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Inactivation of the Genotoxic Aldehyde Acrolein by Human Glutathione Transferases of Classes Alpha, Mu, and Pi

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SUMMARY

Acrolein, a genotoxic aldehyde released in the metabolic activation of the cytostatic drug cyclophosphamide, is inactivated by glutathione transferases either by conjugation with reduced glutathione or by covalent binding to the enzymes in the absence of glutathione. The catalytic efficiency (k_{cat}/K_m) with acrolein as a substrate was determined for representatives of the three classes Alpha, Mu, and Pi of human glutathione transferases. Transferase π exhibited the highest and transferase ϵ the lowest catalytic efficiencies, respectively. As measured by the k_{cat}/K_m value, acrolein ranks among the most active substrates known for transferase π. The irreversible binding of acrolein to the enzymes was monitored as the inactivation of the enzyme activity. Transferase π reacted significantly more rapidly with acrolein than did transferases μ and ϵ.

Acrolein (prop-2-enal) is a toxic aldehyde that occurs as an environmental pollutant as well as a metabolite generated within living cells. For example, acrolein is found in tobacco smoke (1) and engine exhaust of cars (2), and it is a metabolite of cyclophosphamide (3, 4), a cytostatic drug used in the chemotherapy of cancer (5). Moreover, acrolein is generated from allyl alcohol (6, 7) and nitropropanol (8) by alcohol dehydrogenase-catalyzed reactions, and it is produced during lipid peroxidation (9).

Acrolein reacts spontaneously with free sulfhydryl groups of macromolecules (10), and covalent binding is believed to be a major mechanisn by which acrolein exerts its toxic action in the cell (7). The ability of acrolein to inhibit glutathione transferase activity in the rat has been investigated (11). Acrolein has also been shown to cause single-strand DNA breaks, an effect which is believed to play a role in the cytotoxicity of cyclophosphamide (12).

Glutathione transferases (EC 2.5.1.18) catalyze the conjugation of a number of electrophilic compounds with reduced glutathione (13). Various α,β-unsaturated carbonyl compounds have been reported to be substrates for the glutathione transferases in a crude cytosol fraction from rat liver (14).

The major role of glutathione transferases is believed to be detoxication of xenobiotics (15) as well as toxic compounds that arise as metabolic products in the cell (16–18). Moreover, the glutathione transferase level has been found to be elevated in a cyclophosphamide-resistant Yoshida carcinoma cell line, as compared with a drug-sensitive cell line (19). This observation, together with the broad number of possible substrates, makes the assumption that the transferases are involved in drug resistance reasonable.

Glutathione transferases are abundant in the cytosol fraction of cells and occur in three functionally and structurally distinct classes termed Alpha, Mu, and Pi (20). The tissue distributions of the multiple forms of glutathione transferase are distinct for the different isoenzymes. Some tissues may contain representatives of all three classes, but others are dominated by a single glutathione transferase. In view of the distinct functional properties of the different forms of glutathione transferase, the differential tissue distribution may have consequences for tissue-selective toxicity. For example, cells lacking class Mu glutathione transferases, which are particularly active in the detoxication of epoxides (21), may be more sensitive to this group of genotoxic compounds.

In the present study, representatives of each of the three classes of glutathione transferase have, therefore, been used to investigate the role of human glutathione transferases in the inactivation of acrolein. Classes Alpha, Mu, and Pi are represented by glutathione transferases ϵ, μ, and π, respectively. Their efficiencies in catalyzing the conjugation reaction with reduced glutathione as well as their abilities to covalently bind acrolein have been investigated as alternative mechanisms of detoxication.

Materials and Methods

Chemicals. All chemicals were standard commercial products. Acrolein was obtained from Serva (New York).

Enzymes. Glutathione transferases ϵ and μ were isolated from

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ABBREVIATION: CDNB, 1-chloro-2,4-dinitrobenzene.

human adult liver (22) and transferase π from placenta (23) by use of affinity chromatography on immobilized *S*-hexylglutathione and chromatofocusing. Glutathione transferase ϵ is the major form in liver, also referred to as transferase B_1B_1 (24).

Assay of enzyme activity. CDNB activity was based on the reaction between CDNB (1 mM) and glutathione (1 mM) in a 1-ml reaction system containing 0.1 M sodium phosphate buffer, pH 6.5 at 30° (final volume, 1 ml) (25). The conjugation of acrolein with glutathione in the same reaction system was measured spectrophotometrically at 215 nm, using an extinction coefficient of 15 mM^{-1} cm^{-1}. Due to the high absorbance of the substrates at 215 nm, their concentrations were limited to 0.1 mM acrolein and 0.5 mM glutathione in the reaction system. A Varian 2290 spectrophotometer was used for the measurements.

Inactivation of enzyme activity by acrolein. Glutathione transferases ϵ, μ, and π were incubated with 0.25 and 0.5 mM acrolein in 0.1 M sodium phosphate buffer, pH 7.4, at 25°. Aliquots of 10 μl of the incubation mixture were taken at intervals for measurements of remaining CDNB activity (at pH 6.5) in the system described above. An incubation mixture that did not contain acrolein was used as a control in the experiment.

Results

Acrolein as a substrate for glutathione transferases. Table 1 shows activities of representatives of each of the three classes of human glutathione transferases. The specific activities with acrolein as substrate range from 1 to 25% of the corresponding specific activities with the standard substrate CDNB. Glutathione transferase π showed the highest and transferase ϵ the lowest activity.

The specific activities obtained with the two alternative electrophilic substrates are not comparable, because the substrate concentrations used in the assays are significantly different. A more relevant comparison can be made by use of the specificity constants, k_{cat}/K_m, which reflect the catalytic efficiencies at low substrate concentrations. Fig. 1 shows the linear plots of initial rates divided by total enzyme concentration ($v/[E_o]$) versus substrate concentration, from which specificity constants can be determined (cf. Ref. 18). Using this parameter, acrolein is comparatively a better substrate than is indicated by the specific activities. Thus, for all transferases the ratio of k_{cat}/K_m values for acrolein and CDNB is higher than the corresponding ratio of specific activities for acrolein and CDNB (Table 1). In the case of glutathione transferase π, the specificity constants show that acrolein is indeed a 2.7-fold more active substrate than is CDNB. In contrast, almost all previous comparisons of specificity constants made to date with a large number of glutathione transferases have demonstrated CDNB to be the most active substrate.

It should be noted that the k_{cat}/K_m values were determined at saturating glutathione concentrations; no significant difference between values obtained at 0.1 and 0.5 mM glutathione could be detected. The nonenzymatic reaction between glutathione and acrolein was also studied for comparison. It was found to be strictly second-order with respect to the two reactants. The rate constant was determined as 2.3×10^3 M^{-1} sec^{-1} at pH 6.5 and 30°.

The effect of pH on the enzymatic reaction with acrolein was also investigated. The nonenzymatic reaction rate was found to increase strongly with pH, whereas the enzymatic reaction rate was essentially constant in the range of pH 6 to 7.4. The specific activities for glutathione transferase π determined at pH 6.0, 6.5, 7.0, and 7.4 were 25.0 ± 1.9. 26.3 ± 2.1, 26.0 ± 9.3, and 25.0 ± 3.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively.

Inactivation of glutathione transferases by acrolein. Fig. 2 shows time-dependent inactivation of glutathione transferases by acrolein. Enzyme activity was not recovered by dialysis for 24 hr at 4° against 10 mM Tris·HCl, pH 7.8, indicating a covalent modification of the protein. The inactivation of the enzymes was a pseudo-first-order rate process under the condition investigated, and the corresponding $t_{1/2}$ values were found to be inversely proportional to the acrolein concentration used. From the dependence of $t_{1/2}$ on the inactivator concentration, the second-order rate constants of inactivation were calculated for the different enzymes. It was found that the three transferases were significantly different in their sensitivities to acrolein. Glutathione transferase π was the most sensitive enzyme, with an inactivation rate constant of 11.4 $M^{-1} \cdot sec^{-1}$ at pH 7.4 and 22°. Glutathione transferases μ and ϵ were inactivated with corresponding rate constants of 0.11 $M^{-1} \cdot sec^{-1}$ and 0.002 $M^{-1} \cdot sec^{-1}$, respectively. The process was strongly pH dependent for all enzymes. At pH 8, the rate of inactivation was 4–7-fold higher than at pH 7.4, whereas at pH 6.5 the rate was lower by more than a factor of 2; pH 7.4 was taken to approximate physiologically relevant conditions. Increases of the temperature of the reaction system also gave higher rates. At 37° the rate constant for the reaction between acrolein and glutathione transferase π was determined as 117 $M^{-1} \cdot sec^{-1}$. The enzyme could be protected by glutathione; after addition of 0.5 mM glutathione to the incubation mixture containing 0.1 mM acrolein, no further inactivation was noted, and after dialysis the remaining enzyme activity was not significantly different from that at the time of addition.

TABLE 1

Specific activities and k_{cat}/K_m values for human glutathione transferases using acrolein and 1-chloro-2,4-dinitrobenzene as electrophilic substrates

k_{cat}/K_m values were determined by measurements at low concentrations of electrophilic substrates (20 and 40 μM) and a saturating concentration of glutathione (0.5 mM). Specific activities were obtained by measurements at pH 6.5, 30°, in the standard assay systems (see Materials and Methods); the values for CDNB are from Ref. 21. The values given are the means of seven experiments; corresponding standard deviations are in general ≤10% of the means.

GST	Specific activity		k_{cat}/K_m	
	Acrolein	CDNB	Acrolein	CDNB
	$\mu mol \cdot min^{-1} \cdot mg^{-1}$		$M^{-1} \cdot sec^{-1}$	
ϵ	0.86	82	0.01	0.1
μ	7.05	187	0.06	0.26
π	26.3	105	0.35	0.13

Discussion

The results of the present investigation show that glutathione transferases may contribute to the detoxication of acrolein. Two mechanisms may lead to the inactivation of the toxic electrophile, transferase-catalyzed conjugation with glutathione and covalent binding of acrolein to the enzyme protein. At high glutathione concentrations, the enzyme-catalyzed reaction dominates, as judged by the lack of inactivation of the enzyme during catalytic experiments. At limiting glutathione concentrations, direct binding of acrolein to the enzyme may be significant, in view of the high intracellular concentrations of the glutathione transferases; the subunit concentration of the enzymes in liver tissue can be estimated as approaching 0.05 mM. Both in the enzyme-catalyzed conjugation with glutathi-

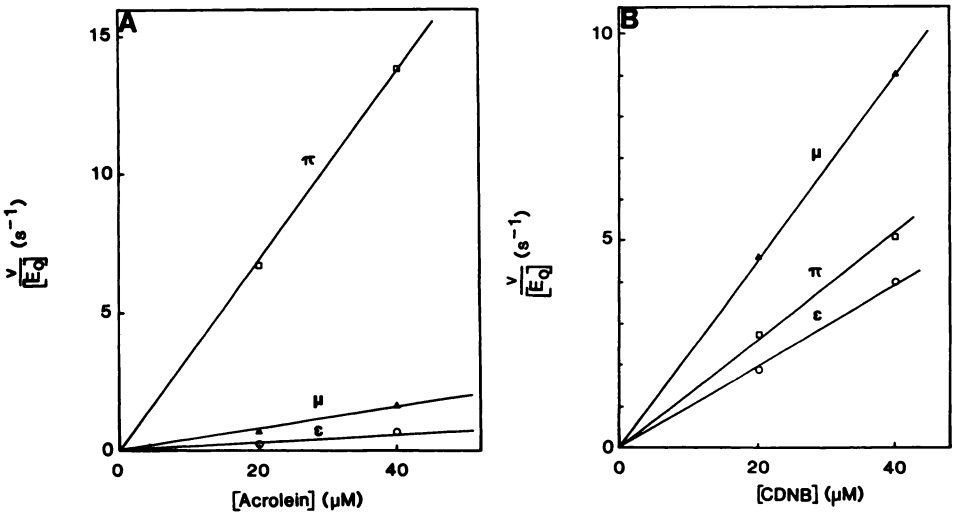

Fig. 1. Enzymatic activities determined at low concentrations of acrolein (A) and CDNB (B). Glutathione transferases π (□), μ (▲), and ε (○) were assayed with 500 μM reduced glutathione at pH 6.5 and 30°. The slopes of the graphs of $v/[E_o]$ (initial rates divided by total enzyme concentration) versus substrate concentration correspond to the k_{cat}/K_m values of the respective enzyme forms. The *points* represent means of 6–10 replicate experiments (standard deviations, ≤10% of the values).

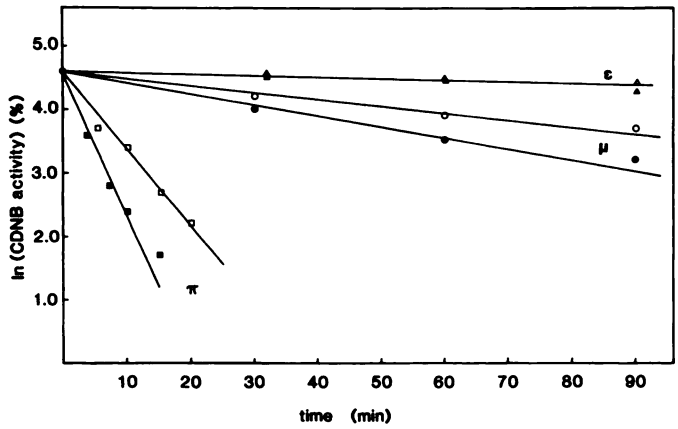

Fig. 2. Irreversible inactivation of glutathione transferases by acrolein. The incubation mixture at pH 7.4 and 22° contained 250 μM (□) or 500 μM (■) acrolein and 0.6 μM glutathione transferase π, 250 μM (○) or 500 μM (●) acrolein and 0.1 μM glutathione transferase μ, or 250 μM (△) or 500 μM (▲) acrolein and 3 μM glutathione transferase ε. Remaining enzyme activity was measured in the standard assay system using CDNB as electrophilic substrate.

one and in the covalent binding, as measured by irreversible inhibition of activity, glutathione transferase π was found to be the most active of the human enzymes tested. Glutathione transferase ε displayed the lowest catalytic activity and the lowest degree of inactivation, whereas glutathione transferase μ had intermediate properties in both respects.

In evaluating the kinetic efficiency of enzymes involved in detoxication reactions, the parameter k_{cat}/K_m is the most appropriate measure, because this value gives the catalytic activity at the low substrate conditions that are normally expected to occur intracellularly. Using this parameter it was found that acrolein is a very good substrate for the transferases, as gauged by corresponding values obtained with CDNB. The latter substrate, in almost all cases investigated, has been found to display an activity which is higher by at least 1 or 2 orders of magnitude than that of alternative substrates (21). Therefore, it is noteworthy that the k_{cat}/K_m value determined for transferase π with acrolein is higher than the value obtained with CDNB. The structure of the conjugate of acrolein and glutathione has not been determined in this investigation, but the thioether formed by addition of sulfur to the β-carbon of the allylic double bond of acrolein is generally considered to be the product both *in vivo* and *in vitro* (cf. Ref. 3).

The high activity obtained with acrolein (k_{cat}/K_m = 0.35 $\mu M^{-1} \cdot sec^{-1}$) should be compared with the high activities previously observed with 4-hydroxyalkenals (18), a related group of α,β-unsaturated carbonyl compounds. In the case of the latter substrates, however, glutathione transferase π is among the least active enzymes; k_{cat}/K_m values for substrates of different chain lenghs range between 0.01 and 0.09 $\mu M^{-1} \cdot sec^{-1}$ (18). The 4-hydroxyalkenal molecules have longer carbon chains (C_5–C_{15}) and may be too bulky for optimal fitting to the active site of glutathione transferase π. Alternatively, the 4-hydroxy substituent adjacent to the reactive carbon-carbon bond makes these molecules too polar for optimal binding to the active site.

It should be noted that the diuretic ethacrynic acid, another α,β-unsaturated carbonyl compound, has been used as a class Pi-distinguishing substrate (20). However, the specific activity of transferase π with ethacrynic acid, 0.86 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (26), is lower by 1 order of magnitude than the value for acrolein (Table 1).

From the toxicological point of view, the high catalytic efficiency of glutathione transferase π in the inactivation of acrolein may be of considerable significance. Acrolein is genotoxic and carcinogenic and may occur in humans owing to environmental exposure, lipid peroxidation, or metabolism of xenobiotics or chemical compounds endogenous to the cell. From this point of view it becomes significant that transferase π has a broad organ distribution. Tissues such as the lung, the kidney, and the gastrointestinal tract, which may be primary targets of toxicity related to xenobiotics, all contain relatively high concentrations of glutathione transferase π (27–29). However, it is noteworthy that in human liver the enzyme occurs only during the fetal period and not in the adult state (30).

A clinical aspect of the detoxication of acrolein relates to the activation of cyclophosphamide. This cytostatic drug is metabolically activated to a phosphoramide mustard, with concomitant release of acrolein (4). The mustard moiety is believed to exert its cytostatic effect by cross-linking the strands of DNA. The acrolein product is considered to give rise to urotoxic side effects, especially hemorrhagic cystitis, in patients undergoing chemotherapy (31), and its toxic action may also contribute to cell killing in tumors. Thus, it is possible that the inactivation

of acrolein effected by glutathione transferases may not only provide protection of normal tissues but also confer resistance to tumor cells. In this connection, it is particularly noteworthy that glutathione transferase π is usually expressed at high levels in tumor cells (32, 33). The protective effect may be exerted by catalysis of glutathione conjugation as well as by covalent binding of acrolein to the enzyme. In rodent tumor models, acquired drug resistance has previously been associated with glutathione transferases distinct from class Pi (34). Acquired resistance against chlorambucil has been associated with overexpression of a class Alpha enzyme (35), and resistance against bis-chloroethylnitrosourea has been linked to elevated levels of rat isoenzymes of the class Mu (36).

The results of the present investigation show that overexpression of glutathione transferase π will probably lead to increased resistance against acrolein, one of the products of the metabolic activation of cyclophosphamide. Whether glutathione transferase π may also protect against the toxic effect of the mustard part of cyclophosphamide remains to be demonstrated. In any event, the present results show properties of human glutathione transferase π that can be functionally linked to a potential role in drug resistance. This suggested role is presently being probed by transfection of sensitive cells with DNA encoding glutathione transferase π and study of the effect of gene expression on the resistance properties of the cell.

References

1. Beauchamps, R. O. Jr., D. A. Andjelkovich, A. D. Kligerman, K. T. Morgan, and H. d'A. Heck. A critical review of the literature on acrolein toxicity. *Crit. Rev. Toxicol.* **14**:309–380 (1985).
2. Tanimoto, M., and H. Uehara. Detection of acrolein in engine exhaust with microwave cavity spectrometer of stark voltage sweep type. *Environ. Sci. Technol.* **9**:153–154 (1975).
3. Alarcon, R. A. Studies on the *in vivo* formation of acrolein: 3-hydroxypropyl mercapturic acid as an index of cyclophosphamide (NSC-26271) activation. *Cancer Treat. Rep.* **60**:327–335 (1976).
4. Alarcon, R. A., and J. Meienhofer. Formation of the cytotoxic aldehyde acrolein during *in vitro* degradation of cyclophosphamide. *Nature New Biol.* **233**:250–252 (1971).
5. Friedman, O. M., I. Wodinsky, and A. Myles. Cyclophosphamide (NSC-26271)-related phosphoramide mustards: recent advances and historical perspective. *Cancer Treat. Rep.* **60**:337–346 (1976).
6. Reid, W. D. Mechanism of allyl alcohol-induced hepatic necrosis. *Experientia (Basel)* **28**:1058–1061 (1972).
7. Rando, R. R. Allyl alcohol-induced irreversible inhibition of yeast alcohol dehydrogenase. *Biochem. Pharmacol.* **23**:2328–2331 (1974).
8. Alston, T. A., S. P. Seitz, and H. J. Bright. Conversion of 3-nitro-1-propanol (miserotoxin aglycone) to cytotoxic acrolein by alcohol dehydrogenase. *Biochem. Pharmacol.* **30**:2719–2720 (1981).
9. Schauenstein, E., and H. Esterbauer. Formation and properties of reactive aldehydes. *Ciba Found. Symp.* **67**:225–224 (1979).
10. Esterbauer, H., H. Zollner, and N. Scholz. Reaction of glutathione with conjugated carbonyls. *Z. Naturforsch. Teil C Biochem. Biophys. Biol. Virol.* **30**:446–473 (1975).
11. Scott, T. R., and R. E. Kirsch. Inhibition of rat glutathione *S*-transferase isoenzymes by acrolein. *Biochem. Int.* **16**:439–442 (1988).
12. Crook, T. R., R. L. Souhami, and A. E. M. McLean. Cytotoxicity, DNA cross-linking and single strand breaks induced by activated cyclophosphamide and acrolein in human leukemia cells. *Cancer Res.* **46**:5029–5034 (1986).
13. Jakoby, W. B. The glutathione *S*-transferases: a group of multifunctional detoxication proteins. *Adv. Enzymol.* **46**:383–414 (1978).
14. Boyland, E., and L. F. Chasseaud. Enzyme-catalyzed conjugations of glutathione with unsaturated compounds. *Biochem. J.* **104**:95–102 (1967).
15. Jakoby, W. B., and W. H. Habig. Glutathione transferases, in *Enzymatic Basis of Detoxication* (W. B. Jakoby, ed.), Vol. 2. Academic Press, New York, 63–94 (1980).
16. Esterbauer, H. Aldehydic products of lipid peroxidation, in *Free Radicals, Lipid Peroxidation and Cancer* (D. C. H. McBrien and T. F. Slater, eds.). Academic Press, London, 101–122 (1982).
17. Ålin, P., U. H. Danielson, and B. Mannervik. 4-Hydroxyalk-2-enals are substrates for glutathione transferase. *FEBS Lett.* **179**:267–270 (1985).
18. Danielson, U. H., H. Esterbauer, and B. Mannervik. Structure-activity relationships of 4-hydroxyalkenals in the conjugation catalysed by mammalian glutathione transferases. *Biochem. J.* **247**:707–713 (1987).
19. McGown, A. T. and B. W. Fox. A proposed mechanism of resistance to cyclophosphamide and phosphoramide mustard in a Yoshida cell line *in vitro*. *Cancer Chemother. Pharmacol.* **17**:223–226 (1986).
20. Mannervik, B., P. Ålin, C. Guthenberg, H. Jensson, M. K. Tahir, M. Warholm, and H. Jörnvall. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. *Proc. Natl. Acad. Sci. USA.* **82**:7202–7206 (1985).
21. Mannervik, B., and U. H. Danielson. Glutathione transferases: structure and catalytic activity. *Crit. Rev. Biochem.* **23**:283–337 (1988).
22. Warholm, M., C. Guthenberg, C. von Bahr, and B. Mannervik. Glutathione transferases from human liver. *Methods Enzymol.* **113**:499–504 (1985).
23. Mannervik, B., and C. Guthenberg. Glutathione transferases (human placenta). *Methods Enzymol.* **77**:231–235 (1981).
24. Stockman, P. K., L. I. McLellan, and J. D. Hayes. Characterization of the basic glutathione *S*-transferase B_1 and B_2 subunits from human liver. *Biochem. J.* **244**:55–61 (1987).
25. Habig, W. H., and W. B. Jakoby. Assays for differentiation of glutathione *S*-transferases. *Methods Enzymol.* **77**:398–405 (1981).
26. Guthenberg, C., and B. Mannervik. Glutathione *S*-transferase (transferase π) from human placenta is identical or closely related to glutathione *S*-transferase (transferase ρ) from erythrocytes. *Biochim. Biophys. Acta* **661**:255–260 (1981).
27. Tateoka, N., S. Tsuchida, Y. Soma, and K. Sato. Purification and characterization of glutathione *S*-transferases in human kidney. *Clin. Chim. Acta* **166**:207–218 (1987).
28. Peters, W. H. M., H. M. J. Roelofs, F. M. Nagengast, and J. H. M. van Tongeren. Human intestinal glutathione *S*-transferases. *Biochem. J.* **257**:471–476 (1989).
29. Mannervik, B. The isoenzymes of glutathione transferase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **57**:357–417 (1985).
30. Guthenberg, C., M. Warholm, A. Rane, and B. Mannervik. Two distinct forms of glutathione transferase from human foetal liver: purification and comparison with isoenzymes isolated from adult liver and placenta. *Biochem. J.* **235**:741–745 (1986).
31. Hill, D. L. *A Review of Cyclophosphamide.* C. Thomas, Springfield, IL (1975).
32. Mannervik, B., V. M. Castro, U. H. Danielson, M. K. Tahir, J. Hansson, and U. Ringborg. Expression of class *pi* glutathione transferase in human malignant melanoma cells. *Carcinogenesis (Lond.)* **8**:1929–1932 (1987).
33. Shea, T. C., S. L. Kelley, and W. D. Henner. Identification of an anionic form of glutathione transferase present in many human tumors and human tumor cell lines. *Cancer Res.* **48**:527–533 (1988).
34. Wang, A. L., and K. D. Tew. Increased glutathione *S*-transferase activity in a cell line with acquired resistance to nitrogen mustards. *Cancer Treat. Rep.* **69**:677–682 (1985).
35. Lewis, A. D., I. D. Hickson, C. N. Robson, A. L. Harris, J. D. Hayes, S. A. Griffiths, M. M. Manson, A. E. Hall, J. E. Moss, and C. R. Wolf. Amplification and increased expression of *alpha*-class glutathione *S*-transferase-encoding genes associated with resistance to nitrogen mustards. *Proc. Natl. Acad. Sci. USA* **85**:8511–8515 (1988).
36. Smith, M. T., C. G. Evans, P. Doane-Setzer, V. M. Castro, M. K. Tahir, and B. Mannervik. Denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosourea by class *mu* glutathione transferases and its role in cellular resistance in rat brain tumor cells. *Cancer. Res.* **49**:2621–2625 (1989).

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