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# REACTION OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU) WITH GUANOSINE: EVIDENCE FOR A NEW MECHANISM OF DNA MODIFICATION

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When guanosine reacts with 1,3-bis(2-chloro-2,2-dideuteroethyl)-1-nitrosourea in a mixture of pH 7.1 buffer and DMSO, the 7-chloroethylguanosine which is isolated contains two deuterium atoms located next to the guanine ring and beta to the chlorine atom as shown by electron impact mass spectrometry. It is proposed that initial attack by DNA bases occurs on the number 2 carbon of the haloethylnitrosourea with displacement of the chloride ion. In accordance with this proposed mechanism, 7-bromoethylguanosine is isolated as a major product when BCNU is reacted with guanosine in the presence of high concentrations of KBr. These results suggest that the antitumor activity of various haloethylating antitumor agents may be determined by structural changes which affect their mechanisms of reaction with DNA. 

The haloethylnitrosoureas, including 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), have an established clinical role as antitumor agents (1). Evidence that the antitumor activity of the 2-haloethylnitrosoureas is related to the transfer of 2-haloethyl groups to nucleophilic sites within DNA has been important, both in explaining the antitumor activity of the 2-haloethylnitrosoureas (2,3) and in providing a rational basis for new drug synthesis (4,5).

Several lines of evidence indicate that an intermediate 2-chloroethylating species in this reaction is 2-chloroethyldiazohydroxide, and that DNA substitution takes place by nucleophilic attack on the carbon beta to the chlorine in

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this compound (1,3). We now present evidence which suggests that under certain conditions substitution may also occur at the carbon adjacent to the chlorine. Evidently, a molecular rearrangement takes place after this initial attack to produce the 2-haloethyl derivitives which are observed as final products in DNA. This additional mechanism may explain the variations in DNA modifications which have been noted for different haloethylnitrosoureas and for other chloroethylating agents, and should be taken into account in designing new antitumor agents which contain chloroethyl groups.

## MATERIALS AND METHODS

Materials: Crystalline BCNU was obtained from the National Cancer Institute, Division of Cancer Treatment. Deuterated BCNU (1,3-bis(2-chloro-2,2-dideuteroethyl)-1-nitrosourea), BCNU-2,2,2',2'-$d_4$, was synthesized as described previously (6); the position of the deuteriums was confirmed by mass fragmentation analysis which showed loss of $-CD_2Cl$ fragments from the deuterated BCNU (data not shown). HPLC markers for the modified guanosines were synthesized by published methods (7-9).

Reactions of guanosine with BCNU: Weighed amounts of normal or deuterated BCNU and of guanosine were dissolved in dimethyl sulfoxide (DMSO) and an equal volume of 25 mM sodium cacodylate buffer, pH 7.1, was added to produce a final concentration of 0.11 M BCNU and 0.11 M guanosine. Reactions performed in the presence of KBr contained 1.3 M KBr. Reaction mixtures were incubated at 37° for 22 hours, and then diluted with $H_2O$; the DMSO was removed by lyophilization.

Isolation of nucleoside derivatives: Guanosine derivitives were separated by HPLC on a 5 micron, 4.6 X 250 mm, Spherisorb ODS column eluted isocratically with 50 mM $KH_2PO_4$, pH 6, containing 5% acetonitrile. Derivatives were identified by their HPLC retention times and ultraviolet spectra by comparison with known standards as described previously (3). Modified bases were released from the guanosine nucleosides by hydrolysis in 0.1 N HCl for 30 min at 100°. Hydrolysates were neutralized with 1 N KOH and each modified base was purified further by HPLC on a C18 column eluted with 10 mM triethylammonium formate, pH 4.5, containing 2.5% acetonitrile. The identity of the modified bases were again checked by comparison of retention times and ultraviolet spectra with known standards.

Mass spectrometry: Mass fragmentation analysis was performed on a Varian MAT 311A instrument with the electron impact technique using 70eV electrons. Analysis was performed on the substituted bases rather than the nucleosides in order to obtain a clearer fragmentation pattern.

## RESULTS AND DISCUSSION

The mass fragmentation patterns of 7-chloroethyguanine prepared from BCNU and BCNU-2,2,2',2'-$d_4$ are shown in Figure 1. The pattern from non-deuterated 7-chloroethylguanine (Panel A) shows an $M^+$ peak at the expected m/z = 213

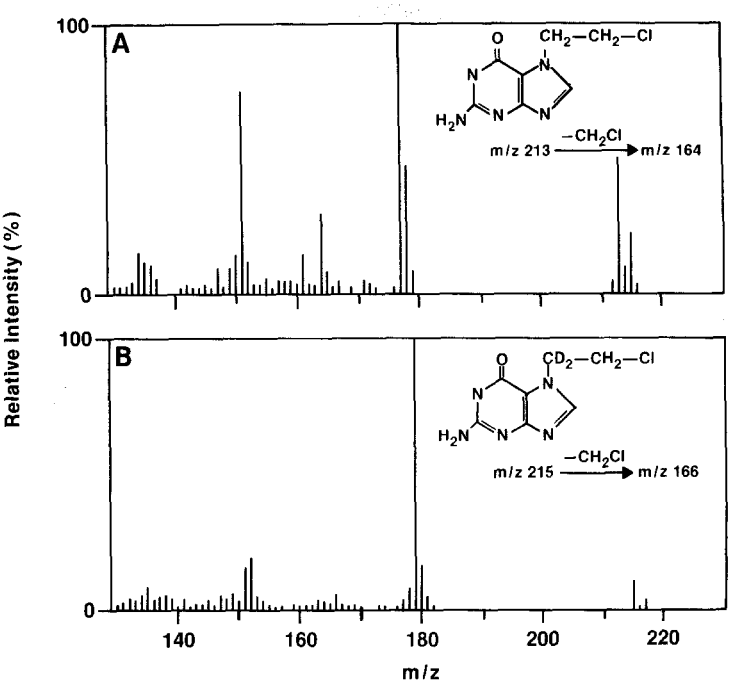


Figure 1. Mass spectra of 7-chloroethylguanine prepared from: (A) normal BCNU and (B) deuterated BCNU.

with a chlorine isotope peak at m/z = 215. The large peak at m/z = 177 corresponds to the loss of HCl; the peak at m/z = 178, to the loss of Cl alone; and the peak at m/z = 164, to the loss of $-CH_2Cl$. The peak at m/z = 151 is guanine. The mass spectrum in panel B for 7-chloroethylguanine prepared from BCNU-2,2,2',2'-$d_4$ shows an $M^+$ peak at m/z = 215 with a chlorine isotope peak at m/z = 217, showing the presence of 2 deuterium atoms in this derivative. The fragmentation pattern shows a loss of the same fragments with the same mass as from non-deuterated 7-chloroethylguanine. Thus, the peak at m/z = 179 represents the loss of HCl and the peak at m/z = 166, the loss of $-CH_2Cl$. This peak is critical because if $-CD_2Cl$ had been lost, it would have been found at m/z = 164. The peak at m/z = 152 probably represents the transfer of one deuterium atom to guanine.

These results were entirely unsuspected and suggested that the chlorine in BCNU was being displaced by nucleophilic attack at the carbon adjacent to chlorine. Presumably, the chloride ion remains in a caged ion pair and ultimately displaces some moiety derived from the nitrosourea to become reattached to the

Table 1. GUANOSINE MODIFICATION BY BCNU IN THE PRESENCE AND ABSENCE OF ADDED KBr

| Guanosine Modification | Retention Time (min) | Percent in Absence of KBr | Percent in Presence of KBr |
|---|---|---|---|
| 7HEG | 8.1 | 10.0 | 6.5 |
| 7CEG | 25.5 | 1.4 | 0.7 |
| 7BEG | 31.0 | 0 | 9.1 |

other carbon atom. To test this possibility, the reactions of guanosine with BCNU and BCNU-2,2,2',2'-$d_4$ were carried out in the presence of about 10 equivalents of KBr. A new derivative appeared that had the same retention time and ultraviolet spectra as the previously described 7-bromoethylguanosine (9).

Table 1 shows the distribution of the 7-alkylguanosines isolated from reactions performed in the presence and absence of KBr. These data, expressed as the percent of total ultraviolet absorbance at 254 nm, show that 10% of the guanosine is converted to 7-hydroxyethylguanosine (7-HEG) and 1.4% is converted to 7-chloroethylguanosine (7-CEG) in the absence of KBr. In the presence of KBr, the percentages of 7-HEG and 7-CEG both fall and 9.1% of the guanosine is converted into 7-bromoethylguanosine (7-BEG). Thus, the bromide ion can compete with chloride in the rearrangement mechanism proposed above.

It follows from this mechanism that the deuterium atoms in 7-bromoethylguanine obtained from reaction with deuterated BCNU should be located next to the guanine ring. This is confirmed by the mass fragmentation patterns shown in Figure 2 A and B, respectively. The mass spectrum of normal 7-bromoethylguanine shows a molecular ion peak at m/z = 257 with an accompanying bromine isotope peak at m/z = 259. The mass spectrum for 7-bromoethylguanine prepared from deuterated BCNU shows a molecular ion at m/z = 259 with an accompanying bromine isotope peak at m/z = 261, establishing the presence of two deuterium atoms. The location of the deuterium atoms was confirmed by comparison of the characteristic fragmentation patterns of 7-CEG and 7-BEG which are similar to those reported for 7-ethylguanine (10).

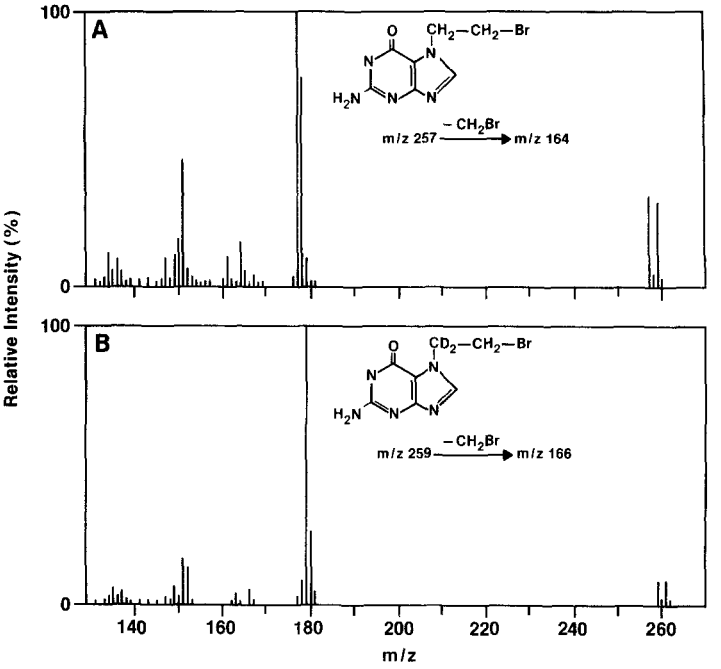


Figure 2. Mass spectra of 7-bromoethylguanine prepared from: (A) normal BCNU and (B) deuterated BCNU.

The loss of $-CH_2Cl$ or $-CH_2Br$ from 7-CEG and 7-BEG prepared from 13,-bis (2-chloro-2,2-dideuteroethyl)-1-nitrosourea strongly suggests that a rearrangement has occurred in the reaction of guanosine with BCNU. Initial substitution has evidently occurred on carbon atom number 2 with displacement of the chloride ion. Confirmation that this has occurred is provided by reaction in the presence of KBr. Under these conditions, the chlorine from BCNU is displaced from the reaction complex and 7-bromoethylguanosine is obtained instead of 7-chloroethylguanosine. Since the amount of 7-hydroxyethylguanosine also decreases, this also suggests that 7-hydroxyethylguanosine is normally produced by the displacement of this same leaving group by water.

It is not clear from these studies what portion of the original BCNU molecule is involved in the initial reaction with guanosine or how much of the original BCNU molecule is attached to the 7 position of guanosine after the initial displacement of the chloride ion. The reactive species could be 2-chloroethyldiazohydroxide as postulated previously (1,4), the parent 2-haloethylnitrosourea, or some other reactive intermediate. The fact that the dis-

tribution of lesions is affected by the structure of the parent 2-haloethyl-nitrosourea would seem to indicate that alternative mechanisms are operating in the reactions of these different agents with DNA. To the extent that DMSO may be viewed as a satisfactory model solvent for events occurring in hydrophobic environments in the cell, the reaction pathway selected may also be influenced by the medium.

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