAD-fmk also inhibited PETC-Cys-induced apoptosis completely. This suggests that caspases are involved in apoptosis of HL60 cells induced by PEITC and PETC-Cys, where activation of caspase-8 is critical for the induction of apoptosis. Activation of caspase-3 may be necessary for optimum proteolytic processing in apoptosis, but caspase-3-independent pathways can still achieve apoptotic cell death: the specific caspase-3 inhibitor Ac-DEVD-CHO did not inhibit apoptosis but did inhibit caspase-3 activity effectively. Ac-DEVD-CHO is also an effective inhibitor of caspase-1 and caspase-7 [28] and it inhibited PEITC-induced apoptosis in HeLa cells [29]. Caspase-1 activity was not increased during PETC-Cys-induced apoptosis of HL60 cells, and the caspase-1 inhibitor Ac-YVAD-CHO did not inhibit apoptosis. This suggests that caspase-1 was not involved in PEITC-induced apoptosis of HL60 cells. Z-VAD-fmk inhibited PETC-Cys-induced apoptosis of HL60 cells herein and inhibited apoptosis of HL60 cells induced by several antitumour agents where caspase-3 inhibitors were ineffective [30–32]. It was suggested that Z-VAD-fmk may inhibit a caspase upstream (involved in the processing of pro-caspase-3) and downstream of caspase-3 [30, 31]. Pro-caspase-3 is a major physiological target of caspase-8 [33]; Z-VAD-fmk is a potent inhibitor of caspase-8 [34]. Caspase-8 may be activated during apoptosis induced by PEITC. This was indeed found: caspase-8 activity was increased and 180 μM Z-IETD-fmk inhibited PEITC-induced apoptosis, a caspase-8 inhibitor that at similar concentration inhibited processing of pro-caspase-3 and apoptosis of HL60 cells induced by a singlet oxygen [23]. Caspase-8 also targets other proteins, however: one such protein, Bid, a BH3 domain-containing proapoptotic Bcl2 family member, is a major mediator of mitochondrial cell damage in apoptosis [35], which can induce caspase-3-independent pathways of apoptosis [33]. The mechanism of activation of caspase-8 by PEITC remains unexplained and is under investigation.

The antiproliferative and cytotoxic effects of PEITC and PETC-Cys were identical in serum-free medium, but the potency of PETC-Cys decreased more markedly with the increase in serum than did the potency of PEITC. Decrease of the antiproliferative and cytotoxic effects by increasing concentrations of serum may not be due to non-specific reaction with serum proteins, since incubation of PEITC and PETC-Cys in serum-free medium with 5 mg/mL of BSA had no significant effect on the antiproliferative activity (Fig. 2). Rather, it is suggested that the dithiocarbamoyl group of the cysteine conjugate may be hydrolysed by serum esterases, and the potency of cysteine conjugates may, therefore, be influenced by the serum esterase activity.

Dietary isothiocyanates have been of interest recently for their ability to inhibit the phase I metabolic activation of procarcinogens and enhance the phase II conjugation and elimination of carcinogens [36]. They are prospective chemopreventive agents for cancer [37], although some concern as to their ability to promote carcinogenesis has appeared recently [38]. The antiproliferative, antitumour activity of dietary isothiocyanates and their cysteine conjugates is a further development. PEITC and AITC, and particularly their cysteine conjugates, were 10- to 20-fold more toxic to human leukaemia cells than to proliferating lymphocytes. These selective antitumour effects may suppress the growth of preclinical tumours and thereby make additional contributions to the well-established decreased cancer incidence associated with a vegetable-rich diet [39].

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