Biochemical Pharmacology, Vol. 27, pp. 77–81. Pergamon Press, 1978. Printed in Great Britain.

MECHANISM OF ACTION OF THE NITROSOUREAS—1.

ROLE OF FLUOROETHYLCYTIDINE IN THE REACTION OF *BIS*-FLUOROETHYL NITROSOUREA WITH NUCLEIC ACIDS

WILLIAM P. TONG and DAVID B. LUDLUM*

Department of Pharmacology and Experimental Therapeutics, Albany Medical College, Albany, NY 12208, U.S.A.

(*Received* 21 *February* 1977; *accepted* 27 *April* 1977)

Abstract—The nitrosoureas are known to react covalently with nucleic acids and have been shown to decompose in aqueous solution to generate the equivalent of haloethyl carbonium ions. Evidence is presented in this paper that these carbonium ions react with nucleosides to form intermediate haloethyl derivatives. One such haloethyl nucleoside, 3-β-fluoroethylcytidine, has been identified as a reaction product of *bis*-fluoroethyl nitrosourea (BFNU) and cytidine. Fluoroethylcytidine undergoes an unusual intramolecular cyclization reaction to form 3,N^4-ethanocytidine. This is a simple intramolecular crosslinking reaction in the terminology of alkylating agent chemistry, and haloethyl nucleosides formed by the nitrosoureas can probably undergo either inter- or intrastrand cross-linking reactions in analogy with the classical alkylating agents. It seems probable that these reactions are important to the cytotoxic and mutagenic actions of these agents.

Although the nitrosoureas have achieved an important place in cancer chemotherapy, their mechanism of action has not been completely established. As a class, they are structurally related to the alkylating agents; indeed, Wheeler and Chumley [1] showed that typical nitrosoureas, such as *bis*-(2-chloroethyl) nitrosourea (BCNU) and *bis*-(2-fluoroethyl) nitrosourea (BFNU), alkylate nitrobenzylpyridine. Clinically, however, they are somewhat different from the classical alkylating agents and have sometimes been useful for treating tumors resistant to those agents.

We have been interested in the interaction of the nitrosoureas with nucleic acids for two reasons. Their relationship to the alkylating agents, which are thought to produce a therapeutic response by modifying nucleic acids, has led us to determine what modifications are caused in DNA and RNA by the nitrosoureas. Furthermore, the chemical similarity of these agents to a well-known group of carcinogens emphasizes the importance of determining whether the modifications produced by therapeutic nitrosoureas are in any way similar.

Although it was known at the time we initiated these studies that the nitrosoureas react covalently with nucleic acids [2], the type of modifications which were produced had not been elucidated. In a series of investigations [3, 4], we have identified three modified nucleosides in hydrolysates of synthetic polynucleotides reacted with BCNU: 3-β-hydroxyethylcytidine, 7-β-hydroxyethylguanosine, and 3,N^4-ethanocytidine. The identification of these derivatives led us to re-investigate the aqueous decomposition of BCNU [5, 6]. These studies and those of other workers [7–10] indicate that the haloethyl nitrosoureas decompose at neutral pH in aqueous solution to generate haloethyl carbonium ions.

The addition of a haloethyl carbonium ion to a nitrogen in a nucleoside would, of course, convert that nucleoside into an alkylating species. Subsequent reactions could occur, as they do with bifunctional alkylating agents, to produce either interstrand or intrastrand crosslinks. These reactions could easily play an important role in the action of the nitrosoureas.

Evidence for the formation of such an alkylating nucleoside, 3-β-fluoroethylcytidine, is presented here. Preliminary studies with 3-β-chloroethylcytidine indicated that the instability of this compound would make it difficult to identify as a product of BCNU reaction. Accordingly, we prepared 3-β-fluoroethylcytidine by direct organic synthesis and then successfully identified this compound as an intermediate in the reaction of BFNU with cytidine. The synthesis and properties of this unusual alkylating nucleoside and its possible role in the mechanism of action of BFNU are described below.

MATERIALS AND METHODS

Crystalline BFNU was kindly supplied by Dr. Harry B. Wood, Jr. (Division of Cancer Treatment, Drug Research and Development, National Cancer Institute, Bethesda, MD). Reagent grade 1-bromo-2-fluoroethane and cytidine were purchased from the Columbia Organic Chemicals Co. (Columbia, SC) and the Aldrich Chemical Co. (Milwaukee, WI) respectively. *Bis*(trimethyl-silyl)trifluoroacetamide came from the Regis Chemical Co. (Morton Grove, IL); all other materials were from standard sources.

Fluoroethylcytidine was prepared in the following manner. A sealed vial containing 1 g cytidine, 5 ml dimethyl sulfoxide (DMSO), and 1 ml bromo-fluoroethane was put in a constant temperature bath at 55° and incubated overnight. At the end of this period, the reaction mixture was diluted with 20 ml

* To whom reprint requests should be addressed.

of 0.05 M sodium formate, pH 4.5. The mixture was then applied to a SP-Sephadex C-25 column, 7 × 30 cm, which had been pre-equilibrated with the same buffer. This column was eluted with 0.05 M sodium formate, pH 4.5, at a flow rate of 1.5 ml/min to yield two ultraviolet-absorbing peaks. The first, near the front, contained DMSO and unchanged cytidine, while the second, which appeared between 375 and 525 ml, contained fluoroethylcytidine. The fractions which contained this product were collected and desalted on an AG 50 × 8, 200–400 mesh, 1.5 × 15 cm column. Fluoroethylcytidine was eluted with 0.1 N HCl and lyophilized to yield 410 mg of a glassy material which was characterized by ultraviolet and mass spectrometry, and by high-pressure liquid chromatography as described below.

Ultraviolet spectra were obtained on a Beckman DB-G spectrophotometer in acid, neutral and alkaline solution. Trimethylsilyl derivatives were prepared for mass spectrometry by dissolving approximately 0.3 mg fluoroethylcytidine in 20 μl anhydrous pyridine and reacting with 20 μl bis(trimethylsilyl)trifluoroacetamide for 1 hr at room temperature. Solvent and excess reagent were removed under vacuum, and mass spectra were obtained on the residue with a CEC 21-110 instrument. Samples were introduced directly on the probe, and spectra were obtained with a source temperature at 225°, using 70 eV electrons.

High-pressure liquid chromatography was performed on a μ-Bondapak C_{18} column, 4 mm × 30 cm, from Waters Associates (Milford, MA) and on a Partisil-10 SCX column, 4.6 mm × 25 cm, from H. Reeve Angel & Co. (a Division of Whatman, Inc., Clifton, NJ). Elution profiles were followed on an LDC u.v. monitor and recorded on a Heath–Schlumberger recorder.

RESULTS

Properties of fluoroethylcytidine. The synthesis of 3-β-fluoroethylcytidine as described above proceeded smoothly and in an overall yield of approximately 40 per cent. The product was eluted as a single peak upon high-pressure liquid chromatography on a reverse phase system (μ-Bondapak C_{18}) and on a cation exchange system (Partisil SCX). It was then identified by a combination of ultraviolet and mass spectrometry.

The ultraviolet spectrum of 3-β-fluoroethylcytidine is shown in Fig. 1. Characteristics of this spectrum are very similar to those of 3-methylcytidine [11] and led us to conclude that substitution had occurred in the 3 position of cytidine. The acid and neutral spectra are very similar, since the pK for 3-β-fluoroethylcytidine is above 7. An exact determination of this pK is difficult because of the rapid cyclization reaction in alkaline medium which is described below.

A mass spectrum of a silylated derivative of 3-β-fluoroethylcytidine is shown in Fig. 2. The molecular weight is given by the molecular ion peak at m/e 505 and confirmed by the M-15 peak at 490, which is generated by the elimination of CH_3. In comparison with the mass spectrum of Tris(trimethylsilyl)cytidine published earlier [4], it is evident that the molecular weight of 505 corresponds to the addition of a fluoroethyl substituent. The fluoroethyl group is placed at the 3 position primarily on the basis of the ultraviolet data, and the final structural assignment is 3-β-fluoroethylcytidine.

Also evident in the sprectrum is a molecular ion peak at 485 with an M-15 peak at 470 corresponding to the Tris(trimethylsilyl) derivative of 3,N^4-ethanocytidine [4]. Presumably, the cyclization reaction which forms 3,N^4-ethanocytidine occurred in the alkaline silylation medium.

The cyclization reaction was, in fact, useful in confirming the structure of 3-β-fluoroethylcytidine. Marker amounts of 3,N^4-ethanocytidine were available from a previous synthesis [12], and a high-pressure liquid chromatography system was developed to separate 3-fluoroethylcytidine from 3,N^4-ethanocytidine. We were able to obtain a rapid and convenient separation of these two compounds using a Partisil SCX column with 0.1 M potassium phosphate buffer at pH 4.5.

This system was used to study the rate of the cycli-

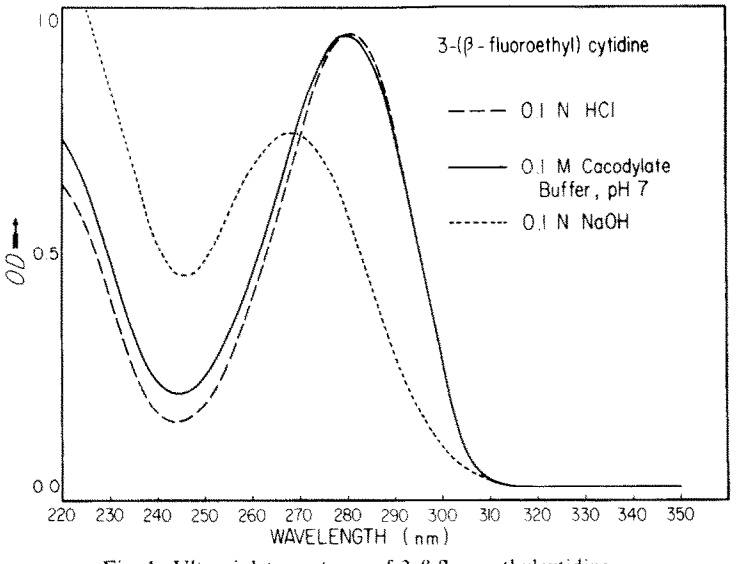

Fig. 1. Ultraviolet spectrum of 3-β-fluoroethylcytidine.

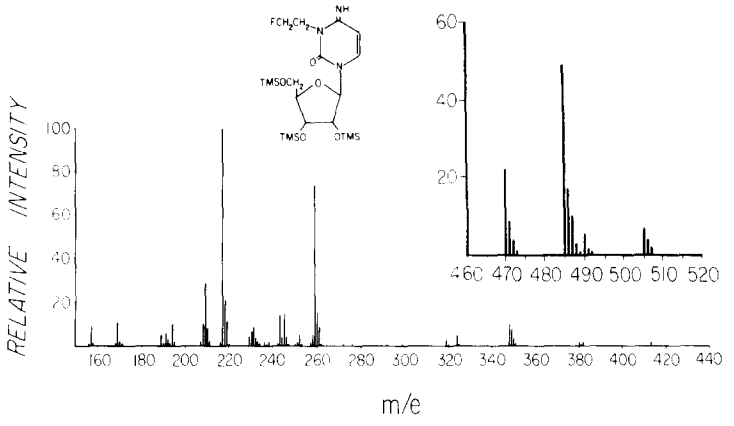

Fig. 2. Mass spectrum of the trimethylsilyl derivative of 3-β-fluoroethylcytidine.

zation reaction as shown in Fig. 3. Approximately 20 μg of 3-β-fluoroethylcytidine and 20 μg of uridine, which was added as an internal marker, were incubated together in 2 ml of 0.05 M potassium phosphate buffer, pH 7.4, at 37°. At 15-min intervals, samples were removed and analyzed by high-pressure liquid chromatography under the conditions stated in the legend to Fig. 3. Since the amount of material injected on the column varied somewhat, areas were compared to the marker uridine. As shown clearly in Fig. 3, the area of the fluoroethylcytidine peak diminishes rapidly, while the area of the 3,N^4-ethanocytidine peak increases.

These data were used to calculate the half-life of 3-β-fluoroethylcytidine in phosphate buffer. Ratios of the peak area of fluoroethylcytidine divided by the peak area of uridine were calculated, and the logarithm of this ratio was plotted, as shown in Fig. 4. This figure amounts to a graph of the logarithm of a normalized fluoroethylcytidine concentration vs time. A linear relationship is observed over a 2-hr period, yielding a half-life of 80 min for 3-β-fluoroethylcytidine. Accordingly, 3-β-fluoroethylcytidine would have an appreciable life time *in vivo*.

It is interesting that hydroxyethylcytidine was not produced from fluoroethylcytidine under these conditions, although it is formed when cytidine reacts with BCNU [3] or BFNU (Fig. 5). This may mean that hydroxyethylcytidine arises from BFNU by some mechanism not involving fluoroethylcytidine or that a fluoroethylcytidine intermediate in the transitional state is more susceptible to hydrolysis than is the compound itself.

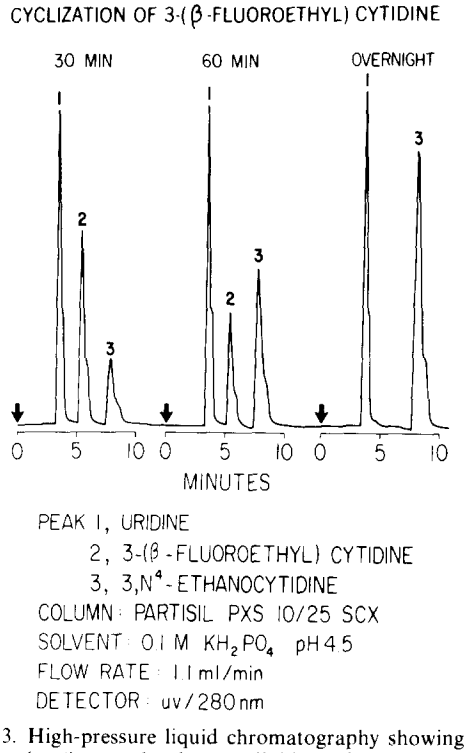

Fig. 3. High-pressure liquid chromatography showing the cyclization (intramolecular crosslinking) of 3-β-fluoroethylcytidine. The figure shows an increase in peak 3,3,N^4-ethanocytidine, at the expense of peak 2,3-β-fluoroethylcytidine. Uridine is added as an internal marker.

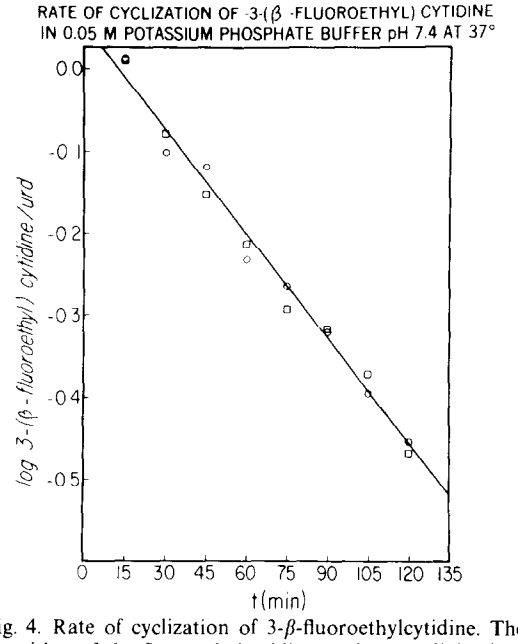

Fig. 4. Rate of cyclization of 3-β-fluoroethylcytidine. The logarithm of the fluoroethylcytidine peak area, divided by the area of the marker uridine peak, is plotted vs time to show a linear pseudo first order decomposition with a half-life of 80 min.

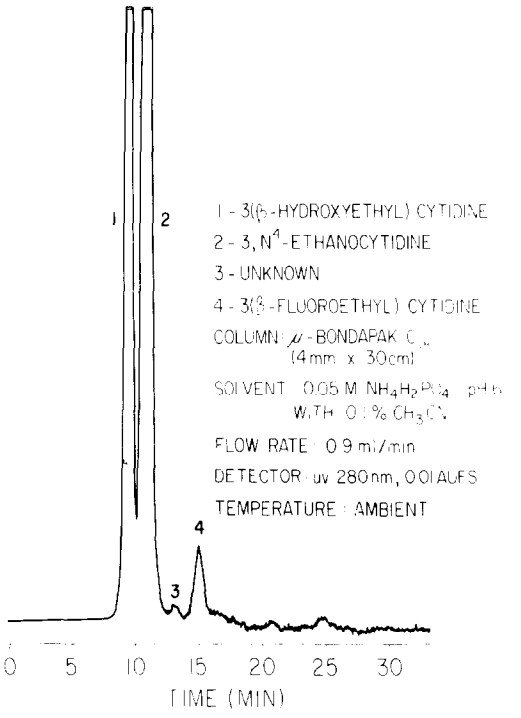

Fig. 5. High-pressure liquid chromatogram showing the formation of 3-β-fluoroethylcytidine from BFNU and cytidine.

Fluoroethylcytidine as a product of BFNU reaction. A reverse phase high-pressure liquid chromatography system with high resolving power for substituted nucleosides was developed to demonstrate the presence of fluoroethylcytidine in a reaction mixture of cytidine and BFNU. Samples were applied to a μ-Bondapak C_{18} column and eluted at pH 6 with a 0.05 M ammonium phosphate buffer containing 0.1% acetonitrile. This system resolved all of the substituted nucleosides of interest as shown in Table 1, and was used to establish fluoroethylcytidine as a product of the reaction of cytidine with BFNU.

To demonstrate this, 10 mg BFNU was reacted with 10 mg cytidine in 1 ml of 0.05 M potassium phosphate buffer, pH 6, at 37°. The mixture was incubated overnight, acidified with 5 drops of 2 N HCl, and applied to an SP-Sephadex C-25 column, 0.9 × 20 cm. This column was then eluted with 0.05 M sodium formate, pH 4.5, at 0.7 ml/min. Ten-min fractions were collected and three major u.v. absorbing peaks were observed. The last peak, which appeared between 100 and 130 min, contained most of the substituted nucleotides. One ml of the central fraction of this peak was lyophylized to dryness, redissolved in 150 μl water, and applied to a μ-Bondapak C_{18} column, as shown in Fig. 5. Four peaks were detected by high-pressure liquid chromatography. The first peak contained 3-β-hydroxyethylcytidine; the second peak, $3,N^4$-ethanocytidine; a third peak, an unidentified cytidine derivative; and the fourth peak, 3-β-fluoroethylcytidine. The identity of this last peak was established by its correct retention time and by the fact that it converted on further incubation to $3,N^4$-ethanocytidine.

Table 1. High-pressure liquid chromatography retention time of bases and nucleosides*

Compound	Time (min)
Cytosine	6.0
Uracil	7.0
Cytidine	9.0
3-β-Hydroxyethylcytidine	9.5
$3,N^4$-ethanocytidine	11.5
Uridine	11.8
N^4-β-hydroxyethylcytidine	15.3
3-β-Fluoroethylcytidine	15.5
3-β-Hydroxyethyluridine	26.3

* Conditions of experiment: column: μ-Bondapak C_{18}, 4 mm × 30 cm; solvent: 0.1% CH_3CN in 0.05 M $NH_4H_2PO_4$, pH 6; flow rate: 0.9 ml/min; detector: u.v. 280 nm; and temperature: ambient.

We have calculated from the peak areas that approximately 0.1 per cent of the cytidine was converted to fluoroethylcytidine by BFNU under the conditions used here.

DISCUSSION

The data presented above show that BFNU reacts with cytidine in aqueous solution to produce fluoroethylcytidine. In general, the reaction of haloethyl nitrosoureas with nucleosides can probably generate a variety of alkylating nucleosides. Thus, there is an interesting parallel in the molecular action of the nitrosoureas and the classical alkylating agents. If the haloethyl group is transferred to a nucleoside which is already contained in a DNA molecule, the possibilities for intrastrand and interstrand crosslinking obviously exist, as they do for classical agents. Data are already available which indicate that both reactions actually occur.

Ewig and Kohn [13] have demonstrated a delayed crosslinking reaction by BCNU in the DNA of L1210 cells. This reaction evidently takes place in two steps, which would be consistent with the formation of a haloethyl nucleoside followed by an intermolecular crosslink. On the other hand, the cyclization reaction reported above can be considered the simplest of all possible intramolecular crosslinking reactions.

Differences from the classical alkylating agents would exist in both types of reaction, however. The original site of electrophilic attack may well be different in the same manner that the site of attack by simple nitrosoguanidines and nitrosoureas is different from the site of attack by the corresponding sulfates and methanesulfonates [14–16]. Once formation of the haloethyl nucleosides has occurred, the possible secondary reactions would also very likely be different from those of the classical alkylating agents.

The possibilities for interstrand crosslinking would clearly depend differently on DNA geometry, since the haloethyl group would have to form a crosslink with two carbon atoms. Similarly, the intrastrand crosslinks that could be formed would be different in the two cases. The internal cyclization reaction elucidated here is probably unique for the haloethyl nitrosoureas.

Thus, there are some interesting similarities and differences in the molecular action of the nitrosoureas and the classical alkylating agents. It seems probable that the particular modifications induced in nucleic acids by these agents help to explain their different biological effects.

Acknowledgements—This research was supported by Grants CA 20129 and CA 20292 from the National Cancer Institute, DHEW, and by Grant 74-B from the American Cancer Society.

REFERENCES

1. G. P. Wheeler and S. Chumley, *J. med. Chem.* **10,** 259 (1967).
2. C. J. Cheng, S. Fujimura, D. Grunberger and I. B. Weinstein, *Cancer Res.* **32,** 22 (1972).
3. B. S. Kramer, C. C. Fenselau and D. B. Ludlum, *Biochem. biophys. Res. Commun.* **56,** 783 (1974).
4. D. B. Ludlum, B. S. Kramer, J. Wang and C. Fenselau, *Biochemistry* **14,** 5480 (1975).
5. M. Colvin, J. W. Cowens, R. B. Brundrett, B. S. Kramer and D. B. Ludlum, *Biochem. biophys. Res. Commun.* **60,** 515 (1974).
6. M. Colvin, R. B. Brundrett, W. Cowens, I. Jardine and D. B. Ludlum, *Biochem. Pharmac.* **25,** 695 (1976).
7. H. E. May, R. Boose and D. J. Reed, *Biochemistry* **14,** 4723 (1975).
8. J. A. Montgomery, R. James, G. S. McCaleb, M. C. Kirk and T. P. Johnston, *J. med. Chem.* **18,** 568 (1975).
9. D. J. Reed and H. E. May, *Life Sci.* **16,** 1263 (1975).
10. D. J. Reed, H. E. May, R. B. Boose, K. M. Gregory and M. A. Beilstein, *Cancer Res.* **35,** 568 (1975).
11. R. Hall, in *The Modified Nucleosides in Nucleic Acids*, p. 118. Columbia University Press, New York (1971).
12. M. J. Murphy, E. J. Goldman and D. B. Ludlum, *Biochim. biophys. Acta*, **475,** 446 (1977).
13. R. A. G. Ewig and K. W. Kohn, *Proc. Am. Ass. Cancer Res.* **17,** 147 (1976).
14. B. Singer and H. Fraenkel-Conrat, *Biochemistry* **8,** 3260 (1969).
15. B. Singer and H. Fraenkel-Conrat, *Biochemistry* **14,** 772 (1975).
16. P. D. Lawley and C. J. Thatcher, *Biochem. J.* **116,** 693 (1970).