# Forward and reverse catalysis and product sequestration by human glutathione S-transferases in the reaction of GSH with dietary aralkyl isothiocyanates

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The reversible reaction of GSH with two dietary anticarcinogens, benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC), has been studied in the absence and presence of human glutathione S-transferases (GSTs). The spontaneous reaction at pH 7.4 and 37 °C yielded values for $k_2$ of 17.9 and 6.0 $M^{-1} \cdot s^{-1}$ for GSH conjugation of BITC and PEITC respectively (forward reaction), and $k_1$ values of $6.9 \times 10^{-4}$ and $2.4 \times 10^{-4}$ $s^{-1}$ for dissociation of the respective GSH conjugates, BITC-SG and PEITC-SG (reverse reaction). GSTs A1–1, A2–2, M1a–1a and P1–1 catalysed both the forward and reverse reactions with specific activities (μmol/min per mg at 30 μM isothiocyanate or GSH conjugate) ranging from 23.1 for the GSH conjugation of BITC by GST P1–1 to 0.03 for the dissociation of BITC-SG by GST A1–1. When present at similar concentration to substrates (12 μM), GSTs A1–1 and A2–2 but not GST M1a–1a shifted the equilibrium in favour of BITC-SG or PEITC-SG. Kinetic studies confirmed that GST A1–1 interacted selectively with the GSH conjugates in the micromolar range ($K_m$ 6.9 μM, $K_i$ 4.3 μM), whereas GST M1a–1a interacted with BITC-SG and PEITC-SG with approx. 5-fold lower affinity. In conclusion, GSTs are true catalysts; at high intracellular concentration they also sequester GSH conjugates, promoting GSH conjugation, whereas trace extracellular GSTs promote dissociation of effluxed organic isothiocyanate–GSH conjugates.

## INTRODUCTION

Soluble glutathione S-transferases (GSTs) are a widely distributed group of enzymes concerned with the detoxification of electrophiles by GSH [1]. Enzymes of the Alpha, Mu and Pi classes [2] generally occur at relatively high intracellular concentration (5–100 μM). They have low $K_m$ values for GSH (compared with cellular [GSH]) and generally even lower $K_i$ values for GSH conjugates (products) which may reduce their catalytic efficiency. This suggests that these GSTs are adapted not only for catalysis of the reaction of GSH with a variety of electrophiles, but also for product binding [3].

Such product retention might function in hepatic vectorial transport whereby compounds are delivered to the efflux pump(s) for excretion in bile [4]. Product retention might also function in the sequestration of reactive conjugates (e.g. the monoglutathionyl conjugate of chlorambucil [5]) or, in the case of readily reversible GSH-conjugation reactions, it might shift the equilibrium in favour of the GSH conjugate. Compounds that fall into the last category are organic isothiocyanates [6,7].

Many organic isothiocyanates occur as glucosinolates in common dietary vegetables [8] and are released by thioglucosidase on mastication [9]. The metabolism of dietary organic isothiocyanates, e.g. benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and sulphoraphane, is of special interest because of their anticancer properties [10–16]. In order to test whether GSTs act as true catalysts, i.e. by accelerating the approach to equilibrium from either direction, and whether they alter equilibria by selective binding of GSH conjugate, we have examined the reaction of BITC and PEITC with GSH in the presence and absence of purified human GSTs.

## EXPERIMENTAL

### Materials

GSH was obtained from Sigma (Poole, Dorset, U.K.). BITC and PEITC were from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). *S*-(*N*-Benzylthiocarbamoyl)glutathione (BITC-SG) was synthesized from BITC and GSH in aqueous ethanol [17], and *S*-(*N*-phenethylthiocarbamoyl)glutathione (PEITC-SG) was synthesized in an analogous fashion. Compounds were stored desiccated at −20 °C.

GSTs A1–1, A2–2 and M1a–1a were prepared from the soluble fraction of liver homogenate, and GST P1–1 was prepared from the soluble fraction of kidney homogenate [18]. Enzyme concentration in the assays refers to the molarity of subunits (catalytic centres).

### Assay of reversible reaction of GSH with aralkylisothiocyanates

Fresh stock solutions (1–10 mM) of the isothiocyanates and dithiocarbamates were prepared in dimethylformamide. Solutions of BITC, BITC-SG, PEITC and PEITC-SG (50 μM) were prepared by addition of stock solution to the assay buffer (0.1 M KCl, 1 mM EDTA, 30 mM sodium phosphate, pH 7.4). Comparison of their u.v. absorption spectra showed that the absorption peak/shoulder at 270 nm [19] could be used to monitor formation or loss of dithiocarbamate ($\epsilon = 8178$ $M^{-1} \cdot cm^{-1}$). All experiments were carried out in this assay buffer at 37 °C.

### Chemical kinetics

Second-order rate constants ($k_2$) for the spontaneous reaction of BITC/PEITC with GSH (forward reaction) were obtained

Abbreviations used: GST, glutathione S-transferase (EC 2.5.1.18); BITC, benzyl isothiocyanate; BITC-SG, *S*-(*N*-benzylthiocarbamoyl)glutathione; PEITC, phenethyl isothiocyanate; PEITC-SG, *S*-(*N*-phenethylthiocarbamoyl)glutathione; CDNB, 1-chloro-2,4-dinitrobenzene.

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from the pseudo-first-order rate constant obtained by mixing 30 $\mu$M isothiocyanate with 0.7 mM GSH and monitoring the increase in $A_{270}$. First-order rate constants for the spontaneous dissociation of BITC-SG and PEITC-SG (reverse reaction) were obtained from the decrease in $A_{270}$ of 0.1 mM solutions of the conjugates or calculated from the equilibrium position and the $k_2$.

### Enzyme kinetics

Specific activities of GSTs with BITC, PEITC, BITC-SG and PEITC-SG were obtained from the initial rate of change in $A_{270}$ with each substrate (30 $\mu$M) and, for the forward reaction, 0.7 mM GSH. Dimethylformamide was 1.5% (v/v). Duplicate or triplicate assays were carried out.

Kinetic data were obtained with concentrations of isothiocyanates or dithiocarbamates ranging from 4 to 200 $\mu$M. Dimethylformamide was less than 2.5% (v/v) which did not appear to affect enzyme activity. A saturating concentration of GSH (1.5 mM) was used in the assays with isothiocyanates.

Kinetics of inhibition of GST activity by BITC-SG with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate were measured in 0.1 M sodium phosphate, pH 6.5 [20]. Either [GSH] was varied with 0, 5 or 15 $\mu$M BITC-SG, or BITC-SG was varied at two levels of GSH. CDNB was 1 mM in all assays. Inhibition type was determined from plotting $s/v$ versus $s$ or $1/v$ versus $I$. Organic solvent was kept below 3% (v/v).

Kinetic data were analysed by fitting to a rectangular hyperbola (Kinenort program, supplied by Dr. A. G. Clark, Victoria University of Wellington, New Zealand). Standard errors of $K_m$, $V_{max.}$ and $K_i$ values were less than 14%.

### Sequestration of GSH conjugate

Alterations in the equilibria between PEITC and BITC and their GSH conjugates resulting from possible sequestration of the GSH conjugates (dithiocarbamates) by GSTs were studied as follows. The equilibrium was determined by mixing, in 2 × 0.5 ml quartz cuvettes, assay buffer plus 200 $\mu$M GSH plus a catalytic amount (0.25 $\mu$M) of GST M1a–1a. After the spectrophotometer had been set to zero, 30 $\mu$M BITC or PEITC was added to the sample cuvette, an equal volume of dimethylformamide was added to the reference cuvette, and equilibration was monitored from the increase in, and subsequent stabilization of, $A_{270}$. GSTs were transferred to assay buffer by rapid gel filtration (PD-10; Pharmacia Biotech, St. Albans, Herts., U.K.) and their concentrations adjusted to 12 $\mu$M by dilution with assay buffer. The effect of sequestration by GST on the equilibrium was then determined by repeating the above experiment but replacing assay buffer by each GST solution in turn (final GST concentration 11.7 $\mu$M).

To check whether any excess apparent GSH conjugate seen at equilibrium in the presence of GST was due to covalent reaction of isothiocyanate with protein thiol rather than sequestration of dithiocarbamate, GST A1–1 (12 $\mu$M in assay buffer) was treated with 1.2 mM *N*-ethylmaleimide at 20 °C for 20 min. The excess *N*-ethylmaleimide was removed by rapid gel filtration, and the protein concentrated back to 12 $\mu$M using a Centriprep (Amicon, Danvers, MA, U.S.A.). The sequestration experiment using GSH and BITC was then repeated as described above.

As the excess GSH resulted in an equilibrium that greatly favoured the dithiocarbamate, more sensitivity was obtained by studying the reverse reaction. Thus experiments were carried out as above using 30 $\mu$M BITC-SG (in the absence of added GSH) and monitoring the decrease in $A_{270}$ to equilibrium.

## RESULTS

### Spontaneous reaction of BITC and PEITC with GSH

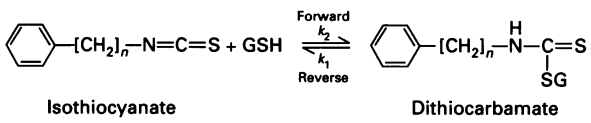
The reaction of BITC or PEITC with GSH at physiological pH and temperature yielded values for $k_2$ of 17.9 and 6.0 $M^{-1} \cdot s^{-1}$ respectively. Values for $k_1$ for the reverse reaction, i.e. the dissociation of the dithiocarbamates BITC-SG and PEITC-SG, were $6.9 \times 10^{-4}$ and $2.4 \times 10^{-4}$ $s^{-1}$ respectively. In reactions of this form ($A + B \rightleftharpoons C$, Scheme 1), the equilibrium 'constants' are concentration-dependent: $2.6 \times 10^4$ and $2.5 \times 10^4$ $M^{-1}$ for the reaction of GSH with BITC and PEITC respectively.

### GST activity for forward and reverse reactions

The specific activities of purified human GSTs A1–1, A2–2, M1a–1a and P1–1 for the conjugation of GSH with BITC or PEITC, and the reverse reactions, are shown in Table 1. The substrate concentration of 30 $\mu$M used was not saturating; it was limited by the high absorbance. All four GSTs catalysed dithiocarbamate formation from BITC and PEITC, and each enzyme also catalysed the reverse reaction. GST P1–1 was the most active and GST A1–1 the least active with all four substrates.

### Effect of GSTs on the equilibrium

Figure 1 shows the amount of BITC-SG present at equilibrium after mixing 30 $\mu$M isothiocyanate with 200 $\mu$M GSH in the presence or absence of a kinetically significant concentration (11.7 $\mu$M) of GSTs. Equilibration was accelerated in the absence of a high concentration of GST by the addition of a trace amount of GST M1a–1a. The data suggest that more BITC-SG is present at equilibrium in the presence of GST A1–1, but not in the presence of GST M1a–1a. GST A2–2 may have caused a small increase in yield of BITC-SG. Similar effects were seen with PEITC plus GSH (Figure 2). The apparent increased PEITC-SG at equilibrium due to GST A1–1 was also seen with GST A1–1




**Scheme 1 Reaction of GSH with aralkyl isothiocyanates**

$n = 1$, benzyl isothiocyanate; $n = 2$, phenethyl isothiocyanate. $K = k_2/k_1$.

**Table 1 Specific activities of GSTs with BITC, PEITC, BITC-SG and PEITC-SG**

Specific activity was measured using 30 $\mu$M substrate which in most cases is subsaturating. GSH (0.7 mM) was included in assays of the forward reaction. Experimental details were as described in the Experimental section. Results are means of duplicates differing by less than 8%.

| | GST activity ($\mu$mol/min per mg of protein) | | | |
|---|---|---|---|---|
| GST | BITC | PEITC | BITC-SG | PEITC-SG |
| A1–1 | 1.8 | 1.9 | 0.03 | 0.4 |
| A2–2 | 14.9 | 6.2 | 0.1 | 0.3 |
| M1a–1a | 12.9 | 10.7 | 0.3 | 0.2 |
| P1–1 | 23.1 | 12.8 | 1.6 | 1.3 |

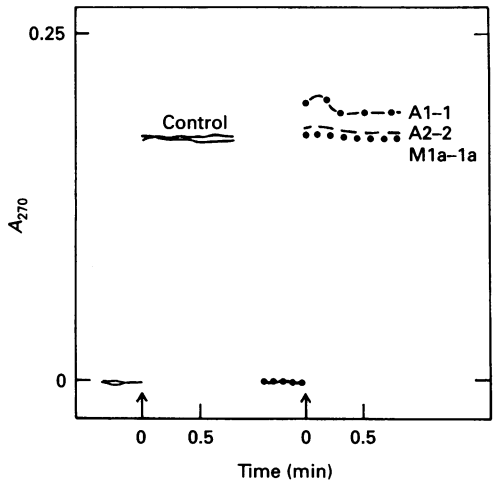



**Figure 1 Effect of GSTs on the BITC–BITC-SG equilibrium**

Initially, reference and sample cuvettes contained assay buffer, 200 $\mu$M GSH and GST as specified below in 0.5 ml at 37 °C. The $A_{270}$ was set to zero and BITC in dimethylformamide (5 $\mu$l) was added to the sample cuvette to 30 $\mu$M, and 5 $\mu$l of dimethylformamide to the reference cuvette. ——, Control equilibrium which is rapidly achieved with a catalytic amount (0.25 $\mu$M) of GST M1a–1a; · · · ·, 11.7 $\mu$M GST M1a–1a; – – – –, 11.7 $\mu$M GST A2–2; –·–·, 11.7 $\mu$M GST A1–1.

pretreated with the thiol-blocking agent, *N*-ethylmaleimide. Therefore reaction with protein thiol is unlikely to be the cause of excess dithiocarbamate. The apparent sequestration of BITC-SG is seen with more sensitivity when the reverse reaction is examined. The absence of excess GSH yields an equilibrium more in favour of the isothiocyanate, hence the GST-dependent sequestration effect is more pronounced. The results, in Figure 3, clearly show the apparent sequestering effect of both GST A1–1 and A2–2. In the case of GST A2–2, the apparent excess of the dithiocarbamate at equilibrium could not be due to reaction

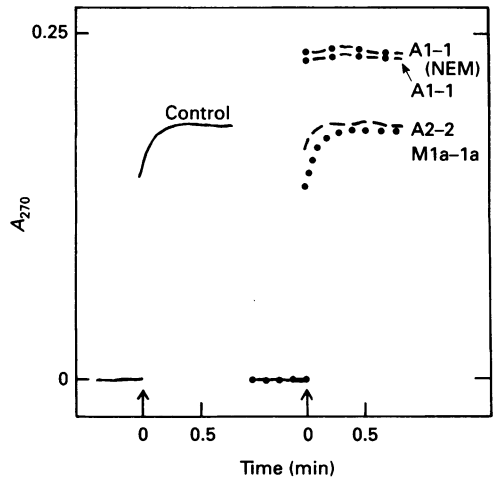



**Figure 2 Effect of GSTs on the PEITC–PEITC-SG equilibrium**

Reference and sample cuvettes were prepared exactly as described in the legend to Figure 1 except that PEITC replaced BITC. Symbols are also the same except that the upper dash–dotted trace contained 11.7 $\mu$M GST A1–1 previously treated with *N*-ethylmaleimide (NEM).

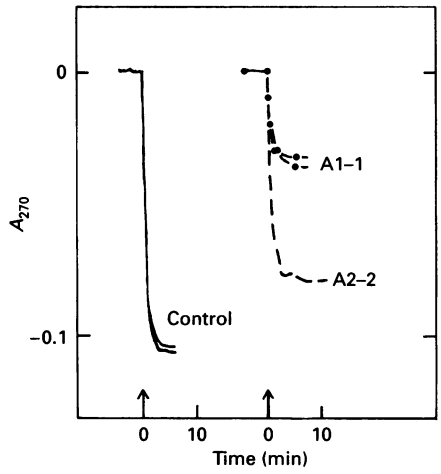



**Figure 3 Effect of GSTs A1–1 and A2–2 on BITC–BITC-SG equilibrium from the reverse reaction in the absence of GSH**

Reference and sample cuvettes were prepared as described in the legend to Figure 1, except that BITC-SG replaced BITC. The symbols correspond to those in Figure 1.

**Table 2 Kinetic data on the interaction of GSTs A1–1 and M1a–1a with isothiocyanates and dithiocarbamates**

Kinetic data for BITC were obtained using excess GSH (1.5 mM); GSH was not added to the kinetic assays with BITC-SG or PEITC-SG. Inhibition of GST activity was obtained with 1 mM CDNB, varied [GSH] and 0, 5, 15 or 50 $\mu$M BITC-SG. Assays were carried out as described in the Experimental section. $K_i$ and $K_m$ values are expressed in $\mu$M and $V_{max.}$ values in $\mu$mol/min per mg of protein. Standard errors are within 14%.

| Kinetic characteristic | GST A1–1 | GST M1a–1a |
|---|---|---|
| $K_m$ for BITC | 166 | 60 |
| $V_{max.}$ for BITC | 12 | 39 |
| $K_m$ for BITC-SG | Not determined | 38 |
| $V_{max.}$ for BITC-SG | Not determined | 0.7 |
| $K_m$ for PEITC-SG | 6.9 | 27 |
| $V_{max.}$ for PEITC-SG | 0.4 | 0.5 |
| $K_i$ for BITC-SG as competitive inhibitor with respect to GSH | 4.3 | 45 |
| $K_m$ for GSH | 220 | 160 |

with protein thiol because the enzyme contains no cysteine residue [21].

## Kinetic analysis

Kinetic analysis was carried out to obtain data that might correlate with the apparent ability of GST A1–1, but not GST M1a–1a, to alter isothiocyanate–dithiocarbamate equilibria by dithiocarbamate sequestration. GST M1a–1a yielded similar $K_m$ values for BITC-SG and PEITC-SG and these were similar to the $k_i$ for BITC-SG in the inhibition of GST activity (27–45 $\mu$M range; Table 2). The $K_m$ for BITC was only slightly higher. In contrast, GST A1–1 yielded a $K_i$ for BITC-SG and a $K_m$ for PEITC-SG in the micromolar range, about 30-fold lower than the $K_m$ for BITC. (Note that the activity with BITC-SG was too

low for simple kinetic analysis.) The $K_i$ for BITC-SG with GST P1–1 was approx. 250 $\mu$M, much higher than that of the other GSTs. The data suggest that only the interaction of GST A1–1 with BITC-SG and PEITC-SG occurs at sufficiently low concentration (5 $\mu$M) to have a significant sequestration effect under the conditions of the experiments described above, i.e. isothiocyanate plus dithiocarbamate, 30 $\mu$M.

## DISCUSSION

By definition, enzymes are catalysts; hence, at low concentration, they accelerate a reaction without altering its stoichiometry. However, many enzymes occur at such a high concentration with respect to their substrate(s) that they have to be considered as components of the reaction. In the case of the reaction of GSH with hydrophobic electrophiles, GSTs of the Alpha, Mu and Pi classes often occur at concentrations similar to or greater than the electrophiles and their GSH conjugates (products). For enzymes, they also have unusually low $K_m$ values for GSH relative to normal cellular levels of GSH (approximately one-fiftieth) which are associated with a relatively high affinity for GSH conjugates and poor catalytic efficiency [3]. Therefore product binding may be an important function of these enzymes *in vivo*. In order to characterize the ability of human GSTs to act as both catalysts and product binders in a single reaction, we have studied the reversible reaction of GSH with BITC and PEITC.

The data in Table 1 show that GSTs A1–1, A2–2, M1a–1a and P1–1, when present at low concentration (less than 1 $\mu$M), accelerate both the forward and reverse reactions, as expected for true catalysts. This has not previously been shown for any GST-dependent reaction.

With regard to the proposed ability of GSTs to shift equilibria by GSH-conjugate sequestration [3], kinetic analysis (Table 2) suggested that GST A1–1 interacted with PEITC-SG and BITC-SG in the micromolar range, strongly favouring GSH-conjugate binding over that of either electrophile or GSH substrates. In contrast, GST M1a–1a interacted with the GSH conjugates at higher concentration (30–60 $\mu$M), only weakly favouring interaction with GSH conjugates over that of GSH. The activity of GST P1–1 was relatively insensitive to inhibition by BITC-SG. From these results, GST A1–1 is predicted to sequester the GSH conjugates when both are present in micromolar or greater concentration, and shift the equilibrium. Studies showing apparent increases in PEITC-SG and BITC-SG at equilibrium in the presence of GST A1–1 but not GST M1a–1a (Figures 1–3) are consistent with the above prediction. The data in Figure 3 may be used to calculate an approximate association constant for BITC-SG with GST A1–1 [22]. The value obtained of $1.7 \times 10^5$ $M^{-1}$, corresponding to a dissociation constant of 6 $\mu$M, is comparable with the $K_i$ value for BITC-SG for GST A1–1 (Table 2). It is concluded that GST A1–1 and, to a lesser extent, GST A2–2 act as both catalysts and product sequestrators in these reactions at the concentrations of substrates and products selected (10–30 $\mu$M). GST M1–1 is also expected to sequester BITC-SG and PEITC-SG and significantly alter the equilibria but at higher product concentrations (approx. 50 $\mu$M).

It was previously suggested [3] that a high affinity for product (GSH conjugate) might contribute to the poor catalytic efficiency of GSTs of the Alpha, Mu and Pi classes. It is worth noting in this regard that there is an inverse correlation between specific activity (GST P1–1 high, GST A1–1 low) and affinity of interaction with the GSH conjugates (GST P1–1 low, GST A1–1 high). Data in Table 2 show that the catalytic specificity and rate enhancements for GST M1a–1a for reversible catalysis of BITC conjugation are characteristically low [$k_{cat.}/K_m$ $2.7 \times 10^5$ $M^{-1} \cdot s^{-1}$; $(K_{cat.}/K_m)/k_2$ $1.5 \times 10^4$].

The utility of the dual role of GSTs (catalysis and product sequestration) may be illustrated by analysis of the metabolism of, e.g., BITC in blood and liver. Consider hepatocytes containing 5 mM GSH and 70 $\mu$M Alpha-class GSTs (A1–1 plus A1–2 plus A2–2) to which 100 $\mu$M BITC is introduced. GST catalysis should cause the reaction to reach equilibrium (99.2 $\mu$M BITC-SG, 0.8 $\mu$M BITC) in less than 1 s. Sequestration by excess GSTs A1–1, A1–2 and A2–2 should further deplete the free BITC to approx. 0.4 $\mu$M resulting in negligible alkylation of protein thiols in the hepatocyte except for those showing unusually high specificity for BITC, e.g. mouse and rat cytochromes *P*-450 metabolizing 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [23,24]. In the blood plasma, the low level of GSH and other low-molecular-mass thiols [25] means that formation of BITC-SG or similar low-molecular-mass dithiocarbamate from 100 $\mu$M BITC would be very slow ($< 0.6$ $\mu$M/min). The predominant reaction is likely to be with the most abundant thiol, serum albumin (0.5 mM). The second-order rate constant for the reaction of BITC with human serum albumin is comparable with that for reaction with GSH (results not presented), and the initial rate of reaction of 100 $\mu$M BITC with plasma albumin would be 1.5 $\mu M \cdot s^{-1}$. If BITC enters erythrocytes, haemoglobin thiol (20 mM) is also likely to be a significant early target, particularly as GST concentration is unusually low in the erythrocyte (approx. 0.07 $\mu$M [26]).

Low levels of GSTs are detectable in human serum and, interestingly, may be significantly increased by eating Brussels sprouts which contain aralkyl isothiocyanates [27]. Because of the low plasma level of GSH, plasma GSTs can be predicted to accelerate the back reaction, i.e. the formation of free isothiocyanate from their respective GSH conjugates, the latter arising from intracellular GSH conjugation and subsequent export into plasma. Hence plasma GSTs combined with low plasma [GSH] would promote reaction of isothiocyanates with serum albumin. Reaction of the isothiocyanates with abundant thiols in blood and sequestration of the GSH conjugates by GSTs, particularly GSTs A1–1, A1–2, A2–2, in the liver and kidney should result in a relatively slow excretion of such compounds. This is seen in a detailed study of metabolism of PEITC in the mouse [28].

The above discussion omits the possibility that product binding by GSTs is involved in transport and delivery to cellular efflux pump(s). We have tested the effect of GST A1–1 on ATP-dependent uptake of the GSH conjugate of bromosulphophthalein by inverted human erythrocyte ghosts. Rather than stimulating uptake, the GST inhibited uptake (J. M. Harris and D. J. Meyer, unpublished work). However, much remains to be done before GST-dependent GSH conjugate delivery, perhaps specific to liver or kidney, can be excluded.

The anticancer properties of organic isothiocyanates have been attributed to several effects including a selective toxicity to tumour cells [29], blocking of procarcinogen activation by inhibition of phase-I enzymes [11,23,24] and induction of phase-II enzymes including GSTs [15,16,30–32]. Cellular GSH and GSTs have been implicated in numerous cases as contributory factors in resistance of tumours to therapy. Naturally occurring relatively non-toxic compounds such as PEITC and BITC might be useful in preventing such resistance by virtue of their GST-mediated GSH depletion, and concomitant GST inhibition by the GSH conjugates which may prevent detoxication of the cytotoxic therapeutic agent. As GST P1–1, which is the major GST in many drug-resistant tumours, is not very sensitive to inhibition by BITC-SG or PEITC-SG, a more potent isothiocyanate should be sought. A mixture of isothiocyanates yielding

GSH conjugates displaying a combined broad inhibitory specificity towards the various human GSTs might be very effective.

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