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A CHEMICAL BASIS FOR THE ANTITUMOR ACTIVITY OF CHLOROETHYLNITROSOUREAS

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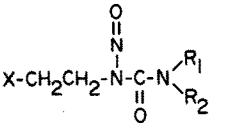
Abstract—A comparison of the aqueous decomposition products of several haloethylnitrosoureas has led to a suggested mode of decomposition and antitumor effect for these compounds. 1,3-Bis-chloroethyl-1-nitrosourea (BCNU), 1-chloroethyl-3-cyclohexyl-1-nitrosourea (CCNU), 1,3-bis-fluoroethyl-1-nitrosourea (BFNU) and 1-chloroethyl-1-nitrosourea (CNU) decompose in buffered aqueous solution to yield haloethanol, vinyl halide, dihaloethane and acetaldehyde. Evidence is presented that these products are derived from an intermediate haloethyl carbonium ion. On the other hand, 1-chloroethyl-3,3-dimethyl-1-nitrosourea decomposes slowly in aqueous solution, generates acetaldehyde, but not the other volatile compounds described above, and is not toxic to murine L1210 leukemia cells *in vitro*. In contrast to the disubstituted nitrosoureas, chloroethylnitrosourea does not generate an organic isocyanate on aqueous decomposition, but is a very active antitumor agent both *in vitro* and *in vivo*. These observations support the hypothesis that the antitumor activity of the chloroethylnitrosoureas is due to the facile decomposition of the parent molecule to form a chloroethyl carbonium ion (or diazonium precursor).

1,3-Bis-chloroethyl-1-nitrosourea (BCNU) and 1-chloroethyl-3-cyclohexyl-1-nitrosourea (CCNU) are antitumor agents which have been used effectively in the treatment of lymphomas [1] and a variety of solid tumors, including brain tumors [2, 3]. BCNU, the most studied chloroethylnitrosourea, crosses the blood–brain barrier readily [4], kills tumor cells which are not actively dividing [5, 6], and exhibits an unusual delayed hematologic toxicity in man [7]. Because of these distinctive properties, it is of particular interest to understand the pharmacology of the chloroethylnitrosoureas.

Early studies on the aqueous decomposition of BCNU demonstrated that an organic isocyanate was generated, and it was suggested that reaction of this isocyanate with the amine groups of macromolecules might play a role in the biologic effects of the compound [8]. Cheng *et al.* [9] studied the binding to macromolecules of CCNU labeled with ^{14}C in either the chloroethyl group or the cyclohexyl moiety. These studies, carried out with solutions of macromolecules and with murine L1210 leukemia cells both *in vitro* and *in vivo*, demonstrated that the cyclohexyl moiety was bound almost exclusively to proteins, while the carbons of the chloroethyl group were bound to both proteins and nucleic acids. The binding of the cyclohexyl group to proteins was shown to be due to carbamoylation of the lysine residues by cyclohexyl isocyanate [10]. These authors proposed that the dual capacity to alkylate nucleic acids and to alter proteins by carbamoylation might explain the pharmacologic effects of the chloroethylnitrosoureas.

Kramer *et al.* [11] studied the products of the reaction between BCNU and polycytidylic acid. The two products identified were 3-hydroxyethyl-CMP and 3,N^4-ethano-CMP. These products are consistent with the transfer of a chloroethyl group from BCNU to generate a transient 3-β-chloroethyl-CMP. These findings led us to investigate further the aqueous decomposition of BCNU. We recently reported that the decomposition of BCNU in aqueous media at physiologic pH generates chloroethanol, acetaldehyde, vinyl chloride and dichlorethane [12], and presented evidence that these volatile materials are derived from a chloroethyl carbonium ion. We have now extended these observations with several analogues of BCNU (Fig. 1) and report here studies which are consistent with the hypothesis that chloroethyl carbonium ion generation is responsible for the cytotoxicity of chloroethylnitrosoureas against murine L1210 leukemia cells.



Compound	R_1	R_2	X
BCNU	$-CH_2CH_2Cl$	H	Cl
CCNU		H	Cl
CNU	H	H	Cl
BFNU	$-CH_2CH_2F$	H	F
dMCNU	$-CH_3$	$-CH_3$	Cl

Fig. 1. Structures of the haloethylnitrosoureas studied.

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MATERIALS AND METHODS

Source of drugs. BCNU, CCNU, 1,3-bis-fluoroethyl-1-nitrosourea (BFNU), and 1-chloroethyl-1-nitroso urea (CNU) were kindly provided by Dr. Harry Wood of the Drug Development Branch of the National Cancer Institute. CCNU, labeled with ^{14}C in either the chloroethyl carbons or the cyclohexyl moiety, was obtained through Dr. Robert Engle of the Drug Development Branch of the National Cancer Institute.

1-Chloroethyl-3,3-dimethylurea was synthesized by reacting chloroethyl isocyanate with one equivalent of dimethylamine (40% aqueous solution) at 0° for 1 hr and purified by chromatography on Alumina with ethyl acetate. An 85 per cent yield of white crystals was obtained: m.p. 84° (literature, m.p. 86°) [13], NMR ($CDCl_3$) δ 5·6 (1 H, broad s), 3·6 (4 H, m) and 2·9 (6 H, s). The above urea (0·5 g) was nitrosated in glacial acetic acid (10 ml) at 0° with two equivalents of sodium nitrite dissolved in water (1 ml) for 2 hr. 1-Chloroethyl-3,3-dimethyl-1-nitrosourea (dMCNU) was isolated by washing an ether solution of the reaction mixture with aqueous bicarbonate, drying with sodium sulfate and evaporating to a yellow oil: 60 per cent yield, NMR ($CDCl_3$) δ 4·2 (2 H, t), 3·7 (2 H, t) and 3·2 (6 H, s).

Decomposition studies. The nitrosourea (0·12 m-mole) was heated at 37° with 0·1 M phosphate buffer (2·5 ml, pH 7·4) in a sealed vial fitted with a serum cap until complete decomposition of the nitrosourea, as measured by the Bratton–Marshall determination [14] had occurred. Diethyl ether (2·5 ml) was then injected into the vial, and both the ether and aqueous phases were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC–MS). In the case of BFNU, no ether was injected into the vial because it interfered with the determination of fluoroethanol.

To determine the relative amount of volatile decomposition products from the different parts of the molecule, CCNU labeled with ^{14}C in either the chloroethyl or the cyclohexyl moieties was incubated in pH 7·4 buffer, 0·05 M, under the conditions shown in Fig. 2 (see Results). Aliquots were withdrawn at varying times, spotted on filter papers, air dried and counted in a liquid scintillation counter.

Deamination studies. Either chloroethylamine or fluoroethylamine (1 m-mole) was dissolved in a mixture of ether (10 ml) and 0·5 M hydrochloric acid (2 ml). Then sodium nitrite (1 m-mole) was added. After gas evolution ceased, additional portions of acid and nitrite were added to a total of 10 ml and 5 m-moles respectively. Both the ether and aqueous phases were analyzed by GC and GC–MS. Fluoroethylamine was also deaminated in the absence of ether for the determination of fluoroethanol and chlorofluoroethane.

GC and GC–MS studies. Gas chromatographic analyses were carried out in a Varian 2400 instrument equipped with flame ionization detectors. A 10-ft glass column packed with Chromosorb 101 with no liquid phase was used isothermally at 120°. For mass spectrometric studies, a similar column was used in a DuPont 491 gas chromatograph–mass spectrometer.

Cytotoxicity studies. Murine L1210 leukemia cells were harvested from female C57BL/6 × DBA/2 F_1 mice 4 days after inoculation with 10^6 cells. The cells were then incubated in Dulbecco's physiologically buffered saline at a concentration of 10^6 cells/ml at 37° for 30 min with the desired concentrations of drug. At the end of the incubation period, 1 ml (10^6 cells) of the cell suspension was injected into each of five recipient mice. The mean survival time of these mice was then compared to the survival of mice injected with the same number of cells incubated with medium alone.

RESULTS

In Fig. 2 are shown the results of the spontaneous decomposition of [^{14}C]CCNU in buffered solution. As can be seen, when the ^{14}C label is in the chloroethyl carbons, a majority of the radioactivity is converted to volatile products and the amount of non-volatile radioactivity gradually decreases. When the ^{14}C label is in the cyclohexyl ring, essentially all of the radioactivity remains non-volatile.

In the experiment described in Fig. 3, CCNU was allowed to decompose in a Teflon-lined, rubber-stoppered vial. Ether was then injected into the vial and both the ether and aqueous phases were analyzed on a Chromosorb 101 gas chromatographic column. The elution pattern of both phases was identical to those obtained from the decomposition of BCNU [12] under similar conditions, and peaks with the appropriate retention times for vinyl chloride, acetaldehyde, chloroethanol and dichloroethane were present. The identity of these compounds was confirmed by GC–MS. Each of the peaks shown in Fig. 3 yielded a mass spectrum consistent with the assigned structure and identical to the mass spectrum of an injected standard [12].

The decomposition products of CNU, BCNU, dMCNU and BFNU were also analyzed in a similar fashion and the products quantitated from the areas under the peaks of the gas chromatogram. As shown in Table 1, CCNU, BCNU and CNU all yielded vinyl chloride, acetaldehyde, chloroethanol and dichloroethane. BFNU yields the corresponding fluoro compounds, fluoroethanol and vinyl fluoride, as well as acetaldehyde. Vinyl fluoride was identified by the

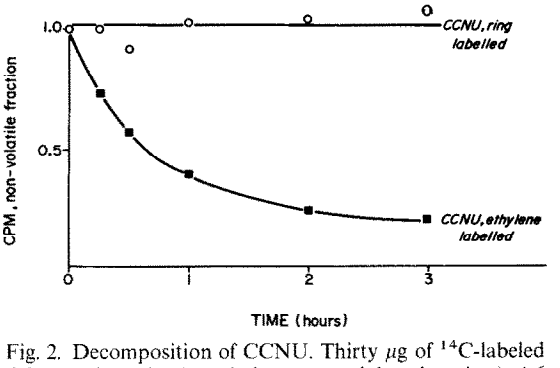

Fig. 2. Decomposition of CCNU. Thirty μg of ^{14}C-labeled CCNU (either in the ethylene or cyclohexyl moiety), 1·6 Ci/μmole, dissolved in 25 μl ethanol, was added to 0·475 ml of 0·05 M phosphate buffer, pH 7·4. Aliquots (50 μl) were withdrawn at the indicated time points and analyzed as described in the text.

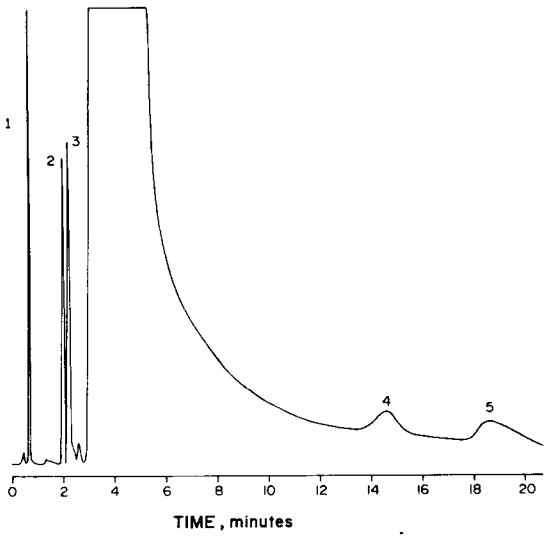

Fig. 3. Gas chromatographic analysis of ether extract of the volatile decomposition products of CCNU. See Methods for experimental and analytical conditions. Peaks 2, 3, 4 and 5 are vinyl chloride, acetaldehyde, dichlorethane and chloroethanol respectively. Peak 1 is not identified and the very broad peak is the ether solvent.

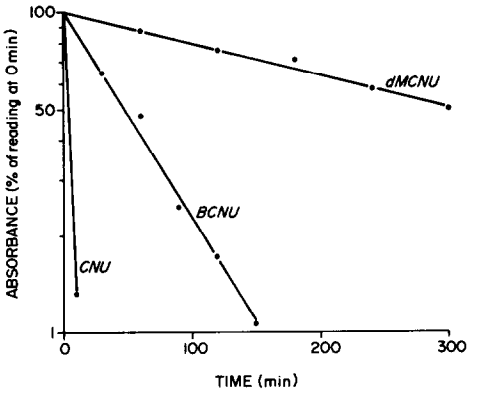

Fig. 4. Rate of decomposition of chloroethylnitrosoureas in 0·1 M sodium phosphate buffer, pH 7·4, containing 1% ethanol. The compounds (0·1 mg/ml) were incubated at 37°, and at the indicated intervals, aliquots were removed and analyzed for the intact nitrosourea by the Bratton–Marshall technique [14].

mass spectrum from GC–MS, since a standard was not available. Decomposition of dMCNU did not yield vinyl chloride, chloroethanol or dichloroethane, but produced acetaldehyde along with smaller quantities of two other unidentified volatiles.

Also shown in Table 1 are the yields of volatile materials produced by the nitrosative deamination of chloroethylamine and fluoroethylamine. This reaction generates the same spectrum of volatile products as the buffered aqueous decomposition of the halonitrosoureas. 1-Chloro-2-fluoroethane was identified by the mass spectrum from GC–MS, as no standard was available.

The rate of decomposition of CNU, BCNU and dMCNU in aqueous solution at physiologic pH has been studied and is illustrated in Fig. 4. As can be seen, CNU decomposes very rapidly, BCNU somewhat less so, and dMCNU very slowly.

The comparative cytotoxic effects of CNU, BCNU and dMCNU against murine L1210 cells *in vitro* were also studied and the results are shown in Table 2. Both CNU and BCNU were very cytotoxic to the L1210 cells at 25 nmoles/ml, but dMCNU had no effect on the viability of these cells at 100 nmoles/ml.

DISCUSSION

We have previously reported that BCNU decomposes in buffered aqueous solution to generate vinyl chloride, acetaldehyde, chloroethanol and dichloroethane [12]. That these products may be generated by the formation of a chloroethyl carbonium ion has been shown by demonstrating that the same products

Table 2. Antitumor activity *in vitro* of chloroethylnitrosoureas

Agent	Concn (μM)	M.S.T. (days)	Survival >30 days
Control*		7·6	0/5
CNU	25		5/5
CNU	50		5/5
BCNU	25		5/5
BCNU	50		5/5
dMCNU	50	8·2	0/5
dMCNU	100	8·0	0/5

* L1210 cells incubated with medium alone.

Table 1. Yields of volatile products*

Product	Haloethylnitrosourea decompositions				Haloethylamine deaminations	
	CNU	BCNU	CCNU	BFNU†	$ClCH_2CH_2NH_2$	$FCH_2CH_2NH_2$
$CH_2 = CHX$	2	2	4	Detected	3	Detected
CH_3CHO	14	26	37	15†	18	12‡
$ClCH_2CH_2X$	1	1	3		13	Detected
XCH_2CH_2OH	83	71	56	85†	66	67‡†

* Mole per cent of total recovered volatiles, except where noted. In all cases, recoveries were greater than 70 per cent of theoretical.

† No ether extraction was used for these determinations (see Methods).

‡ Mole per cent of fluoroethylamine reacted. 1-Chloro-2-fluoroethane, a major product, and vinyl fluoride, a minor product, were not quantitated due to lack of standards.

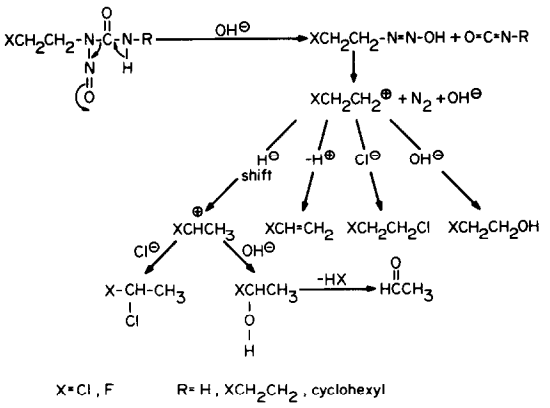

Fig. 5. Suggested scheme for the aqueous decomposition of haloethylnitrosoureas in the presence of buffer.

are generated from the nitrosative deamination of chloroethylamine, a reaction known to generate carbonium ions [15]. The products obtained from BFNU are consistent with a fluoroethyl carbonium ion and the same products were obtained from the deamination of fluoroethylamine.

In this work, we have demonstrated that the buffered aqueous decomposition of CCNU and CNU also generates the same volatile products. As shown by the experiments illustrated in Fig. 2, the volatile products come from the chloroethyl group of CCNU, and presumably from the nitroso-bearing chloroethylamino portion of BCNU.

A suggested scheme for the decomposition of haloethylnitrosoureas in buffered aqueous solution is shown in Fig. 5. Consideration of this scheme suggested that 1,1-dichlorethane should be produced during the decomposition of the chloroethylnitrosoureas. When the decomposition of BCNU was carried in the pressence of 2 M chloride ion, the yield of 1,2-dichlorethane was markedly increased and a small peak eluting from the gas chromatograph shortly before 1,2-dichlorethane was seen. This material had the same retention time as authentic 1,1-dichlorethane on both Chromosorb 101 and 0·4% Carbowax 1500 on Carbopack A gas chromatographic columns. The finding of 1,1-dichlorethane as a product of decomposition supports the concept that acetaldehyde is produced through a hydride shift of the chloroethyl carbonium ion, as shown in Fig. 5.

Wheeler and Bowdon [6] reported that BCNU inhibited DNA nucleotidyltransferase activity in L1210 cells, and found that this was due to an effect on the enzyme system by the chloroethyl isocyanate produced by the decomposition of BCNU. Baril *et al.* [17] have subsequently found that DNA polymerase II, but not DNA polymerase I, is inhibited by the alkyl isocyanates produced from BCNU and CCNU. The inhibition of the repair of irradiation-produced DNA strand breaks by BCNU and chloroethyl isocynate was described by Kann *et al.* [18] Kann *et al.* [19] have recently reported that the organic isocyanates generated by nitrosoureas inhibited RNA synthesis and processing in L1210 cells, but that potassium cyanate did not. In this report, it was pointed out that CNU did not generate an organic isocyanate moiety and did not inhibit RNA synthesis and processing, yet was an active antitumor agent. On this basis, it was suggested that organic isocyanate generation is not required for antitumor activity of the nitrosoureas.

CNU, on aqueous decomposition, would yield isocyanic acid ($\rightleftharpoons$ cyanic acid, $K_a = 2{\cdot}2 \times 10^{-4}$), which at physiologic pH would be ionized to cyanate and thus be a much poorer carbamoylating agent than the organic isocyanates. This interpretation is supported by the results of Kann *et al.* [19] cited above. Wheeler *et al.* [20] have shown that *N*-methyl-*N*-nitrosourea (which would also release isocyanic acid on hydrolysis) will inhibit the sickling *in vitro* of sickle trait red blood cells, but is considerably less effective than BCNU. Since the anti-sickling effect is presumably due to carbamoylation of hemoglobin S, these results suggest that isocyanic acid has some carbamoylating effect at physiologic pH, but this activity is much less than that of the alkyl isocyanates.

Our data indicate that CNU is at least as cytotoxic *in vitro* against L1210 cells as BCNU, and Schabel *et al.* [4] reported that the optimal dose of CNU against L1210 *in vivo* is one-fourth that of BCNU, on a molar basis. Thus, the relative anti-L1210 effect of the two agents does not correlate with their carbamoylating activity. Both agents, however, decompose rapidly to generate chloroethyl carbonium ions, CNU faster than BCNU.

In contrast to CNU and BCNU, dMCNU is relatively stable in buffered aqueous solutions. This is presumably due to the disubstitution of the 3-nitrogen, which prevents proton abstraction and rearrangement to form the isocyanate and release the diazonium hydroxide, as illustrated in Fig. 5. As shown in Table 2, dMCNU is not toxic to L1210 cells *in vitro* at concentrations 4-fold higher than the effective level of CNU or BCNU. This lack of activity is consistent with the concept that decomposition of the parent molecule is necessary for the cytotoxic effect. The slow decomposition of dMCNU to form acetaldehyde, but not the other products expected from the aqueous reactions of a chloroethyl carbonium ion, suggests that this molecule degrades by a different pathway.

Our observations support the hypothesis that the active chloroethylnitrosoureas generate a cytotoxic effect against L1210 cells by virtue of decomposition to a chloroethyl carbonium ion (or diazonium hydroxide precursor). The degradation of these nitrosoureas also generates carbamoylating moieties which affect nucleic acid-synthesizing enzymes and may contribute to or modify the therapeutic effect and toxicities of some or all of these agents.

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REFERENCES

1. V. DeVita, P. Carbone, A. Owens, G. L. Gold, M. J. Krant and J. Edmonson, *Cancer Res.* **25**, 1876 (1965).

2. S. K. Carter, F. A. Schabel, Jr., L. E. Broder and T. P. Johnson, in *Advances in Cancer Research* (Eds. G. Klein, S. Weinhouse and A. Haddow), Vol. 16, p. 273. Academic Press, New York (1972).
3. M. D. Walker and B. S. Hurwitz, *Cancer Chemother, Rep.* **54,** 263 (1970).
4. F. M. Schabel, T. P. Johnston, G. S. McCaleb, J. A. Montgomery, W. R. Laster and H. E. Skipper, *Cancer Res.* **23,** 725 (1963).
5. F. M. Schabel Jr., H. E. Skipper, M. W. Trader and W. S. Wilcox, *Cancer Chemother. Rep.* **48,** 17 (1965).
6. S. C. Barranco, J. K. Novak and R. M. Humphrey, *Cancer Res.* **33,** 691 (1973).
7. H. Lessner, *Cancer, N.Y.* **22,** 2 (1968).
8. J. A. Montgomery, R. James, G. S. McCaleb and T. P. Jonnston, *J. med. Chem.* **10,** 668 (1967).
9. C. J. Cheng, S. Fujimura, D. Grunberger and I. B. Weinstein, *Cancer Res.* **32,** 22 (1972).
10. B. Schmall, C. J. Cheng, S. Fujimura, N. Gersten, D. Grunberger and I. B. Weinstein, *Cancer Res.* **33,** 1921 (1973).
11. B. S. Kramer, C. C. Fenselau and D. B. Ludlum, *Biochem. biophys. Res. Commun.* **56,** 783 (1974).
12. M. Colvin, J. W. Cowens, R. B. Brundrett, B. S. Kramer and D. B. Ludlum, *Biochem. biophys. Res. Commun.* **60,** 515 (1974).
13. T. P. Johnston, G. S. McCaleb and J. A. Montgomery, *J. med. Chem.* **6,** 669 (1965).
14. T. L. Loo and R. L. Dion, *J. pharm. Sci.* **54,** 809 (1965).
15. I. T. Millar and H. D. Springall, *A Shorter Sidgewick's Organic Chemistry of Nitrogen*, pp. 44–5. Clarendon Press, Oxford (1969).
16. G. P. Wheeler and B. J. Bowdon, *Cancer Res.* **28,** 52 (1968).
17. B. B. Baril, E. F. Baril, J. Lazlo and G. P. Wheeler, *Cancer Res.* **35,** 1 (1975).
18. H. E. Kann, Jr., K. W. Kohn and J. M. Lyles, *Cancer Res.* **35,** 398 (1974).
19. H. E. Kann, Jr., K. W. Kohn, L. Widerlite and D. Gullion, *Cancer Res.* **34,** 1982 (1974).
20. G. P. Wheeler, B. J. Bowdon and W. J. Hammack, *Biochem. biophys. Res. Commun.* **54,** 1024 (1973).