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Inactivation of Glutathione Reductase by 2-Chloroethyl Nitrosourea-Derived Isocyanates

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Summary: The specific inactivation of yeast glutathione reductase (GSSG-reductase) by 2-chloroethyl isocyanate and cyclohexyl isocyanate derived from their respective 2-chloroethyl nitrosoureas has been demonstrated. Titration of the enzyme with 2-chloroethyl isocyanate or [^{14}C] labeling with 1-(2-chloroethyl)-3-(1-^{14}C-cyclohexyl)-1-nitrosourea or 1,3-bis(2-^{14}C-chloroethyl)-1-nitrosourea resulted in near stoichiometric inactivation and/or covalent labeling of the enzyme. In addition to 1,3-bis (2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea, several other 2-chloroethyl nitrosoureas were capable of inactivation of not only purified GSSG-reductase, but also the activity of this enzyme in cell-free extracts of murine lymphoma L5178Y ascites tumor cells and murine bone marrow.

The 2-chloroethyl nitrosoureas are some of the most promising antineoplastic agents in use today. Interest is being focused on the alkylating and carbamylating intermediates generated during their degradation under physiological conditions. These studies have indicated (5,10,11,14) that the carbamylating moieties generated are isocyanates. Certain alkyl isocyanates have been shown to be active-site directed inactivators of several enzymes (1,2,7,15) and CCNU has been shown to be capable of stoichiometric inactivation of chymotrypsin by virtue of its cyclohexyl isocyanate intermediate (1). Thus it seemed reasonable to postulate the interaction of a 2-chloroethyl nitrosourea-generated isocyanates with an enzymatic active site *in vivo*. Frisher and Ahmand (6) observed reduced erythrocyte GSSG-reductase activity in patients administered BCNU but saw

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no effect on the activity of nineteen other erythrocyte enzymes. This finding suggested that the myelosuppressive BCNU, via a 2-chloroethyl isocyanate, might be interacting with GSSG-reductase in a highly specific fashion. This study describes the specific carbamylation of the NADPH-reduced GSSG-reductase by 2-chloroethyl isocyanate and cyclohexyl isocyanate which resulted in the near stoichiometric inactivation of the enzyme. BCNU and several other 2-chloroethyl nitrosoureas were found to inactivate the enzyme, presumably after they had degraded to form their respective isocyanates. Of the 2-chloroethyl nitrosoureas used in this work those which inactivated GSSG-reductase also exhibit myelosuppressive activity. This work demonstrates the potential of alkylisocyanates as active site probes of glutathione reductase.

Materials and Methods

Reagents. Highly purified yeast GSSG-reductase was obtained from Sigma Chemical Co. All reagents and solvents were reagent grade and commercially available. Isocyanates from Eastman, were redistilled and maintained anhydrous under nitrogen prior to use. Nitrosoureas and [^{14}C] nitrosoureas were supplied by the National Cancer Institute.
General Conditions for GSSG-reductase Incubations. Conditions used throughout this study for GSSG-reductase incubations were 0.1M KPO_4 pH 7.6 with 0.2M KCl and 1 mM EDTA at 37°C. Enzyme activity was determined by the method of Colman (4). Protein concentrations were determined by the method of Lowry et al. (9). The quantitation of enzyme throughout this paper is based on the monomer molecular weight of 55,000 daltons.
Inactivation of GSSG-reductase by Various Nitrosoureas. Various nitrosoureas (50-250 nmoles) were added to solutions containing GSSG-reductase (4.5×10^{-6}M - 6.9×10^{-5}M). Enzyme activity was determined after one half-life of the nitrosourea added had elapsed or in the case of BCNU every 10 min. The half-lives were determined by the decrease in absorbance of the nitroso group in the above incubation buffer, pH 7.6 at 37°C. The nitrosoureas used in this study are abbreviated as follows: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(4-trans-methylcyclohexyl)-1-nitrosourea; cis-2-OH-CCNU, 1-(2-chloroethyl)-3-(cis-2-hydroxycyclohexyl)-1-nitrosourea; trans-4-OH-CCNU, 1-(2-chloroethyl)-3-(trans-4-hydroxycyclohexyl)-1-nitrosourea; GANU, 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea; chlorozotocin, 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-

glucopyranose; ACNU, 1-(4-amino-2-methylpyrimidin-5-yl) methyl-3-(2-chloroethyl)-3-nitrosourea; and [Chx-1-^{14}C]CCNU, 1-(2-chloroethyl)-3-(1-^{14}C-cyclohexyl)-1-nitrosourea.

Titration of GSSG-reductase with 2-Chloroethyl Isocyanate. GSSG-reductase (4.5×10^{-6}M - 6.9×10^{-5}M) was titrated by the sequential addition of 2.5 mM 2-chloroethyl isocyanate according to the procedure of Brown and Wold (2). Cyclohexyl isocyanate was also reacted with the enzyme at these concentrations.

Labeling of GSSG-reductase with [^{14}C] Nitrosoureas. [Chx-1-^{14}C]CCNU (125 nmoles) or [chloroethyl-2-^{14}C]BCNU (125 nmoles) were added to buffered solutions containing 7.2 nmoles (1.7×10^{-5}M) GSSG-reductase in the absence and presence of 2.4×10^{-4}M NADPH. The reactions were incubated at 37°C until approximately 90% enzyme inactivation was achieved. The incubates were diluted 25% with cold 8M urea, cooled rapidly to 4°C, dialyzed overnight against 8M urea in 0.01M KPO_4 pH 7.6 at 4°C and then extensively against 0.01M KPO_4 pH 7.6 at 4°C. The samples were then assayed for radioactivity and protein to determine moles bound [^{14}C] moiety/mole 55,000 MW monomer. The determination of this [^{14}C] labeling stoichiometry takes into consideration the symmetrical [^{14}C] label of the [chloroethyl-2-^{14}C] BCNU.

Inactivation of L5178Y and Expressed Bone Marrow Cell by Various Nitrosoureas. L5178Y cells grown in Gibco growth media or grown *in vivo* were harvested by centrifugation. Cells (1.3×10^6/ml) were washed 2 x with saline, taken up in KPO_4 pH 7.6 with 0.2M KCl and 1 mM EDA, and sonicated 60s with a Kontes sonicator. The sonicate was spun at 39K in a Beckman Ti-50 rotor for 60', and the supernatants used for incubations. Murine bone marrow was expressed from femurs with saline and treated in a similar fashion as above. Various 2-chloroethyl nitrosoureas were incubated at concentrations of 10^{-5}M - 10^{-3}M with the above supernatants. The GSSG-reductase activity was determined at 0 and 60 min after addition of each nitrosourea.

Results

BCNU (50 nmoles) when incubated with NADPH-reduced GSSG-reductase (1.38 nmoles) inactivated the enzyme. The inactivation was time dependent. No enzyme inactivation was observed when BCNU was incubated with GSSG-reductase in the absence of NADPH (Figure 1). In separate experiments BCNU, CCNU, MeCCNU, trans-4-OH-CCNU and to a lesser extent ACNU inactivated NADPH-reduced GSSG-reductase but had no effect on enzyme activity in the absence of NADPH. Chlorozotocin, cis-2-OH-CCNU, and GANU did not inactivate the NADPH-reduced enzyme significantly (Table III). Since these 2-chloroethyl nitrosoureas all generated 2-chloroethyl carbonium ions and the enzyme inactivation was dependent on nitrosourea degradation, the isocyanates

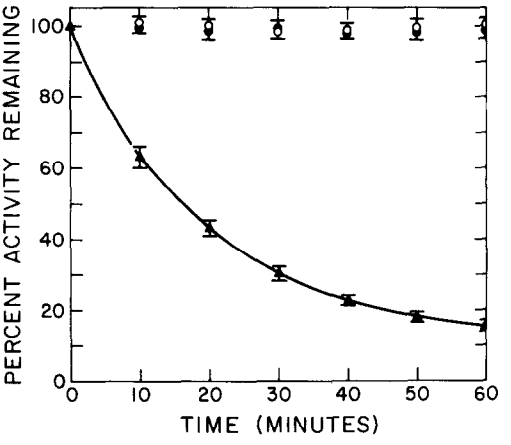

Figure 1: BCNU inactivation of glutathione reductase. BCNU (50 nmoles in anhydrous acetone) was added to solutions containing 1.38 nmoles glutathione reductase (5.48×10^{-6} M) in the presence of 1.58×10^{-4} M NADPH (▲), and absence of NADPH (●). Controls contained acetone, NADPH, and enzyme, but no BCNU (◎).

TABLE I: TITRATION OF GLUTATHIONE REDUCTASE WITH 2-CHLOROETHYL ISOCYANATE. 2-CHLOROETHYL ISOCYANATE TO GLUTATHIONE REDUCTASE MOLE RATIO FOR 50% INACTIVATION.

ENZYME CONCN (M)	2-CHLOROETHYL ISOCYANATE/ GLUTATHIONE REDUCTASE
4.5×10^{-6}	9.94
1.1×10^{-5}	5.52
2.8×10^{-5}	2.64
6.9×10^{-5}	1.15

Titration of glutathione reductase (4.5×10^{-6} M - 6.9×10^{-5} M in 0.1 M KPO_4 pH 7.6 with 0.2 M KCl and 1 mM EDTA at 37°C) by the sequential addition of 2.5 mM 2-chloroethyl isocyanate in anhydrous acetone. Values are given as mole ratios for 50% glutathione reductase inactivation.

generated were assumed to be responsible for the observed enzyme

TABLE II: [*chloroethyl*-2-^{14}C]BCNU AND [*chx*-1-^{14}C]CCNU LABELING OF GLUTATHIONE REDUCTASE

[^{14}C] NITROSOUREA	PERCENT OF TOTAL ENZYME			[^{14}C] MOIETY BOUND/MOL OF TOTAL ENZYME	[^{14}C] MOIETY BOUND/MOL OF NITROSOUREA INACTIVATED ENZYME
	Total Enzyme Inactivation	NADPH Inactivation	[^{14}C] Nitrosourea Inactivated Enzyme		
[*chloroethyl*-2-^{14}C] BCNU					
WITH NADPH	84	14	70	0.74	1.07
WITHOUT NADPH	0	-	0	0.19	-
[*chx*-1-^{14}C] CCNU					
WITH NADPH	95	12	83	1.16	1.41
WITHOUT NADPH	0	-	0	0.39	-

[chx-1-^{14}C]CCNU (125 nmoles) and [chloroethyl-2-^{14}C] BCNU (125 nmoles) were added to separate solutions containing 7.2 nmoles glutathione reductase (1.7×10^{-5} M) in the presence of 2.4×10^{-4} M NADPH and absence of NADPH. The reactions were incubated at 37°C until approximately 90% enzyme inactivation was achieved. The procedure is further described in the text.

inactivation. To determine this, 2-chloroethyl isocyanate or cyclohexyl isocyanate were incubated with NADPH-reduced GSSG-reductase; immediate enzyme inactivation ensued. As Table I indicates, the 2-chloroethyl isocyanate titration of GSSG-reductase became more stoichiometric as the enzyme concentration was increased. The oxidized form of the enzyme was not inactivated by either of these isocyanates. To confirm that the inactivation was due to stoichiometric carbamylation by nitrosourea derived isocyanates, [chx-1-^{14}C]CCNU and [chloroethyl-2-^{14}C]BCNU at a 17-fold molar excess were incubated separately with GSSG-reductase in the absence and presence of NADPH. These additions of radiolabeled 2-chloro-

Table III: Inactivation of Glutathione Reductase by Various Nitrosoureas

Nitrosourea	Concn ($\times 10^{-4}$ M)	t 1/2 (Min)	Percent inactivation at t 1/2	Carbamylating* Activity	Absolute Neutrophile Count
CCNU	1.97	56	97	94 (13)	25 (13)
MeCCNU	1.97	58	96	91 (16)	
BCNU	1.97	42	77	69 (13)	25 (13)
cis-2-OH-CCNU	9.72	48	3	1 (18)	
trans-4-OH-CCNU	1.97	45	81	94 (18)	
Chlorozotocin	9.72	22	0	4 (13)	110 (13)
GANU	9.72	7	2	63 (13)	113 (13)
ACNU	9.72	24	30	3.5 (12)	4 (12)

+ Percent of control peripheral blood neutrophile count on Day 3, WBC at nadir.

* Percent of 2-chloroethylnitrosourea carbamylation of ^{14}C-lysine.

The nitrosoureas above were added to buffered solutions containing glutathione reductase (4.5×10^{-6} M - 6.9×10^{-5} M). Enzyme activity was determined after one half-life of the added nitrosourea had elapsed.

ethyl nitrosoureas to the reduced enzyme led to the time-dependent inactivation and concurrent [^{14}C] labeling of GSSG-reductase. In the absence of NADPH there was no inactivation of enzyme and [^{14}C] labeling was quite low (Table II). The amount of [^{14}C] moiety bound per mole of nitrosourea-inactivated enzyme was stoichiometric. The eight 2-chloroethyl nitrosoureas listed in Table III were incubated for 1 hr with sonicates of L5178Y and murine bone marrow cells and the GSSG-reductase measured at 0' and 60'. Those 2-chloroethyl nitrosoureas which were found to inactivate the purified yeast enzyme also inactivated GSSG-reductase in these sonicates.

Discussion

2-Chloroethyl isocyanate and cyclohexyl isocyanate have been shown to be near stoichiometric inactivators of GSSG-

reductase. Inactivation was observed when these isocyanates were either added directly or generated *in situ* by the degradation of BCNU and CCNU, respectively. These results combined with the observed *in vivo* inactivation of human erythrocyte GSSG-reductase by BCNU (6) suggest that this highly specific inactivation may have important *in vivo* consequences. Loos *et al.* (8) have shown that the lack of erythrocyte GSSG-reductase was compensated for by increased glutathione biosynthesis. However, an oxidative challenge resulted in a severe hemolytic crisis resembling the myelosuppression which accompanies administration of certain 2-chloroethyl nitrosoureas. It is interesting to note that of the nitrosoureas examined, those which inactivated the enzyme also exhibit myelosuppressive activity. The observed stoichiometric inactivation of GSSG-reductase indicates that this and other active site-specific inactivations of enzymes by nitrosourea-derived isocyanates warrant further investigation.

Alkyl isocyanates have been demonstrated to be active site directed inactivators of chymotrypsin (1,2), alcohol dehydrogenase (15), and transglutaminase (7). In the case of chymotrypsin, Brown and Wold have suggested a two-step process. Octyl isocyanate first binds by virtue of its n-alkyl side chain, analogous to the formation of a Michaelis complex, followed by carbamylation of the active site serine (3).

To date the rationale for determining carbamylating potential of 2-chloroethylnitrosoureas has been the extent to which L-lysine is carbamylated (17). The presence of structurally complementary binding sites for isocyanates *in vivo* would preclude their random carbamylation of nucleophiles. L-lysine concentra-

tions up to 10 mM *in vitro* had no effect on the active site directed inactivation of chymotrypsin by CCNU (1). The intramolecular carbamylation which occurs during degradation of ACNU could be responsible for the low extent to which ACNU carbamylates L-lysine (Tanaka et al., work cited by [12]). Yet it was demonstrated that ACNU was capable of inactivating GSSG-reductase presumably via the isocyanate. Thus it seems unreasonable to exclude the possibility of carbamylation by nitrosoureas solely on the basis of their low potential to chemically modify L-lysine. Clearly the structure of the isocyanates' side chain determines the selectivity of the isocyanate for a complementary binding site and therefore determines its role *in vivo*. The specificity of these isocyanates suggest their potential as active site probes. Work is now in progress to assess the value of these isocyanates as probes for the catalytic site of GSSG-reductase.

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