Alkylation of Guanosine and Deoxyguanosine by Phosphoramide Mustard

Jitendra R. Mehta, Michael Przybylski, and David B. Ludlum

Department of Pharmacology and Experimental Therapeutics, Albany Medical College, Albany, New York 12208 [J. R. M., D. B. L.], and Institute of Organic Chemistry, University of Mainz, D-65 Mainz, West Germany [M. P.]

ABSTRACT

Phosphoramide mustard, an active metabolite of cyclophosphamide, has been reacted separately with guanosine and deoxyguanosine in aqueous solution at pH 7.4. The major adduct which was formed in each case has been isolated by reverse-phase high-pressure liquid chromatography. The structure of the major adduct, as determined by a combination of ultraviolet and field desorption mass spectrometry, is that of phosphoramide mustard, one arm of which has reacted with guanosine or deoxyguanosine in position 7. These adducts are much less stable than was 7-methylguanosine, and they decompose with a half-life of 2.3 hr at 37° and pH 7.4. This instability may contribute to the action of phosphoramide mustard at a molecular level.

INTRODUCTION

The alkylating agent cyclophosphamide is singularly effective in the treatment of a wide variety of neoplasms (13). It is a nitrogen mustard, but it differs from the vast majority of such agents in requiring metabolic activation to achieve cytotoxicity. The initial step in this process appears to be formation of 4-hydroxycyclophosphamide by hepatic mixed-function oxidases (11). 4-Hydroxycyclophosphamide then presumably circulates in the blood, enters tumor cells, and decomposes to acrolein (1) and an active alkylating agent, phosphoramide mustard (4). Details of this proposed metabolic scheme have been reviewed by others (6, 8).

Phosphoramide mustard is probably a significant metabolite as far as the antitumor activity of cyclophosphamide is concerned (3, 5, 12). Once formed within the cell, phosphoramide mustard could react with nucleic acids at several sites, including position 7 of guanine. However, details of this reaction have not been reported.

Accordingly, we have investigated the reaction between phosphoramide mustard and both guanosine and deoxyguanosine. As described below, adducts are formed which are surprisingly unstable, suggesting that some of the distinctive properties of cyclophosphamide may be related to the instability of the nucleoside adducts which it forms.

MATERIALS AND METHODS

Crystalline phosphoramide mustard (NSC 69945) was kindly provided by Dr. Harry B. Wood, Jr. (Division of Cancer Treatment, Drug Research and Development, National Cancer Institute, Bethesda, Md.). Guanosine was purchased from Sigma Chemical Co., St. Louis, Mo., and deoxyguanosine was purchased from ICN Pharmaceuticals, Inc., Cleveland, Ohio. Both compounds were purified by high-pressure liquid chromatography before use. UV-grade acetonitrile, distilled in glass, came from Burdick & Jackson Laboratories, Inc., Muskegon, Mich. Analytical reagent-grade KH₂PO₄, purified by our modification of Shmukler’s method (10), was used in making up buffers for high-pressure liquid chromatography.

High-pressure liquid chromatography separations and analyses were performed on a modular apparatus consisting of a Milton Roy 5000-psi minipump, a Laboratory Data Control Model 709 pulse damper, and a Perkin-Elmer LC-55 UV detector interfaced with a Sigma 10 Data System. Preparative separations were performed on a Waters Associates C₁₈-μBondapak column (4 mm x 30 cm), and products were analyzed for purity on a Laboratory Data Control 5-μm Excalibur ODS³ column (4 mm x 25 cm). These columns were protected with a small guard column of CO₂:Pell ODS resin (Whatman, Inc., Clifton, N. J.).

UV spectra were obtained in 0.1 n HCl-0.1 M sodium carbonate buffer, pH 7.0, and 0.1 n NaOH on a Beckman Model 35 spectrophotometer.

Field desorption mass spectrometry was performed with a Varian MAT CH 711 double-focusing spectrometer equipped with an electron impact-field desorption combination ion source. Activated 10-μm tungsten-field ion emitters were prepared by a high-temperature activation procedure developed by Becke and Schulten (2). Purified nucleoside phosphoramide mustard adducts were dissolved in water containing 5% dimethyl sulfoxide, loaded on the emitter by the syringe technique, and run with approximately 10 ma of emitter heating current at a source temperature of 180°. Instrumental conditions were: 1000 (10%) resolution; 11 kV total potential difference between field emitter anode and cathode; 8 kV accelerator voltage; and 2.5 kV electron multiplier voltage. Acquisition of spectra was obtained by an on-line computer Varian SS-100 on magnetic tape. Electron impact spectra of perfluorokerosine (E. Merck, Darmstadt, West Germany) were used for mass calibration.

The kinetics of 7-methylguanosine, guanosine-phosphoramide mustard adduct, and deoxyguanosine-phosphoramide mustard adduct decomposition were studied in 0.05 M potassium phosphate buffer at pH 7.4 and 37°. Aliquots (250 μl) were withdrawn from a 0.1 mm solution over a 24-hr period. The samples were then analyzed by high-pressure liquid chromatography on a C₁₈-μBondapak column. The products were eluted isocratically at a flow rate of 0.6 ml/min with 0.05 M KH₂PO₄, pH 4.5, which contained 2% acetonitrile for the guanosine-phosphoramide mustard adduct and 5% acetonitrile for the deoxyguanosine-phosphoramide mustard adduct.

Received April 28, 1980; accepted August 5, 1980.

1 Supported by Grants CA 20129 and CA 20292 from the National Cancer Institute, Department of Health, Education, and Welfare, and by Grant PTB 8207 for drug development from the Bundesministerium für Forschung und Technologie, Bonn, West Germany.

2 To whom requests for reprints should be addressed.

3 The abbreviation used is: ODS, octadecylsilane.
RESULTS

As might be expected, phosphoramide mustard reacted readily with both guanosine and deoxyguanosine in aqueous solution. Typically, 6 mg of phosphoramide mustard were incubated with 1 mg of guanosine in 1 ml of 0.1 M potassium phosphate buffer, pH 7.4, for 45 min at 37°. The pH was then adjusted to 4.5, and the entire reaction mixture was separated on a C$_{18}$-Bondapak column, as shown in Chart 1. This column was eluted with a linear gradient of acetonitrile (0 to 10%, 50 ml) in 0.05 M KH$_2$PO$_4$ at a flow rate of 0.66 ml/min.

A guanosine-phosphoramide mustard adduct, which amounted to approximately 3% of the original guanosine, appeared as a symmetrical peak at 57 min. Earlier peaks contained small amounts of unstable derivatives which, judging from their UV spectra, arose from the main adduct by scission of the imidazole ring.

Similarly, phosphoramide mustard was reacted with deoxyguanosine and the mixture was separated in the same way, except that the eluent contained 4% acetonitrile. A deoxyguanosine-phosphoramide mustard adduct, which amounted to 2.7% of the original deoxyguanosine, appeared at 22 min.

Fractions containing the adducts were pooled and the products were purified by rechromatography on a C$_{18}$-Bondapak column equilibrated with 1% formic acid, pH 3.5. The products were eluted with this buffer and freed of formic acid by lyophilization.

Purities were established by chromatography on a Laboratory Data Control Excalibur ODS column with both a paired-ion solvent and a phosphate buffer. The resolving power of this system and the appearance time of the products are shown in Table 1. The adducts appeared as single symmetrical peaks in both of the systems described in the footnote to this table.

The structures of the guanosine-phosphoramide mustard and deoxyguanosine-phosphoramide mustard adducts were deduced from UV and mass spectrometry. UV spectra of the guanosine-phosphoramide mustard adduct in acid, neutral, and basic solution are shown in Chart 2. These spectra are typical of a 7-substituted guanosine, as are the irreversible spectral changes which were observed in basic solution (7). Very similar UV spectra were obtained for the deoxyguanosine-phosphoramide mustard adduct.

The nature of the 7 substituent was established by field desorption mass spectrometry as shown in Chart 3 for the guanosine-phosphoramide mustard adduct. There is a molecular ion peak, MH$^+$, at m/e 469, that is consistent with the structure shown in that chart. The peak at m/e 471 can be attributed in part to the 37Cl isotope but evidently, contains multiply protonated species as well. 13C isotopic species also contribute to the cluster of peaks in this region. The peak at m/e 492 may be (MH + Na)$^+$ with concomitant 37Cl isotope and protonated species, while the cluster around m/e 515 probably corresponds to the addition of a formic acid molecule to the MH$^+$ structure, or possibly to (MH + 2Na)$^+$. The peak at m/e 550 may be (MH + 2Na + Cl)$^+$ or (MH + HC00H + Cl)$^+$, and m/e 606 is probably (MH + 3HC00H)$^+$.

Further information concerning the structure of the molecule is obtained by the fragment ions at m/e 284, 151, and 133 which correspond to guanosine, guanine, and ribose residues, respectively. The peak at m/e 420 may be (MH - CH$_2$Cl)$^+$ since loss of CH$_2$Cl is frequently observed for cyclophosphamide and its metabolites (4).
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As shown in Chart 4, a similar pattern was obtained for the deoxyguanosine adduct isolated in the same way. A molecular ion appears at m/e 452 with peaks corresponding to protonated and isotopic species ranging up to m/e 459. Additional ion clusters at higher masses again presumably correspond to complexes with formic acid. Fragments corresponding to the nucleoside (m/e 268, 269), to deoxyribose (m/e 117), and to the base (m/e 151) are observed in relatively high abundance.

Thus, we conclude that we have isolated the structures shown in Charts 3 and 4, guanine nucleosides which have reacted in position 7 with one arm of phosphoramide mustard. Judging from the mass of the molecular ions and the evidence for the presence of chlorine, the other reactive group in phosphoramide mustard is still intact.

The marked instability of these adducts became apparent early in our attempt to isolate them. Success was achieved by using rapid high-pressure liquid chromatographic separations on a reverse-phase column in an acidic medium.

After the adducts had been isolated in purified form, their stability was determined at pH 7.4, as described in "Materials and Methods." These data are shown in Chart 5, where the logarithm of the percentage of adduct remaining is plotted versus time. As is evident from this chart, the adducts decompose by a first-order process with a half-life of 2.3 hr; there is no discernible difference between the ribose and the deoxyribose derivatives. By contrast, over 98% of 7-methylguanosine remains unchanged in 24 hr under these conditions. Thus, the difference in stability between the 7-alkyl-substituted and the phosphoramide mustard-substituted guanine nucleosides is truly striking.

We have not yet obtained any detailed information on the mode of decomposition of these adducts. Several decomposition products which, judging from their position on the C_{18}®Bondapak column, were more polar than the parent adduct appeared simultaneously during the stability studies. An UV spectrum of the mixture of products suggests that much of the decomposition involves scission of the imidazole ring.

**DISCUSSION**

The use of cyclophosphamide in treating a wide range of cancers lends importance to studies of its action at a molecular level. The careful work of other investigators, which is outlined above (1, 3–5, 11, 12), indicates that this agent exerts an effect through its decomposition product, phosphoramide mustard. This would suggest that the reaction of phosphoramide mustard with cellular DNA may be ultimately responsible for the cytotoxicity of cyclophosphamide.

Since it is believed that other nitrogen mustards exert their antitumor effect by cross-linking DNA through guanine moieties, we were particularly interested in the reaction of phosphoramide mustard with guanosine and deoxyguanosine. Earlier attempts in our laboratory to isolate and identify these adducts were unsuccessful because of their instability and their tendency to bind strongly to conventional chromatographic media. Success in isolating them depended on the use of a reverse-phase high-pressure liquid chromatography column, as described above.

The UV spectra of these adducts suggested that they were 7-substituted guanine nucleosides, but attempts to obtain mass spectral data by conventional means were unsuccessful because of their instability and low volatility. Success in elucidating their structure, therefore, depended very much on the use of field desorption mass spectrometry. This "soft" ionization technique, in contrast to conventional mass spectral methods, does not require any volatility of the sample which is ionized and desorbed by the high-external electric field. Using this technique, intact molecular and cluster molecular ion species were obtained. These ions, together with the fragments of the nucleoside, sugar, and base residues which are typically observed in field desorption spectra of nucleosides and nucleotides (9), led to the structural assignments indicated in Charts 3 and 4.
Earlier difficulties in isolating these adducts were fully explained by their instability. With a half-life of only 2.3 hr at pH 7.4 and 37°C, considerable decomposition would be expected under conventional chromatographic conditions.

This instability may be relevant to the antitumor effect of cyclophosphamide. The cytotoxic action of nitrogen mustards is generally thought to depend on the number of cross-links which are produced in cellular DNA and on their resistance to repair. However, the presence of a highly unstable nucleoside might lead to excessive strand scission, which could also be lethal.

The presence of an unstable adduct could also contribute to a mutagenic or carcinogenic outcome. The residual damage might by itself, or after repair by an error-prone process, lead to the transfer of misinformation in a replicative or transcriptive process. Further investigations will, however, be necessary to establish the significance of this lesion at a cellular level.

ACKNOWLEDGMENTS

We gratefully acknowledge the editorial assistance of Suzanne Wissel.

REFERENCES

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