# Influence of Glutathione and Glutathione *S*-transferases on DNA Interstrand Cross-Link Formation by 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine, the Active Anticancer Moiety Generated by Laromustine

Philip G. Penketh,* Eric Patridge, Krishnamurthy Shyam, Raymond P. Baumann, Rui Zhu, Kimiko Ishiguro, and Alan C. Sartorelli

Department of Pharmacology and Yale Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06520, United States

ABSTRACT: Prodrugs of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE) are promising anticancer agents. The 90CE moiety is a readily latentiated, short-lived ($t_{1/2} \sim 30$ s) chloroethylating agent that can generate high yields of oxophilic electrophiles responsible for the chloroethylation of the O-6 position of guanine in DNA. These guanine O-6 alkylations are believed to be responsible for the therapeutic effects of 90CE and its prodrugs. Thus, 90CE demonstrates high selectivity toward tumors with diminished levels of O^6-alkylguanine-DNA alkyltransferase (MGMT), the resistance protein responsible for O^6-alkylguanine repair. The formation of O^6-(2-chloroethyl)guanine lesions ultimately leads to the generation of highly cytotoxic 1-(N^3-cytosinyl),-2-(N^1-guaninyl)ethane DNA interstrand cross-links via N^1,O^6-ethanoguanine intermediates. The anticancer activity arising from this sequence of reactions is thus identical to this component of the anticancer activity of the clinically used chloroethylnitrosoureas. Herein, we evaluate the ability of glutathione (GSH) and other low molecular weight thiols, as well as GSH coupled with various glutathione *S*-transferase enzymes (GSTs) to attenuate the final yields of cross-links generated by 90CE when added prior to or immediately following the initial chloroethylation step to determine the major point(s) of interaction. In contrast to studies utilizing BCNU as a chloroethylating agent by others, GSH (or GSH/GST) did not appreciably quench DNA interstrand cross-link precursors. While thiols alone offered little protection at either alkylation step, the GSH/GST couple was able to diminish the initial yields of cross-link precursors. 90CE exhibited a very different GST isoenzyme susceptibility to that reported for BCNU, this could have important implications in the relative resistance of tumor cells to these agents. The protection afforded by GSH/GST was compared to that produced by MGMT.
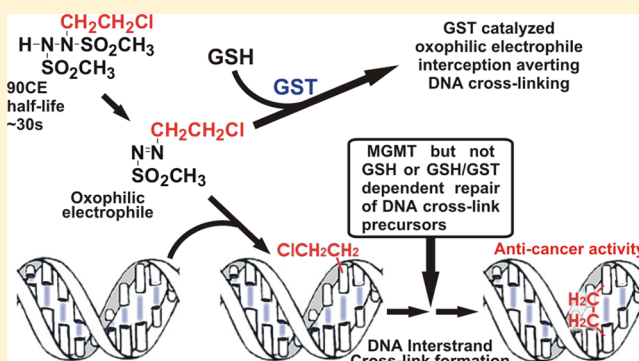
INTRODUCTION

1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE) was developed as a more specific DNA guanine O-6 chloroethylating agent lacking many of the toxicophores contained in agents such as BCNU.[1,2] The short half-life of 90CE in aqueous media does not permit adequate time for effective distribution in animals; thus, prodrug forms must be used for high *in vivo* efficacy.[3-5] Laromustine (cloretazine, onrigin, VNP40101M, 101M) is the most studied 90CE prodrug, and this agent produced 100% cures in several murine tumor model test systems.[5] Laromustine exhibited a therapeutic index (LD_{50}/ED_{50}) against L1210 leukemia of >8, more than double that of the best of over 300 nitrosoureas tested.[5,6] In addition, laromustine has been the subject of multiple late stage clinical trials and has exhibited significant activity against acute myeloid leukemia, small cell lung carcinoma, and glioblastoma tumors.[7-10] In addition, two tumor hypoxic region targeted 90CE prodrugs, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119, VNP40119) and 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(3-phospho-4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119W, VNP40541), have also been extensively studied.[11,12] 90CE rapidly generates ($t_{1/2} \sim 30$ s at 37 °C and pH 7.4) high yields (>80%) of hard oxophilic chloroethylating electrophiles in Tris/HCl buffers.[3,13] In contrast, BCNU reacts far more slowly ($t_{1/2} \sim 40$ min at 37 °C and pH 7.4) and decomposes in a multifarious manner, generating a more complex mixture of electrophiles, including chloroethylating, hydroxyethylating, vinylating, aminoethylating, and carbamoylating species with a wide range of nucleophile preferences.[14-16] The slow reaction of BCNU results in greater difficulty in the segregation of primary alkylation events, resulting in the formation of DNA cross-link precursors, from the secondary alkylation events that
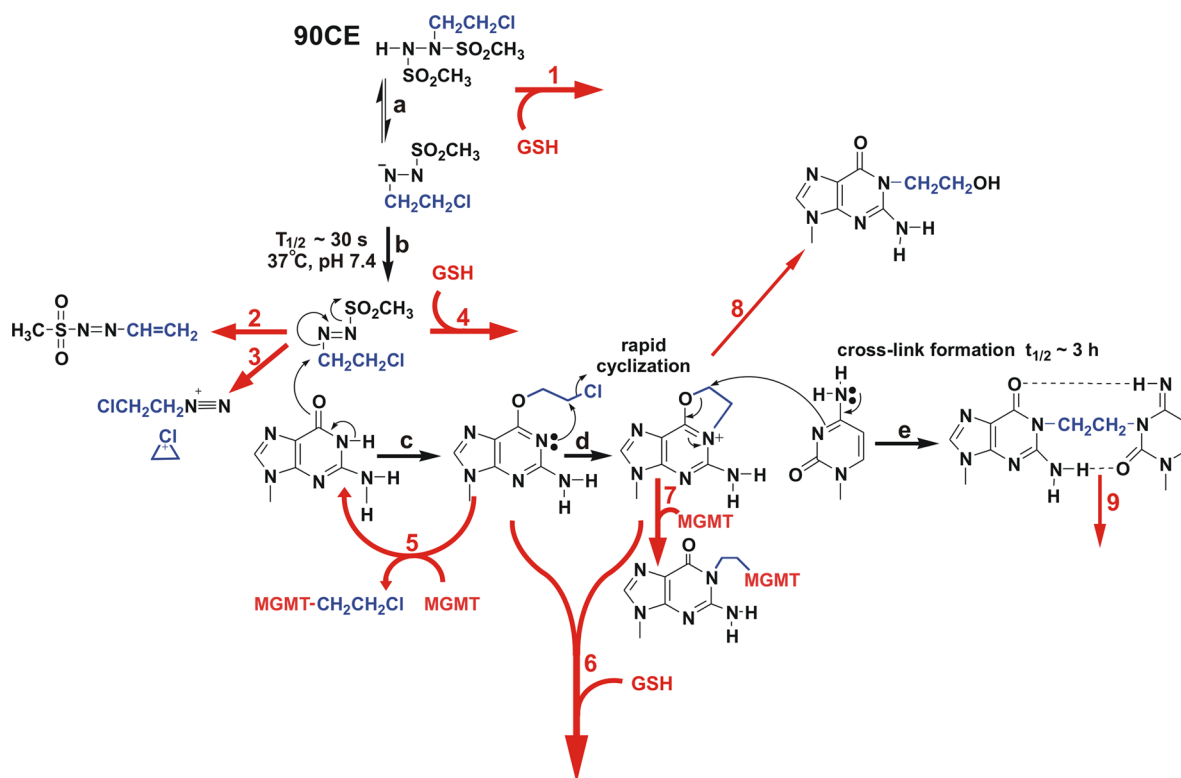
Figure 1. Scheme illustrating the generation of O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine cross-link precursor lesions and their transition into G-C ethane cross-links. Reaction steps leading to cross-link formation are indicated by black arrows (a to e), while those representing known or potential reactions resulting in a net reduction in DNA cross-link formation are shown in red (1 to 9). Reaction steps leading to cross-link formation: (a) 90CE ionization; (b) rate determining elimination of the N-1 methylsulfinate to generate the primary chloroethylating species; (c) the chloroethylation of DNA guanine to form O^6-(2-chloroethyl)guanine; (d) O^6-(2-chloroethyl)guanine cyclization to form N^1,O^6-ethanoguanine; and (e) attack by the N-3 position of cytosine on N^1,O^6-ethanoguanine to form the G-C ethane cross-link. Reaction steps leading to decreases in net cross-linking yields (1) potential GSH and/or GSH/GST reaction with parental 90CE; (2) Brønsted-Lowry base/phosphate-catalyzed alternative decomposition pathway resulting in failure to generate chloroethylating species capable of DNA cross-linking; (3) potential fragmentation to generate harder less guanine O-6 selective electrophiles; (4) GSH and/or GSH/GST interception of oxophilic chloroethylating species; (5) MGMT repair of O^6-(2-chloroethyl)guanine; (6) potential GSH and/or GSH/GST quenching of O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine cross-link precursors; (7) MGMT repair of the intermediate N^1,O^6-ethanoguanine lesion, resulting in tethered MGMT (additional repair steps may be required, but the highly cytotoxic cross-link is averted); (8) hydrolysis of N^1,O^6-ethanoguanine cross-link precursor to form N^1-(2-hydroxyethyl)guanine; and (9) homology directed repair of very limited cellular burden of G-C ethane cross-links. Tumor selectivity arises from tumor deficits in one or more of these repair/interception processes, with MGMT insufficiency likely being the foremost factor in most cases.

produce DNA interstrand cross-links. In addition, BCNU derived electrophiles can result in significant quantities of DNA strand nicks, which can interfere with the quantification of DNA interstrand cross-links.[14,15] These differences make 90CE a superior agent for *in vitro* studies of DNA guanine O-6 chloroethylation and its sequelae.

Chloroethylation of the O-6 position of guanine results in a rapid intramolecular nucleophilic substitution reaction, forming N^1,O^6-ethanoguanine; this product then reacts slowly, with a $t_{1/2}$ at 37 °C and pH 7.4 of ∼3 h, with the N-3 position of an opposing cytosine to form a highly cytotoxic 1-(N^3-2′-deoxycytidinyl)-2-(N^1-2′-deoxyguanosinyl)ethane cross-link (G-C ethane cross-link) (Figure 1).[16] G-C ethane cross-link formation appears to be responsible for the vast majority of the cytotoxicity in responsive tumor models treated with 90CE prodrugs and for a large proportion of the cytotoxicity of BCNU and other chloroethylnitrosoureas (CNUs).[16-20] Selectivity for tumor cells arises predominately from differentials between normal and tumor cells in (1) their O^6-alkylguanine-DNA alkyltransferase (MGMT) content,[16-20] the protein responsible for the repair of DNA O-6 guanine alkyl lesions,[18] and (2) in their ability to repair the cross-links

formed from lesions that escape MGMT repair.[21] Additional resistance may arise from the interception of (or decreased yields of) chloroethylating species by other mechanisms.[13] Each MGMT molecule can only repair a single guanine O-6 lesion.[18] In the case of guanine O-6 methylations, a mutual titration of the lesions and MGMT occurs resulting in potentially deleterious consequences only if the number of methylations exceeds the number of MGMT molecules.[19] However, because secondary reactions leading to cross-link formation compete with repair, in the case of guanine O-6 chloroethylations to avert toxicity, the MGMT must be present at a sufficiently high activity to clear the lesions before a small but lethal number have transitioned to cross-links.[17-19,22] Because of a greater reliance on guanine O-6 alkylation for cytotoxicity, 90CE and its prodrugs exhibit greater selectivity than BCNU toward cells with deficient O^6-alkylguanine repair in many model systems.[17,19,22] DNA-DNA interstrand cross-links cause replication arrest when encountered by a DNA replication fork; this leads to cell death if the cross-link is not repaired. The G-C ethane cross-link generated by 90CE and the CNUs appears to be exceptionally cytotoxic, and <10 such lesions per genome can result in cell death.[22] It is therefore not surprising

that cross-linking CNUs can be 200-fold more cytotoxic than analogues still able to alkylate but incapable of cross-linking DNA.[23] Similar burdens of unrepaired G-C ethane cross-links probably cause the deaths of both MGMT deficient cells and cells with modest to high MGMT levels.[22,23] However, MGMT expressing cells require a much greater exposure to 90CE to acquire a comparable cross-link burden compared to that of their MGMT deficient but otherwise equivalent counterparts.

A pervasive misconception in the biological literature is the ability of the thiol group of glutathione to readily intercept and neutralize almost any electrophilic species with high efficiency. This has likely arisen from the common description of thiols as "strong nucleophiles." Thus, it is often presumed that because "strong thiol nucleophiles" react exceptionally well with "weak electrophiles," thiols must have a very expansive range of electrophile preference. However, this is not the case, and the concept of "hard" and "soft" nucleophiles and electrophiles was introduced as a qualitative predictor of electrophile/nucleophile reaction preference.[24] Hard electrophiles have a high positive charge density and tend to react via S_N1 reaction mechanisms with hard nucleophiles which have a high negative charge density.[24] In contrast, soft electrophiles have a low charge density or are easily polarized and tend to react via S_N2 reaction mechanisms with soft nucleophiles that have a low negative charge density or are easily polarized.[24] The activation energy for reaction is the lowest between pairs of electrophiles and nucleophiles with closely matching degrees of hardness/softness.[24] Thus, the relative hardness/softness of electrophiles determines the range of preferred nucleophiles for reaction.[24] Hard oxophilic chloroethylating electrophiles with a preference for the O-6 position of guanine, the hardest base centered nucleophilic site in DNA,[24] will have a relatively low affinity for thiols. Therefore, thiols acting as competing nucleophiles should afford little net protection to guanine O-6 targets from attack by hard oxophilic electrophiles. However, softer electrophiles favoring the N-7 position of guanine may have considerable overlap in their nucleophile preferences with thiols. Thus, thiols can exhibit strong competitive inhibition toward N-7 guanine alkylation. One of the functions of glutathione S-transferases (GSTs), in addition to xenobiotic binding,[25] is to catalyze the conjugation of GSH with a wide range of electrophiles, effectively extending the electrophile preference range of GSH.[26] GSTs can constitute up to 4% of the total soluble protein in the liver and comprise a family of isoenzymes with a broad range of overlapping electrophilic substrates.[26,27]

Studies centered on BCNU have suggested that glutathione can result in cellular protection at several key points, including the inactivation of the parental drug,[28] interception of the electrophiles generated,[28] and direct attenuation of cross-link yields by the quenching of DNA cross-link precursors within the DNA.[29] In addition, glutathione S-transferases have been shown to facilitate at least some of these processes such as the inactivation of BCNU by dehalogenation and denitrosation.[30,31] In view of the commonality between 90CE and CNUs, in terms of DNA guanine O-6 chloroethylation leading to G-C cross-link generation, it would be anticipated that they should at least share equivalent cross-link precursor quenching by GSH, as these precursors should be identical in both cases.

In this study, we have examined the ability of GSH to attenuate the final yields of cross-links when added pre or post the initial chloroethylation step, in the presence and absence of various GSTs, and we compared this to the protection

produced by MGMT. The ~80-fold shorter $t_{1/2}$ of 90CE compared to BCNU and its high yield of hard oxophilic chloroethylating electrophiles favoring guanine O-6 alkylation, coupled with the relative absence of nicking and additional complicating electrophile species, simplifies the segregation of the primary alkylation event from the slower subsequent cross-linking reaction and the interpretation of the data.[3,13,14,32]

■ MATERIALS AND METHODS

Caution: *90CE is potentially carcinogenic and mutagenic and should be handled carefully using personal protective equipment.*
All chemicals, reagents, and enzymes including equine liver GST (G6511) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification, with the exceptions of the fluorescent dye Hoechst 33258, which was purchased from Molecular Probes, Inc., (Eugene, OR), 90CE, which was synthesized as previously described,[33] and purified recombinant human MGMT, which was a kind gift from Dr. Joann Sweasy (Yale Medical School, New Haven, CT). In addition, L1210 DNA and various GSTs were produced as described below.

Preparation of Murine L1210 Cell DNA. L1210 murine leukemia cell lines were grown in suspension culture in RPMI 1640 medium supplemented with 10% FBS in air/5% CO_2 at 37 °C. The cells were subcultured as required every 2–3 days, and the DNA was isolated using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) using procedures recommended by the manufacturer.

Preparation of Recombinant GSTs. Primers were obtained through Integrated DNA Technologies (Coralville, IA). Human GSTA1 (hGSTA1), GSTM1 (hGSTM1), and GSTP1 (hGSTP1) cDNA were gifts from Charles S. Morrow (Wake Forest University School of Medicine, Winston-Salem, NC). Human GSTT1 (hGSTT1) cDNA was a gift from George Georgiou (University of Texas at Austin, Austin, TX). Expression plasmid kits and expression cell lines were obtained from EMD Millipore (Madison, WI). Cobalt TALON Superflow Metal Affinity Resin was obtained from Clontech Laboratories (Mountain View, CA).

Cloning, Expression, and Purification of GSTs. Genes encoding hGSTA1, hGSTM1, hGSTP1, and hGSTT1 were amplified from cDNA, and each gene was cloned into the Novagen pET46 Ek/LIC vector, yielding plasmids pET46-hGSTA1, pET46-hGSTM1, pET46-hGSTP1, and pET46-hGSTT1. Protein production from the pET46-hGSTP1 and pET46-hGSTT1 vectors was optimized via silent codon mutation, yielding the final expression vectors pET46-hGSTP1-EXP and pET46-hGSTT1-EXP. Each vector (pET46-hGSTA1, pET46-hGSTM1, pET46-hGSTP1-EXP, and pET46-hGSTT1-EXP) was transformed for heterologous expression using the Novagen BL21(DE3)pLysS cell line.

Transformed cell lines were cultured at 37 °C in terrific broth (Terrific broth capsules; RPI Corporation; Mount Prospect, IL), containing 50 μg/mL of ampicillin and 17 μg/mL of chloramphenicol. When the optical density at 600 nm reached 0.6, protein production was induced by the addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG) at 37 °C. After 5 h, cells were harvested by centrifugation, weighed, and lysed using a microfluidizer M-110P (Microfluidics Corp; Westwood, MA). Cell debris was pelleted by centrifugation, and the cleared lysate was filtered, flash frozen, and stored at −80 °C.

Each GST protein was purified at room temperature by His-Tag affinity chromatography, using TALON Superflow Metal Affinity Resin. Lysate from approximately 10 g of cell paste was thawed, diluted to 120 mL with equilibration buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM imidazole, and 10% glycerol, pH 8), and loaded onto a gravity flow column packed with His-Tag resin. The affinity column was washed with 500 mL of wash buffer (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, and 10% glycerol, pH 8), and bound GST protein was eluted from the column with 200 mL of elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, and 10% glycerol, pH 8). Gel electrophoresis (12% SDS−PAGE) was used to visualize protein expression, and fractions containing GST proteins were

pooled. Aggregated fractions were dialyzed for 4 h in 4 L of dialysis buffer (50 mM MOPS and 100 mM NaCl, pH 7.0), concentrated to 3 mL, and dialyzed again for 16 h in 4 L of dialysis buffer. After measuring specific activity, each GST protein was flash frozen and stored at −80 °C. Protein concentration was determined spectrophotometrically for GSTA1 ($\varepsilon 280$ = 20.4 mM^{-1}cm^{-1}), GSTM1 ($\varepsilon 280$ = 40 mM^{-1}cm^{-1}), GSTP1 ($\varepsilon 280$ = 29 mM^{-1}cm^{-1}), and GSTT1 ($\varepsilon 280$ = 38 mM^{-1}cm^{-1}). The specific activity of each GST protein was determined at 25 °C in 100 mM potassium phosphate, pH 6.5, in the presence of 1 mM 1-chloro-2,4-dinitrobenzene ($\varepsilon 340$ = 9.6 mM^{-1}cm^{-1}) and 1 mM GSH (prebuffered in 100 mM potassium phosphate, pH 6.5). Values of 8.3 μmol min^{-1}mg^{-1}, 42 μmol min^{-1}mg^{-1}, 17.4 μmol min^{-1}mg^{-1}, and 1.0 μmol min^{-1}mg^{-1} were obtained for GSTA1, GSTM1, GSTP1, and GSTT1, respectively.

Influence of DTT, TG, and GSH on 90CE Dependent DNA Cross-Linking. The assay used in these experiments to determine DNA cross-linking is based upon the fact that DNA molecules containing one or more covalent interstrand cross-links rapidly renature upon snap cooling following thermal denaturation.[15,22] This is because the cross-links hold the complementary DNA strands in close proximity and in register. Since H33258 forms a highly fluorescent complex with double stranded but not mispaired/denatured DNA, DNA molecules containing cross-links will yield highly fluorescent complexes following a heat/chill cycle with H33258 dye; whereas DNA molecules devoid of such cross-links will not. Since all assay steps are conducted at neutral pH values, potential problems caused by base catalyzed lesion hydrolysis are avoided.[15,22]

L1210 DNA (∼32 μg/mL) in 10 mM Tris-HCl buffer (pH ∼7.4) was treated with 50 μM 90CE at 37 °C in the presence or absence of thiols (1.0 mM DTT, 10 mM TG, and 10 mM GSH), which were either added immediately prior to 90CE addition or at various times thereafter up to 5 min. At various time points up to 24 h, 50 μL aliquots were assayed for their levels of DNA interstrand cross-links by diluting these to a volume of 1.5 mL with 5 mM Tris-HCl, 1.0 mM EDTA, and 1.0 mM NaN$_3$ buffer (pH 8.0), containing 0.1 μg/mL (final concentration) of Hoechst H33258 fluorescent dye, and the fluorescence was then measured using a Hoefer Scientific Instruments TKO 100 fluorometer. The diluted mixture was then heated in a 100 °C hot-block for 5 min and plunged into a water bath at room temperature for 5 min before the fluorescence was measured again. The percentage of DNA molecules that were cross-linked (i.e., containing at least one cross-link per molecule) was then calculated from the change in fluorescence in comparison to DNA not treated with cross-linking agent as previously described.[15,22]

Influence of GSTs on 90CE dependent DNA cross-linking. The reactions were carried out in 200 μL of final reaction volumes in 10 mM Tris-HCl and 1 mM EDTA buffer at pH 7.4 and 37 °C containing ∼8 μg/mL of L1210 DNA; reactions were initiated by adding 90CE to give final concentrations of 50 μM from 1 mM stocks made up in DMSO. Additional components were present in some reaction mixtures; these included GSH to give a final concentration of 10 mM (added from 100 mM at pH 7.4 stock solutions), GSTs to give a final concentration of ∼0.5 mg/mL (added from ∼20 mg/mL stocks), and BSA to give a final concentration of ∼0.5 mg/mL (added from ∼20 mg/mL stock). As indicated in some experiments, the GSTs were added at various times (0, 30 s, 2, and 5 min) after the reaction was initiated by the addition of 90CE. Reaction mixtures were incubated for 15 min at 37 °C, 0.1 mg/mL of proteinase K was added, and the sample was then incubated for a further 10 min at 37 °C. GSTs are known to bind to dsDNA, creating an artifactual background cross-linking signal, but this artifact can be completely eliminated by the inclusion of a short proteinase K digestion step. Therefore, proteinase K 10 min incubations (0.1 mg/mL final concentration) were used to digest the GST prior to assay for DNA cross-linking. The samples were then brought to 0.5 mL and incubated at 50 °C for 2.5 h to allow the remaining cross-link precursors to progress to cross-links (this processes requires >12 h to approach completion if performed at 37 °C) and diluted to a volume of 1.5 mL with 5 mM Tris-HCl, 1.0 mM EDTA, and 1.0 mM NaN$_3$ buffer, pH 8.0, containing 0.1 μg/mL

(final concentration) of Hoechst H33258 fluorescent dye and assayed for cross-linking as previously described.[15,22]

Investigation of GSTA1 Reaction Point via Effects on the Kinetics of H$^+$ Generation during the Decomposition of 90CE. The normal decomposition of 90CE in Tris-HCl buffer involves the liberation of two H$^+$ ions and can be followed using protonometric assays.[3,13,34] One of these H$^+$ ions is liberated during the chloroethylation of nucleophiles and occurs after the rate-limiting elimination of the N-1 sulfonyl moiety from the 1,2-bis(sulfonyl)-1-alkylhydrazine anion that results in the formation of the primary chloroethylating species ClCH$_2$CH$_2$N═NSO$_2$CH$_3$.[3,13] We utilized a simple colorimetric assay that relies upon the measurement of the relatively linear change in absorption at 560 nm of phenol red that occurs in proportion to the generation/addition of small quantities of hydrogen ions when a weakly buffered solution of this pH indicator is subjected to incremental acidification over a narrow pH range (ΔpH < 0.1 unit) close to the pK_a values of the buffer/indicator components.[34] Using a 20 μg/mL solution of phenol red in 10 mM Tris-HCl/10 mM GSH buffer, the absorbance at 560 nm was followed at an initial pH value of 7.4 at 37 °C upon addition of 100 μM 90CE (10 μL/mL of 10 mM 90CE in DMSO), in the presence and absence of GSTA1 0.5 mg/mL. The assay mixtures were sealed with parafilm in 1 mL cuvettes to minimize changes in pH due to CO$_2$ exchange, and they were brought to the appropriate temperature prior to the addition of agent by injection through the parafilm and rapid mixing. The assay sensitivity is influenced by the buffering capacity of all of the major components (Tris/GSH/GST); therefore, calibrations were performed using HCl standards for mixtures with and without GSTA1. Some of these components (Tris in particular) exhibit significant temperature dependent shifts in their pK_a values; therefore, the reaction mixture pH must be set at the experimental temperature.[13,34]

MGMT Quenching of 90CE Derived DNA Cross-Link Precursors. L1210 DNA (∼160 μg/mL) was reacted in 10 mM Tris-HCl buffer (pH ∼7.4) with 50 μM 90CE at 37 °C; this initially generates DNA containing only cross-link precursors (O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine lesions) but no significant numbers of cross-links. This is because at 37 °C the $t_{1/2}$ for nucleophile chloroethylation by 90CE is ∼30 s, but the $t_{1/2}$ for the progression of these lesions to cross-links is ∼3 h. A 10 μL aliquot of this "substrate DNA" contains ∼12.5 fmol of cross-link precursors based on the minimum quantity of MGMT required to essentially completely block cross-linking when added to freshly prepared (3 min post-90CE treatment) substrate DNA. If this substrate DNA is incubated in the absence of MGMT for ∼24 h at 37 °C or ∼3 h at 50 °C, the cross-link precursors will transition into cross-links resulting in ∼35–40% of the DNA molecules containing one or more interstrand cross-links. Substrate DNA can be stored at 0 °C for over 8 h without developing significant levels of cross-links. Substrate DNA prepared as described above was incubated at 37 °C, and at various times (0, 1, 3, 5 h) after the initiation of this incubation, the mixture was split, and an ∼ 4-fold molar excess of MGMT (in MGMT stabilization buffer) with respect to the initial DNA cross-link precursor content added to the daughter portion, and the incubation of the parental mixture and the daughter sample continued. At various time intervals (0, 1, 3, 5, 7, and 9 h), small aliquots containing equivalent quantities of DNA (1.6 μg) were removed from all of the incubation samples (parental and daughter) and diluted to a volume of 1.5 mL with 5 mM Tris-HCl, 1.0 mM EDTA, and 1.0 mM NaN$_3$ buffer (pH 8.0), containing 0.1 μg/mL (final concentration) of Hoechst H33258 fluorescent dye, and the level of DNA interstrand cross-links measured as described above.[15,22] MGMT stabilization buffer (pH 7.4) has the following composition: 10 mM Tris-HCl, 0.5 mM EDTA, 0.5 mg/mL of bovine serum albumin, 50 μM dithiothreitol, and 1 μg/mL of L1210 DNA; the L1210 DNA greatly contributes to the MGMT stability but does not add significantly to the total DNA in the final assay. The concentration of MGMT in these reaction mixtures corresponds to ∼60 ng/mL.

■ RESULTS AND DISCUSSION

The quantity of cross-link precursors generated per unit length of DNA is proportional to the initial concentration of 90CE but is largely independent of the DNA concentration.[22] This is because the hard chloroethylating electrophile exposure (concentration multiplied by time) that each DNA molecule experiences is controlled primarily by the reaction of the former with water to produce 2-chloroethanol since only a relatively small fraction reacts with DNA or other solutes.[3,13,22] The additional copresence of a competing soft nucleophilic thiol would therefore be expected to have a limited direct impact on the yields of cross-link precursors. Our experimental observations with TG, GSH, and DTT match this expectation (Figure 2). At the relatively high concentration of 10 mM, TG and
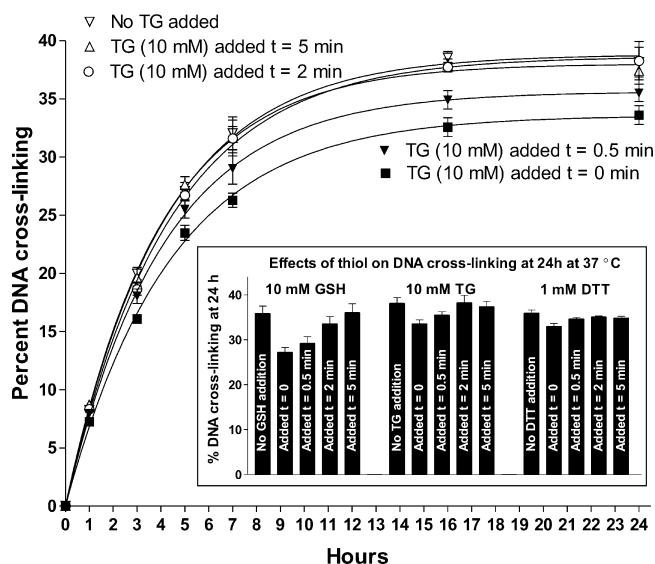

Figure 2. Inhibition of DNA cross-linking by free thiols. The effects of the presence/absence of 10 mM TG on the kinetics of L1210 DNA (~32 μg/mL) cross-linking by 50 μM 90CE in 10 mM Tris-HCl (pH 7.4, 37 °C) when added at various time points (0, 0.5, 2, and 5 min) after 90CE addition. Insert: resultant DNA cross-linking levels 24 h after treatment with 50 μM 90CE in the presence/absence of either 10 mM TG, 10 mM GSH, or 1 mM DTT when the thiols are added at 0, 0.5, 2, and 5 min after 90CE addition. The best fit curves were modeled using GraphPad Prism software (version 3.02) and fitted to a one phase exponential association equation: $Y = Y_{max} \cdot (1 - e^{-K \cdot X})$. All values are the result of at least 3 determinations ± SE.

GSH resulted in ~15% and ~25% inhibitions of eventual DNA cross-link formation, respectively, while <4% inhibition was observed with 1 mM of the dithiol DTT. The greater effect of GSH could be due in part to its two carboxyl groups which could possibly function as competing hard nucleophilic targets. Cellular carboxyl groups have been previously reported to be preferential targets for alkylation by nitrosoureas.[35] TG was chosen because it is an uncharged, highly soluble, small thiol thought likely to have good access to the cross-link precursor lesions. GSH was chosen because of its biological relevance and to allow comparison with BCNU studies that utilized this thiol.[29] The DTT was used largely as a control because a small quantity of DTT (50 μM) is present as a stabilizer in our MGMT preparations. In addition to the direct interception of a minor proportion of the chloroethylating electrophiles by nucleophilic moieties in these molecules, other potentially contributing factors toward these relatively small inhibitions

include a direct reaction of the thiols with the parental 90CE molecule (90CE's short half-life and lack of thiol-reactive moieties make this unlikely); and a Brønsted-Lowry base catalyzed diversion of a small proportion of the decomposition pathway flux away from the production of hard oxophilic chloroethylating electrophiles;[2] this latter effect is more likely to occur with GSH. While the precise contributions of these different mechanisms to the small observed inhibitions of DNA cross-linking is uncertain, it can nevertheless be concluded that thiols even at 10 mM do not efficiently intercept the oxophilic chloroethylating electrophiles derived from 90CE. It should also be noted that for these relatively small inhibitions of DNA cross-linking to occur the thiol had to be present during the primary alkylation phase (Figure 2). Inhibition of DNA interstrand cross-linking by thiols added after the first few minutes when the initial chloroethylation reactions have been completed would represent quenching of the cross-link precursors generated in the DNA (Figure 1) since at this juncture the DNA contains cross-link precursors but undetectable cross-link levels. It can be seen (Figure 2) that none of the thiols had any significant effects on the yields of cross-links when added post the initial chloroethylation events. This strongly implies that any thiol-dependent cross-link precursor quenching reaction either does not occur with these thiols or occurs far too slowly under these conditions to measurably compete with the slow interstrand cross-linking reaction. It might at first glance be expected that a small low molecular weight thiol might compete modestly against cytosine N-3 for reaction with the N^1,O^6-ethanoguanine cross-link precursors since cross-link formation involves a softer electrophile/nucleophile pair than the primary chloroethylation event. However, cross-link formation involves a reaction between two groups locked in very close proximity greatly enhancing their effective concentrations. Thus, even a small very permeable thiol would need to be present at an excessive concentration to compete with the intramolecular reaction of cytosine N-3 with N^1,O^6-ethanoguanine in this modified base pair. The extent to which the cross-linking reaction is favored in double stranded DNA over the equivalent reaction in free solution is illustrated by the reaction O^6-(2-fluoroethyl)guanine and cytosine.[36] In DNA, this reaction is ~80-fold faster than that with the concentrated reactants in free solution in DMSO (150 mM O^6-(2-fluoroethyl)guanosine and 440 mM deoxycytidine).[36] In the slow DMSO reaction, hydrolysis of the N^1,O^6-ethanoguanine intermediate to give 1-(2-hydroxyethyl)guanosine predominated over cross-link formation, even if small quantities of water were present in the reaction mixture.[36] However, with the much faster intramolecular reaction within the confines of the DNA helix, G-C ethane cross-link formation predominates over hydrolysis to form 1-(2-hydroxyethyl)guanosine.[37] The lack of cross-link precursor quenching by GSH strongly contrasts with that reported in earlier studies using BCNU.[29] These conclusions were based in part on the covalent binding of [glycine-³H]GSH to BCNU treated DNA.[29] Cross-link lesions and consequently their precursors are of a low frequency in both BCNU treated DNA and cells, and if alkylations to the phosphate residues of the DNA backbone are included, cross-link precursors constitute <1% of the total lesions generated.[38] It is therefore likely that the efficient binding of labeled GSH to BCNU treated DNA involves reaction with other more abundant BCNU derived lesion(s) that are not involved in G-C ethane cross-link formation. The direct reaction between concentrated aqueous solutions of cysteine (1.0 M) and O^6-(2-

fluoroethyl)guanosine (0.1 M) generates 1-(guan-1-yl)-2-(cystein-S-yl)ethane.[39] This reaction models the expected tethered product formed by cross-link precursor quenching by thiol containing molecules.[29,39,40] This high concentration reaction in free aqueous solution proceeds at ∼1/10th the rate of G-C cross-linking reaction in DNA with relatively little hydrolysis to generate 1-(2-hydroxyethyl)guanosine.[39] It is likely that the added steric hindrance from confinement within DNA would result in even slower reaction rates between cysteine and cross-link precursors within DNA than in free solution. These considerations further support the insignificant quenching of cross-link precursors within DNA by physiological concentrations of simple thiols.

The abilities of various GSTs in the presence and absence of glutathione to block the formation of cross-link precursors generated in DNA samples (or their subsequent repair) treated with 50 μM 90CE were examined. The GSTs were given a 15 min repair window at 37 °C, then the samples were treated with proteinase K (10 min, 37 °C), then incubated at 50 °C for 2.5 h to speed the conversion of any remaining cross-link precursors to cross-links. In a manner analogous to the previous series of experiments involving thiols, the addition of the potential protectant (GST) was delayed in some experiments to determine its major point(s) of action. A relatively high GST concentration of 0.5 mg/mL was used; this corresponds to a subunit concentration of ∼20 μM (GST subunit ∼25 kDa) or 40% of the 90CE concentration. GSTs have two major actions involved in xenobiotic detoxification: (a) they catalyze the conjugation of GSH with xenobiotics, increasing the rate and range of electrophiles GSH readily reacts with, and (b) GSTs possess strong promiscuous nonactive site binding pocket(s) for xenobiotics and toxins.[25−27] This latter property was responsible for their initial characterization as ligandins[25] and potentially allows them to offer some protection against agents even in the absence of GSH. In our initial experiments, we utilized Sigma equine liver GST (G6511), which is probably a mixture of GSTs but is expected to contain a significant proportion of α class GST because of its organ of origin.[26,27,41] This preparation also contains a small contamination of GSH/GSSG. It can be seen in Figure 3 that the equine liver GST preparation in the copresence of 10 mM GSH is able to
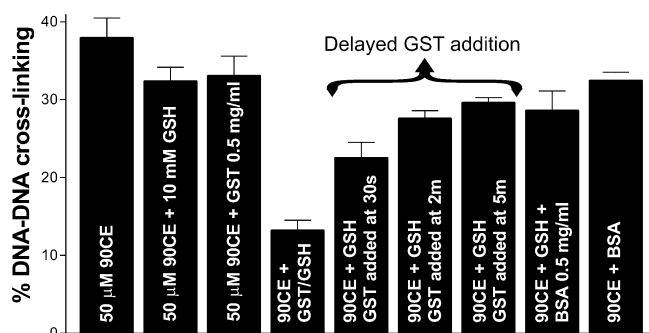

Figure 3. Inhibition of DNA cross-linking by commercial equine liver GST. The effects of 10 mM GSH, 0.5 mg/mL Sigma (G6511) equine liver GST, and GSH/GST combinations on maximal L1210 DNA cross-linking levels after treatment with 50 μM 90CE. In some of the GSH/GST combination experiments, the addition of GST was delayed and added at 0.5, 2, or 5 min after the 90CE addition. As controls for nonspecific protein effects, 0.5 mg/mL BSA and GSH/BSA (0.5 mg/mL) combinations were also run. All values are the result of at least 3 determinations ± SE.

attenuate the eventual cross-linking produced by 50 μM 90CE by ∼60% compared to that by 10 mM GSH alone when present at the time of 90CE addition. However, this protection is confined to the short temporal window after 90CE addition, corresponding to the residual presence of parental 90CE ($t_{1/2}$ ∼30 s) and the extremely short-lived chloroethylating electrophiles 90CE generates. Thus, the addition 30 s later than 90CE approximately halved the protective value of the GST/GSH combination and additions after 2 and 5 min conferred very little if any additional protection (Figure 3). This finding implies that the GST/GSH combination was able to decrease the initial chloroethylation of the DNA but had relatively insignificant ability to quench the cross-link precursors once generated within the DNA. The small protection seen with the initial presence of GST (0.5 mg/mL) without additional GSH could be due to contaminating GSH, nonactive site binding of 90CE (or subsequently generated alkylating species), or to competing nucleophilic moieties on the protein itself, or a group on the protein acting as a Brønsted-Lowry base and diverting a small proportion of the decomposition pathway away from the formation of chloroethylating species.[13] In support of the latter two explanations was the observation that bovine serum albumin (BSA) at 0.5 mg/mL offered a similar degree of protection (Figure 3). These experiments were repeated using the following recombinant human GSTs (Figure 4) that had been extensively dialyzed during their purification and therefore should be free of significant GSH contamination: GSTA1, GSTM1, GSTP1, and GSTT1. Overall, these GSTs exhibited behavior very similar to that of the Sigma equine liver preparation. The rank order of activity was GSTA1 > GSTM1 ≈ GSTP1 > GSTT1, with GSTT1 displaying very marginal if any activity (Figure 4). The trivial protective activity of GSTT1 against 90CE contrasts with its very efficient inactivation of BCNU by denitrosation.[42] This could lead to marked activity differences between CNUs and 90CE prodrugs against tumor cells expressing high levels of GSTT1. GSTP1 elicited a noteworthy ∼39% decrease in DNA cross-linking in the absence of added GSH since the GSTP1 subunit concentration is 40% of that of the initial 90CE concentration in these experiments; this effect may be largely associated with its ligandin (nonsubstrate binding) abilities.[43]

With all of the GSTs examined, the protective activity was essentially confined to within the first few minutes of 90CE addition, implying relatively little or no activity toward 90CE derived cross-link precursors. The GST/GSH inhibition of DNA guanine O-6 chloroethylation during the first few minutes of reaction could potentially be the result of several processes. These include a direct reaction with parental 90CE (such as via dehalogenation) or a reaction with subsequent chloroethylating species generated after the rate-limiting elimination of the N-1 methylsulfonyl moiety. In view of the large inhibitory (∼85%) effect seen with GSTA1/GSH over that of GSH alone (Figure 4), this combination was chosen to distinguish between these possibilities by observing the influence of GSTA1/GSH on the kinetics of H$^+$ ion generation during 90CE decomposition (Figure 5). During the decomposition of 90CE, 2 mol of H$^+$ ions are normally liberated per mole of 90CE in a biphasic manner.[3,13] The first mole of H$^+$ ions is released instantaneously and corresponds to the ionization of the acidic N-2 proton to form the 90CE anion. The second mole of H$^+$ ions released is largely dependent upon the chloroethylation of water or other nucleophiles (∼80% of the reaction flux in low phosphate buffers) or is formed during Brønsted-Lowry base
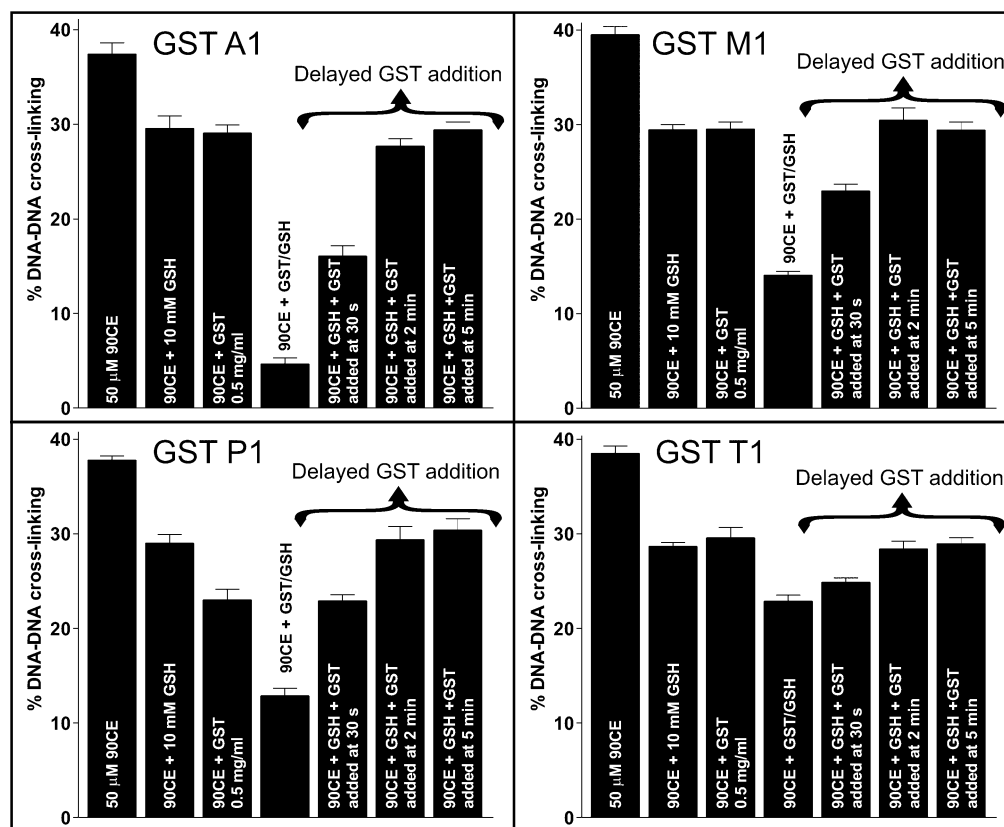
Figure 4. Inhibition of DNA cross-linking by human GST enzymes. Comparison of the effects of various recombinant human GSTs (GSTA1, GSTM1, GSTP1, and GSTT1) at 0.5 mg/mL in the presence and absence of 10 mM GSH, on the maximal DNA cross-linking levels produced after treatment with 50 μM 90CE. In some of the GSH/GST combination experiments, the addition of GST was delayed and added at 0.5, 2, or 5 min after the 90CE addition. All values are the result of at least 3 determinations $\pm$ SE.

catalyzed chloride loss (~20% of the reaction flux in low phosphate buffers). The sum of these two pathways account for the second mole of H$^+$ ions generated, and because they both occur after the rate-limiting elimination step (Figure 5), they are indistinguishable based on the kinetics of H$^+$ ions formation. If the GSTA1 decreased DNA cross-linking by catalyzing the reaction of GSH with chloroethylating electrophiles (or by Brønsted-Lowry base catalyzed chloride loss) after the rate-determining elimination step, no changes in the overall kinetics or total magnitude of H$^+$ liberation would be expected in this time frame. However, if the GSTA1 catalyzed an attack prior to the rate-determining step on the parental 90CE at a sufficient rate as to decrease the potential for DNA cross-linking by ~85% (e.g., by catalyzing halide loss), effects on the kinetics and magnitude of H$^+$ liberation would be expected. The presence of GSTA1 was found to have little or no effect on the kinetics of H$^+$ ion liberation by 90CE in the presence of 10 mM GSH (Figure 5); therefore, it is likely that GSTA1's inhibitory effects on DNA cross-linking do not primarily involve an interaction with the parental 90CE molecule. Inactivation of the more slowly decomposing BCNU by GST catalyzed chloride loss and denitrosation has been previously reported.[30,42] As a positive control, we examined the ability of GSTA1 to catalyze the rapid dehalogenation of methyl iodide by GSH. The presence of GSTA1 markedly changed the kinetics of H$^+$ ion liberation by the methyl iodide/GSH reaction (Figure 5). It should be noted that methyl iodide is a far more ideal substrate for nucleophilic substitution than 90CE

since it contains a halide with greater leaving group ability and a less sterically hindered alkyl (i.e., methyl) moiety.

MGMT plays a major role in resistance to the cytotoxicity of CNUs and 90CE and its prodrugs.[16−20] This resistance is believed to result from the quenching/repair of the O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine cross-link precursors which are generated by both these classes of agents.[16−20] In contrast to GSH/GST, MGMT had the ability to rapidly quench 90CE generated cross-link precursors within DNA and blocked DNA cross-linking after the primary alkylation phase was completed. Thus, when MGMT in excess of the cross-link precursor content was added to DNA containing 90CE derived cross-link precursors, cross-linking was essentially completely blocked. Furthermore, when MGMT was added to partially cross-linked DNA, it efficiently quenched any remaining cross-link precursors that had not yet transitioned to cross-links (Figure 6). MGMT is very effective at dealing with this type of damage despite its repair capacity limitation of a single lesion per protein molecule;[18] this is because most of the hard oxophilic chloroethylating electrophiles generated react with water, and there are relatively few of these highly cytotoxic lesions to repair.[22] Thus, while MGMT does not confer the broad spectrum electrophile protection of GSH/GST,[18,27] against this specific damage type in our model system it is more than 8,000 times as effective in terms of protein mass (0.5 mg/mL of GSTA1 being less effective than 60 ng/mL of MGMT). However, since GST/GSH acts primarily to reduce the number of cross-link precursors generated in the first place, while MGMT cleans up those
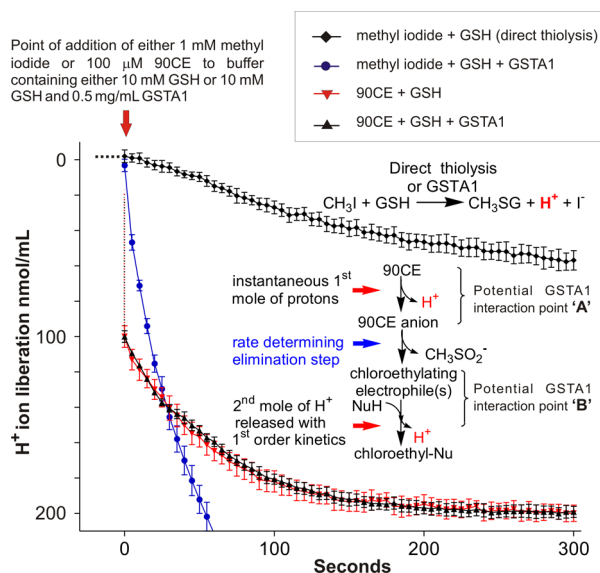
Figure 5. Effect of GSTA1 on H$^+$ ion liberation during the decomposition of 90CE (and methyl iodide) in the presence of GSH. Effects of the presence and absence of GSTA1 (0.5 mg/mL) on the kinetics of H$^+$ ion generation during 90CE decomposition in the presence of 10 mM GSH. If the GSTA1/GSH decreased DNA cross-linking by intercepting chloroethylating electrophiles (reaction after the rate-determining elimination step, point 'B'), no changes in the overall kinetics or total magnitude of H$^+$ liberation would be expected. If GSTA1/GSH attacked parental 90CE (interception point 'A'), an increase in the overall rate of H$^+$ liberation (and possibly magnitude, depending on the point of attack) would be expected. As a positive control, the ability of GSTA1/GSH to catalyze the dehalogenation of methyl iodide by GSH was examined. All values are the result of at least 3 determinations ± SE.

which clear this initial hurdle, these two protective mechanisms are expected to complement each other.

The proteinase K digestion step used to degrade the GSTs was required prior to assaying for DNA cross-linking because these proteins bind to dsDNA creating an artifactual background cross-linking signal not seen with most other proteins. While this may be merely an artifactual consequence of the "ligandin activity" of GST in the presence of "naked" DNA, this binding could also imply some possible function of GSTs in the repair of some classes of DNA lesions. Moreover, there are several reports in the literature indicating that some GSTs are specifically located in the nucleus.[44]

In addition to the generation of DNA interstrand cross-links, DNA protein cross-links could also be generated by 90CE and CNUs by at least three potential mechanisms. One well documented mechanism involves the tethering of MGMT during the repair of the N^1,O^6-ethanoguanine cross-link precursor lesion (Figure 1).[40] While a DNA−DNA interstrand cross-link is averted by this action, a DNA protein cross-link is produced instead. MGMT is strongly protective even when present at relatively low levels when more tethered products are likely to form due to a slower rate of lesion clearance.[19] Thus, tethered proteins appear to be relatively nontoxic when compared to G-C ethane cross-links where <10 lesions per cell can result in lethality.[22] DNA protein cross-links are generally thought to be of lesser importance compared to DNA interstrand cross-links in the mode of action of CNUs and similar agents. MGMT uses a specialized "finger" mechanism to flip/displace the modified guanine base out of the shielding
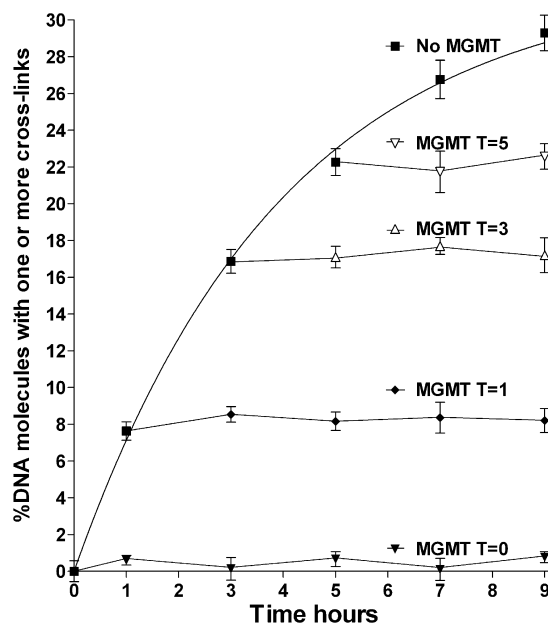

Figure 6. Quenching of DNA cross-link precursors by MGMT. The effects of the addition of MGMT at various time points on the progression of cross-link precursors to fully formed cross-links at 37 °C at pH 7.4. The best fit curve for the time course of DNA cross-linking in the absence of MGMT was modeled using GraphPad Prism software (version 3.02) and fitted to a one phase exponential association equation: $Y = Y_{max} \cdot (1 - e^{-K \cdot X})$. All values are the result of at least 3 determinations ± SE.

DNA double helix base-stack to gain access to this lesion.[45] In view of this requirement, it would appear unlikely that significant levels of other proteins could become tethered by interacting directly with this lesion. A second DNA protein cross-linking mechanism could result from the chloroethylation of protein thiols. A small proportion of the hard oxophilic chloroethylating species generated by 90CE and the CNUs will react with both protein and nonprotein thiols even though these nucleophiles are not highly favored targets. The resulting chloroethylated thiols are then expected to rapidly eliminate chloride to form a reactive cyclic sulfonium ion via an intramolecular nucleophilic substitution reaction and then react further with water and other surrounding nucleophiles, potentially including sites within DNA resulting in DNA proteins cross-links. In addition, it is also possible that chloroethylated DNA backbone phosphate moieties could also react with some protein thiol groups to produce DNA proteins cross-links.

A comprehensive understanding of the factors involved in the sensitivities of cells to 90CE prodrugs is fundamental to predicting their relative cytotoxities toward tumors and different host tissues. Host tissue resistance factors would be of lower importance in the case of prodrugs with precise tumor delivery strategies in view of 90CE's short half-life minimizing escape from sites of liberation. Factors in the sensitivity of cells to 90CE prodrugs in addition to MGMT expression (absent in 5−20% of different tumor types)[46] and DNA cross-link repair competence[21] are likely to include their GST expression levels (particularly those of GSTA1 type) and their net catalytic Brønsted-Lowry base content.[13] Examination of these factors may allow for personalized cancer therapy by the selection of candidate patients with highly sensitive tumor subsets that are expected to respond exceptionally well to easily host tolerated

doses of 90CE prodrugs, largely avoiding wide reaching normal tissue toxicities.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8066, USA. Tel: 203-785-4533. Fax: 203-737-2045. E-mail: philip.penketh@yale.edu.

Notes

Three of the authors (P.G.P., K.S., and A.C.S.) are listed as inventors on U.S. Patent 5637619, which includes laromustine, a 90CE prodrug. Laromustine has been licensed to Nanotherapeutics Inc. by Yale University.

The authors declare no competing financial interest.

■ ABBREVIATIONS

90CE, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine; BCNU (carmustine), 1,3-bis(2-chloroethyl)-1-nitrosourea; CNU, chloroethylnitrosoureas; DTT, dithiothreitol; HDR, homology directed repair; MGMT, O^6-alkylguanine-DNA alkyltransferase; G-C ethane cross-link, 1-(N^3-cytosinyl),-2-(N^1-guaninyl)ethane; GSH, glutathione; GST, glutathione S-transferase; TG, α-thioglycerol

■ REFERENCES

(1) Shyam, K., Hrubiec, R. T., Furubayashi, R., Cosby, L. A., and Sartorelli, A. C. (1987) 1,2-Bis(sulfonyl)hydrazines: 3. Effects of structural modification on antineoplastic activity. *J. Med. Chem. 30*, 2157−2161.

(2) Shyam, K., Penketh, P. G., Loomis, R. H., Rose, W. C., and Sartorelli, A. C. (1996) Antitumor 2-Aminocarbonyl-1,2-bis-(methylsulfonyl)-1-(2-chloroethyl)hydrazines. *J. Med. Chem. 39*, 796−801.

(3) Penketh, P. G., Shyam, K., and Sartorelli, A. C. (1994) Studies on the mechanism of decomposition and structural factors affecting the aqueous stability of 1,2-bis(sulfonyl)-1-alkylhydrazines. *J. Med. Chem. 37*, 2912−2917.

(4) Shyam, K., Penketh, P. G., Divo, A. A., Loomis, R. H., Rose, W. C., and Sartorelli, A. C. (1993) Synthesis and evaluation of 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines as antineoplastic agents. *J. Med. Chem. 36*, 3496−3502.

(5) Finch, R. A., Shyam, K., Penketh, P. G., and Sartorelli, A. C. (2001) 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino)-carbonylhydrazine (101M): a novel sulfonylhydrazine prodrug with broad-spectrum antineoplastic activity. *Cancer Res. 61*, 3033−3038.

(6) Johnston, T. P., and Montgomery, J. A. (1986) Relationship of structure to anticancer activity and toxicity of the nitrosoureas in animal systems. *Cancer Treat. Rep. 70*, 13−31.

(7) Giles, F., Verstovsek, S., Thomas, D., Gerson, S., Cortes, J., Faderl, S., Ferrajoli, A., Ravandi, F., Kornblau, S., Garcia-Manero, G., Jabbour, E., O'Brien, S., Karsten, V., Cahill, A., Yee, K., Albitar, M., Sznol, M., and Kantarjian, H. (2005) Phase I study of cloretazine (VNP40101M), a novel sulfonylhydrazine alkylating agent, combined with cytarabine in patients with refractory leukemia. *Clin. Cancer Res. 11*, 7817−24.

(8) Badruddoja, M. A., Penne, K., Desjardins, A., Reardon, D. A., Rich, J. N., Quinn, J. A., Sathornsumetee, S., Friedman, A. H., Bigner, D. D., Herndon, J. E., Cahill, A., Friedman, H. S., and Vredenburgh, J. J. (2007) Phase II study of Cloretazine for the treatment of adults with recurrent glioblastoma multiforme. *Neuro− Oncology (Cary, NC, U. S.) 9*, 70−74.

(9) Gururangan, S., Turner, C. D., Stewart, C. F., O'Shaughnessy, M., Kocak, M., Poussaint, T. Y., Phillips, P. C., Goldman, S., Packer, R., Pollack, I. F., Blaney, S. M., Karsten, V., Gerson, S. L., Boyett, J. M., Friedman, H. S., and Kun, L. E. (2008) Phase I trial of VNP40101M (Cloretazine) in children with recurrent brain tumors: a pediatric brain tumor consortium study. *Clin. Cancer Res. 14*, 1124−1130.

(10) Mekhail, T., Gettinger, S., Blumenschein, G., Jr., Axelrod, R., Haigentz, M., Guarino, M. J., Cahill, A. L., Spigel, D., and Greco, F. A. (2007) A phase II trial of VNP40101M in patients with relapsed or refractory small cell lung cancer (SCLC) with or without brain metastases. *J. Clin. Oncol. 25*, 7724.

(11) Seow, H. A., Penketh, P. G., Shyam, K, Rockwell, S., and Sartorelli, A. C. (2005) 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine: an anticancer agent targeting hypoxic cells. *Proc. Natl. Acad. Sci. U.S.A. 102*, 9282−9287.

(12) Kim, E. Y., Liu, Y., Akintujoye, O. M., Grove, T. A., Shyam, K., Sartorelli, A. C., and Rockwell, S. (2012) Preliminary studies with a new hypoxia-selective cytotoxin, KS119W, *in vitro* and *in vivo*. *Radiat. Res. 178*, 126−137.

(13) Penketh, P. G., Shyam, K, Zhu, R., Baumann, R. P., Ishiguro, K., and Sartorelli, A. C. (2014) Influence of phosphate and phosphoesters on the decomposition pathway of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE), the active anticancer moiety generated by laromustine, KS119 and KS119W. *Chem. Res. Toxicol. 27*, 818−833.

(14) Penketh, P. G., Shyam, K., and Sartorelli, A. C. (2000) Comparison of DNA lesions produced by tumor-inhibitory 1,2-bis(sulfonyl)hydrazines and chloroethylnitrosoureas. *Biochem. Pharmacol. 59*, 283−291.

(15) Penketh, P. G., Shyam, K., and Sartorelli, A. C. (1997) Fluorometric assay for the determination of DNA-DNA cross-links utilizing Hoechst 33258 at neutral pH values. *Anal. Biochem. 252*, 210−213.

(16) Ludlum, D. B. (1997) The chloroethylnitrosoureas: sensitivity and resistance to cancer chemotherapy at the molecular level. *Cancer Invest. 15*, 588−598.

(17) Ishiguro, K., Shyam, K., Penketh, P. G., and Sartorelli, A. C. (2005) Role of O^6-alkylguanine-DNA alkyltransferase in the cytotoxic activity of cloretazine. *Mol. Cancer. Ther. 4*, 1755−1763.

(18) Gerson, S. L. (2002) Clinical relevance of MGMT in the treatment of cancer. *J. Clin. Oncol. 20*, 2388−2399.

(19) Ishiguro, K., Zhu, Y. L., Shyam, K., Penketh, P. G., Baumann, R. P., and Sartorelli, A. C. (2010) Quantitative relationship between guanine O^6-alkyl lesions produced by Onrigin and tumor resistance by O^6-alkylguanine-DNA alkyltransferase. *Biochem. Pharmacol. 80*, 1317−1325.

(20) Ishiguro, K., Shyam, K., Penketh, P. G., Baumann, R. P., Sartorelli, A. C., Rutherford, T. J., and Ratner, E. S. (2013) Expression of O^6-methylguanine-DNA methyltransferase examined by alkyl-transfer assays, methylation-specific PCR and western blots in tumors and matched normal tissue. *J. Cancer Ther. 4*, 919−931.

(21) Rockwell, S., Liu, Y., Seow, H. A., Ishiguro, K., Baumann, R. P., Penketh, P. G., Shyam, K., Akintujoye, O. M., Glazer, P. M., and Sartorelli, A. C. (2012) Preclinical evaluation of laromustine for use in combination with radiation therapy in the treatment of solid tumors. *Int. J. Radiat. Biol. 88*, 277−285.

(22) Penketh, P. G., Baumann, R. P., Ishiguro, K., Shyam, K., Seow, H. A., and Sartorelli, A. C. (2008) Lethality to leukemia cell lines of DNA interstrand cross-links generated by cloretazine derived alkylating species. *Leukemia Res. 32*, 1546−1553.

(23) Bodell, W. J., Aida, T., Berger, M. S., and Rosenblum, M. L. (1985) Repair of O^6-(2-chloroethyl)guanine mediates the biological effects of chloroethynitrosoureas. *Environ. Health Perspect. 62*, 119−126.

(24) Coles, B. (1985) Effects of modifying structure on electrophilic reactions with nucleophiles. *Drug Metab. Rev. 15*, 1307−1334.

(25) Litwack, G., Ketterer, B., and Arias, I. M. (1971) Ligandin: a hepatic protein which binds steroids, bilirubin, carcinogens and a number of exogenous organic anions. *Nature 234*, 466−467.

(26) Salinas, A. E., and Wong, M. G. (1999) Glutathione S-transferases−a review. *Curr. Med. Chem. 4*, 279−309.

(27) David, L., Eaton, D. L., and Bammler, T. K. (1999) Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol. Sci. 49*, 156−164.

(28) Ali-Osman, F., Caughlan, J., and Gray, G. S. (1989) Decreased DNA interstrand cross-linking and cytotoxicity induced in human brain tumor cells by l,3-bis(2-chloroethyl)-l-nitrosourea after *in vitro* reaction with glutathione. *Cancer Res. 49*, 5954−5958.

(29) Ali-Osman, F. (1989) Quenching of DNA cross-link precursors of chloroethylnitrosoureas and attenuation of DNA interstrand cross-linking by glutathione. *Cancer Res. 49*, 5258−5261.

(30) Talcott, R. E., and Levin, V. A. (1983) Glutathione-dependent denitrosation of N,N'-bis(2-chloroethyl)N-nitrosourea (BCNU): nitrite release catalyzed by mouse liver cytosol *in vitro*. *Drug Metab. Dispos. 11*, 175−176.

(31) Weber, G. F., and Waxman, D. J. (1993) Denitrosation of the anti-cancer drug 1,3-bis(2-chloroethyl)-1-nitrosourea catalyzed by microsomal glutathione S-transferase and cytochrome P450 mono-oxygenases. *Arch. Biochem. Biophys. 307*, 369−78.

(32) Wheeler, G. P., Bowdon, B. J., Grimsley, J. A., and Lloyd, H. H. (1974) Interrelationships of some chemical, physicochemical and biological activities of several 1-(2-haloethyl)-1-nitrosoureas. *Cancer Res. 34*, 194−200.

(33) Shyam, K., Penketh, P. G., Divo, A. A., Loomis, R. H., Patton, C. L., and Sartorelli, A. C. (1990) Synthesis and evaluation of 1,2,2-tris(sulfonyl)hydrazines as antineoplastic and trypanocidal agents. *J. Med. Chem. 33*, 2259−2264.

(34) Penketh, P. G., Shyam, K., Patton, C. L., and Sartorelli, A. C. (1996) Spectrophotometric assay for processes involving changes in hydrogen ion concentration in aqueous solution. *Anal. Biochem. 238*, 46−49.

(35) Tew, K. D., et al. (1984) Nitrosoureas, in *Antitumor Drug Resistance* (Fox, B. W., and Fox, M., Eds.) pp 425−442, Springer-Verlag, Berlin, Germany.

(36) MacFarland, J. G., Kirk, M. C., and Ludlum, D. B. (1990) Mechanism of action of the nitrosoureas-IV. Synthesis of the 2-haloethylnitrosourea-induced DNA cross-link 1-(3-cytosinyl),2-(1-guanyl)ethane. *Biochem. Pharmacol. 39*, 33−36.

(37) Bodell, W. J. (2003) Repair of DNA alkylation products formed in 9L cell lines treated with 1-(2-chloroethyl)-1-nitrosourea. *Mutat. Res. 522*, 85−92.

(38) Bodell, W. J., Tokuda, K., and Ludlum, D. B. (1988) Differences in DNA alkylation products formed in sensitive and resistant human glioma cells treated with N-(2-chloroethyl)-N-nitrosourea. *Cancer Res. 48*, 4489−4492.

(39) Niu, T., Yu, D., Kirk, M. C., and Ludlum, D. B. (1993) Synthesis of the prototype DNA-protein cross-link, 1-(guan-1-yl)-2-(cysteine-S-yl)ethane, and its role in the reactions of the haloethylnitrosoureas. *Carcinogenesis 14*, 195−198.

(40) Gonzaga, P. E., and Brent, T. P. (1989) Affinity purification and characterization of human O^6-alkylguanine-DNA alkyltransferase complexed with BCNU-treated, synthetic oligonucleotide. *Nucleic Acids Res. 17*, 6581−6590.

(41) D'Silva, C. (1990) Inhibition and recognition studies on the glutathione-binding site of equine liver glutathione S-transferase. *Biochem. J. 271*, 161−165.

(42) Lien, S., Larsson, A. K., and Mannervik, B. (2002) 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. *Biochem. Pharmacol. 63*, 191−197.

(43) Oakley, A. J., Bello, L. B., Nuccetelli, M., Mazzetti, A. P., and Parker, M. W. (1999) The ligandin (non-substrate) binding site of human Pi class glutathione transferase is located in the electrophile binding site (H-site). *J. Mol. Biol. 291*, 913−926.

(44) Johnson, J. A., el Barbary, A., Kornguth, S. E., Brugge, J. F., and Siegel, F. L. (1993) Glutathione S-transferase isoenzymes in rat brain neurons and glia. *J. Neurosci. 13*, 2013−2023.

(45) Jena, N. R., and Bansal, M. (2011) Mutagenicity associated with O^6-methylguanine-DNA damage and mechanism of nucleotide flipping by AGT during repair. *Phys. Biol. 8*, 1−12.

(46) Christmann, M., Verbeek, B., Roos, W. P., and Kaina, B. (2011) O^6-Methylguanine-DNA methyltransferase (MGMT) in normal tissues and tumors: enzyme activity, promoter methylation and immunohistochemistry. *Biochim. Biophys. Acta 1816*, 179−190.