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Inhibition of Human Leukaemia 60 Cell Growth by Mercapturic Acid Metabolites of Phenylethyl Isothiocyanate

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Abstract—Mercapturic acid pathway metabolites of phenylethyl isothiocyanate inhibited the growth of human leukaemia 60 (HL60) cells *in vitro*. The adduct with L-cysteine, *S*-(*N*-phenylethylthiocarbamoyl)cysteine, was the most potent with strong antileukaemic activity: the median growth inhibitory concentration (GC_{50}) value was 336 ± 1 nM ($N = 18$) compared with GC_{50} values of the precursor formed from dietary glucosinolates, phenylethyl isothiocyanate, 1.49 ± 0.01 μM ($N = 8$), and the initial mercapturic acid pathway metabolite *S*-(*N*-phenylethylthiocarbamoyl)glutathione 5.46 ± 0.36 μM ($N = 18$). *S*-(*N*-Benzylthiocarbamoyl)cysteine and *S*-(*N*-phenylpropylthiocarbamoyl)cysteine also had antiproliferative activity but *S*-(*N*-phenylethylthiocarbamoyl)cysteine was the most potent compound studied. The latter induced DNA fragmentation in HL60 cells but DNA laddering characteristic of apoptosis was not observed. It had low toxicity to corresponding differentiated cells, neutrophils, in culture, and therefore the cytotoxicity had selectivity for leukaemia cells. The antiproliferative activity of *S*-(*N*-phenylethylthiocarbamoyl)cysteine was lost during preincubation with culture medium, attributed to *S*-thiocarbamoyl transfer to serum proteins, which may decrease its effectiveness *in vivo*. The antiproliferative activity of *S*-(*N*-phenylalkylthiocarbamoyl)cysteine derivatives, by inhibiting tumour growth in pre-clinical development, may contribute to the association of decreased cancer incidence with dietary glucosinolate consumption.

INTRODUCTION

Phenylalkyl isothiocyanate glucosinolates are present in cruciferous vegetables (Fenwick *et al.*, 1983; Fenwick and Heaney, 1983), particularly gluconasturtiin, the glucosinolate from which phenylethyl isothiocyanate is formed (Fenwick *et al.*, 1983; VanEtten *et al.*, 1976). Gluconasturtiin is present in Chinese cabbage, turnip and rutabaga (up to 30–50 mg per 100 g fresh weight) (Carlson *et al.*, 1982; Daxenbichler *et al.*, 1979; Mullin *et al.*, 1980). It is hydrolysed to an unstable aglycone by myrosinase (thioglucoside glucohydrolase; EC 3.2.3.1) during chopping and chewing of uncooked vegetables (Fenwick *et al.*, 1983) which degrades to phenylethyl isothiocyanate and other products with an estimated yield of 21% (Chung *et al.*, 1992).

Recent interest in the dietary intake of phenylethyl isothiocyanate has arisen from the ability of phenylalkyl isothiocyanates to prevent the development of tumours in animals exposed to chemical carcinogens. Dietary phenylethyl isothiocyanate was found to prevent the development of tumours in laboratory rodents exposed to chemical carcinogens: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, *N*-nitrosomethylbenzylamine and *N*-nitrosodimethylamine (Chung *et al.*, 1985; Morse *et al.*, 1991 and 1993; Stoner *et al.*, 1991). This is thought to be due to the inhibition of carcinogen-activating isozymes of cytochrome *P*-450 (Chung *et al.*, 1984) and the induction of increased activity of detoxifying glutathione *S*-transferases (Zheng *et al.*, 1992). Little attention has been paid to the anti tumour activity of phenylethyl isothiocyanate and its major metabolites (Hasegawa *et al.*, 1993; Musk and Johnson, 1993).

The major excretory metabolites of phenylethyl isothiocyanate in mice were 4-carboxy-4-hydroxy-3-phenylethylthiazolidine-2-thione and *N*-acetyl-*S*-(*N*-phenylethylthiocarbamoyl)-L-cysteine (Eklind *et al.*, 1990); only the latter was detected in human urine after a meal of watercress, rich in gluconasturtiin (Chung *et al.*, 1992). Both these metabolites originate from the cysteine adduct *S*-(*N*-phenylethylthiocarbamoyl)cysteine (PETC-Cys), formed directly from the reaction of phenylethyl isothiocyanate with cysteine and indirectly from the reaction of phenylethyl isothiocyanate and reduced glutathione with metabolism by γ-glutamyl transferase and dipeptidase

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Abbreviations: GC_{50} = median growth inhibitory concentration; GSH = reduced glutathione; IC_{50} = median inhibitory concentration; NBT = nitroblue tetrazolium; PETC-cys = *S*-*N*-phenylethylthiocarbamoyl)cysteine; TC_{50} = median toxic concentrations.

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Table 1. Median growth inhibitory concentration GC_{50} values and median toxic concentration TC_{50} values of mercapturic acid pathway metabolites of phenylethyl isothiocyanate and analogous compounds

Compound	GC_{50} (μM) mean ± SD	n mean ± SD	(N)	TC_{50} (μM) mean ± SD	n mean ± SD	(N])
Phenylethyl isothiocyanate	1.49 ± 0.01	1.97 ± 0.01	(8)	4.95 ± 0.28	2.86 ± 0.53	(8)
S-(*N*-Phenylethylthiocarbamoyl) glutathione	5.46 ± 0.36	4.46 ± 1.70	(18)	14.80 ± 1.95	1.79 ± 0.28	(18)
S-(*N*-Phenylethylthiocarbamoyl) cysteinylglycine	1.93 ± 0.01	3.87 ± 0.13	(9)	2.83 ± 0.27	8.11 ± 1.92	(9)
S-(*N*-Phenylethylthiocarbamoyl) cysteine	0.336 ± 0.001	3.26 ± 0.01	(18)	0.785 ± 0.002	1.97 ± 0.01	(18)
N-Acetyl-*S*-(*N*-phenylethyl thiocarbamoyl)cysteine	5.68 ± 0.01	4.39 ± 0.01	(8)	9.70 ± 0.06	4.50 ± 0.24	(8)
S-(*N*-Benzylthiocarbamoyl) cysteine	0.74 ± 0.01	4.85 ± 0.36	(9)	2.32 ± 0.08	2.63 ± 0.18	(9)
S-(*N*-Phenylpropylthiocarbamoyl) cysteine	4.04 ± 0.22	4.08 ± 0.89	(18)	7.18 ± 0.33	2.97 ± 0.42	(18)

(Hasegawa *et al.*, 1993). Recent studies on the antileukaemic activity of cysteine derivatives (Edwards *et al.*, 1995) suggested that cysteine adducts of dietary isothiocyanates may also have antileukaemic activity.

In this report, we describe the potent inhibition of growth of human leukaemia 60 (HL60) cells *in vitro* by PETC-Cys.

MATERIALS AND METHODS

Chemicals

Benzyl isothiocyanate, 2-phenylethyl isothiocyanate, 3-phenylpropyl isothiocyanate, L-cysteine, *N*-acetyl-L-cysteine, L-cysteinylglycine, reduced glutathione, Trypan blue, the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate and dimethylsulfoxide were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Tissue culture medium RPMI 1640 and foetal calf serum were purchased from Gibco Europe (Paisley, UK). [^{3}H]-Thymidine (80 mCi/mmol) was purchased from Amersham International (Amersham, Bucks, UK).

Mercapturic acid metabolites of phenylalkyl isothiocyanates

PETC-Cys, *S*-(*N*-phenylethylthiocarbamoyl) cysteinylglycine, *N*-acetyl-*S*-(*N*-phenylethylthiocarbamoyl)cysteine, *S*-(*N*-benzylthiocarbamoyl)cysteine and *S*-(*N*-phenylpropylthiocarbamoyl)cysteine were prepared by reaction of the appropriate phenylalkyl isothiocyanate and cysteine, cysteinyl glycine or reduced glutathione (GSH) in ethanolic sodium phosphate buffer at room temperature (20°C) (Brusewitz *et al.*, 1977). The ^{1}H and ^{13}C NMR spectra, FAB mass spectra and TLC analysis data of the compounds (Adesida, 1995) were consistent with the assigned structures.

Cell culture conditions

HL60 cells were incubated at 37°C in RPMI 1640 media containing 10% foetal calf serum under an atmosphere of 5% CO_2 in air, 100% humidity (Edwards *et al.*, 1995). HL60 cells were seeded at an initial density of 5×10^4/ml and incubated with 0.1–100 μM of the test compound. Stock solutions of the test compounds (100 mM) were prepared in dimethyl sulfoxide and diluted into the growth medium such that the final concentration of dimethyl sulfoxide did not exceed 0.1% (v/v), a concentration which does not induce differentiation or toxicity in HL60 cells (Collins *et al.*, 1978). The effect of the compounds on cell growth was determined by addition of the compounds to HL60 cell cultures and counting of viable cell number, assessment of cell viability and cell maturation status after incubation for 48 hr. The concentration of the compound that decreased the viable cell number by 50% of the control value, the median growth inhibitory concentration (GC_{50}) value, was determined by logistic regression. Cell viability was judged by the ability of

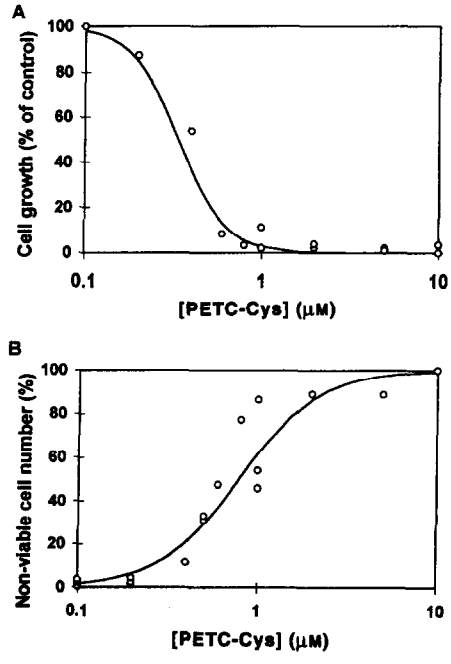

Fig. 1. Inhibition of HL60 cell growth by *S*-(*N*-phenylethylthiocarbamoyl)cysteine (PETC-Cys). (A) PETC-Cys concentration–cell growth curve, $GC_{50} = 0.336 \pm 0.001$ μM, n = 3.26 ± 0.01 (N = 18). (B) PETC-Cys concentration–cytotoxicity curve, $TC_{50} = 0.785 \pm 0.002$ μM, n = 1.97 ± 0.01 (N = 18).

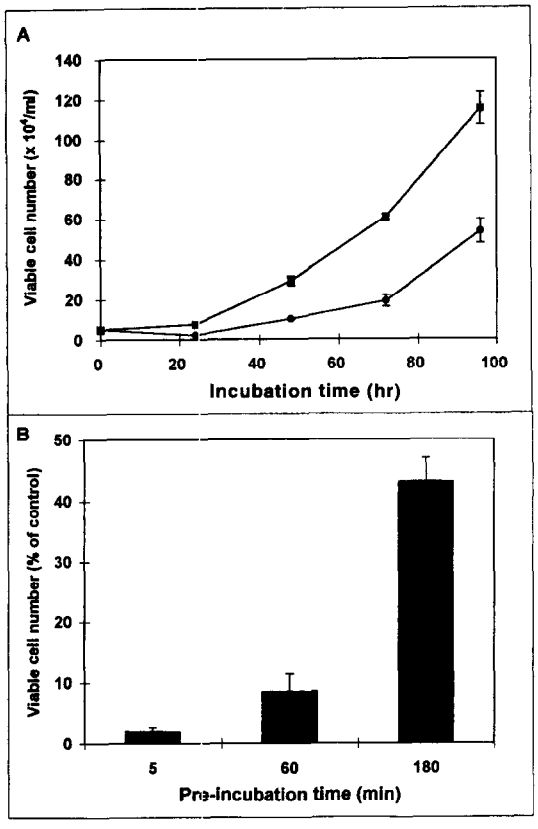

Fig. 2. (A) Effect of *S*-(*N*-phenylpropylthiocarbamoyl)cysteine on HL60 cell growth. HL60 cells (5×10^4/ml) were incubated in RPMI 1640 with 10% foetal calf serum: ■, control; ●, + 4 μM *S*-(*N*-phenylpropylthiocarbamoyl)cysteine. Data are mean ± SD of three determinations. (B) Loss of growth inhibitory activity of PETC-Cys by pre-incubation with the culture medium. Data are mean ± SD of three determinations.

cells to exclude Trypan blue (Kaltenbach *et al.*, 1958). The concentration of the compound which decreased the cell viability by 50%, the median toxic concentration (TC_{50}) value, was determined by logistic regression. Cell differentiation was estimated from the development of maturation-dependent activity of the superoxide-forming enzyme NADPH oxidase, by staining for the superoxide-mediated reduction of nitroblue tetrazolium (NBT) to formazan, in the presence of phorbol ester (Mendelson *et al.*, 1980). The effect of the compounds on the rate of DNA synthesis in HL60 cells was estimated by measuring the rate of incorporation of [^{3}H]thymidine into DNA. HL60 cells (5.0×10^4/ml) were incubated with 0–100 μM PETC-Cys in RPMI 1640 with 10% foetal calf serum for 2 hr. [^{3}H]Thymidine (2.5 μCi, 2.5 μl) was added and the incubation continued for 1 hr. The DNA was then extracted and counted (Wilson, 1986). The concentration of the compound that decreased the rate of DNA synthesis by 50% of the control value, the median inhibitory concentration (IC_{50}) value, was determined by logistic regression.

Isolation and culture of human neutrophils

Mature human neutrophils were isolated from venous blood and separated from other leukocytes using Ficoll–Hypaque (Boyum, 1976). The viability of the isolated neutrophils was greater than 99%. Neutrophils (5×10^4/ml) were incubated with and without PETC-Cys for 24 hr and cell viability assessed by the Trypan blue exclusion technique.

Morphological examination and fragmentation of DNA

HL60 cells (5×10^4 cells/ml; 1×10^6 cells) were incubated for 0–10 hr in the absence and presence of PETC-Cys (1 μM). The cells were washed with phosphate buffered saline (0.9% sodium chloride, 10 mM potassium phosphate buffer, pH 7.4), and the cell morphology and DNA fragmentation analysed as described (Edwards *et al.*, 1995).

Data analysis

Data of viable cell number *V* (expressed as a percentage of control), and corresponding phenylalkyl

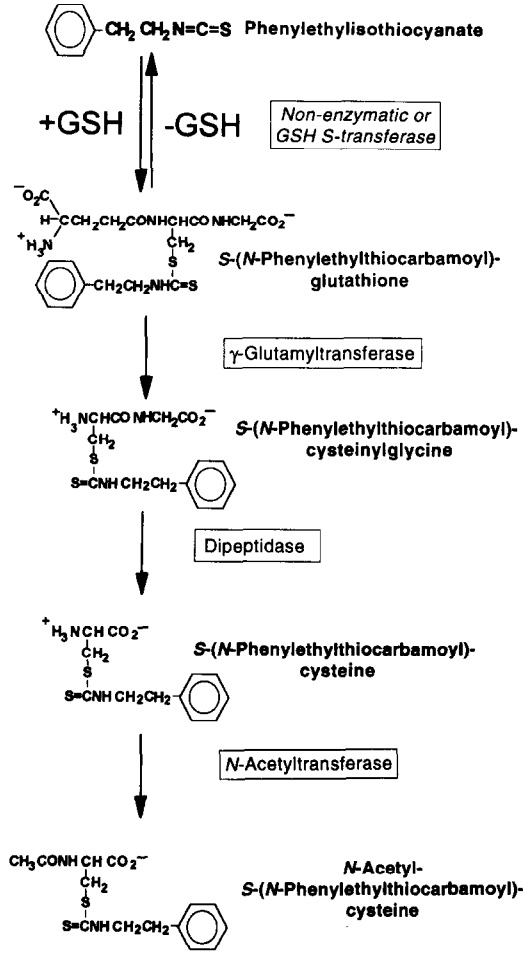

Fig. 3. Metabolism of phenylethyl isothiocyanate by the mercapturic acid pathway.

isothiocyanate derivative concentration [D] 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. Data from the study of the effect of PETC-Cys on cell viability and DNA synthesis were analysed similarly to determine the TC_{50} and IC_{50} values, respectively. Data were fitted by non-linear regression using ENZFITTER programme (Biosoft, Cambridge, UK).

RESULTS

Effect of mercapturic acid pathway metabolites of phenylethyl isothiocyanate on the growth of human leukaemia 60 cells

When phenylethyl isothiocyanate and its mercapturic acid pathway metabolites were incubated with HL60 cells (5×10^4 cells/ml) for 48 hr there was a concentration-dependent decrease in viable cell number. All these compounds were inhibitors of HL60 cell growth (Table 1) but the most potent compound was the cysteine adduct, PETC-Cys, for which the GC_{50} value was 336 nM (Fig. 1A). The TC_{50} value of PETC-Cys was 785 nM (Fig. 1B). PETC-Cys also inhibited DNA synthesis in HL60 cells, studied in the third hour of culture during which there was no decrease in cell viability and no significant change in labelling of the cellular thymidine pool. The IC_{50} value of PETC-Cys for DNA synthesis was $6.47 \pm 0.14\ \mu$M, $n = 2.26 \pm 0.11$ ($N = 6$). PETC-Cys was the most potent inhibitor of HL60 cell growth studied (Table 1).

There was decreased toxicity of PETC-Cys to human neutrophils under the same culture conditions. The TC_{50} value of PETC-Cys was $20.4 \pm 1.3\ \mu$M, $n = 1.36 \pm 0.13$ ($N = 8$).

A study of the time course of the effect of *S*-(*N*-phenylalkylthiocarbamoyl)cysteine derivatives on HL60 cell growth showed that HL60 viable cell number was decreased in the initial 48 hr of culture but thereafter residual surviving cells resumed normal growth kinetics—the time course of the effect of *S*-(*N*-phenylpropylthiocarbamoyl)cysteine on HL60 cell growth is shown in Fig. 2A. Studies of the fragmentation of the adducts of benzyl isothiocyanate with cysteine and GSH (Bruggeman *et al.*, 1986) suggested that PETC-Cys might fragment and lose its potent antiproliferative activity in the incubation medium. When PETC-Cys, final concentration 10 μM, was preincubated in RPMI 1640 with 10% foetal calf serum for 5 min before addition to HL60 cells, the growth of the HL60 cells over the subsequent 48 hr was inhibited by more than 97%, relative to control values. Similar preincubation of PETC-Cys in RPMI 1640 with 10% foetal calf serum for 1 hr and 3 hr gave a progressive decrease in the inhibition of HL60 cell growth with increasing preincubation time (Fig. 2B).

DNA fragmentation in HL60 cells induced by S-(N-phenylethylthiocarbamoyl)cysteine

When HL60 cells were incubated for 6 hr with PETC-Cys, there was condensation and fragmentation of nuclear DNA within cells with an intact plasma membrane. Some cells had plasma membrane with extensive budding, with buds containing fragments of DNA in the process of developing apoptotic bodies (Plate 1A). The cellular DNA was also analysed by agarose gel electrophoresis (Plate 1B). When HL60 cells were incubated for 6 hr with 1 μM PETC-Cys, the cellular DNA was cleaved to small fragments: the most intense fragment band was *c.* 23,000 base pairs, tailing to fragments *c.* 4000 base pairs. After incubation of HL60 cells with 1 μM PETC-Cys for 10 hr, the DNA fragments had decreased in size to an intense band of *c.* 10,000 base pairs, tailing to fragments of less than 2000 base pairs.

DISCUSSION

Phenylethyl isothiocyanate is a dietary isothiocyanate. It reacts with GSH non-enzymatically and enzymatically, catalysed by glutathione *S*-transferase, to form *S*-(*N*-phenylethylthiocarbamoyl)glutathione in the cytosol of cells. This is exported from cells across the plasma membrane by glutathione *S*-conjugate transporters where it is converted successively into *S*-(*N*-phenylethylthiocarbamoyl)cysteinylglycine by γ-glutamyl transferase, and to PETC-Cys by dipeptidase. In the kidney, PETC-Cys is converted into the *N*-acetyl-*S*-(*N*-phenylethylthiocarbamoyl)cysteine by *N*-acetyl transferases, and is excreted in the urine (Eklind *et al.*, 1990) (Fig. 3). Pharmacological activities of mercapturic acid metabolites of phenylethyl isothiocyanate and analogous isothiocyanates are therefore of nutritional and prospective clinical interest.

Phenylalkyl isothiocyanates have long been known to exhibit antiproliferative activities against fungi and bacteria—particularly gram-positive bacteria (Dornberger *et al.*, 1975; Virtanen *et al.*, 1963; Zsolnai, 1966), and more recently antitumour and anticarcinogenic activities. Benzyl isothiocyanate and phenylethyl isothiocyanate inhibited the growth of HeLa cells *in vitro* where the IC_{50} values were *c.* 3 μM. There was an accumulation of cells in the G_2/M phase of the cell cycle associated with growth inhibition (Hasegawa *et al.*, 1993). Phenylalkyl isothiocyanates inhibited the growth of SV_{40}-transformed Indian muntjac cell line *in vitro*. The GC_{50} value of phenylethyl isothiocyanate was *c.* 2.5 μM but benzyl isothiocyanate was more potent with a GC_{50} value of *c.* 1.0 μM (Musk and Johnson, 1993); in comparison, the GC_{50} value of phenyl isothiocyanate with HL60 cells was 1.49 μM. Phenylalkyl isothiocyanates were also clastogenic, inducing chromatid abberations in SV_{40}-transformed Indian muntjac cells (Musk and Johnson, 1993), and mutagenic in *Salmonella*

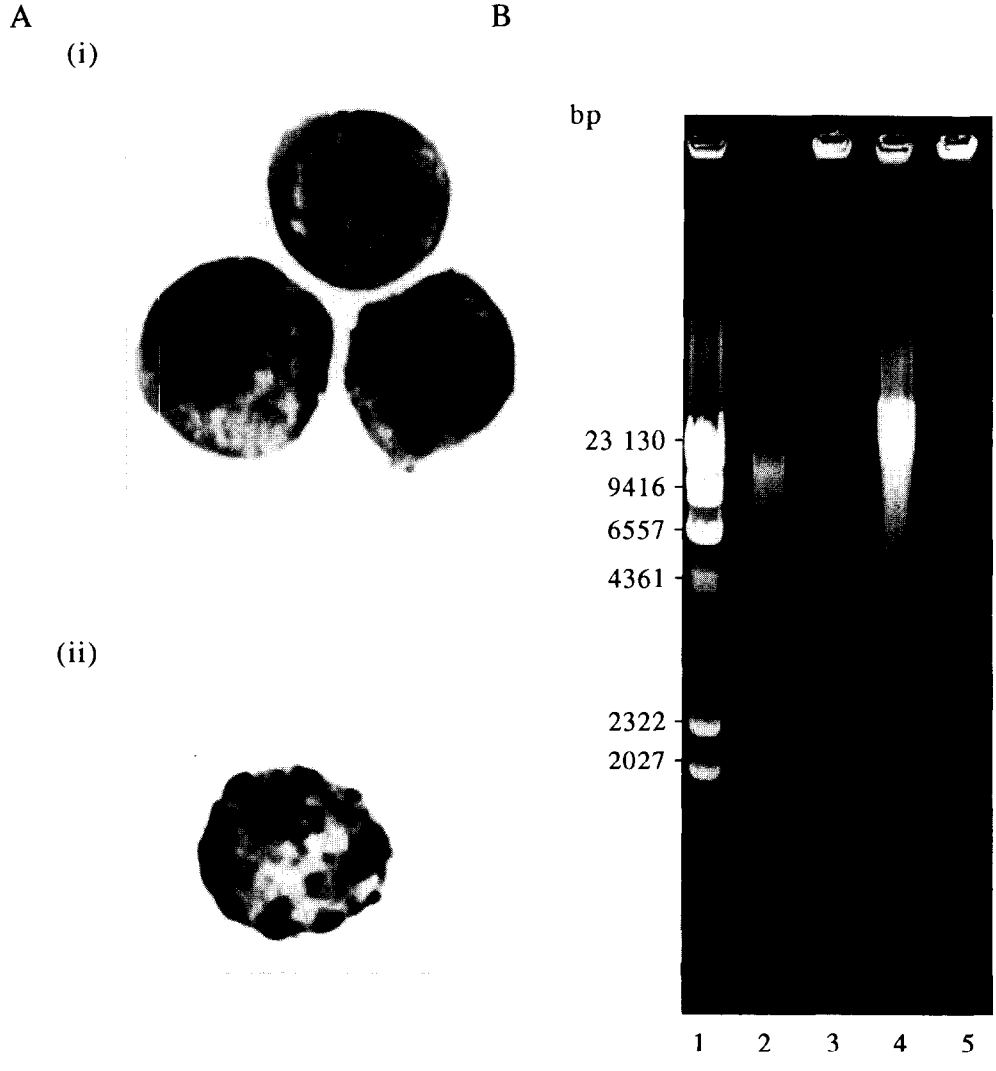

Plate 1. (A) Morphological examination of HL60 cells incubated for 6 hr in the absence (i) and presence (ii) of 1 μM PETC-Cys. (B) Agarose gel electrophoresis of DNA of HL60 cells: lane 1, standards (bands 23, 130, 9416, 6557, 4361, 2322 and 2027 bp); lane 2, + 1 μM PETC-Cys (10 hr); lane 3, control (10 hr); lane 4, + 1 μM PETC-Cys (6 hr); lane 5, control (6 hr).

typhimurium (Yamaguchi, 1980). Clastogenic effects and the inhibition of the metabolism of carcinogens (associated with antitumorigenesis effects) of phenylethyl isothiocyanate *in vitro* occurred at *c.* 20–100 times the antiproliferative GC_{50} value of the cysteine conjugate determined herein (Doerr-O'Rourke *et al.*, 1991; Murphy *et al.*, 1991; Musk and Johnson, 1993; Stoner *et al.*, 1991).

Cysteine adducts of phenylalkyl isothiocyanates were prepared previously and their anticarcinogenic activity evaluated. They had decreased toxicity to mice compared with the parent phenylalkyl isothiocyanate, and were more potent inducers of glutathione *S*-transferase—which was implicated in their anticarcinogenic activity (Zheng *et al.*, 1992). Little is known of the concentration and stability of PETC-Cys in the systemic circulation arising from dietary intake of phenylethyl isothiocyanate. The cysteine and GSH adducts of benzyl isothiocyanate were found to liberate the parent isothiocyanate in aqueous solution at pH 7.4, and the *N*-benzylthiocarbamoyl moiety could be exchanged from the thiol groups of cysteine to GSH and vice versa (Bruggeman *et al.*, 1986). PETC-Cys had the most potent antiproliferative activity. The inhibition of HL60 cell growth by *S*-(*N*-phenylethylthiocarbamoyl) glutathione has been previously reported (Barnard *et al.*, 1993). *S*-(*N*-phenylethylthiocarbamoyl) glutathione is expected to form PETC-Cys by interaction with γ-glutamyl transpeptidase and dipeptidase on the extracellular surface of the HL60 cell plasma membrane. Some *S*-(*N*-phenylethylthiocarbamoyl)glutathione may, however, interact with the low activity of glutathione *S*-transferase from foetal calf serum in the culture medium which catalyses the fragmentation of *S*-(*N*-phenylethylthiocarbamoyl) glutathione to GSH and phenylethyl isothiocyanate (Meyer *et al.*, 1995), leading to modification of serum proteins by phenylethyl isothiocyanate. Fragmentation of *S*-(*N*-phenylethylthiocarbamoyl)glutathione in the extracellular medium, rather than metabolism to PETC-Cys may decrease the antiproliferative activity.

PETC-Cys has reported low toxicity *in vivo* (Zheng *et al.*, 1992). Its antiproliferative activity may be mediated by *N*-phenylethylthiocarbamoylation of critical nucleophilic groups in the active sites of enzymes and functionally critical groups of other proteins associated with nucleic acid and DNA synthesis inside cells. PETC-Cys may be an effective vehicle for the delivery of the *N*-phenylethylthiocarbamoyl moiety to these groups. Structurally related *S*-(*N*-alkylcarbamoyl)cysteine derivatives which had antiproliferative, antitumour activity however, were glutamine antagonists, inhibiting glutamine-utilizing enzymes of purine and pyrimidine synthesis (Jayaram *et al.*, 1990). Similar effects of PETC-Cys may contribute to the antileukaemic activity.

The inhibition of HL60 cell growth by PETC-Cys was associated with the inhibition of DNA synthesis but the GC_{50} value was markedly lower than the IC_{50} value for DNA synthesis. This indicates that mechanisms other than inhibition of DNA synthesis are involved in the inhibition of HL60 cell growth by PETC-Cys, or that DNA synthesis is inhibited later than the third hour of exposure to PETC-Cys. Cellular DNA was fragmented, and the mean fragment size decreased with increasing incubation time in HL60 cells incubated with PETC-Cys. There was, however, no DNA ladder effect indicative of internucleosomal fragmentation of DNA found in apoptosis. Rather, DNA fragmentation appears non-specific and may be due to modification of DNA and/or histone protein by PETC-Cys activating irregular fragmentation and cell death. An alternative mechanism of action is the inhibition of protein kinases involved in signal transduction for cell growth, which was found in the antiproliferative effects of cysteine derivatives on activated T lymphocytes (Jeitner *et al.*, 1994).

When PETC-Cys was incubated in the culture medium prior to exposure to cells, the antiproliferative activity was lost. This may be attributed in part to the *N*-thiocarbamoylation of serum proteins and, therefore, diminished delivery of the *N*-phenylethylthiocarbamoyl moiety into cells. The extracellular instability of PETC-Cys and similar compounds is an important factor to be considered in the development of these agents for antitumour therapy *in vivo*. Moreover, the contribution of the antiproliferative activity of this metabolite to the dietary effects on cancer incidence related to isothiocyanate consumption from vegetables is not known and is of future interest.

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