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MOLECULAR PHARMACOLOGY OF THE HALOETHYL NITROSOUREAS: FORMATION OF 6-HYDROXYETHYLGUANINE IN DNA TREATED WITH BCNU (N,N^1-BIS[2-CHLOROETHYL]-N-NITROSOUREA)

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SUMMARY

The substituted base, 6-(β-hydroxyethyl)guanine, has been identified in DNA which has been treated with the antitumor agent, N,N^1-bis(2-chloroethyl)-N-nitrosourea (BCNU). This finding provides support for the suggestion that interstrand crosslinks may involve substitution at this position. The presence of 6-hydroxyethylguanine in DNA could also explain the carcinogenic potential of the haloethyl nitrosoureas since this DNA modification is considered mutagenic

INTRODUCTION

The haloethyl nitrosoureas have useful antitumor activity against a variety of neoplasms including lymphomas and certain solid tumors. The mechanism by which they produce cytotoxicity has not been fully established, but it is probably related to nucleic acid alkylation.

In previous studies, we have isolated several modified bases from DNA which has been reacted with N,N^1-bis(2-chloroethyl)-N-nitrosourea (BCNU) (1,2). Some of these bases contain a haloethyl group which has led us to suggest that cross-links could form by a two-step reaction in which a haloethyl group is first transferred to a base in one strand of DNA and then reacts with a base in the opposite strand (3).

Physical evidence for interstrand crosslinking reaction has been obtained in other laboratories (4,5), but the chemical nature of the crosslink has not

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been established. Although we have identified diguanylethane in DNA which has been reacted with BCNU (6), this structure is not necessarily involved in an interstrand crosslink.

As Kohn has pointed out (4), the helical nature of DNA would favor a guanine cytosine crosslink. Data from his laboratory suggest that the 6 position of guanine might be involved because tissue culture cells which can repair this lesion are resistant to the nitrosoureas (7). Substitution of guanine in the 6 position, if it did occur, would have additional significance because this modification is probably mutagenic (8,9) and could explain the carcinogenic potential of the haloethyl nitrosoureas.

We would like to report evidence here that guanine is, in fact, substituted in the 6 position by the haloethyl nitrosoureas. We have isolated and identified 6-(β-hydroxyethyl)guanine in the purine fraction released from DNA which has been treated with BCNU. This finding provides a molecular basis for suggestions that both the cytotoxic and mutagenic reactions of the haloethyl nitrosoureas involve substitution at the 6-position of guanine.

MATERIALS AND METHODS

Crystalline BCNU was kindly provided by the National Cancer Institute, Division of Cancer Treatment. A 50% dispersion of sodium hydride in oil was obtained from the Alfa Division of Ventron Corporation; 6-chloro-2-aminopurine-9-riboside came from Waldhof, West Germany; and calf thymus DNA came from Worthington. Modified purines, except for O^6-(β-hydroxyethyl)guanine whose synthesis is described below, were prepared previously (6).

Synthesis of O^6-(β-Hydroxyethyl)guanosine: 50 mg of sodium hydride dispersion was added cautiously with stirring to 2 ml of ethylene glycol to form monosodium ethylene glycolate, and then 50 mg of 6-chloro-2-aminopurine-9-riboside was added to the mixture. After the reaction (displacement of Cl by OCH_2CH_2OH) had proceeded for 1 hr at 45°, 3 ml of water was added and the mixture was neutralized with concentrated HCl. The solution was applied to a Sephadex G-10 column (2 x 100 cm) and eluted with water at 1 ml/min. Ten min fractions were collected and the absorbance peak between fractions 35 to 53 was pooled and lyophilized to give 1100 A_{280} of O^6-(β-hydroxyethyl)guanosine.

Depurination of O^6-(β-Hydroxyethyl)guanosine: 300 A_{280} units of the nucleoside were dissolved in 3 ml of 0.1 N HCl. The solution was heated at 80° for 1 hr and then neutralized with 1 N NH_4OH. The solution was chromatographed on a G-10 column as described above and fractions 75 to 93 were pooled to give 240 A_{280} units of O^6-(β-hydroxyethyl)guanine.

High Pressure Liquid Chromatography: HPLC separations were performed on a Spherisorb ODS 5μ column (4.6 x 250 mm) as described previously (1,2). Purines were eluted at 1 ml/min with 3 different solvent systems: (1) isocratic 0.05 M KH_2PO_4, pH 4.5, containing 5% CH_3CN; (2) isocratic 0.01 M PIC-B-7 reagent containing 10% CH_3CN; and (3) a linear 0-10% gradient of CH_3CN in 0.05 M KH_2PO_4 for 40 min followed by isocratic elution with 10% CH_3CN in 0.05 M KH_2PO_4.

Eluents were monitored for ultraviolet absorbance at 280 nm with a Perkin Elmer LC-55 UV detector, and for fluorescence with a Fluoromonitor III (Laboratory Data Control, Riviera Beach, FL). Fluorescence was excited at 254 nm and monitored with an emission filter for wavelengths of 300 to 400 nm.

Spectroscopy: Ultraviolet spectra were obtained in 0.1 N HCl; in 0.1 M sodium cacodylate buffer, pH 7; and in 0.1 N NaOH on a Beckman Model 35 spectrophotometer. Mass spectroscopy was performed on a Varian MAT 311A mass spectrometer with the electron impact technique. The compound was introduced directly on the probe, and spectra were obtained with 70 ev electrons at a source temperature of 140°.

Identification of 6-(β-Hydroxyethyl)guanine in BCNU-Treated DNA: 20 mg of BCNU in 200 μl of ethanol was added to 2 ml of a calf thymus DNA solution, 8 mg/ml, in 0.025 M sodium cacodylate at pH 7. A DNA blank received 200 μl of ethanol alone. The solutions were incubated for 3 hr at 37° and then the DNA was precipitated by the addition of 100 μl of 6 M NaCl and 2 vol. of ice cold ethanol. The precipitate was collected by centrifugation, washed with 95% ethanol, redissolved, and reprecipitated to remove unreacted BCNU. Finally, 5 ml of 0.1 N HCl was added to the precipitated DNA and substituted purines were released by incubation at 37° overnight. This solution was brought to neutrality with 0.5 ml of 1 N NH_4OH and passed through a small DEAE-Sephadex A-25 column (0.5 x 3 cm) to remove oligonucleotides. The eluent was collected and analyzed for purine content by HPLC.

RESULTS

The first step in identifying 6-(β-hydroxyethyl)guanine from BCNU-treated DNA was the preparation and characterization of marker as described above. O^6-(β-hydroxyethyl)guanine isolated from the G-10 column consisted of one major absorbance peak on reverse phase and cation exchange high pressure liquid chromatography. Material purified on the Spherisorb column was used for ultraviolet and mass spectrometric confirmation of structure.

The ultraviolet spectrum which was obtained is typical of a 6-substituted guanine (10), having a λ_{max} of 286 nm in 0.1 N HCl, λ_{max} of 250 and 282 nm at pH 7, and a λ_{max} of 284 nm in 0.1 N NaOH. The mass spectrum showed a molecular ion at m/e = 195 which is consistent with the expected product, 6-(β-hydroxyethyl)-guanine. The fragmentation pattern also revealed peaks at m/e = 151 corresponding to $(M^+ - CH_2CH_2OH + H^+)$ and at m/e = 134 corresponding to $(M^+ - OCH_2CH_2OH)$.

The use of high pressure liquid chromatography with fluorescence detection made it feasible to demonstrate the presence of 6-(β-hydroxyethyl)guanine in BCNU-treated DNA. The strong fluorescence of 6-(β-hydroxyethyl)guanine allowed us to detect this compound in the presence of much larger amounts of unsubstituted bases. Chromatographic systems for the separation of 6-(β-hydroxyethyl)guanine from the other purines are shown in Table 1 below. With the markers and the appropriate HPLC systems in hand, it became a relatively simple matter to demonstrate that 6-(β-hydroxyethyl)guanine was indeed found in DNA which had been reacted with BCNU.

Figure 1 shows the elution profile for the purines released from untreated DNA and from DNA which had been incubated with BCNU as described above. In this figure, absorbance was monitored at 280 nm and a small absorbance peak 4 was observed at a position corresponding to 6-(β-hydroxyethyl)guanine in this gradient system. Peak 4 was collected and rechromatographed in the two isocratic solvent systems listed in Table 1 and was found to have the correct retention time for 6-(β-hydroxyethyl)guanine. The UV spectrum of the material released from DNA was also identical with that of the marker material.

Table I. RETENTION TIMES IN MINUTES FOR THE HPLC SEPARATION* OF PURINES

Compound	Solvent A	Solvent B
Guanine	5.5	6.0
Adenine	10.3	9.4
7-(β-hydroxyethyl)guanine	7.0	7.1
O^6-(β-hydroxyethyl)guanine	14.5	13.4
7-(β-chloroethyl)guanine	26.1	18.0
Diguanylethane	17.8	15.3

*Separations on a Spherisorb ODS 5μ column (4.6 x 250 mm) eluted at 1 ml/min with solvent A: 0.05 M KH_2PO_4, pH 4.5, containing 5% CH_3CN and with solvent B: 0.01 M Pic-B-7 reagent containing 10% CH_3CN.

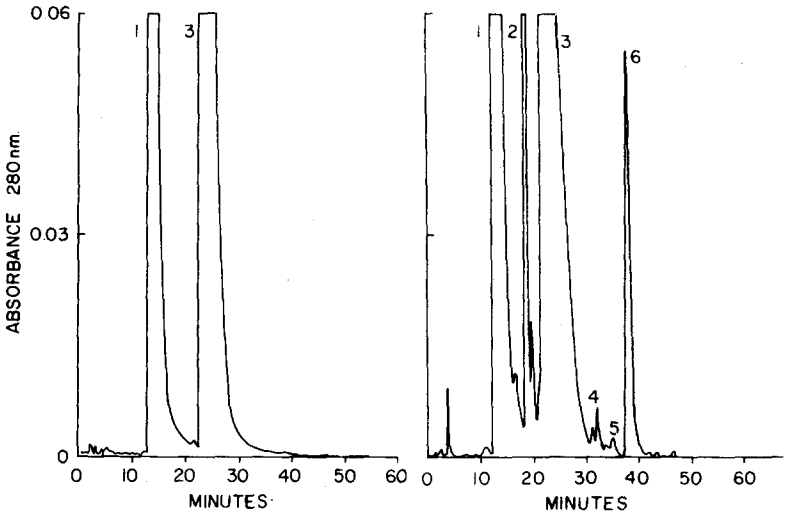

Figure 1. HPLC separation of purines released from control DNA (left) and DNA which had been reacted with BCNU (right). Derivatives were separated on a Spherisorb ODS 5μ column eluted at 1 ml/min with a KH_2PO_4-acetonitrile gradient (system 3 in Methods). The eluent was monitored for ultraviolet absorbance at 280 nm. Peak 1, guanine; peak 2, 7-(β-hydroxyethyl)guanine; peak 3, adenine; peak 4, O^6-(β-hydroxyethyl)-guanine; peak 5, 1,2-(diguan-7-yl)ethane; peak 6, 7-(β-chloroethyl)-guanine.

The importance of monitoring with a fluorescence detector is shown in Figure 2. The same peaks are apparent here, but the magnitude of peak 4 is much greater in relationship to the other peak sizes.

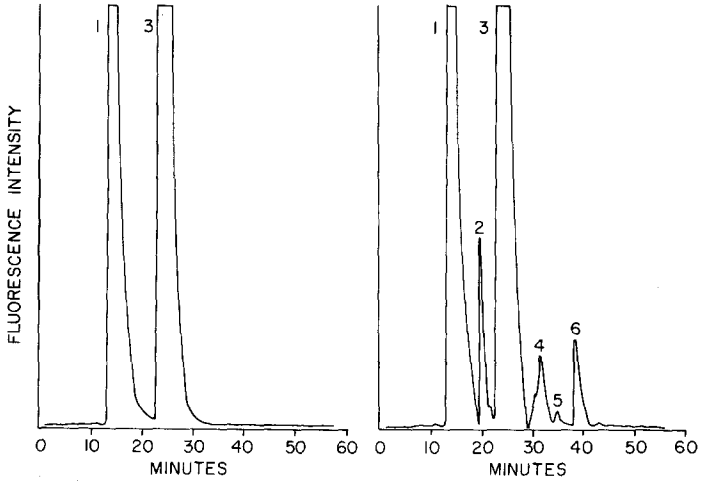

Figure 2. HPLC separation of purines as in Figure 1, except that eluent was monitored for fluorescence.

DISCUSSION

The potential importance of substitution at the 6 position of guanine has led us to make a careful and determined search for these products. Since hydroxyethyl derivatives have been found in much larger amounts than the corresponding chloroethyl derivatives, we prepared 6-(β-hydroxyethyl)guanine as the derivative most likely to be detected. Synthesis of this compound and its characterization proved to be relatively straightforward.

With the aid of fluorescence detection, it was possible to develop chromatographic systems which separate this derivative from the other larger peaks released from BCNU-treated DNA. The sample of DNA shown in Figures 1 and 2 had approximately 4% of its purines substituted; O^6-(β-hydroxyethyl)guanine amounted to approximately 4% of this total.

Thus, substitution does occur at the 6 position of guanine and crosslinks could be formed through 6-(β-chloroethyl)guanine which would probably be a highly reactive compound. Further studies will be required to demonstrate the existence of this compound or of crosslinked bases involving substitution at the 6 position of guanine.

The presence of 6-(β-hydroxyethyl)guanine might also explain the carcinogenic action of BCNU. In practical terms, however, it may be that those cells which cannot repair this lesion would be so sensitive to the cytotoxic action of the nitrosoureas that they would usually not survive.

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