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MECHANISM OF ACTION OF 2-HALOETHYLNITROSOUREAS ON DEOXYRIBONUCLEIC ACID

PATHWAYS OF AQUEOUS DECOMPOSITION AND PHARMACOLOGICAL CHARACTERISTICS OF NEW ANTICANCER DISULFIDE-LINKED NITROSOUREAS*

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Abstract—We have examined the pharmacological characteristics of three dinitrosated isomers of *N,N'*-bis[*N*(2-chloroethyl)-*N*-carbamoyl]cystamine [CNCC-(D), 1C1G1325] differing in the relative positions of the nitroso substituents [CNCC-(C), (1,1'dinitroso); CNCC-(S), 3,3'dinitroso); and CNCC-(M), (1,3'-dinitroso)] and which were designed to be subject to preferential bioreductive activation in hypoxic tumors. The decomposition products of the isomers formed under physiological conditions [both in the absence and in the presence of dithiothreitol (DDT)] were identified and quantified. For example, CNCC-(S) in phosphate buffer, pH 7.0, and 37° gave rise to 2-chloroethylisocyanate, bis(2-chloroethyl)urea and bis(2-hydroxyethyl)disulfide, whereas in the presence of DTT it afforded 2-chloroethylisocyanate, bis(2-chloroethyl)urea, bis(2-hydroxyethyl) disulfide, thiirane and 2-mercaptoethanol. Control aqueous decomposition profiles were performed with two known metabolites of CNCC, namely 3-(2-chloroethyl)-1-(2-thioethyl)-1-nitrosourea and 3-(2-chloroethyl)-1-(2-methylthioethyl)-1-nitrosourea. CNCC-(C) caused 20% interstrand cross-linking of λ-DNA in 2 hr, whereas in the presence of DTT the extent of cross-linking increased to 38% in the same time period. In contrast, isomer (S) showed no detectable cross-linking in 7 hr. This thiol potentiation of cross-linking which is observed with other 2-chloroethylnitrosoureas is explained by nucleophilic attack at the carbonyl group and subsequent stereoelectronically controlled decomposition of the tetrahedral intermediate. The relative extents of carbamoylating activity of the CNCC isomers were obtained using a [^{14}C]-lysine assay which showed (S) ≈ (M) > (C). Inhibition of glutathione reductase for both Walker 256 resistant (WR) and Walker 256 sensitive (WS) strains showed that isomer (S) inactivated the enzyme more effectively than isomer (C) in accord with the carbamoylating activity results. The higher carbamoylators (S) and (M) also showed greater effects on the intracellular thiol pools in both WR and WS cells indicative of sulfhydryl conjugation and efflux and/or inhibition of the GSH metabolic enzymes. *In vitro* cytotoxicity studies with human DU 145 prostatic carcinoma cells showed the isomer cytotoxicity was (M) > (C) > (S) over a 24-hr incubation period. The reduced cytotoxic potential of CNCC-(S) in both the Walker 256 cells and in the human prostatic carcinoma cells may be a function of an interaction between GSH and the drug thereby protecting other more critical nucleophilic targets within the nucleus.

2-Chloroethylnitrosoureas (CENUs)¶, including BCNU, CCNU, MeCCNU and chlorozotocin, have widespread clinical application in the treatment of human malignancies, including Hodgkin's disease, Burkitt's lymphoma, cerebral neoplasms and cancer of the gastrointestinal tract [1–6]. Their therapeutic efficacies appear to be related to their spontaneous decompositions under physiological conditions to

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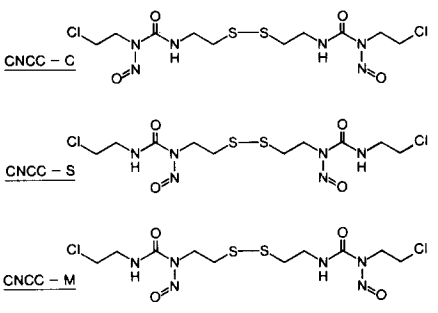
¶ Abbreviations: CENU, 2-chloroethylnitrosourea; BCNU, 1,3 bis(2-chloroethyl)nitrosourea; CCNU, 3-cyclohexyl-1-(2-chloroethyl)-1-nitrosourea; MeCCNU, 3-(4'-methylcyclohexyl)-1-(2-chloroethyl)-1-nitrosourea; CNCC-(D), mixture of the three dinitrosated isomers of *N,N'*-bis[*N*(2-chloroethyl)-*N*-carbamoyl]cystamine, code number: 1C1G1325; NADPH, nicotinamide adenine dinucleotide triphosphate; DTT, dithiothreitol; NBP, 4-(4'-nitrobenzyl)pyridine; GSH, glutathione; GR, glutathione reductase; WR, Walker 256 carcinoma strain resistant to bifunctional nitrogen mustards; WS, Walker 256 carcinoma strain sensitive to bifunctional nitrogen mustards; HPLC, high performance liquid chromatography; CLC, covalently linked complementary; GC, gas chromatography; GC-MS, gas chromatography analysis linked to mass spectrometry; and CIMS, chemical ionization mass spectrometry.



CNCC – D is a 1:1 mixture of S and M, and traces of C.

Fig. 1. Structural formulas of the isomeric dinitrosated derivatives of *N'N'*-bis[*N*(2-chloroethyl)-*N*-carbamoyl]-cystamine.

generate both electrophilic species which alkylate cellular macromolecules (principally nucleic acids) and isocyanates which can carbamoylate proteins [1, 5, 7, 8]. Intensive study of the extraordinarily rich chemistry of CENUs has revealed alternative pathways of decomposition, the preference for which depends on the structure of a given CENU [7–11]. These studies combined with others on the biochemical pharmacology of CENUs demonstrated that certain lesions including DNA alkylation and interstrand cross-linking correlate in many cases with cytotoxicity and *in vivo* antileukemic properties [8, 12, 13]. Because of the inherent difficulties in treating solid tumors [14], CENUs have also been designed to be subject to preferential bioreductive activation in hypoxic tumors. Promising agents prepared on this basis include 3,3'-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyldisulfide [13] and a mixture of dinitrosated derivatives of *N,N'*bis[*N*(2-chloroethyl)-*N*-carbamoyl]cystamine [15; *, †, ‡]. The availability of the latter in three isomeric forms (Fig. 1) together with comparative cytotoxicity and other pharmacological data [16] afford an opportunity for a more critical evaluation of the relationship of given macromolecular lesions to cytotoxicity. Accordingly, we report an examination of the alternative pathways of decomposition under physiological conditions of isomeric forms of CNCC, their products, and interactions with DNA. Pertinent biochemical and pharmacological studies of carbamoylating and alkylating properties, inhibition of glutathione reductase effects on intracellular glutathione pools, and *in vitro* activity of the CNCC isomers against a DU 145 human prostatic carcinoma cell line are also described.

MATERIALS AND METHODS

Materials. The pure isomeric forms of CNCC were prepared by the methods previously described [15; *, †, ‡]. Prior to each experiment, drug purity and concentration were monitored by HPLC. Chlorozotocin was a gift from Dr. Gerald Goldenberg, Manitoba Institute of Cell Biology, Winnipeg, Manitoba. CCNU was prepared by a literature procedure [3]. Dithiothreitol, 2-mercaptoethanol, and authentic samples used in the gas chromatographic analysis, 2-chloroethanol, vinyl chloride, thiirane and acetaldehyde were obtained from the Aldrich Chemical Co., Milwaukee, WI. Ethidium bromide was from Sigma; λ-DNA (mol. wt 31×10^6) was obtained from Miles Laboratories.

General procedure for analysis of products of aqueous decomposition of nitrosoureas. Solutions of the nitrosoureas (0.1 mmole/m) in 40 mM potassium phosphate buffer (pH 7.2) (and containing 0.6 mmole/m of dithiothreitol where applicable) in 3 m air-tight Reacti-vials equipped with Teflon septums were incubated at 37° for a standard reaction time of 24 hr. The reaction contents were analyzed for (i) volatile and gaseous products, (ii) extractable organic products, and (iii) water soluble organic products.

Gaseous and volatile components. Samples of the gaseous and volatile products were withdrawn with a hypodermic syringe from the pressurized space in the Reacti-vial and analyzed in a Hewlett–Packard 584A analytical gas chromatograph using a temperature program in the range of 45–100°.

Extractable organic components. A volume of 0.25 m of chloroform was injected into the aqueous layer in the Reacti-vial and vigorously shaken for a few minutes. The chloroform layer was withdrawn with a syringe and dried (Na_2SO_4), and then the contents were analyzed on the analytical GC using a temperature program in the range 60–160°.

Water soluble components. The remaining aqueous layer in the Reacti-vial was lyophilized and the residue was examined by chemical ionization mass spectrometry (CIMS) using NH_3 as reagent gas.

GC-Mass spectral analytical procedure. The gaseous, volatile and chloroform extracted components were analyzed by GC-MS. Individual components were identified by GC retention times and characteristic MS fragmentation patterns by comparison with authentic samples. Additional analyses were carried out with a direct inlet system. For example, the chloroform extract was introduced by direct inlet and, after removing the solvent by vacuum, the temperature was slowly raised and MS scans were recorded at 10° intervals.

Ethidium binding assay for determining CLC sequences in DNA produced by nitrosoureas. The fluorometric method to determine CLC sequences in linear DNA has been described [17, 18]. The basis of this assay is that, whereas a linear DNA (e.g. phage λ) will denature completely to single strands upon heating in the ethidium bromide assay solution at pH 11.8, if an interstrand cross-link has been introduced as a result of chemical reaction, this serves as a nucleation site in a subsequent 96° heat denaturation and cooling cycle so that treated DNA returns to duplex register and thus provides intercalation sites for ethidium. That this assay procedure detects the formation of CLC-DNA as a result of a cross-linking event has been confirmed by experi-

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ments with endonuclease-S_1 [18]. This enzyme specifically cleaves single-stranded DNA; therefore, it distinguishes DNA which is renaturable by virtue of a chemical cross-link and DNA which separates into single strands on heating [17, 18].

All measurements were performed on a G. K. Turner and Associates 430 spectrofluorometer equipped with a cooling fan to minimize fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One centimeter-square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. The 100× scale of medium sensitivity was generally used, and the water was circulated between the cell compartment and a thermally regulated bath at 22°. Reactions were carried out in a total volume of 200 μl containing 50 mM potassium phosphate, pH 7.0, 1.0 A_{260} unit of λ-DNA, 5 mM nitrosourea at 37° (and 30 mM dithiothreitol where applicable). A 10-μl aliquot was taken at intervals from the reaction mixture and added to 2 ml of the standard assay mixture (which was 200 mM potassium phosphate, pH 11.8, 0.4 mM EDTA and 0.5 μg/ml of ethidium). The fluorescence reading after heat denaturation (96°/4 min) and rapid cooling and equilibration at 22° compared with the control times 100 gives the percentage of CLC-DNA, i.e. DNA containing at least one cross-link per molecule. For a standard set of conditions (i.e. type and concentration of DNA, pH, ionic strength and the temperature), the accuracy of the CLC assay is determined by the precision of the fluorescence readings. Overall accuracy of the CLC assay is estimated at ± 2% [17, 18]. Alkylation of DNA, unaccompanied by interstrand cross-linking was determined by the characteristic suppression of ethidium binding sites revealed by the concomitant decrease of ethidium fluorescence in the before heat denaturation readings [17, 18]. This method of measuring alkylation has been confirmed by independent studies using radiolabeled drugs [18].

Thiol analysis. Aspects of glutathione metabolism were studied using Walker 256 rat breast carcinoma cells. Selected from the parent cell line (WS), a resistant sub-line (WR) has been shown previously to express an approximate 15-fold resistance to a diverse range of bifunctional nitrogen mustards and other alkylating agents, but to lack cross-resistance to nitrosoureas [19].

Total intracellular thiol pools. Cells were harvested by centrifugation. The pellet was suspended in 5% trichloroacetic acid (TCA) for 30 min. Deproteinated supernatant fraction (500 μl) was added to 4.5 ml of 0.1 mM bis-(3-carboxy-4-nitrophenyl) disulfide in 0.1 M potassium phosphate, pH 8.0 [20]. The samples were then read at 412 nm after 25 min. Standardization was achieved by concurrent measurements of glutathione (Sigma, St. Louis, MO) standard in acid.

Assay for glutathione reductase. Glutathione reductase activity was measured by the method of Mize and Langdau [21]. Enzyme was prepared from either drug-treated or untreated log phase cells suspended in 0.1 M K_2HPO_4/KH_2PO_4, pH 7.6; 0.2 M KCl; 1 mM EDTA. This suspension was sonicated at a power setting of 60 using 3 × 30 sec exposures, and centrifuging at 34,000 rpm for 1 hr. The crude enzyme was adjusted to 1.5 mg protein/ml prior to use. All data are expressed as percent of untreated enzyme activity. Protein values were determined by the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA) with bovine gamma globulin as a standard.

Chemical alkylating activity. An aliquot with 0.20 to 4.0 μmoles of each nitrosourea dissolved in acetone was added to 1.5 ml of 5% (w/v) 4-(*p*-nitrobenzyl)pyridine in acetone. Four milliliters of 0.025 M acetate buffer, pH 6, was added, and the solution was incubated at 37° for 2 hr; 2 ml of acetone and 3 ml of ethyl acetate were then added. The mixture was made alkaline by the addition of 1.5 ml of 0.25 N NaOH, shaken vigorously, and centrifuged at 3000 rpm for 15 sec. The absorbance of the ethyl acetate layer was determined at 540 nm. The relative alkylating activity of CNCC is expressed as a percentage of the activity of chlorozotocin [22].

Chemical carbamoylating activity. To measure the chemical carbamoylating activity of each nitrosourea, the following procedure was used: 4.2 μmoles of cold lysine, 0.4 μCi of [U-^{14}C]lysine (Amersham Corp., sp. act. 300 mCi/mmole), 120 μl of 0.1 M sodium phosphate buffer, pH 7.4, 160 μl of ethanol, and 4.2 μmoles of each nitrosourea were incubated together for 20 hr at 37°. The reaction mixture without nitrosoureas served as a control. Ten microliters of each reaction mixture was spotted on Whatman No. 3 chromatography paper (Whatman, Inc., Clifton, NJ) that had been prewetted with 0.1 M sodium phosphate buffer, pH 6. Separation was accomplished using a Savant high-voltage flat plate electrophoresis tank (Savant Instruments, Inc., Hicksville, NY) at 3000 V for 75 min. Radioactivity in 1-inch squares was counted. A distinct peak of radioactivity not associated with the parent [U-^{14}C]-lysine represented products of carbamoylation. The carbamoylated products have been identified previously as a mixture of an α-carbamoyl derivative of lysine, and ε-carbamoyl derivative of lysine, and a combined α-carbamoyl and ε-carbamoyl derivative of lysine. Carbamoylating activity is expressed as the percentage of the total radioactivity associated with the carbamoylated product [23].

Cytotoxicity assay DU 145 human prostatic carcinoma cells. Human prostatic carcinoma cells (DU 145, obtained from AB Leo, Helsingborg, Sweden) growing as monolayers in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (KC Biologicals, Kansas), 2 mM glutamine and penicillin/streptomycin (MA Bioproducts, Maryland) were seeded at 2×10^2 cells/ml in Corning polystyrene 25 cm^3 tissue culture flasks (final volume 10 ml). Cells were allowed to attach by incubating for 24 hr at 37° under 5% CO_2. At this time various concentrations of the isomeric forms of CNCC were added to the cultures and incubated for 2 hr. Following drug exposure, the medium was decanted and replaced by 10 ml of fresh medium. Following an 8-day growth period, attached colonies were fixed with absolute ethanol–glacial acetic acid (9:1) and stained for 1 hr with an aqueous solution of 10% blood giemsa (MCB, New Jersey). Survival was expressed as percentage of control growth.

Table 1. Products of aqueous decomposition of CNCC isomers and metabolites

Compound	Reaction conditions	Decomposition products	Retention time (min)	% Yield	*m/e* (relative intensity fragments)
CNCC-C	Phosphate buffer, pH 7.0, 37°	Acetaldehyde	1.4	2	44(M^+,62), 43(M-H,30), 29(CHO,100)
		Vinyl chloride	1.6	5	64(M+2,34), 62(M^+,100), 27(M^+—Cl,91)
		2-Chloroethanol	23	10	82(M+2,1.4), 80(M^+,4.3) 31(CH_2^+ OH,100)
		Bis(2-cyanatoethyl)-disulfide		15–20	205(MH^+,100), 149(MH^+—CH_2NCO,66)
CNCC-C	Phosphate buffer, pH 7.0, plus 30 mM dithiothreitol	Acetaldehyde	1.45	3	44(M^+,39), 43(M—H,24), 29(CHO,100)
		Vinyl chloride	1.6	5	64(33.9,M+2), 62(M^+,100), 27(M—Cl,89)
		2-Chloroethanol	23	3	82(M+2,1.4), 80(M^+,4.3), 31 (CH_2OH,100)
		N,N'-bis(2-mercaptoethyl)urea		Trace	181(MH^+,1.2), 180(MH^+—,0.9), 179(MH^+—2,0.5), 134(MH^+—CH_2SH,1.0), 121(MH^+—CH_2CH_2S,5.2) 105(HS—CH_2CH_2NH(0.1(
CNCC-S	Phosphate buffer, pH 7.0, 37°	2-Chloroethylisocyanate	8	10–15	107(M+2,1.7), 105(M^+,5.1), 70(M—Cl,7.9), 56(M—CH_2Cl,100), 49(CH_2Cl,5.8)
		Bis(2-chloroethyl)urea		5–10	188(M^++4,5.2), 186(M+2,32.2), 184(M^+,53.1), 137(M—CH_2Cl^{35},33.2), 135(M—CH_2Cl^{37},100)
		Bis(2-hydroxyethyl)-disulfide*		20–30	155(MH^+,3), 124(MH^+—CH_2OH,7) 105(CH_2=S^+—S—CH=CH_2,100)
CNCC-S	Phosphate buffer, pH 7.0, plus 30 mM dithiothreitol	2-Chloroethylisocyanate	7.5	10–15	107(M+2,1.1), 105(M^+,5.7), 70(M—Cl,7.8), 56(M—CH_2Cl,100), 45(CH_2Cl,4.1)
		Bis(2-hydroxyethyl) disulfide*		20–30	155(MH^+,0.5), 124(MH^+=CH_2OH,3), 105(CH=S^+SCH_2=CH_2 ,100)
		2-Mercaptoethanol	18.5	2	78(M^+,38), 60(M—H_2O,100), 47(CH_2=S^+H,87), 46(CH_2S^+,48), 45(CH≡S^+,51)
		Thiirane	3.1	3	60(M^+,87), 59(M—H,91), 47(CH_2=S^+H,67), 46(CH_2S^+,48), 4.5(CH≡S^+,100)
		Bis(2-chloroethyl)urea		5–10	188(M+4,5.1), 186(M+2,32.8), 184(M^+,51.8), 137(M—CH_2Cl^{35},33.5), 135(M—CH_2Cl^{37},100)
Monomer-thiol	Phosphate buffer, pH 7.0, 37°	2-Chloroethanol	24	2	82(M+2,0.4), 80(M^-1.2), 31(C^+H_2OH,100)

Table 1—*continued*

Compound	Reaction conditions	Decomposition products	Retention time (min)	% Yield	*m/e* (relative intensity fragments)
Monomer-thiol	Phosphate buffer, pH 7.0, 37°	Vinyl chloride	1.3	10	64(M+2,34.5), 62(M^+,100), 27(M—Cl,80)
		2-Mercaptoethylamine		3	78(MH^+,100), 61(MH—NH_3,2.9)
		Bis(2-thioethyl)urea*		15–25	181(MH^+,44), 147(MH^+—H_2S,26.8), 122(MH^+—C_2H_4S,100)
		2-Thioethylisocyanate*		Trace	104(MH^+,38), 61(MH^+—HNCO,27)
Monomer-thiol	Phosphate buffer, pH 7.0, 37°, plus 30 mM dithiothreitol	Vinyl chloride	1.2	1	64(M+2.33.5),
		2-Chloroethanol	21	2–5	62(M^+,100), 27(M—Cl,73) 82(M+2,0.5), 80(M^+,1.6), 31(CH^+_2OH,100)
		2-Thioethylamine*		2–5	78(MH^+,100), 61(MH—NH_3,2.9)
		Bis(2-thioethyl)urea*		20.22	181(MH^+,1.5), 147(MH^+—H_2S,23), 122(MH^+-C_2H_4S,100)
Monomer-thiol methyl ether	Phosphate buffer, pH 7.0, 37°	2-Chloroethanol	22.5	10–15	82(M+2,3.1), 80(M^+,9.5), 31(C^+H_2OH,100)
		Acetaldehyde	1.45	1	44(M^+,51), 43(M—H,29), 29(C^+HO,100)
		Vinyl chloride	1.6	3	64(M+2,34), 62(M+2,100), 27(M—Cl,90)
		1,3-Bis(2-methylthio) ethylurea*		20–25	209(MH^+,50), 161(MH^+—CH_3SH,13), 92($CH_3SCH_2CH^+_2NH_3$,100) 75($CH^+_2SCH_3$,15)

* Molecular formula confirmed by mass spectrometry.

RESULTS

Decomposition pathways of CNCC isomers under physiological conditions. The products of the decomposition of CNCC-(S) at pH 7.0 and 37° and in the absence of added thiol were 2-chloroethylisocyanate, bis(2-chloroethyl)urea and bis(2-hydroxyethyl)-disulfide (Table 1, Fig. 2). In the presence of added dithiothreitol, CNCC-(S) under similar conditions afforded 2-chloroethylisocyanate, bis(2-chloroethyl)-urea, bis(2-hydroxyethyl)disulfide, thiirane, and 2-mercaptoethanol (Table 1, Fig. 3). Isomer CNCC-(C) under physiological conditions gave acetaldehyde, vinyl chloride, 2-chloroethanol and bis(2-cyanatoethyl)disulfide (Table 1, Fig. 4). In the presence of added dithiothreitol CNCC-(C) afforded acetaldehyde, vinyl chloride, 2-chloroethanol, and *N,N'*-bis (2-mercaptoethyl)urea (Table 1, Fig. 5).

Control experiments were also performed with two of the known metabolites of CNCC-(C)*, namely 1-(2-chloroethyl)-3-(2-thioethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(2-methylthioethyl)-1-nitrosourea. The former compound is the anticipated initial product of bioreductive activation of CNCC-(C) and under physiological conditions leads to 2-chloroethanol, vinyl chloride, 2-mercaptoethylamine, 2-thioethylisocyanate, thiazolidinone

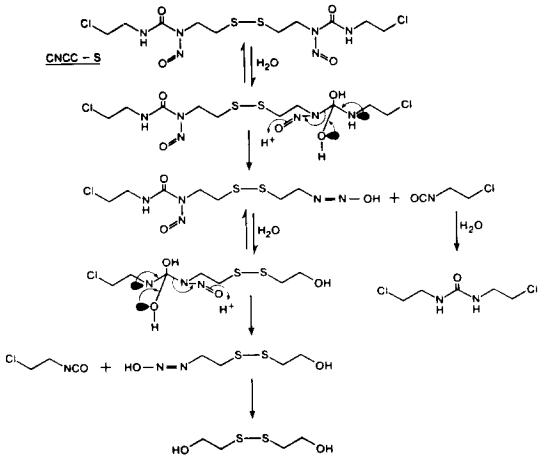

Fig. 2. Pathways of aqueous decomposition of CNCC-S.

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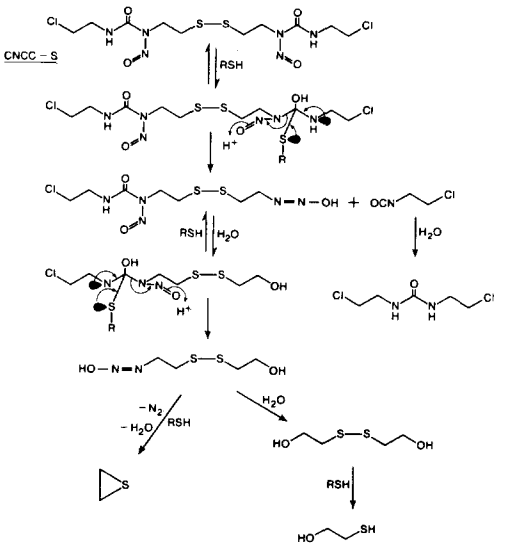

Fig. 3. Pathways of aqueous decomposition of CNCC-S in the presence of dithiothreitol.

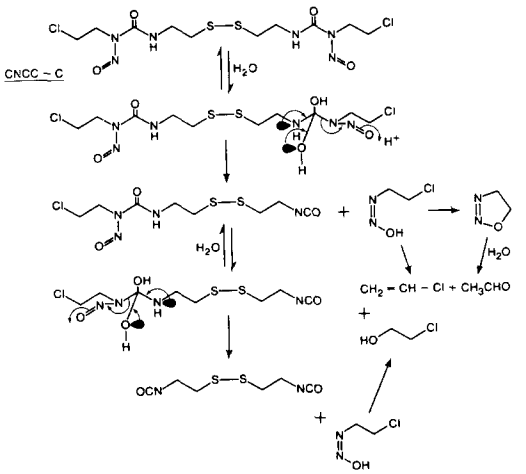

Fig. 4. Pathways of aqueous decomposition of CNCC-C.

and bis(2-thioethyl)urea. 1-(2-Chloroethyl)-3-(2-methylthioethyl)-1-nitrosourea results from the *in vivo* methylation of 1-(2-chloroethyl-3-(2-thioethyl)-1-nitrosourea presumably by *S*-adenosylmethionine. Upon aqueous decomposition, it affords 2-chloroethanol, acetaldehyde, vinyl chloride and 1,3-bis(2-methylthio)ethylurea (Table 1).

DNA interstrand cross-linking by CNCC isomers in the absence and in the presence of added dithiothreitol. Isomer CNCC-(C) at a concentration of 5 mM caused 20% interstrand cross-linking of λ-DNA in 2 hr, whereas in the presence of 30 mM dithiothreitol the extent of cross-linking increased to 38% in the same time period (Table 2). Isomer CNCC-(S) at a concentration of 5 mM caused no detectable interstrand cross-linking in 7 hr (Table 2). In the presence of added 30 mM dithiothreitol, there was extensive alkylation of the DNA as evidenced by suppression of ethidium binding sites although DNA interstrand cross-linking did not result since there was no interference with heat-induced denaturation. Isomer CNCC-(M) afforded 15% interstrand cross-linking which increased to 30% in the presence of 30 mM dithiothreitol (Table 2). For comparison purposes it was found that 5 mM concentrations of CCNU caused 37% DNA interstrand cross-linking at pH 7.0 and 37° which increased to ~41% in the presence of thiol in 2 hr. The pronounced accelerating effects on the extents of DNA interstrand cross-linking produced by added thiol in the cases of the nitrosoureas BCNU and chlorozotocin over longer incubation times may be seen in Fig. 6. In the case of the thiomethyl ether, one observes a progressive enhancement in the extent of DNA interstrand cross-linking up to a maximum of about 46% with increasing proportions of dithiothreitol (Fig. 7). In contrast with the monomer-thiol, one sees quite efficient DNA cross-linking in the absence of dithiothreitol which is progressively suppressed with increasing proportions of dithiothreitol down to a limiting value which is comparable with that produced by CH_3-S-R (see Fig. 7).

Alternative determination of in vitro *alkylating activity.* The relative extents of alkylation of 4-(4′-nitrobenzyl)pyridine (NBP) [22] by electrophilic species generated from the isomers of CNCC were measured using a modified assay. Table 3 shows that in this assay the extent of alkylation was (S) > (D) > (M) > (C). Each CNCC isomer posessed an alkylating potential in excess of chlorozotocin.

Table 2. Interstrand cross-linking and alkylation of λ-DNA by CNCC isomers and related compounds in the presence and absence of thiol

Nitrosourea	% Interstrand cross-linking/alkylation in 2 hr	
	Without thiol	With 30 mM dithiothreitol
CNCC-C	20 ± 0.4*	38 ± 0.8*
CNCC-S	0†‡	≃0
CNCC-M	15 ± 0.3*	30 ± 0.6*†
CNCC-monomer-thiol	>95 ± 4*	45 ± 2*
CNCC-monomer-thiomethyl ether	20 ± 0.4*	45 ± 2*
CCNU	37 ± 0.8*	41 ± 2

* Interstrand cross-linking.
† DNA alkylation.
‡ Seven hours.

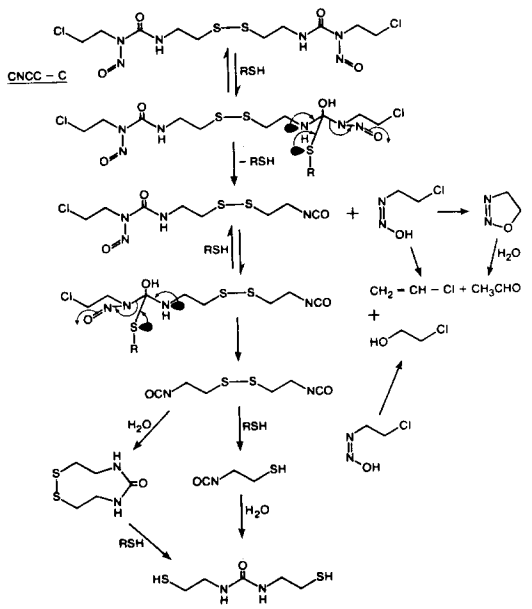

Fig. 5. Pathways of aqueous decomposition of CNCC-C in the presence of dithiothreitol.

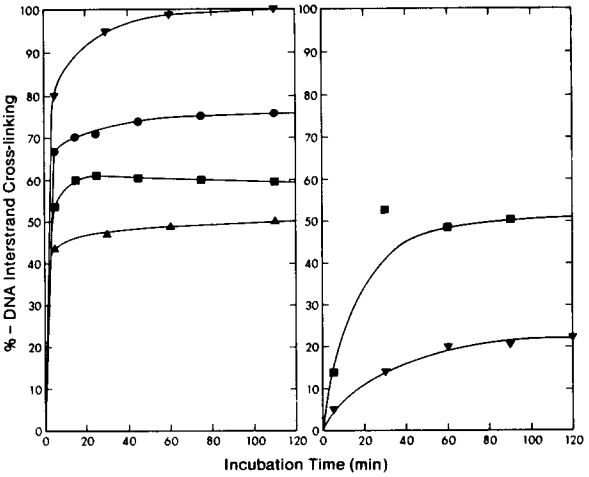

Fig. 7. Percentage of interstrand cross-linking in λ-DNA as a function of time at 37° in the presence of various proportions of DTT. Left-hand diagram with 5 mM CNCC-monomer thiol with (ratio of CNCC-SH:DTT) (▼) 1:0, (●) 1:1.2, (■) 1:3 and (▲) 1:6. Right-hand diagram with 5 mM CNCC-monomer thiomethyl ether (ratio of CNCC-SMe:DTT) (▼) 1:0, and (■) 1:6.

Table 3. *In vitro* alkylating activity of CNCC isomers employing the 4-(4′-nitrobenzyl)pyridine (NBP) assay

Isomer	Relative alkylating activity (% of chlorozotocin)
C	118 ± 8*
S	145 ± 17
M†	126 ± 12
D†‡ (1C1G1325)	139 ± 7

* Results are the mean ± S.D. of four determinations.
† Unsymmetrically substituted CNCC-isomer.
‡ Mixture (1:1) of CNCC-M and CNCC-S with traces of CNCC-C.

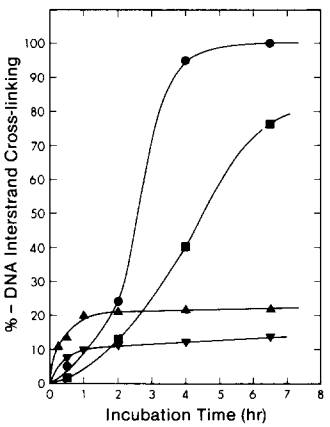

Fig. 6. Percentage of interstrand cross-linking in λ-DNA as a function of time at 37° produced by BCNU: (▲) in the absence of thiol, (●) in the presence of 30 mM 2-mercaptoethanol, and by chlorozotocin alone (▼), and (■) with chlorozotocin in the presence of 30 mM 2-mercaptoethanol.

In vitro *carbamoylating activity*. The comparative extents of modification of lysine by the three CNCC isomers and the mixture were obtained using a standard [^{14}C]lysine assay [23], and the results are shown in Table 4. A direct comparison with other nitrosoureas was not attempted since the usual incubation period of 6 hr (drug and lysine) gave little measurable carbamoylation. The data shown are based on a 20-hr incubation at 37°. The isomers (S) and (M) were more effective than isomer (C).

Inhibition of glutathione reductase. Table 5 shows the percentage inhibition of GR from both Walker resistant (WR) and Walker sensitive (WS) cells treated with 250 or 500 μM CNCC for 2 hr. Isomer CNCC-(C) inactivated the enzyme less effectively than isomer (S) or the mixture (D). This conclusion is in agreement with the *in vitro* carbamoylating estimates given in Table 4.

Effect of CNCC isomers on intracellular GSH pools. Intracellular thiol pools were estimated in both WS and WR cells following injection of micromolar concentrations of the three isomers (Table 6). Reduction in GSH pools, as seen with isomer (S), is

Table 4. CNCC *in vitro* carbamoylating activity*

Isomer	Carbamoylating activity (% carb. [^{14}C]lysine)	% of Carbamolyating products with net positive charge
C	5.8	20
S	12.3	25
M	15.2	27
D	13.3	17

* Assays were performed as described in the Experimental section. Incubation of isomers with lysine was for 20 hr at 37°.

Table 5. Effects of CNCC isomers on intracellular glutathione reductase (GR) activity in Walker 256 carcinoma cells

Isomer	Concn (μM)	% Inhibition of glutathione reductase activity* WR	WS
C	250	32	40
	500	89	60
S	250	95	92
	500		97
M	250	95	96
	500	95	96
D	250	97	96
	500	95	96

* Untreated values for GR activity: WS = 8.67 nmoles NADPH oxidized/μg protein/min, and WR = 3.98 nmoles NADPH oxidized/μg protein/min.

indicative of sulfhydryl conjugation and efflux, and/or an inhibition of the GSH metabolic enzymes. It may be seen from Table 6 that isomer S had the greatest effect on GSH depletion and that the WR cells were more depleted by drug treatment than the WS cells.

Cytotoxicity assays. These were carried out in a human DU 145 prostatic carcinoma cell line. It may be seen from Fig. 8 that the isomer toxicity shows (M) > (C) > (D) > (S) over a 2-hr incubation period.

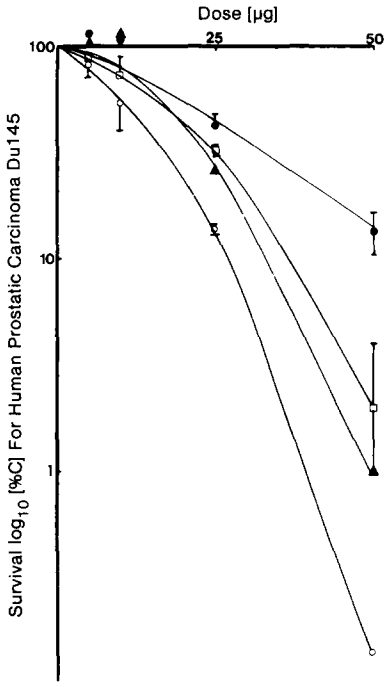

Fig. 8. Cytotoxicity of CNCC-D and its isomers in the human prostatic carcinoma cell line DU 145. Survival is expressed as log percentage of control following a 2-hr incubation at micromolecular concentrations: (●) isomer S; (○) isomer M; (▲) isomer C; and (□) CNCC-(D).

DISCUSSION

The products of decomposition of CNCC-(C) under physiological conditions are compatible with reversible addition of water to the amide carbonyl bond with formation of a tetrahedral intermediate. This has been proven in similar cases by ^{18}O-exchange in $H_2{}^{18}O$ [10, 24]. The direction of decomposition of the latter is dictated by stereoelectronic control requiring the orbitals of the participating heteroatoms to be aligned antiperiplanar to the breaking C—N_1 bond [25] giving rise to bis(2-thioethylisocyanate), and the products derived from the 2-chloroethyl cation, namely vinyl chloride, acetaldehyde and 2-chloroethanol. Release of the bifunctional electrophilic species accounts, as in the case of other CENUs [8, 12], for the observed interstrand cross-linking. A similar spectrum of products is obtained from CNCC-(C) in the presence of added dithiothreitol (Table 1, Fig. 4). Notably absent is any evidence for products resulting from reductive scission of the disulfide link in CNCC-(C), including the thiazolidinone which is detected in the separate aqueous decomposition of the monomer thiol corresponding to CNCC-(C). The striking increase (*ca.* 95%) in the rate and extent of interstrand DNA cross-linking produced by CNCC-(C) in the presence of added thiol, therefore, cannot be attributed to intramolecular thiol participation. Instead, it is tentatively attributed to intermolecular nucleophilic attack by dithiothreitol via the intermediate shown. Stereoelectronically controlled decomposition of the latter, which may involve antiperiplanar alignment of the sulfur lone pair orbitals [10, 25] leads to the observed products. Under these conditions, the excess of nucleophilic thiol will mask the total extent of DNA cross-linking observable in that it competes with the DNA nucleophiles for the electrophiles generated from the activated CNCC. Thus, the observed 38% interstrand cross-linking, which is comparable with that produced by 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyl disulfide [13], represents a minimum of the ability of CNCC-(C) to cross-link DNA (see below).

Table 6. Suppression of glutathione (GSH) by CNCC isomers in WR and WS cells*

Drug concn (μM)	WR† C	S	M	D	WS‡ C	S	M	D
1	67	30	50	61	92	59	127	144
2	55	36	33	49	71	58	99	85
5	47	48	49	55	59	49	118	91
10	57	20	40	46	33	32	117	104
25	55	17	34	26	65	35	78	91
50	36	8	25	14	64	10	50	86

* Data are expressed as percent of controls.
† Control levels of GSH:WR 1.79×10^{-5} M corrected/mg cell protein.
‡ Control levels of GSH:WS 1.54×10^{-5} M corrected/mg cell protein.

The pathway of decomposition of CNCC-(S) in the absence of dithiothreitol is in accord with Fig. 2, again involving formation of a tetrahedral intermediate. Bifunctional electrophiles are evidently not produced in significant concentration since no detectable interstrand cross-linking or alkylation was observed by CNCC-(S) under these conditions. However, in the presence of 30 mM dithiothreitol there was a substantial acceleration in the extent of decomposition and electrophile production capable of reacting covalently with DNA (Fig. 3). Significantly, there is little evidence for reductive disulfide cleavage in CNCC-(S) which would be expected to lead to *N*-nitrosothiazolidinone and thiazolidine and large amounts of 2-mercaptoethanol derivatives.

The effect of CNCC-(M) on DNA was as expected in that intermediate extents of interstrand cross-linking of λ-DNA were observed which increased in the presence of added dithiothreitol.

A control decomposition of the CNCC-(C) monomer thiol under physiological conditions was carried out. In this case, thiazolidinone was detected (in addition to the electrophilic products). Surprisingly little 2-chloroethanol was detected in any of the aqueous decompositions. This may indicate that, owing to the effective bulk of the N_3 substituent in CNCC-(C) and CNCC-(S), [26] the N-nitroso group adopts the preferred conformation [26] indicated in Figs. 1–4. This would require that the CNCC isomers give rise largely to the *Z* 2-chloroethyldiazohydroxide. We have shown by ^{18}O labeling that the latter gives the unstable 1,2,3-oxadiazoline and then acetaldehyde but no 2-chloroethanol [27]. The traces of the latter detected with CNCC could arise from the 2-chloroethyl cation intermediate.

In contrast to the isomers of CNCC, the corresponding monomers R—SH and R—S—CH_3 decompose smoothly under physiological conditions. There is also a marked contrast in the effects of added DTT on the rates of DNA cross-linking. In the case of R—S—CH_3, one observes an enhancement in the rate and extent of DNA interstrand cross-linking as was observed for BCNU and chlorozotocin, indicating intermolecular nucleophilic thiol attack at the amide carbonyl group. In contrast, with the monomer RSH one sees efficient DNA interstrand cross-linking in the absence of DTT which is progressively suppressed as the proportion of DTT increases down to a limiting value which is comparable with that produced by the methyl thioether. These data suggest a competition in the case of RSH between intermolecular and intramolecular thio group addition to the amide carbonyl. The results do not preclude reductive scission of the disulfide bond in the CNCC isomers in the *in vivo* situation. However, there is a close parallel between these *in vitro* results and those on CNCC metabolism [20], suggesting these interpretations may also hold substantially in the latter case.

It should be borne in mind that the proportion of DNA alkylation to DNA cross-linking is relatively high for CENUs (estimates are 10:1 or greater [1]). Interstrand cross-linking dominates in the first few minutes (since only one molecular event is required for each DNA molecule) after which extensive alkylation begins to destroy potential ethidium binding sites (both by steric hindrance and by depurination and/or depyrimidination [1, 13, 17] causing a decrease in the net ethidium fluorescence; see also Table 3). It is this latter portion of the overall reaction, i.e. dominated by alkylation, which will be sensitive to and suppressed by excess external thiol which competes for the electrophiles.

The carbamoylating activities estimated in Table 4 indicate that they are comparable for the CNCC isomers which is in accord with the decomposition product analysis given in Table 1 which indicates formation of comparable amounts of isocyanates. The data given in Table 4 are based on a 20-hr incubation and, therefore, probably represent maximal carbamoylation. The data given in Table 5, representing relative extents of inhibition of the enzyme glutathione reductase, are a more biologically relevant estimate of carbamoylating activity of the CNCC isomers. Isomer CNCC-(C) inactivated the enzyme less effectively than the others. This result is in accord with the *in vitro* carbamoylating reactivities in Table 4 and may reflect the greater reactivity of 2-choloroethylisocyanate compared with bis(2-thioethylisocyanate).

In interpreting the results in Table 6, it is possible that the reduced cytotoxic potential of CNCC-(S) in both the Walker 256 [16] and in the human prostatic carcinoma (Fig. 7) may be a function of an interaction between GSH and the drug, thereby protecting other more critical nucleophilic targets within the nucleus. The cumulative evidence summarized in Table 6 is in accord with the above studies, indicating a specific role for thiols in the biochemical mode of action of CENUs.

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