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The polymorphic human glutathione transferase T1-1, the most efficient glutathione transferase in the denitrosation and inactivation of the anticancer drug 1,3-bis(2-chloroethyl)-1-nitrosourea

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Abstract

A member of the Theta class of human glutathione transferases (GST T1-1) was found to display the greatest catalytic activity towards the cytostatic drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) of the GSTs studied. In this investigation (the most extensive to date), enzymes from four classes of the soluble human GSTs were heterologously expressed, purified, and kinetically characterized. From the 12 enzymes examined, only GST M2-2, GST M3-3 and GST T1-1 had significant activities with BCNU. This establishes that the activity is not a characteristic of a particular class of GSTs. Although GST M3-3 was previously reported to have the greatest activity with BCNU, the current investigation demonstrates that GST M2-2 is equally active and that GST T1-1 has an approximately 20-fold higher specific activity than either of the Mu class enzymes. A more rigorous kinetic analysis of GST T1-1 gave the following parameters with BCNU: a k_{cat} of 0.035 ± 0.003 s^{-1} and a K_M of 1.0 ± 0.1 mM. The finding that GST T1-1 has the highest activity towards BCNU is significant since GST T1-1 is expressed in the brain, a common target for BCNU treatment. Furthermore, the existence of a GST T1-1 null allele in up to 60% in some populations, may influence both the sensitivity of tumors to chemotherapy and the severity of adverse side-effects in patients treated with this agent.

Keywords: Glutathione transferase; BCNU; Denitrosation; Drug resistance

1. Introduction

The antitumor reagent known as BCNU is a member of the nitrosourea family of cytostatic drugs. Since 1975 this group of compounds has been used in the treatment of melanomas, lung carcinomas, lymphomas and brain tumors [1]. They are thought to exert their antitumor effect mainly by causing the formation of DNA interstrand cross-links, but there is also evidence that they can directly inhibit cellular functions by the covalent modification of enzymes such as thioredoxin reductase, glutathione reductase and ribonucleotide reductase [2]. Upon introduction to the body, the nitrosoureas rapidly undergo a spontaneous hydrolytic decomposition, resulting in the release of bifunctionally alkylating chloroethylcarbonium ions and organic isocyanates. These chemically reactive degradation products then cause the formation of chloroethyl adducts at the O^6 position of guanine bases in the DNA, as well as generating alkyl- and carbamoyl-modified proteins (reviewed by [3]).

The efficacy of BCNU treatment is limited by the outgrowth of drug resistant tumor cell populations. Resistance to BCNU can arise by a variety of mechanisms, primarily ascribed to the overexpression of the DNA repair protein O^6-alkylguanine DNA alkyltransferase [4]. It can also be due to the overexpression of drug detoxifying enzymes which are thought to act by preventing the formation of alkylation and carbamoylating species by consuming the parental BCNU. These detoxifying enzymes include enzymes such as the cytochrome P450s and GSTs. It has been established that both cytochrome P450s and GSTs catalyze the denitrosation of BCNU in the presence of NADPH and GSH, respectively [5]. However, the drug-metabolizing cytochrome P450s are found primarily in the liver and are, therefore, unlikely to be a predominant cause of the BCNU resistance seen in many different

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Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GST, glutathione transferase (EC 2.5.1.18); GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; EPNP, 1,2-epoxy-3-(4-nitrophenoxy)propane.

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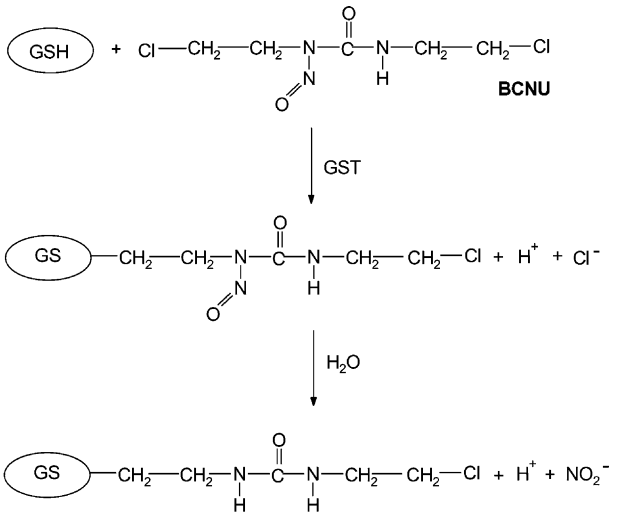

Fig. 1. Reaction scheme proposed for glutathione (GSH) conjugation and denitrosation of the cytostatic drug BCNU catalyzed by GSTs [7].

tumor types. In contrast, mammalian GSTs are found both in membranes and as a family of soluble proteins and are expressed in tissues throughout the body. The cytosolic GSTs have been divided into several classes, based on their primary structures. The most well characterized classes have been named Alpha, Mu, Pi and Theta and, with the exception of the Pi class, each of these classes contains several different isoenzymes [6].

The reaction proposed to be catalyzed by the GSTs [7] is shown in Fig. 1. We have chosen to focus our study on the soluble GSTs since these enzymes are well known to be upregulated in a variety of tumor cells. Several investigations have already been conducted to examine the relationship between the expression of GSTs (particularly Pi and Mu class enzymes) and resistance to BCNU [8–11]. The results suggest that GSTs in tumor cells provide protection against the cytostatic drug by catalyzing its degradation. However, some of these investigations were performed using animal models and only one of the studies featured a systematic investigation of the denitrosation of BCNU with purified human GSTs [9].

We now report a survey of the denitrosation activities of human cytosolic GSTs with BCNU. The enzymes were heterologously expressed and purified, allowing kinetic comparisons of their activities with BCNU to be performed. It is demonstrated that a member of the Theta class GSTs has the highest activity with BCNU. This finding sheds new light on the possible relationship between GST phenotype and clinical resistance to BCNU.

2. Materials and methods

2.1. Materials

All enzymes used were recombinant proteins obtained by heterologous expression in *Escherichia coli*. Our colleagues in the laboratory kindly provided many of the GST preparations. The plasmids for expression of GST M4-4 [12] and GST M5-5 [13] were a gift from Y. Patskovsky and I. Listowsky (Albert Einstein College of Medicine, New York). BCNU was provided by S. Vitols (Karolinska Hospital, Stockholm). All other chemicals used were of the highest purity available.

2.2. Expression and purification of GSTs

Human GST T1-1 was purified as described by Jemth and Mannervik [14] and rat GST T2-2 as described by Jemth *et al.* [15]. For the expression of human GST M4-4, bacteria carrying the plasmid pET3a(hGSTM4) were used as described by Gustafsson and Mannervik [16] with the exceptions that the expression of hGST M4-4 was induced at an OD_{600} of 0.4 and the bacteria were then grown for 3 hr before being harvested. The bacterial cell pellet was resuspended in 50 mM sodium phosphate buffer pH 6.0, 0.02% (w/v) azide and lysis was performed by adding lysozyme (1 mg/mL for 30 min at 25°), followed by addition of DNase I (100 ng/mL), freeze-thawing and sonication. After centrifugation the supernatant fraction was loaded onto a GSH–Sepharose affinity column equilibrated with 50 mM sodium phosphate buffer pH 6.0, supplemented with 0.02% (w/v) azide. The column was washed with the same buffer, and GST M4-4 was eluted with 50 mM glycine pH 10. The pH was immediately neutralized by the addition of 0.1 volume of 2 M Tris–HCl pH 7.2 to each fraction. Elution of GST M4-4 was much more efficient with glycine than the 5 mM GSH in phosphate buffer usually used [17].

For the expression of human GST M5-5, bacteria with the plasmid pET3a(hGSTM5) were grown in the same manner as described for cells containing pET3a(hGSTM4) with the exception that they were grown overnight without addition of an inducer. Cell harvesting, lysis and binding of GST M5-5 to the GSH–Sepharose column were performed as for the GST M4-4 purification. Pure human GST M5-5 was eluted from the affinity gel with 5 mM GSH in 50 mM sodium phosphate buffer pH 6.0, containing 0.02% (w/v) azide, since the use of glycine resulted in a rapid loss of enzymatic activity with this protein.

2.3. Standard assays of GST activity

All the GST specific activity measurements were performed at 30° on a Shimadzu UV2501PC spectrophotometer (Shimadzu Scientific Instruments). The activities of the GSTs were determined with their standard substrates. For the majority (all except GST T1-1 and GST T2-2) the activity was measured with 1 mM CDNB and 1 mM GSH in 0.1 M sodium phosphate pH 6.5 as previously described [18]. The progress of the reaction was followed at 340 nm. For GST T1-1 catalytic activity was measured at 360 nm in the same buffer with 0.5 mM EPNP and 10 mM GSH [18].

The activity of GST T2-2 was monitored at 298 nm with 0.5 mM 1-menaphthyl sulfate (MS) and 10 mM GSH in 0.1 M sodium phosphate pH 7 [19]. Specific activities were calculated and expressed as μmol of product formed per min/mg of enzyme (μmol/min mg).

2.4. Standard BCNU denitrosation assay

The extent of BCNU denitrosation was determined by measuring the amount of nitrite formed upon incubation at 37° with 100–300 μg/mL GST in the presence of 0.1 M sodium phosphate pH 7.4 and 5 mM GSH. This was spectrophotometrically monitored at 540 nm after reacting with sulfanilamide and *N*-(1-naphthyl)ethylenediamine [5]. Each 4 mL reaction was performed in a 50 mL Erlenmeyer flask. A blank reaction without enzyme and a standard curve, with known concentrations of sodium nitrite in phosphate buffer and GSH but no BCNU, were always included. The denitrosation reaction was started by the addition of BCNU to a final concentration of 2 mM from a 200 mM stock solution freshly prepared in 95% (v/v) ethanol. Duplicate samples (750 μL) were withdrawn 0, 5, 10, 20 and 30 min after the addition of substrate. Each sample was transferred to an Eppendorf tube containing 750 μL chloroform and immediately vortexed to stop the reaction by removing intact BCNU and denaturing GST. After 5 min centrifugation at 18,000 *g*, 550 μL of the aqueous phase was extracted with 750 μL chloroform to remove any remaining traces of BCNU. After the second extraction 200 μL of the aqueous phase was transferred to an Eppendorf tube containing 400 μL of 50 mM sulfanilamide and 0.5 mM *N*-(1-naphthyl)ethylenediamine dihydrochloride dissolved in 3 M HCl. After vortexing the mixtures were incubated for 20 min at 55°. The samples were cooled to room temperature before measuring A_{540} using a ThermoMax microtitre plate reader (Molecular Devices). The amount of nitrite formed by GST-catalyzed denitrosation of BCNU was calculated from the sodium nitrite standard curve when the contribution from the non-enzymatic reaction had been subtracted. Specific activities were expressed as nmol/min mg.

2.5. Determination of kinetic parameters of BCNU denitrosation

Kinetic parameters for GST T1-1 were obtained by varying the BCNU concentration between 0.25 and 4 mM at a constant GSH concentration of 5 mM. The measurements were performed in 0.1 M sodium phosphate pH 7.4 at 37° in the same way as in the standard denitrosation assay, except that sampling was performed between 0 and 9 min after the addition of substrate (BCNU).

Rates of the denitrosation reactions were adjusted for non-enzymatic background reactions. Kinetic parameters were obtained by non-linear regression analysis in GraphPad Prism Version 2.01 (GraphPad Software). K_M and k_{cat} values were determined by fitting the Michaelis–Menten equation to the experimental data. Catalytic efficiency (k_{cat}/K_M) was determined by fitting the equation $v = (k_{cat}/K_M)[S]/(1 + (1/K_M)[S])$ to the data. Values were expressed as mean ± SD.

3. Results

3.1. Establishing BCNU assay conditions

The linearity of nitrite formation in the BCNU denitrosation reaction under the assay conditions used was demonstrated with all active GSTs. For example, linear time-dependence is illustrated with 300 μg/mL GST M2-2 (Fig. 2). It was also shown that the rate of the reaction was linear with enzyme concentration (data not shown and [9]). High enzyme concentrations and prolonged incubation times resulted in deviations from linearity, as expected when substrate depletion and product inhibition become noticeable.

3.2. BCNU denitrosation activities of GSTs

A survey of the BCNU denitrosation activities of purified GSTs was performed. Members of four GST classes, Alpha, Mu, Pi and Theta, were studied. The Pi class was represented by two allelic variants differing by one amino acid residue (GST P1-1/Ile 105 and GST P1-1/Val 105), which are known to differ in catalytic activity [20]. In addition to the human GSTs, a rat enzyme, rGST T2-2, was investigated. The specific activities of the enzymes are shown in Fig. 3. Most of the GSTs studied did not possess detectable activity towards BCNU under the assay condi-

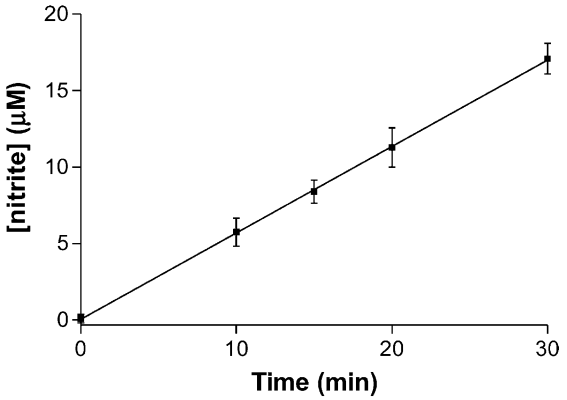

Fig. 2. Demonstration of the linearity of BCNU denitrosation with time. The denitrosation reaction was catalyzed by 300 μg/mL GST M2-2 in an assay with 2 mM BCNU and 5 mM GSH in 0.1 M sodium phosphate buffer pH 7.4 at 37°. Formation of nitrite was determined spectrophotometrically at 540 nm after reaction with sulfanilamide and *N*-(1-naphthyl)ethylenediamine as described in Section 2. Duplicate samples were taken in each of three independent experiments. Values are shown as mean ± SEM.

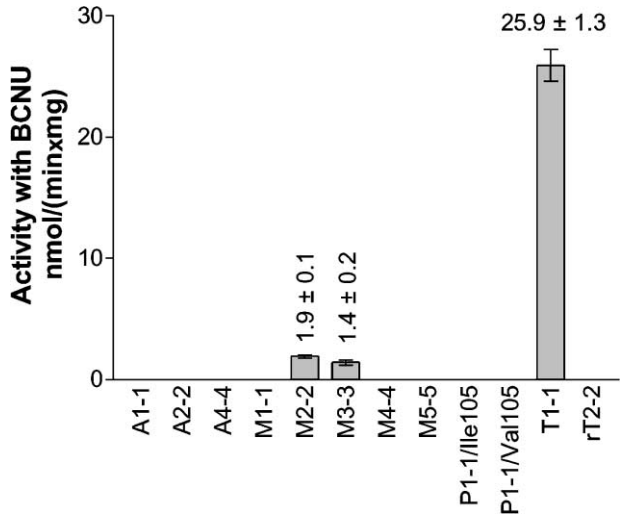

Fig. 3. Specific activities of GSTs assayed with BCNU as a substrate. The activities were determined as the rate of nitrite formation in an assay with 100–300 μg/mL GST, 2 mM BCNU and 5 mM GSH in 0.1 mM sodium phosphate buffer pH 7.4 at 37°. The formation of nitrite was determined spectrophotometrically as described in Section 2. Duplicate samples were taken in three or more independent experiments for each GST. Values are expressed as mean ± SEM. The ratio of the activities with BCNU and the standard substrate CDNB was 4.6×10^{-6} and 1.9×10^{-4} for GST M2-2 and GST M3-3, respectively; the ratio of the activities with BCNU and EPNP, the standard substrate for GST T1-1, was 4.0×10^{-3}. Data for GST M3-3 are from Berhane *et al.* [9] and Comstock *et al.* [39].

tions used. In contrast, the human Theta class enzyme GST T1-1 displayed a specific activity towards BCNU that was more than one order of magnitude greater than that of GST M3-3, the enzyme which had previously been reported to have the highest specific activity with BCNU [9]. Another Mu class enzyme, GST M2-2, which had not been tested for activity with BCNU before, had a similar specific activity to that of GST M3-3. Other members of the Mu class (GSTs M1-1, M4-4 and M5-5) did not share this activity. In order to complete the study of the Theta class family, the rat Theta class enzyme rGST T2-2 was tested for activity with BCNU, since the human GST T2-2 clone was not available. The rat enzyme, which differs from human GST T2-2 in its active site by only one out of 12 H-site residues (Table 1), did not possess detectable activity towards BCNU.

To ascertain that the GSTs without detectable activity towards BCNU were indeed fully active, their specific activities were measured spectrophotometrically against a ''standard substrate''. For most of the enzymes the substrate used was CDNB, but for the Theta class enzymes GST T1-1 and GST T2-2, which have low catalytic activity with CDNB [14,15,21], EPNP and MS were used, respectively. The experimentally determined specific activities for the enzymes were generally greater than or equal to values previously reported in the literature (data not shown), verifying that the lack of detectable activity towards BCNU was due to a real inability to catalyze the denitrosation reaction.

Table 1
Comparison of amino acid residues in the hydrophobic substrate binding site (H-site) of rat GST T2-2, human GST T2-2 and human GST T1-1[a]

Rat GST T2-2	Human GST T2-2	Human GST T1-1
Leu 10	Val 10	Leu 10
Gln 12	Gln 12	Gln 12
Leu 35	Leu 35	Leu 35
Arg 107	Arg 107	Arg 107
Gly 108	Gly 108	Arg 108
Gly 111	Gly 111	Leu 111
Leu 114	Leu 114	Leu 114
Trp 115	Trp 115	Trp 115
Leu 119	Leu 119	Met 119
Glu 175	Glu 175	His 176
Arg 239	Arg 239	Val 235
Arg 242	Arg 242	Met 238

[a] GST T2-2 active site residues were identified by examination of the crystal structure of human GST T2-2 and alignment of the rat primary structure. No crystal structure is available for human GST T1-1. GST T1-1 active site residues are derived from a primary structure alignment and homology modeling based on the crystal structure of human GST T2-2 [26].

3.3. Steady state kinetics of GST T1-1 with BCNU

Given that GST T1-1 has a relatively high catalytic activity with BCNU, it could well be of clinical relevance in the outgrowth of BCNU-resistant tumors. The kinetic behavior of this enzyme with BCNU was, therefore, further characterized at a physiologically relevant concentration of glutathione (5 mM). A typical BCNU saturation curve is shown in Fig. 4. Non-linear regression analysis of the results gave a k_{cat} of $0.035 \pm 0.003\ s^{-1}$, a K_M of 1.0 ± 0.1 mM and a k_{cat}/K_M of (36 ± 2)/M s.

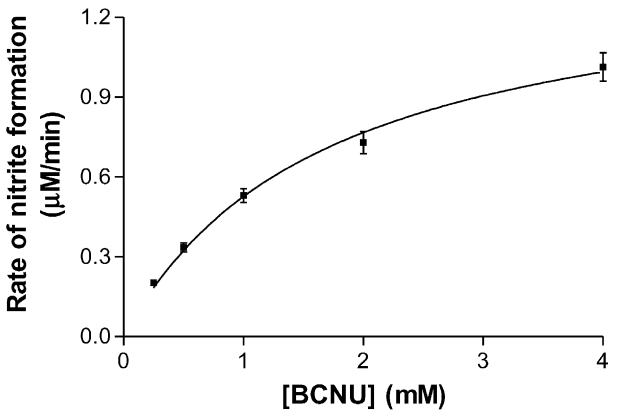

Fig. 4. Kinetics of the denitrosation of BCNU by GST T1-1. The rate of nitrite formation in a reaction with 30 μg/mL GST T1-1 was determined as a function of BCNU concentration. The measurements were performed in the presence of 5 mM GSH in 0.1 M sodium phosphate pH 7.4 at 37°. The graph corresponds to one of three independent experiments. Kinetic parameters ($k_{cat} = 0.035 \pm 0.003\ s^{-1}$ and $K_M = 1.0 \pm 0.1$ mM) were obtained by fitting the Michaelis–Menten equation to the data sets of all three experiments.

4. Discussion

In this study, we have shown that members of the Theta and Mu class isoenzymes are relevant in the GST-catalyzed denitrosation of BCNU. The Theta class enzyme GST T1-1 was demonstrated to be the most efficient catalyst of the reaction, from the many enzymes tested. GST T1-1 had a specific activity with BCNU which was 14-fold greater than that of GST M2-2. GST M3-3, which had previously been known as the GST with the highest activity towards BCNU [9], had a specific activity similar to that of GST M2-2. In comparison, most of the GSTs tested had no detectable activity with BCNU under the assay conditions used.

Despite the significant activities of GST M2-2 and GST M3-3 towards BCNU, the three other members of the Mu class were not found to catalyze this denitrosation to a detectable level. Furthermore, rGST T2-2 did not share the BCNU denitrosation ability of hGST T1-1, implying that activity with this substrate is not a general feature of the Theta class enzymes. It appears that neither allelic variant of GST P1-1 is involved in BCNU resistance *in vivo* (this work and [9]), despite the fact that the GST P1-1 isoenzyme is upregulated in many different cancer types [22–25]. The Alpha class enzymes, with the possible exception of GST A3-3 (not tested), also seem unlikely to contribute to clinical resistance to BCNU.

In order to investigate GST T1-1 further, we examined the steady state kinetics of the denitrosation reaction, with results that were comparable to those of Talcott and Levin [7]. Using mouse liver cytosolic extracts, these workers estimated an apparent K_M for BCNU of 0.8 mM from a Lineweaver–Burk plot, whereas our findings, using purified recombinant protein and non-linear regression algorithms gave a K_M of 1.0 ± 0.1 mM. Furthermore, we determined a k_{cat} of $0.035 \pm 0.003\ s^{-1}$ and a catalytic efficiency (k_{cat}/K_M) of (36 ± 2)/M s. These parameters show that despite the relatively rapid denitrosation of BCNU by GST T1-1, this reaction is quite slow compared to several other reactions catalyzed by the enzyme [14]. On the other hand, another alkylhalogenide substrate, methylene chloride, also gives a low activity with GST T1-1 [14]. We did not investigate the kinetic parameters for glutathione in the denitrosation reaction, but the K_M values for GSH of 1–9 mM found for GST T1-1 in reactions with other substrates [14] suggest that the reaction can be effectively catalyzed at physiological concentrations of glutathione.

It is difficult to identify the active site residues that give rise to the denitrosation activity of GST T1-1 as opposed to GST T2-2, due to the extensive differences in this region of the enzymes (Table 1). Alignments of the residues which define the hydrophobic substrate binding sites (H-sites) of rGST T2-2, hGST T2-2 and hGST T1-1 show that the rat and human GST T2-2 enzymes differ by only a single conservative mutation (Leu10 → Val) in a total of 12 H-site residues. It is, therefore, unlikely that hGST T2-2 exhibits a detectable denitrosation activity with BCNU. In contrast, hGST T1-1 is far more dissimilar to hGST T2-2, with a relative deletion and several non-conservative mutations in the C-terminal region, which is known to be essential for activity in GST T2-2 [26,27].

It is not possible to draw definitive conclusions from prior studies about the most catalytically active GST with BCNU *in vivo*, since in these studies detection of the GSTs was performed by immunoblotting with antisera distinguishing the Alpha, Mu and Pi classes, or by bulk activity measurements with transferase substrates such as CDNB. Given that the Theta class enzymes have very little activity towards CDNB, the most commonly used GST substrate [14,15,21] and were characterized fairly recently [28,29], their influence on the model systems so far reported has been undetected. However, studies have shown that GST T1-1 is expressed throughout the body, in tissues including the kidney and liver and in erythrocytes [21,30]. Notably, GST T1-1 is also expressed in the brain [21,30], a clinical target of BCNU treatment [11,31].

As has been mentioned earlier, brain tumor recurrence and the outgrowth of BCNU-resistant populations following chemotherapy are quite common. Scheck *et al.* [32] demonstrated that malignant human gliomas were typically heterogeneous before BCNU treatment, but that the loss of chromosome 22 was a common feature. In contrast, after chemotherapy the outgrowing BCNU-resistant tumor cell population often had an aneuploid karyotype with an overrepresentation of chromosome 22. The selection for this abnormality, particularly given that the majority of cells in the untreated tumors displayed a loss of the same chromosome, implies that there is strong selective pressure during BCNU treatment for increased dosage of one or several genes found on chromosome 22. Although GST M2-2 and GST M3-3 are expressed in the brain [33,34], these genes have been mapped to chromosome 1 [35,36]. In contrast, GST T1-1 is located on chromosome 22 [37].

On the other hand, it is noteworthy that GST T1-1 is polymorphic such that a null allele is present in approximately 20% of the Caucasian, as opposed to 60% in the Chinese and Korean populations [38]. The absence of GST T1-1 in some patients may be relevant both to their ability to develop drug resistance and be subjected to adverse side-effects of BCNU.

In conclusion, the results from this investigation show a distinctive ability of GSTs of both the Mu and the Theta classes for the denitrosation of BCNU. It would be valuable to find out whether these enzymes are also the most active against related nitrosoureas, such as 3-cyclohexyl-1-(2-chloroethyl)-1-nitrosocarbamide (Lomustine), which are being developed to avoid the toxic side-effects of BCNU. Nitrosoureas are hydrolyzed to generate the bifunctional chloroethylcarbonium ion, which cross-links double-stranded DNA and inhibits mitosis. The GST-catalyzed denitrosation reaction studied here prevents formation of

this alkylating species by consuming the parent compound. However, it is also possible that additional protection could be provided by GSTs, if the transient chloroethylcarbonium ion was also a substrate. This possibility should be explored by alternative assay methods. In addition, the relative importance of GST T1-1 and other GSTs to the alternative resistance mechanisms to BCNU *in vivo* remains to be elucidated.

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