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# Involvement of glutathione metabolism in the cytotoxicity of the phenethyl isothiocyanate and its cysteine conjugate to human leukaemia cells *in vitro*

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**Abstract**

The dietary isothiocyanate and cancer chemopreventive agent, phenethyl isothiocyanate, induced apoptosis of human leukaemia HL60 and human myeloblastic leukaemia ML-1 cells *in vitro*. Cytotoxicity was associated with an initial decrease in GSH and GSSG, with a concomitant formation of the GSH adduct *S*-(*N*-phenethylthiocarbamoyl)glutathione inside cells, which was then exported from cells. After 12 hr, the cellular concentration of GSH recovered and then declined after 24 hr. Buthionine sulphoximine prevented the recovery of cellular GSH concentration and potentiated the cytotoxicity of phenethyl isothiocyanate. *S*-(*N*-phenethylthiocarbamoyl)glutathione spontaneously fragmented to GSH and phenethyl isothiocyanate, GSH oxidized to GSSG and glutathionyl–protein disulphides, and phenethyl isothiocyanate hydrolyzed to phenylethylamine. GSH and GSSG depletion was more marked in ML-1 cells than in HL60 cells. Studies with [$^{14}$C]-labelled phenethyl isothiocyanate gave evidence of phenethylthiocarbamoylation of cells that maximized after 2–3 hr. This occurred later than the maximum concentration of *S*-(*N*-phenethylthiocarbamoyl)glutathione, but coincided with the commitment to apoptosis and cytotoxicity which developed later. The cytotoxicity of phenethyl isothiocyanate was prevented by a high concentration of GSH (15 mM) and delayed by the antioxidant and c-Jun N-terminal kinase signalling pathway inhibitor curcumin. GSH prevented and curcumin partly prevented the decrease in cellular GSH. These studies show that the cysteinyl thiol group of GSH is an important site of thiocarbamoylation by phenethyl isothiocyanate during induction of apoptosis and that this may lead to depletion of cellular GSH by efflux of the GSH conjugate. Thiocarbamoylation also occurred at other sites. The recent demonstration of a critical role for activation of caspase-8 in phenethyl isothiocyanate-induced apoptosis suggests that this thiocarbamoylation directly or indirectly leads to functional activation of a cell death receptor/adaptor protein complex. 

*Keywords:* GSH; Oxidative stress; Phenethyl isothiocyanate; HL60; ML-1; Apoptosis

## 1. Introduction

Dietary isothiocyanates have recently been of intense interest for their anticarcinogenic activities and potential use in the chemoprevention of cancer [1]. One such compound is PEITC. Chemopreventive activity is thought to be associated with inhibition of the metabolic activation of carcinogens by cytochrome P450 isozymes [2] and increased excretion of carcinogens by inducing increased activities of quinone reductase (EC 1.6.99.2) and GSH *S*-transferases (EC 2.5.1.18) involved in elimination of carcinogens by the mercapturic acid pathway [3,4]. The cysteine adduct of PEITC, PETC-Cys, had similar chemopreventive activity [4,5]. A further feature of the pharmacological activity of PEITC and other dietary isothiocyanates was their anticancer activity *in vitro* associated with induction of apoptosis in tumour cells. 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. PEITC and PETC-Cys inhibited the growth and induced apoptosis of human leukaemia HL60

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*Abbreviations:* BSO, L-buthionine sulphoximine; DNP-SG, *S*-2,4-dinitrophenylglutathione; DTT, dithiothreitol; GST, glutathione *S*-transferase; HL60, human leukaemia 60; JNK1, c-Jun N-terminal kinase 1; ML-1, human myeloblastic leukaemia-1; PEITC, phenethyl isothiocyanate; PETC, *N*-phenethylthiocarbamoyl; PETC-Cys, *S*-(*N*-phenethylthiocarbamoyl)cysteine; PETC-SG, *S*-(*N*-phenethylthiocarbamoyl)glutathione; and Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone.

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cells (p53−) and ML-1 cells (p53+) *in vitro* [6,7]. Growth inhibition and toxicity was characterized by a rapid interaction of the PEITC with the cells in the first hour of culture or exposure to PEITC liberated from the spontaneous fragmentation of PETC-Cys in the initial 3 hr of culture. Activation of caspase-8 occurred in the initial 3 hr and was critical for induction of apoptosis [7].

The mechanism of induction of apoptosis by dietary isothiocyanates is unknown, but has been linked to induction of oxidative stress, activation of mitogen-activated protein kinases and, more recently, to the activation of caspase-8 [7]. PEITC was found to induce a sustained activation of JNK1 in serum-deprived HeLa cells [8]. This was associated with activation of protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1) [9]. 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 [9], probably to protect mitochondrial function. Activation of caspase-8 was critical for PEITC-induced apoptosis [7]. The mechanism of activation of caspase-8 by PEITC remains unexplained and is under investigation. High concentrations of thiol-containing antioxidants, 2-mercaptoethanol (10 mM) and *N*-acetylcysteine (20 mM), inhibited PEITC-induced apoptosis. This was interpreted as evidence for the involvement of oxidative stress in PEITC-induced apoptosis by the modification of cellular GSH by PEITC [9]. Thiocarbamoylation of particular cellular thiols by PEITC or PEITC derived from PETC-Cys may be an important initiator of apoptosis.

In this report, we describe the effects of PEITC and PETC-Cys on GSH metabolism in human leukaemia HL60 and ML-1 cells during induction of apoptosis. We conclude that dietary isothiocyanates may deplete cells of GSH by formation and expulsion of their GSH conjugates, but binding of isothiocyanate to other sites, probably protein thiols, may initiate signalling for apoptosis.

## 2. Materials and methods

### 2.1. Materials

PEITC was purchased from Aldrich Chem. Co. Ltd. L-Cysteine, *N*-acetyl-L-cysteine, L-lysine, *N*-acetyl-L-lysine, trypan blue, DTT, GSH, GSSG, BSO, sulphosalicylic acid, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), D-penicillamine, *S*-(*p*-chlorophenacyl)glutathione, and dimethylsulphoxide were purchased from Sigma Chemical Co. Ltd. Tissue culture medium RPMI-1640 and foetal bovine serum were purchased from GIBCO Europe Ltd. The inhibitor of caspases Z-VAD-fmk was purchased from Calbiochem. PETC-Cys and PETC-SG were prepared by reaction of the PEITC with L-cysteine and GSH, respectively, in ethanolic sodium phosphate buffer, pH 6.6, at room temperature (20°), isolated and characterized by $^1$H NMR and FAB mass spectrometry as described [10]. [$^{14}$C]PEITC and [$^{14}$C]PETC-Cys were prepared and purified as described [11].

### 2.2. 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° [6,12]. For the study of the cellular concentration of total GSH, GSH and GSSG, cells ($9.1 \times 10^4$ mL) were incubated with and without 5 $\mu$M PEITC for the times indicated. To study the effect of GSH synthesis on the increase of GSH concentration in cells after 12 hr, HL60 cells ($9.1 \times 10^4$/mL) were also incubated with the $\gamma$-glutamylcysteine synthetase inhibitor BSO (200 $\mu$M) [13]. For the study of extracellular GSH, GSSG, PETC-SG, and glutathione–protein mixed disulphides, HL60 cells ($1 \times 10^6$/mL) were preincubated for 1 hr with the $\gamma$-glutamyltransferase inhibitor acivicin (200 $\mu$M) [14], washed with fresh medium, and then incubated with and without 50 $\mu$M PEITC. The inhibition of $\gamma$-glutamyltransferase activity of HL60 cells was studied, assaying enzymatic activity as previously described [15]. A stock solution of PEITC was prepared in dimethylsulphoxide and freshly 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.

For the study of PEITC binding to cells, HL60 cells ($5 \times 10^4$/mL) were incubated with 5 $\mu$M [$^{14}$C]PEITC or 5 $\mu$M [$^{14}$C]PETC-Cys for 0.5–24 hr. The cells were sedimented by centrifugation (215 *g*, 6 min), washed 3 times with PBS, scintillation cocktail added and counted. Subcellular localization of the [$^{14}$C]PEITC-derived moiety at the time point of maximum cell labelling was investigated: cytosolic and membrane fractions were prepared by sonication of $1 \times 10^6$ cells (20 sec, 100 W) in PBS with 0.1% Triton X-100 and sedimentation of membranes by centrifugation (50,000 *g*, 1 hr, 4°). The presence of [$^{14}$C]PEITC-derived moieties in cellular DNA, RNA, and protein was also investigated by preparation of macromolecular extracts as described [16, 17].

### 2.3. Assay of the concentration of total GSH, GSSG, and GSH

HL60 cell or ML-1 cell suspensions ($9.1 \times 10^4$ cell/mL, 4 mL) were incubated with PEITC and PETC-Cys for the times indicated. Cells were sedimented by centrifugation (215 $g \times 6$ min), washed twice in PBS, and re-suspended in 100 $\mu$L of 1% sulphosalicylic acid. The samples were vortex-mixed and left on ice for 15 min, then centrifuged (5000 *g*, 3 min). The supernatant was analyzed for total GSH (GSH + 2GSSG) and GSSG. For total GSH, 20 $\mu$L of

cell extract was added to sodium phosphate buffer solution (100 mM, pH 7.5, containing 1 mM EDTA, 180 μL). An aliquot of this (50 μL) was added into the well of a 96-well microplate. The assay was initiated by addition of 100 μL of GSH reductase solution. This was prepared as follows: sodium phosphate buffer (100 mM, pH 7.5, containing 1 mM EDTA, 0.48 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.97 mL), NADPH (1 mM, 0.63 mL), and GSH reductase (3 units, 3 μL), made up to 2 mL with water. The rate of formation of 2-nitro-5-mercaptobenzoic acid (NMB) was followed at 405 nm over the initial 3 min of reaction time and the rate of increase in absorbance ($dA/dt_o$) determined. The assay was calibrated by reference to a standard curve run simultaneously (range 0–300 pmol/well).

For the assay of GSSG, an aliquot (60 μL) of the cell supernatant fraction was mixed with 30 μL of 4% (v/v) 2-vinylpyridine and 15 μL of 4% (w/v) triethanolamine. An aliquot of this solution (100 μL) was then added to the well of a 96-well microplate. The assay was initiated by addition of 100 μL of GSH reductase solution as described above. The assay was calibrated by reference to a standard curve run simultaneously (range 0–150 pmol GSSG/well). The concentration of GSH was deduced: [GSH] = [Total GSH] − 2[GSSG]. The concentration of GSH metabolites is given in nmol/$10^6$ viable cells where cell viability was assessed by trypan blue exclusion. It is assumed that cells that leak trypan blue through the plasma membrane have also leaked out GSH and GSSG. The method was also used to determine extracellular total GSH, but most of the extracellular GSH was present as glutathione–protein mixed disulphides that were quantified rather by the monobromobimane method described below.

### 2.4. Assay of extracellular GSH and combined total GSH and glutathione–protein mixed disulphides

The concentrations of GSH and combined total GSH and glutathione–protein mixed disulphides were determined by derivatization with monobromobimane without and with prior reduction of disulphides with DTT. HL60 cells ($1 \times 10^6$/mL, 4 mL) were collected by centrifugation, the supernatant removed, and 2 aliquots of 50 μL taken. The first aliquot was derivatized with monobromobimane directly. The second was reduced with DTT (100 mM, 5 μL) at room temperature for 1 hr prior to derivatization. Both aliquots were then treated with sulphosalicylic acid (5%, 20 μL), vortex-mixed, and kept for 10 min on ice. They were then centrifuged (1000 *g*, 5 min), 50 μL then removed and added to the derivatization solution: monobromobimane (100 mM in acetonitrile, 5 μL), *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulphonic acid (EPPS) buffer (1 M, pH 8.0; 80 μL), and internal standard D-penicillamine (0.1 mM, 5 μL). Derivatization was for 10 min at room temperature in the dark. Glacial acetic acid (10 μL) was added to stop the reaction. The sample was filtered (0.2 μm) and analyzed by HPLC. The mobile phase was: solvent A, 44 mM acetic acid in 15% methanol and solvent B, 44 mM acetic acid in 90% methanol. The elution profile was: 0–5 min, isocratic 100% solvent A; 5–25 min, a linear gradient of 0–25% solvent B. The flow rate was 2 mL/min. The eluate was monitored for fluorescence: excitation wavelength 385 nm, emission wavelength 478 nm. The injection volume was 50 μL. The assay was calibrated with authentic GSH.

### 2.5. Assay of S-(N-*phenethylthiocarbamoyl)glutathione*

The concentration of PETC-SG was assayed in HL60 cells and extracellular medium. For assay of the cellular concentration, HL60 cells ($4 \times 10^6$) incubated with PEITC were washed with PBS and the cell pellet was deproteinized by addition of perchloric acid (0.6 M, 200 μL). The internal standard *S*-(*p*-chlorophenacyl)glutathione was added (0.5 mM, 3.2 μL) and the precipitate was sedimented by centrifugation (3000 *g*, 3 min), with the supernatant removed and retained. This was neutralized by addition of sodium carbonate (2 M, 30 μL) to pH 7.4, filtered (0.2 μm), and analyzed immediately by HPLC. For assay of the extracellular concentration, extracellular medium from the culture (200 μL) was deproteinized by addition of perchloric acid (1.2 M, 200 μL), internal standard added (0.5 mM, 6.4 μL), the precipitate sedimented by centrifugation (3000 *g*, 3 min), and the supernatant removed and retained. This was neutralized by addition of sodium carbonate (2 M, 30 μL) to pH 7.4, filtered (0.2 μm), and analyzed immediately by HPLC. The mobile phase was: solvent A, 20 mM glacial acetic acid in $H_2O$, pH 4.8; solvent B, 20 mM glacial acetic acid in 80% acetonitrile, pH 4.8. The elution profile was: 0–30 min, a linear gradient of 0–100% B with a flow rate of 2 mL/min. The eluate was monitored by absorbance at 250 nm. The injection volume was 150 μL. The assay was calibrated with authentic PETC-SG.

### 2.6. Instrumentation and data analysis

HPLC was performed with a Waters HPLC system (600 quaternary pump/gradient controller, Lambda Max 481 LC spectrophotometer, 474 scanning fluorescence detector and 717 autosampler). Significance of changes in measured variables was assessed by Student's *t*-test.

## 3. Results

### 3.1. Effect of PEITC on the viability and GSH metabolism of HL60 cells in vitro

When HL60 cells ($9.1 \times 10^4$/mL) were incubated for 24 hr in RPMI-1640 with 10% foetal bovine serum, the viable cell number increased to $21.4 \pm 2.3 \times 10^4$/mL ($N = 3$, $P < 0.001$) (Fig. 1). Addition of 5 μM PEITC inhibited cell growth, with the viable cell number decreasing from control values in the 9–24 hr period to a final value of $6.6 \pm 1.0 \times$

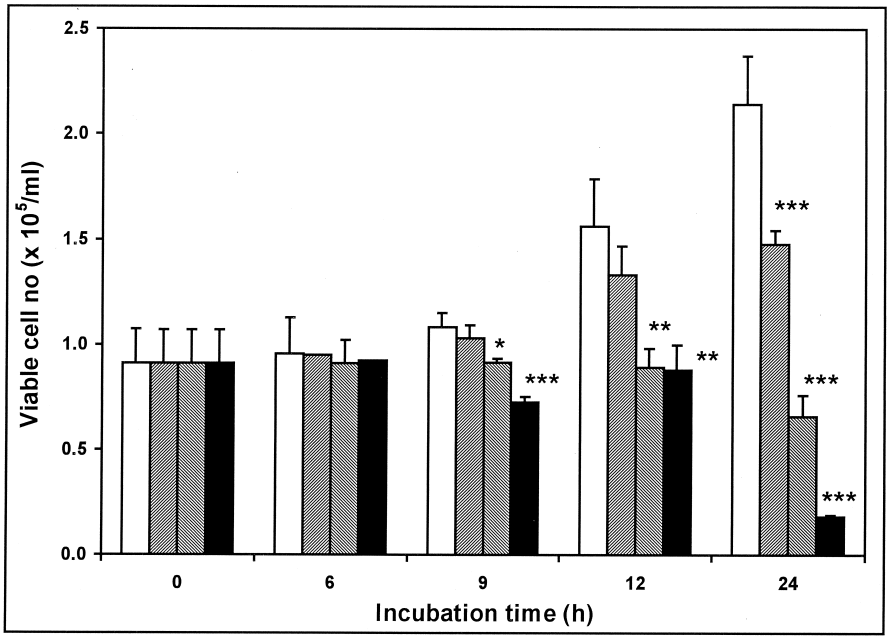


Fig. 1. Effect of PEITC on the growth of HL60 cells *in vitro* in the absence and presence of BSO. Key: □ control; ▨ + 500 $\mu$M BSO; ▧ + 5 $\mu$M PEITC; ■ + 500 $\mu$M BSO and 5 $\mu$M PEITC. Data are means ± SD of 3 determinations. HL60 cells ($9.1 \times 10^4$/mL) were incubated in RPMI-1640 with 10% foetal bovine serum for 0–24 hr and viable cell number determined. Significance with respect to control: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

$10^4$/mL (N = 3, $P < 0.001$) after 24 hr. Concomitant incubation of HL60 cells with 200 $\mu$M BSO in the absence and presence of 5 $\mu$M PEITC decreased the viable cell number to $14.8 \pm 0.6 \times 10^4$/mL and $1.8 \pm 0.1 \times 10^4$/mL, respectively (N = 3, $P < 0.001$). Therefore, PEITC decreased the growth and viability of HL60 cells, and this effect was potentiated by inhibition of GSH synthesis with BSO. There was no significant decrease in cell viability in the initial 6 hr of culture as judged by trypan blue exclusion, although commitment to apoptosis occurred within the initial 1–2 hr [7].

The changes in total GSH, GSH, and GSSG of HL60 cells during these incubations were investigated (Fig. 2a–c). The concentration of total GSH in HL60 cells at time zero was $2.78 \pm 0.18$ nmol/$10^6$ cells, the concentration of GSSG was $0.42 \pm 0.04$ nmol/$10^6$ cells, and the concentration of GSH $1.94 \pm 0.10$ nmol/$10^6$ cells. These values did not change significantly during control incubations for 24 hr. When HL60 cells were incubated with 5 $\mu$M PEITC, there was a decrease in total GSH concentration in the initial 3 hr of incubation. The concentration of total GSH then remained at *ca*. 1 nmol/$10^6$ cells (36% of control values) for 3–9 hr, but by 12 hr had recovered to the control value. Thereafter, a further marked decrease occurred as cell viability also declined severely (Fig. 1). This change in total GSH concentration reflected changes mainly in GSH concentration. The concentration of GSSG also declined markedly in the initial 3 hr of culture, but increased in the 3- to 6-hr period, only to decline again by 9 hr and remain at very low levels thereafter.

Addition of the $\gamma$-glutamylcysteine synthetase inhibitor BSO to HL60 cells decreased the concentration of total GSH, GSSG, and GSH over the initial 9–12 hr of culture; the concentrations of these GSH metabolites were decreased by 50% after 3 hr. Addition of BSO and PEITC to cells produced a more rapid decline in total GSH and GSH than with either of these agents alone, and unlike the PEITC-only incubation, the concentration of GSH did not recover after 12 hr.

Similar effects on GSH metabolism were found in HL60 cells incubated with PETC-Cys and with other human leukaemia cells. When HL60 cells ($9.1 \times 10^4$/mL) were incubated with 5 $\mu$M PETC-Cys, there was a marked decrease in the cellular concentrations of total GSH, GSH, and GSSG in the initial 2–3 hr of culture. The cellular concentration of GSH returned to the control level at 12 hr, but by 24 hr had severely declined again. This was a similar period of recovery of cellular GSH concentration as found with PEITC in the absence of BSO (Fig. 3a, c, and e). ML-1 cells ($9 \times 10^4$/mL) were incubated with 5 $\mu$M PEITC and showed severe decreases in the cellular concentration of total GSH, GSH, and GSSG in the initial 6 hr which were persistent throughout the subsequent 18 hr. No period of recovery of cellular GSH concentration was found in these cells (Fig. 3b, d, and f).

The fate of GSH in HL60 cells was then investigated. A 10-fold higher cell density and PEITC concentration was used to enable direct detection of the GSH conjugate, PETC-SG. GSH metabolites were probably being expelled from the HL60 cells and therefore to preserve them from extracellular degradation by $\gamma$-glutamyltransferase, HL60 cells were preincubated with 200 $\mu$M acivicin for 1 hr. This

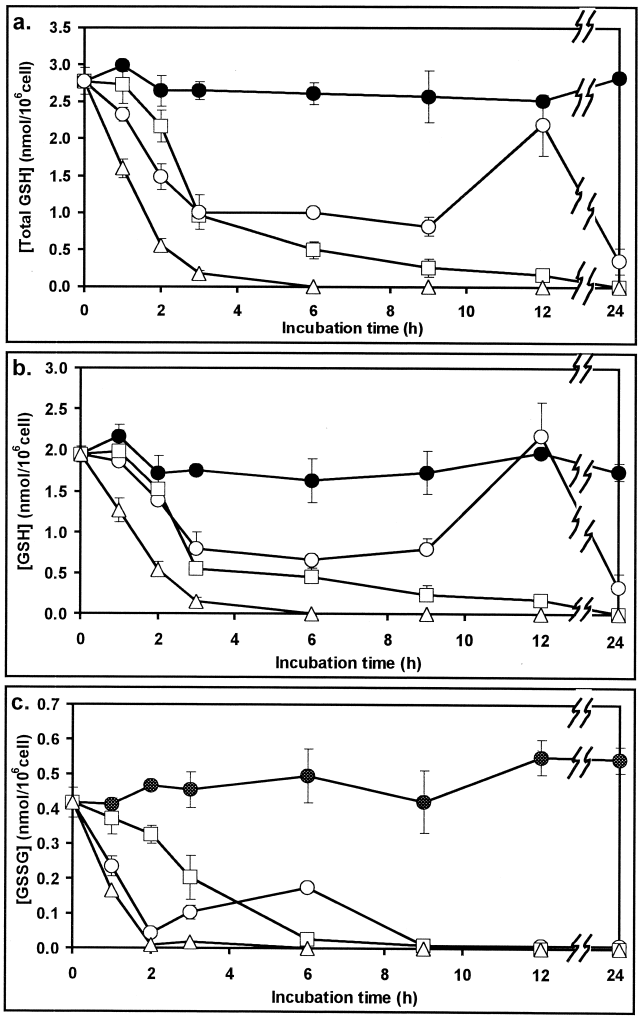


Fig. 2. Effect of PEITC on GSH metabolism of HL60 cells *in vitro* in the absence and presence of BSO. (a) Total GSH, (b) GSH, and (c) GSSG. Key: ●—● control; □—□ + 500 $\mu$M BSO; ○—○ + 5 $\mu$M PEITC; △—△ + 500 $\mu$M BSO and 5 $\mu$M PEITC. HL60 cells ($9.1 \times 10^4$/mL) were incubated in RPMI-1640 with 10% foetal bovine serum for 0–24 hr and cellular total GSH and GSSG concentrations determined. Data are means ± SD of 3 determinations.

preincubation inhibited γ-glutamyltransferase activity of HL60 cells by 88 ± 6% of the control value ($P < 0.001$) with no significant decrease in cell viability or total GSH, GSSG, or GSH concentration ($P > 0.05$). In the initial 3 hr of incubation of HL60 cells ($1 \times 10^6$/mL) with 50 $\mu$M PEITC, the concentration of total GSH decreased from 2.77 ± 0.28 nmol/$10^6$ cells to 1.42 ± 0.31 nmol/$10^6$ cells ($P < 0.01$), which was reflected in a corresponding proportionate decrease in GSSG and GSH (Fig. 4a). There was a concomitant formation of PETC-SG inside cells which maximized after 30 min and thereafter rapidly declined to *ca*. half the maximum concentration after a further 30 min. (Fig. 4b). During this period, GSH metabolites were appearing in the culture medium. At the 30-min time point, PETC-SG was detectable in the extracellular medium, but then declined to below the limit of detection. PETC-SG fragmented spontaneously under physiological conditions. In PBS, the rate constant for fragmentation of PETC-SG was 0.0156 ± 0.0002 $min^{-1}$, which implies a half-life of 44.4 ± 0.6 min. There was an increase of total GSH in the medium, but this only accumulated to 0.15 ± 0.01 $\mu$M, accounting for only *ca*. 11% of the decrease in cellular total GSH. Unlike the cellular total GSH in which GSSG accounted for only 28%, the total GSH in the medium was all detected as GSSG. The remainder of the GSH exported from cells was rather detected as glutathione–protein mixed disulphides. When combined total GSH and glutathione–protein mixed disulphides was determined by the monobromobimane procedure with prior reduction with DTT (no GSH was detected without this reduction), this analyte increased to 1.02 ± 0.15 $\mu$M GSH equivalents after 3 hr in the medium. This now accounted for the decrease in cellular total GSH during the incubation with PEITC.

### *3.2. Binding of* N*-phenethylthiocarbamoyl moieties from PEITC and PETC to HL60 cells* in vitro

The binding of PEITC and PETC-Cys to cells was investigated using the $^{14}$C-labelled derivatives 2-phenyl-[1-$^{14}$C]ethyl isothiocyanate and *S*-(*N*-phenyl-[1-$^{14}$C]ethylthiocarbamoyl)cysteine. PEITC is assumed to interact with cells by reaction with nucleophilic groups to form PETC moieties. PETC adducts with cysteinyl thiols are formed reversibly, with fragmentation half-lives of typically 10–100 min ($t_{1/2}$ for PETC-Cys = 16 min [7] and $t_{1/2}$ for PETC-SG 44 min − this work). PETC adducts with amino groups are typically irreversible forming thiourea derivatives $PhCH_2CH_2NHC(=S)NHR$. PETC-Cys fragmented rapidly to PEITC under physiological conditions and therefore may also interact with cells through the PEITC intermediate. However, there will be an extended period of delivery of PEITC to cells from PETC-Cys, compared to administration of authentic PEITC, as the fragmentation of PETC-Cys proceeds [7].

When HL60 cells were incubated with [$^{14}$C]PEITC or [$^{14}$C]PETC-Cys for 0.5–24 hr and washed 3 times with ice-cold PBS (taking *ca*. 20 min), there was an accumulation of cellular PETC adducts over the initial 2 hr which maximized at 2–3 hr and thereafter decreased. The decline in cellular PETC adduct concentration occurred before onset of decreased cell viability: the decrease in PETC adduct concentration began at 4–8 hr and the decrease in cell viability was initially detected at 12 hr. The time point of maximum PETC adduct concentration corresponded, however, with the time of commitment to apoptosis and the minimum period of exposure to PEITC and PETC-Cys for the growth inhibitory effect [7] (Fig. 5). For HL60 cells incubated with [$^{14}$C]PETC-Cys for 3 hr with maximum

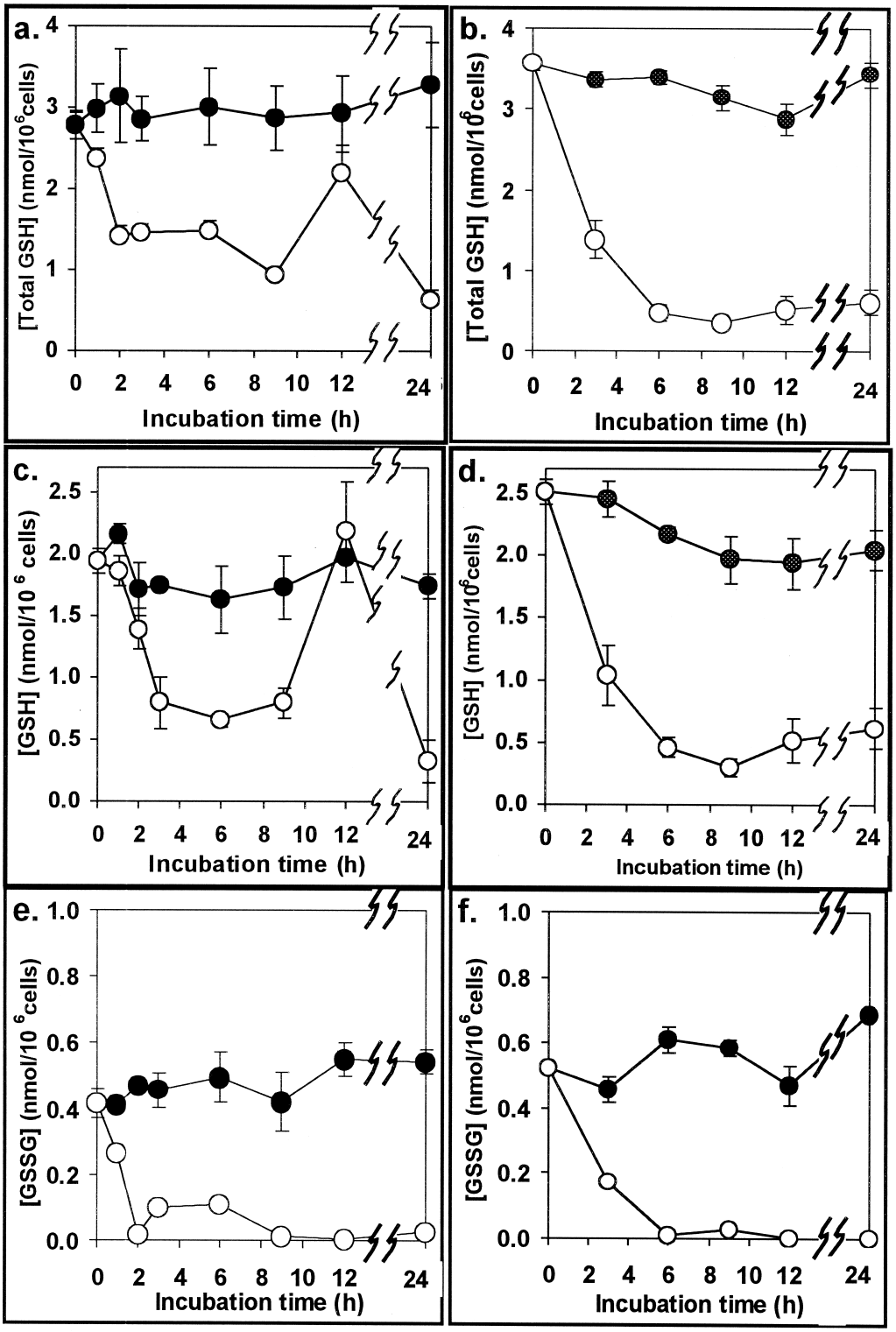


Fig. 3. Effects of PETC-Cys on GSH metabolism of HL60 cells (panels on the left) and of PEITC on GSH metabolism of ML-1 cells *in vitro* (panels on the right). Total GSH (top), GSH (middle), GSSG (bottom). HL60 or ML-1 cells ($9.1 \times 10^4$/mL) were incubated in RPMI-1640 with 10% foetal bovine serum for 0–24 hr and cellular total GSH and GSSG concentrations determined. Key: ●—● control; ○—○ + 5 $\mu$M compound. Data are means ± SD of 3 determinations.

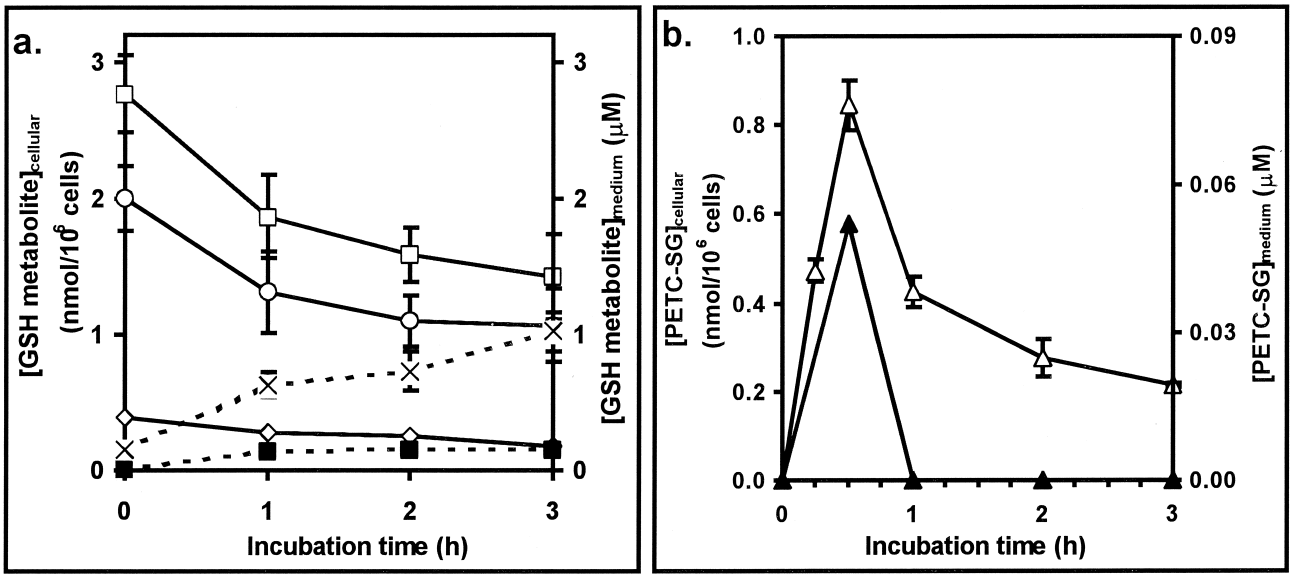


Fig. 4. Effect of PEITC on GSH metabolism of HL60 cells *in vitro*: formation of PETC-SG and increase of extracellular GSH metabolites. (a) Cellular concentrations of GSH metabolites: (□——□) total GSH, (◯——◯) GSH, (◇——◇) GSSG. Concentrations of GSH metabolites in the medium: (■- - -■) GSSG and (×- - - -×) GSSG + glutathione–protein mixed disulphides. (b) Concentration of PETC-SG: (△——△) cellular and (▲——▲ medium. Data are means ± SD of 3 determinations. HL60 cells ($1 \times 10^6$/mL) were incubated with 50 μM PEITC in RPMI-1640 with 10% foetal bovine serum for 0–3 hr. For extracellular GSH metabolite determination, HL60 cells were preincubated with 200 μM acivicin for 1 hr.

radiolabelling, protein, DNA, and RNA extracts were prepared and counted. No significant radiolabelling of extracted cellular protein, DNA, or RNA was found (equivalent to $< 0.002$ nmol PETC/$10^6$ cells). Adducts with cysteinyl thiols of proteins, however, were expected to have fragmented during the extraction procedure and the PEITC thereby liberated lost in the methanol wash.

### *3.3. Prevention of PEITC and PETC-Cys-induced inhibition of HL60 cell growth* in vitro *by high concentrations of exogenous GSH and the cancer chemopreventive agent curcumin*

When HL60 cells ($9 \times 10^4$/mL) were incubated for 24 hr with 5 μM PETC-Cys, there was an inhibition of cell

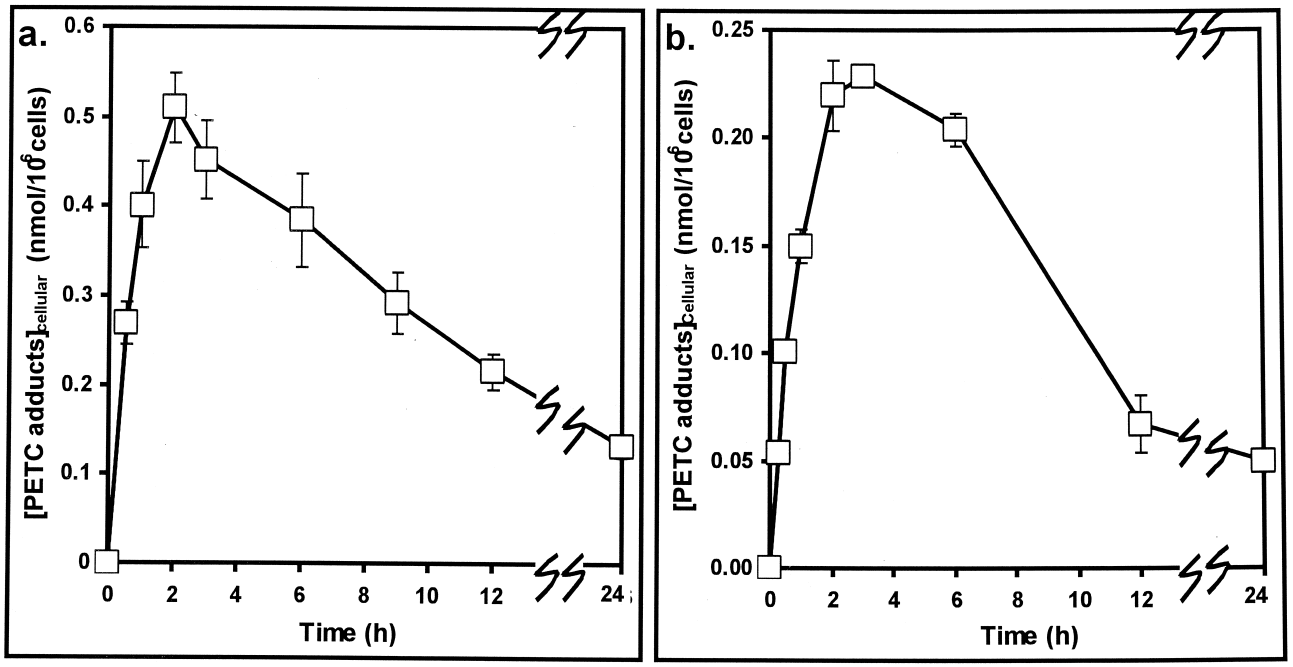


Fig. 5. Thiocarbamoylation of HL60 cells by PEITC (a) and PETC-Cys (b) *in vitro*. HL60 cells ($5 \times 10^4$/mL) were incubated with 5 μM [$^{14}$C]PEITC (a) or 5 μM [$^{14}$C]PETC-Cys (b) in RPMI-1640 with 10% foetal bovine serum for the times indicated, washed 3 times in PBS, and then counted (≤ 30% of PETC adducts may have been lost by fragmentation during PBS washing). Data are means ± SD of 3 determinations.

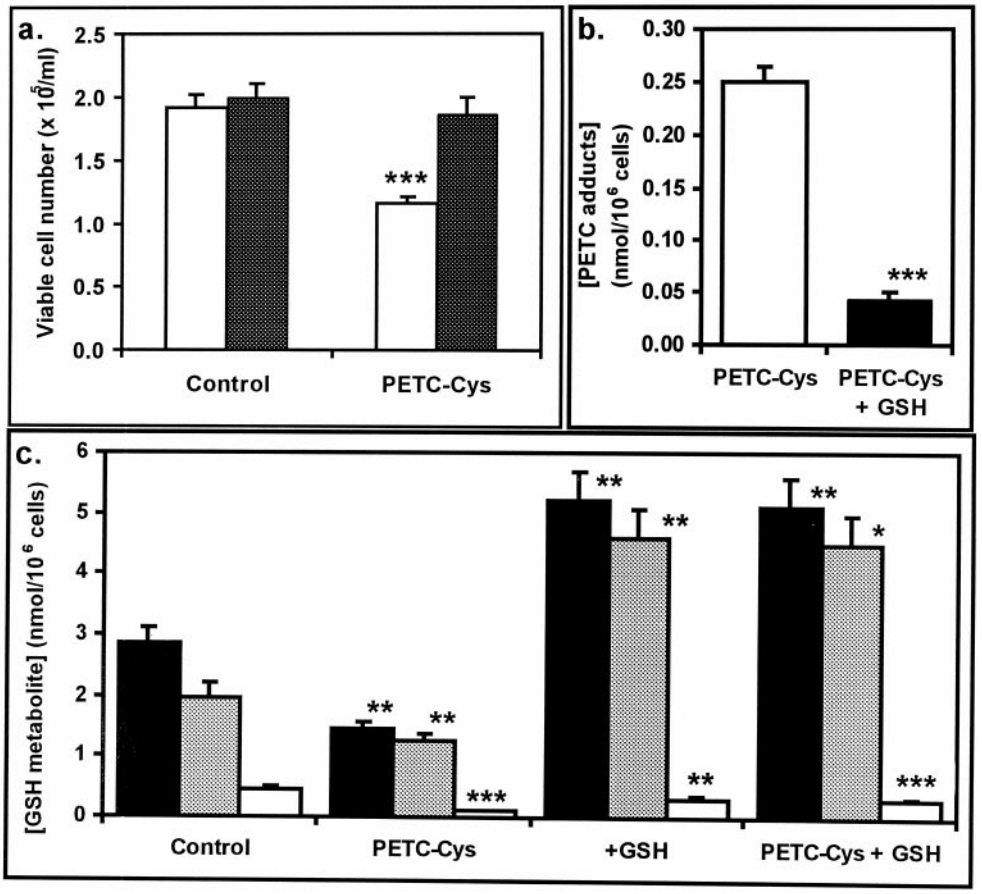


Fig. 6. Prevention of PETC-Cys-induced cytotoxicity to HL60 cells by exogenous GSH *in vitro*. (a) Effect on viable cell number. HL60 cells ($9.1 \times 10^4$/mL) were preincubated with and without 15 mM GSH for 2 hr in RPMI-1640 with 10% foetal bovine serum, and then incubated with and without 5 $\mu$M PETC-Cys for 24 hr. (b) Effect on cell thiocarbamoylation. HL60 cells ($5 \times 10^4$/mL) were preincubated with and without 15 mM GSH for 2 hr in RPMI-1640 with 10% foetal bovine serum, and then incubated with and without 5 $\mu$M PETC-Cys for 3 hr, washed 3 times in PBS and then counted. Key: (□) − GSH, and (■) + GSH. (c) Effect on cellular GSH metabolism. HL60 cells ($9.1 \times 10^4$/mL) were preincubated with and without 15 mM GSH, as indicated, in RPMI-1640 with 10% foetal bovine serum for 2 hr, and then incubated with and without 5 $\mu$M PETC-Cys for 3 hr. The concentrations of total GSH and GSSG determined. Key: (■) total GSH, (▨) GSH, and (□) GSSG. Data are means ± SD of 3 determinations. Significance with respect to control: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

growth, as evidenced by a 40% decrease in viable cell number ($P < 0.001$). When HL60 cells were preincubated for 2 hr with 15 mM GSH, however, the inhibition of HL60 cell growth by PETC-Cys was totally prevented (Fig. 6a). Addition of 15 mM GSH to HL60 cells incubated with 5 $\mu$M [$^{14}$C]PETC-Cys markedly decreased the binding of PETC moieties to cells (Fig. 6b). Addition of 15 mM GSH to HL60 cells significantly increased the cellular concentrations of GSH and GSSG in the absence and presence of 5 $\mu$M PETC-Cys (Fig. 6c). We were not able to demonstrate similar protection with 15 mM GSH ethyl diester, *N*-acetylcysteine, mercaptoethanol, lysine, or *N*-acetyllysine. GSH ethyl diester and mercaptoethanol induced cytotoxicity alone.

A further compound that was protective against PEITC-induced cytotoxicity was the cancer chemopreventive agent curcumin. Curcumin (20 $\mu$M) inhibited the induction of apoptosis of HL60 cells by 5 $\mu$M PEITC after 6 hr (Fig. 7a). Unlike the effect of 15 mM GSH, however, the protective effect of curcumin was not sustained for 24 hr. After incubation for 24 hr, curcumin did not significantly prevent the decrease in viable cell number induced by 5 $\mu$M PEITC (data not shown). Therefore, curcumin delayed rather than prevented PEITC-induced apoptosis of HL60 cells. Curcumin increased the cellular concentration of GSH in HL60 cells and thereby partially prevented the PEITC-induced decrease in cellular GSH concentration (Fig. 7, b–d).

Other agents were investigated for effects on GSH and GSSG depletion in HL60 cells incubated with PEITC and PETC-Cys, but proved to be ineffective. The caspase inhibitor Z-VAD-fmk (50 $\mu$M) inhibits caspase-3 and caspase-8 [18] and prevented PEITC-induced apoptosis [7], but it did not prevent depletion of GSH by PEITC. Methionine (5 mM) in the extracellular medium and the intracellular GSH conjugates *S*-sulfophthaleinylglutathione and DNP-SG, generated intracellularly from non-toxic concentrations of

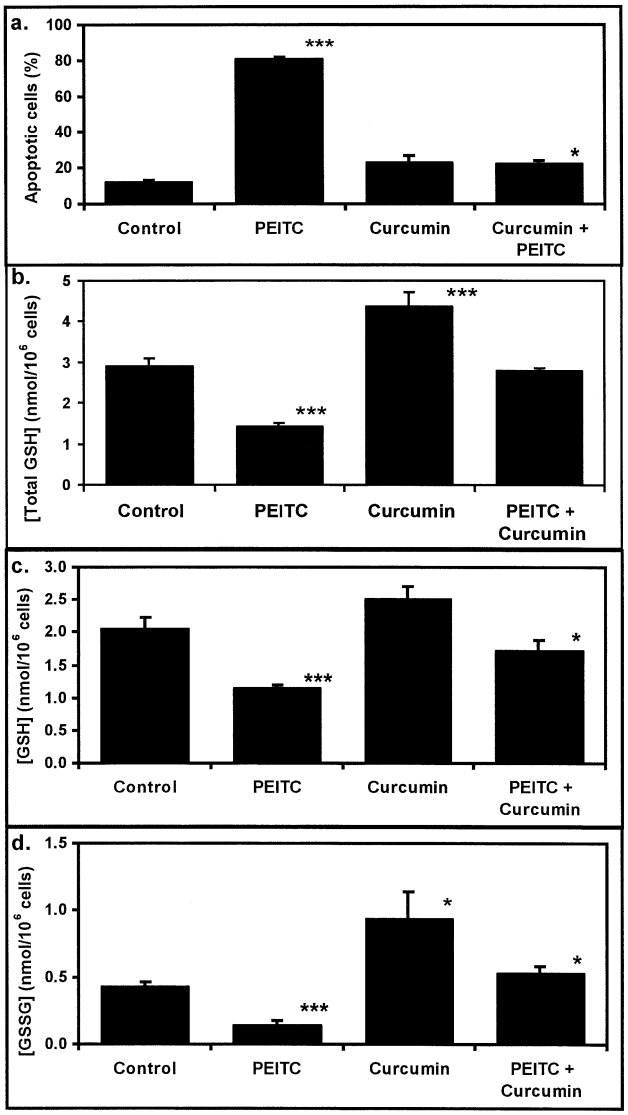


Fig. 7. Delay of PEITC-induced cytotoxicity to HL60 cells by curcumin *in vitro*. (a) Apoptosis. The percentage of apoptotic cells was determined by flow cytometry. (b–d) Cellular glutathione metabolism. The cellular concentrations of total GSH (b), GSH (c), and GSSG (d) were determined. Data are means ± SD of 3 determinations. HL60 cells ($9.1 \times 10^4$/mL) were incubated, with and without 5 $\mu$M PEITC, in RPMI-1640 with 10% foetal bovine serum for 24 hr with and without 20 $\mu$M curcumin, as indicated, for 6 hr (a) or 3 hr (b–d). Significance with respect to control: $^*P < 0.05$; $^{***}P < 0.001$.

bromosulfophthalein and 1-chloro-2,4-dinitrobenzene (CDNB), were not effective in preventing the decrease in cellular GSH and GSSG in HL60 cells incubated with PEITC (data not shown).

## 4. Discussion

The antiproliferative, antitumour activity of dietary isothiocyanates and their cysteine conjugates has been of recent research interest, because these 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 [19]. We reported for the first time the induction of apoptosis in HL60 cells by PEITC, PETC-Cys and related compounds [6] and recently confirmed and extended this discovery in reporting the involvement of caspases in PEITC-induced apoptosis and the inactivation of PEITC by hydrolysis under physiological conditions [7]. In the study of the mechanism of apoptosis induced by PEITC and related compounds, high concentrations of thiol-containing antioxidants have been found to inhibit apoptosis: induction of JNK activation during apoptosis of human Jurkat T-cells induced by PEITC was inhibited by 10 mM mercaptoethanol and 20 mM *N*-acetylcysteine [9]. An independent report of JNK1 activation during PEITC-induced apoptosis of HeLa cells, however, stated that 30 mM *N*-acetylcysteine had no effect on PEITC-induced activation of JNK1 and the pro-oxidants hydrogen peroxide (800 $\mu$M) and diamide (1 mM) inhibited PEITC-induced JNK1 activation, although hydrogen peroxide alone stimulated JNK1 activation [8]. These discordant results deserve further investigation. Unlike oxidants such as hydrogen peroxide and agents that may be stimulated to generate oxidants *in situ* (redox cycling quinones and hydrazine derivatives, for example), isothiocyanates are not intrinsic oxidising agents. The major spontaneous reaction of PEITC under physiological systems is hydrolysis to phenylethylamine via a phenethylmonothiocarbamate intermediate [7]. PEITC reacted rapidly with thiol groups by non-enzymatic reaction with cysteine residues and enzymatic conjugation with GSH catalysed by GSH *S*-transferases [20]. These reactions are rapid and reversible [7], but they may lead to a temporary decrease in cysteinyl thiol groups in cells that provide an oxidative trigger for apoptosis.

In this study, we found that both PEITC and PETC-Cys induced a decrease in cellular concentration of GSH and GSSG in the initial 2–3 hr of culture. In the first hour of culture, there was a rapid formation of PETC-SG inside cells which was then detected in the extracellular medium. We propose that PETC-SG was formed by the reaction of GSH with PEITC catalysed by GSH *S*-transferase and was then exported from cells by a GSH conjugate transporter. PETC-SG was detected in the medium only at the time point of maximum cellular PETC-SG concentration—the statistical limit of detection of PETC-SG in the medium was equivalent to 0.023 $\mu$M. PETC-SG was not stable in the extracellular medium, even when $\gamma$-glutamyltransferase had been inhibited by preincubation of HL60 cells with acivicin. PETC-SG fragmented spontaneously to PEITC and GSH. In the extracellular medium, GSH oxidised and formed GSSG and glutathione–protein mixed disulphides. In the absence of acivicin as well as the $\gamma$-glutamylcysteine synthetase inhibitor BSO, GSH and GSSG formed from the exported PETC-SG may be degraded to cysteine and cystine, re-enter cells, and stimulate GSH synthesis. This may account for

the recovery of cellular GSH concentration in HL60 cells incubated with PEITC for 12 hr. After incubation for 24 hr, however, the concentration of GSH decreased again as apoptosis developed. In the presence of BSO, no recovery of cellular GSH concentration occurred, supporting the assertion that recovery of cellular GSH concentration was due to GSH synthesis *de novo*.

An alternative explanation for the decrease in cellular GSH concentration during PEITC-induced apoptosis is that PEITC-induced apoptosis may stimulate the efflux of GSH from cells, as found in apoptosis induced by hydrogen peroxide, puromycin, cycloheximide, etoposide, and anti-Fas/APO-1 antibody [21,22]. We could find no evidence for marked accumulation of GSH in the extracellular medium ($[GSH]_{medium} < 50$ nM), although the concentrations of GSSG and GSH–protein disulphides increased. Methionine (5 mM) in the extracellular medium and intracellular GSH conjugates *S*-sulfophthaleinylglutathione and DNP-GSH were effective inhibitors of GSH efflux activated in apoptosis [21,22], but were not effective in preventing the decrease in cellular GSH and GSSG in HL60 cells incubated with PEITC in our studies. Unlike GSH, PETC-SG may compete effectively with *S*-sulfophthaleinylglutathione and DNP-SG for GSH transporters and thereby still give rise to a decrease in the cellular GSH concentration in the presence of these substrates of GSH conjugate transporters.

The formation and cellular efflux of PETC-SG decreased GSH and gave a corresponding decrease in GSSG in the initial 2-hr period of incubation of HL60 cells with PEITC. This may be due to GSH/GSSG thiol–disulphide exchange reactions and activation of GSH reductase as the GSSG/GSH concentration ratio increases. In the 2- to 6-hr period, there was an increase in the cellular concentration of GSSG. This may be due to increased oxidative stress associated with the decrease in total GSH. A similar time–course of cellular GSH and GSSG concentration changes was found when GSH was conjugated with 1-chloro-2,4-dinitrobenzene to form DNP-SG in murine $P388D_1$ macrophages, except that in this case there was no later recovery in cellular GSH concentration [23]. The difference is that PETC-SG fragments to GSH in the extracellular medium leading eventually, via γ-glutamyltransferase and dipeptidase processing, to cysteine and cystine, which then re-enter cells for *de novo* GSH synthesis; DNP-SG does not fragment to form cysteine but rather undergoes similar mercapturic acid pathway processing to form *S*-2,4-dinitrophenylcysteine.

PETC-Cys, added at approximately the median toxic concentration, gave similar changes in the concentrations of GSH and GSSG. We have previously found that PETC-Cys rapidly fragmented to form PEITC and cysteine [7], suggesting that the effects of PETC-Cys are probably mediated by the PEITC thereby formed. Since the recovery of GSH concentration after 12 hr in HL60 cells incubated with PEITC was attributed to availability of cysteine from the extracellular degradation, it was surprising that the cellular GSH concentration did not recover more rapidly in HL60 cells incubated with PETC-Cys than PEITC. Other factors, such as availability of ATP, may be rate controlling in the repletion of cellular GSH. Indeed, the time–course of depletion of cellular GSH and GSSG induced by the $TC_{50}$ concentration of PEITC was different in ML-1 compared to HL60 cells. In ML-1 cells, there was a more marked depletion of GSH and GSSG with no subsequent period of GSH repletion.

The depletion of GSH in tumour cells by dietary isothiocyanates and related *S*-thiocarbamoyl metabolites is expected to depend on the activity of GST catalysing the formation of the GSH conjugate [20], GSH *S*-conjugate transporter activity [24], and enzyme and amino acid transporter activities involved in extracellular GSH degradation, and cysteine, cystine and γ-glutamylcystine cell uptake and GSH synthesis [25]. Differential aspects of glutathione metabolism in HL60 cells, ML-1 cells, and lymphocytes may also contribute to the selective toxicity of PEITC to HL60 and ML-1 cells, with respect to concanavalin-A-stimulated peripheral lymphocytes, reported recently [25]. The GST isozymes in HL60 and ML-1 cells catalysing the formation of PETC-SG were mainly of the π-class [26]; lower expression of α- and μ-class enzymes was also detected in HL60 cells [27]. Similar isozymes were found in human B- and T-lymphocytes [28], and similar activities per mg total cell protein were found in HL60 and ML-1 cells and human B- and T-lymphocytes [26,29]. All of these GST isozymes catalyse the GSH conjugation of isothiocyanates, but π-class GST (P1-1) had the highest activity [20]. The GSH content of HL60 and ML-1 cells determined herein ($1.94 \pm 0.10$ and $2.51 \pm 0.1$ nmol GSH/$10^6$ cells, respectively), equivalent to $16.2 \pm 0.8$ and $12.5 \pm 0.5$ nmol GSH/mg protein, was much higher than found in peripheral lymphocytes (*ca*. 2 nmol GSH/mg protein [30]). Glutathione conjugates are expelled from HL60 cells and lymphocytes by the multidrug resistance protein, which has similar expression in leukaemia cells and peripheral lymphocytes [24], increasing markedly in some instances of resistance to antitumour drugs [31]. Cysteine for resynthesis of GSH in cells may arise from: (i) entry of cysteine into cells by the ASC transporter; (ii) entry of cystine by the $X_c^-$ transporter; and (iii) entry of γ-glutamylcystine by the γ-glutamyl amino acid transporter (discussed in [25]). Little is known of these activities in HL60 cells, ML-1 cells, and lymphocytes, except that the activity of the $X_c^-$ transporter is very low in lymphocytes [32]. Since the cellular concentration of GSH of HL60 cells recovered after the initial 3 hr of culture with PEITC but that of ML-1 cells did not (Figs. 2b and 3d), there may have been increased expression of the ASC transporter, $X_c^-$ transporter, and/or γ-glutamyl amino acid transporter in HL60 cells relative to ML-1 cells. It is not clear currently, however, if the differences in GSH metabolism of

HL60 and ML-1 cells, relative to that of lymphocytes, contribute to the selective toxicity of PEITC. This requires further investigation.

Incubation of HL60 cells with PEITC and PETC-Cys radiolabelled in the phenethyl moiety leads to labelling of cells. The modification was reversible and declined to *ca*. 20% of the maximum value before decrease in cell viability. There was no detectable irreversible PETC adduct formation on cells extracts of DNA, RNA, and protein ($< 0.01\%$ of PETC-Cys). A further possible site of modification was basic lipid derivatives such as sphingoid bases and basic phospholipids. We found no evidence of these by mass spectrometric analysis of membrane extracts. Adducts of sphingosine, sphinganine, phosphatidylethanolamine, and phosphatidylserine were prepared: they were formed only very slowly under physiological conditions and had potencies similar to or much less than PEITC itself [33]. Part of the radiolabel of HL60 cells incubated with [$^{14}$C]PEITC was due to PETC-SG formation, but the radiolabelling of HL60 cells was longer lived than PETC-SG in HL60 cells under identical conditions and maximised later than the maximum cellular concentration of PETC-SG. It is likely that other thiol groups, i.e. protein cysteinyl residues, are modified in HL60 cells by PEITC. Indeed, depletion of cellular GSH will enhance this. Modification of cysteinyl thiol groups in the proteins involved in signal transduction may activate PEITC-induced apoptosis.

Maintenance of the cellular concentration of GSH may prevent PEITC and PETC-Cys-induced apoptosis. This was indeed found when cellular GSH was supplemented by a high concentration (15 mM) of exogenous GSH. GSH ethyl diester, which repleted cellular GSH more effectively than GSH [25], did not prevent PETC-Cys-induced apoptosis and was cytotoxic when added at 15 mM (data not shown). Moreover, addition of GSH not only maintained cellular concentrations of GSH and GSSG but also prevented radiolabelling of HL60 cells by [$^{14}$C]PETC-Cys. A probable explanation for this is that 15 mM GSH markedly increases the concentration of extracellular thiol groups which bind PEITC liberated from PETC-Cys. This suppresses entry of PEITC into HL60 cells and decreases free PEITC to subtoxic levels whilst it slowly hydrolyses. Since overexpression of Bcl-2 also increased cellular GSH [34], the prevention of PEITC-induced activation of JNK1 and apoptosis by Bcl-2 [9] may be mediated by both intervention in GSH metabolism and prevention of proapoptotic changes in mitochondrial function [35]. Curcumin, however, delayed but did not prevent the development of cytotoxicity. It only partly prevented the initial decrease in cellular GSH induced by PEITC, however. There are also effects of curcumin not linked to GSH metabolism that may suppress PEITC-induced apoptosis: curcumin inhibited JNK signalling upstream of protein kinase/extracellular signal-regulated kinase kinase 1 [36] that is an important mediating factor of PEITC-induced apoptosis [9].

These studies show that the cysteinyl thiol group of GSH is an important site of thiocarbamoylation by PEITC during the phase of commitment to apoptosis and that this may lead to depletion of cellular GSH by efflux of the PETC-SG. It is, however not the only one; similar active site cysteinyl thiols of enzymes involved in signalling to the JNK pathway may be involved in activation of apoptosis by dietary isothiocyanates [37–39]. Activation of caspase-8, as occurs in PEITC-induced apoptosis [7], is associated with activation of JNK [40]. The caspase inhibitor Z-VAD-fmk did not prevent PEITC-induced depletion of GSH, but did prevent PEITC-induced apoptosis. The critical activation of caspase-8 [7], therefore, occurs downstream of GSH depletion. Caspase-8 is activated by the bringing together of the cytosolic domain of a cell death receptor and the adaptor, protein Fas-associated death domain (FADD), which then binds and processes procaspase-8 [40]. Our working hypothesis is that PEITC modifies one or more of these components to facilitate caspase-8 activation and apoptosis.

Inhibition of malignant cell proliferation and induction of apoptosis by dietary isothiocyanates in preclinical tumours was characteristic of the cancer chemopreventive effect of isothiocyanates *in vivo* [41–43]. Moreover, dietary isothiocyanate-induced signal transduction via the JNK pathway in apoptosis was implicated in the induction of phase II enzymes via activation of activated protein-1 and nuclear factor kappa B binding transcription factors [44]. Identification of the receptor(s) for isothiocyanates that initiate signalling for apoptosis and induction of phase II enzyme expression may lead to improved design of these agents which may find use in chemoprevention of cancer [1], inhibition of metastasis [45], and potentiation of the therapeutic activity of bioreductive anticancer drugs [46].

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