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MECHANISM OF ACTION OF THE NITROSOUREAS—II. FORMATION OF FLUOROETHYLGUANOSINE FROM THE REACTION OF BIS-FLUOROETHYL NITROSOUREA AND GUANOSINE

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Abstract—The haloethyl nitrosoureas evidently decompose in neutral aqueous solution to generate haloethyl carbonium ions. In support of this hypothesis, we have shown that bis-fluoroethyl nitrosourea (BFNU) reacts with guanosine to generate 7-β-fluoroethylguanosine, in addition to larger amounts of 7-β-hydroxyethylguanosine. This nucleoside analog has been synthesized from guanosine and fluorobromoethane, and identified in a BFNU–guanosine reaction mixture by high pressure liquid chromatography. Fluoroethylguanosine, *per se*, has an antitumor effect against P-388 murine leukemia, suggesting that it may have some role in mediating the antitumor or toxic effects of BFNU.

Previous studies from our laboratory have shown that the therapeutic nitrosoureas react covalently with polynucleotides to produce a variety of nucleoside derivatives [1–3]. Such reactions may be related to the antitumor activity of these compounds and should, therefore, be completely elucidated. At the same time, since closely related substances with carcinogenic activity are also known to react with DNA and RNA, nucleoside modifications resulting from the action of the nitrosoureas should be investigated from this point of view as well.

BCNU* (bis-chloroethyl nitrosourea), BFNU (bis-fluoroethyl nitrosourea), and similar nitrosoureas evidently decompose in aqueous solution to generate haloethyl carbonium ions [4–6]. In a previous paper of this series, we demonstrated that BFNU reacts with cytidine in aqueous solution to produce 3-β-fluoroethyl cytidine [3]. Presumably, this compound arises from the attack of a fluoroethyl carbonium ion on the 3-position of cytidine. The resulting 3-β-fluoroethyl cytidine is an active alkylating agent in its own right, and cyclizes to produce 3,N^4-ethanocytidine.

Attachment of a reactive haloethyl group to a nucleoside contained in a DNA or RNA strand could result in intermolecular or intramolecular cross-linking reactions. These cross-links would be similar to those formed by classical alkylating agents, but they would involve somewhat different chemistry. Since these differences could be responsible for some of the unique properties of the nitrosoureas, we are investigating the details of these reactions.

As noted in the first paper of this series, chloroethyl nucleosides, which would be generated be the reaction of nucleosides with BCNU, would be more reactive than the corresponding fluoroethyl nucleosides. Thus, we are currently investigating the reactions of BFNU where it is easier to demonstrate the existence of intermediate haloethyl derivatives.

In this paper, we report the identification of another haloethyl nucleoside derivative, 7-β-fluoroethyl guanosine, which is formed by the reaction of BFNU with guanosine. This compound has been synthesized in gram amounts and tested as an antitumor agent in mice bearing P-388 leukemia. It has definite, but minimal, antitumor activity in this system, and its formation *in vivo* is probably responsible for only a small fraction of the total activity of BFNU. The full antitumor activity of BFNU may result from the contributions of several individually cytotoxic events.

MATERIALS AND METHODS

Crystalline BFNU was kindly provided 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 was purchased from Columbia Chemicals Co. (Columbia, SC); guanosine (Lot No. 061147) from Aldrich Chemical Co. (Milwaukee, WI); and acetonitrile from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). 7-Hydroxyethyl guanosine was synthesized according to Brookes and Lawley [7]. All other reagents were from standard sources.

7-β-Fluorocthylguanosine was synthesized for marker purposes and animal testing. Six g guanosine, 6 ml bromofluoroethane, and 25 ml dimethyl sulfoxide were incubated together in a 250-ml round bottom flask at 55° for 48 hr. The solvent and other volatile components were then removed under vacuum at room temperature. The residue was extracted with 30 ml of 5 mM formic acid and the remaining solids, mostly unreacted guanosine, were separated by centrifugation. The extract was then

* Abbreviations: BCNU, 1,3-bis-chloroethyl-1-nitrosourea; and BFNU, 1,3-bis-fluoroethyl-1-nitrosourea.

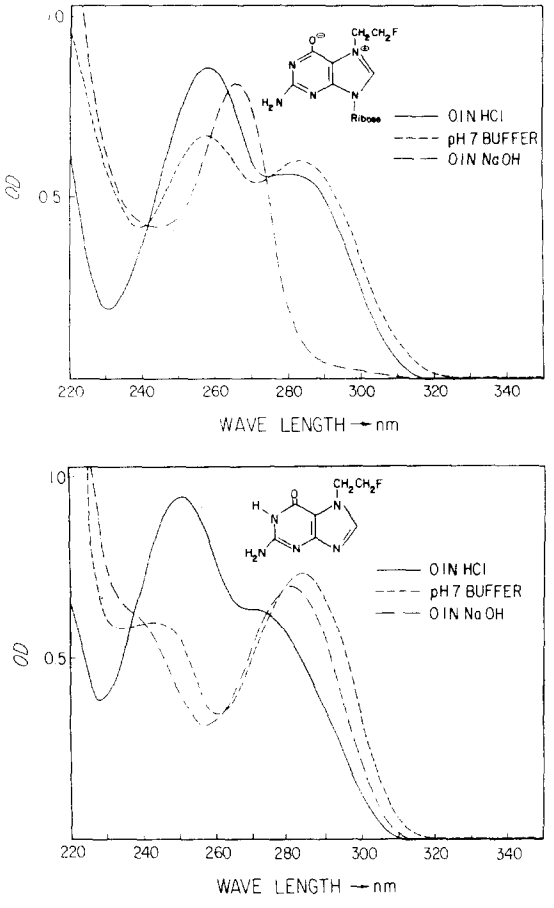

Fig. 1. Ultraviolet spectra of 7-(β-fluoroethyl) guanosine (top panel) and 7-(β-fluoroethyl) guanine (bottom panel).

applied to an Amberlite XAD-4, 20–50 mesh, column (5 × 50 cm) in 5 mM formic acid. The column was eluted with this solution at a flow rate of 1.5 ml/min and 10-min fractions were collected. A peak containing fluoroethylguanosine appeared ahead of guanosine which was more tightly absorbed to the XAD, since it is uncharged at this pH. Fractions 85–160 were collected, lyophilized, and found to contain 1.3 g of product.

To aid in the structural identification of 7-β-fluoroethylguanosine, the compound was subjected to mild acid hydrolysis. Ten mg of the nucleoside was heated in a vial with 0.5 ml of 0.1 N HCl for 1 hr at 100°. The hydrolysate was applied to an SP-Sephadex C-25 column (1.5 × 32 cm) in 0.05 M sodium formate, pH 3.8. The column was eluted with the same buffer at a flow rate of 0.5 ml/min and 10-min fractions were collected. Fractions 33 through 35 contained a substance with the u.v. spectrum of a 7-substituted guanine. These fractions were pooled, HCl was evaporated and the residue was washed three times with 0.5 ml water.

Ultraviolet spectra of 7-β-fluoroethylguanosine and 7-β-fluoroethylguanine were obtained on a Beckman DB-G spectrophotometer in acid, neutral and alkaline solutions. A mass spectrum of 7-β-fluoroethylguanine was obtained on a Hitachi-Perkin Elmer RNU-6E instrument; the sample was introduced directly on the probe and spectra were obtained at a source temperature of 260° with 70 eV electrons.

High pressure liquid chromatography was performed on a μ-Bondapak C_{18} column, 7.9 mm by 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). Column eluents were followed at 254 nm on an LDC u.v. monitor and recorded on a Heath–Schlumberger recorder.

Antitumor activity was measured as the prolongation of survival of mice which carried P-388 lymphocytic leukemia cells. Six-weeks-old BDF_1 mice from Texas Inbred Co. (Houston, TX) received 10^6 tumor cells intraperitoneally on day zero. Treatment was started 24 hr later by the intraperitoneal route with fluoroethylguanosine dissolved in 1 mM HCl. Each treatment group contained six mice and fluoroethylguanosine was administered once daily until death. Control animals received equal amounts of 1 mM HCl. Each treatment group was compared separately with the control group and the significance of the increased survival was evaluated by Student's *t*-test.

RESULTS

Properties of fluoroethylguanosine. Two high pressure liquid chromatographic systems with high resolving power for substituted nucleosides were used to assay fluoroethylguanosine: a Partisil SCX column and a μ-Bondapak C_{18} column, eluted as in the footnote to Table 1. The product described above was found to contain less than 0.5 per cent of ultraviolet absorbing impurities.

Substitution of guanosine in the 7-position is a well-known reaction; in this case, 7-β-fluoroethylguanosine is the predicted product and its structure was confirmed by ultraviolet and mass spectrometry. Ultraviolet spectra of 7-β-fluoroethylguanosine and of the 7-β-fluoroethylguanine released from it by acid hydrolysis are shown in Fig. 1. These spectra are similar to those of the corresponding methyl-substituted compounds, and are characteristic of 7-substituted guanosines and guanines respectively. The spectrum of 7-β-fluoroethylguanosine underwent characteristic changes in alkaline solution

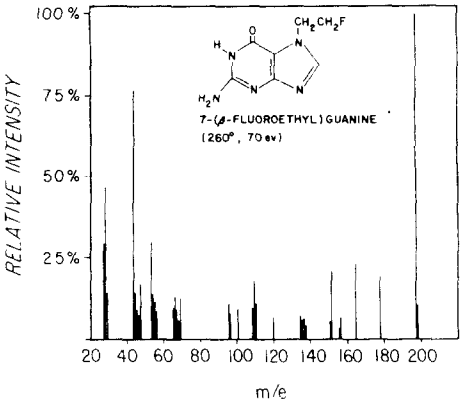

Fig. 2. Mass spectrum of 7-(β-fluoroethyl) guanine.

Table 1. High pressure liquid chromatography retention times (min) of guanosine derivatives

Compound	Partisil SCX*	μ-Bondapak C_{18}†
Guanosine	3.3	11.1
7-Hydroxyethyl-guanosine	12.1	8.2
7-Fluoroethyl-guanosine	16.0	11.3

* Partisil SCX, 4.6 mm × 25 cm, eluted with 0.01 M NaH_2PO_4, pH 4.5, at 1.1 ml/min; ambient temperature.

† μ-Bondapak C_{18}, 7 mm × 30 cm, eluted with 0.05 M NaH_2PO_4, pH 4.5, with 3% acetonitrile (v/v) at 2.3 ml/min; ambient temperature.

typical of the scission of the five member ring of all 7-substituted guanosines [8].

Mass spectrometry was used to show that the substituent in the 7-position was indeed a fluoroethyl group. The mass spectrum of 7-fluoroethylguanine released from fluorotethylguanosine is shown in Fig. 2. The molecular weight is given by the molecular ion peak at m/e = 197 which corresponds to the addition of a $—CH_2CH_2F$ group with the accompanying loss of a proton. The fragmentation pattern shows a large M-20 peak at m/e = 177 corresponding to the elimination of HF, and a M-33 peak at m/e = 164 corresponding to the elimination of $—CH_2F$, both of which confirm the presence of F.

Fluoroethylguanosine as a product of BFNU reaction. With fluoroethylguanosine available as a marker, we developed two high pressure liquid chromatographic systems which could be used to demonstrate its formation from guanosine and BFNU. These are the cation exchange and reverse phase columns described above; retention times for guanosine and its derivatives are shown in Table 1.

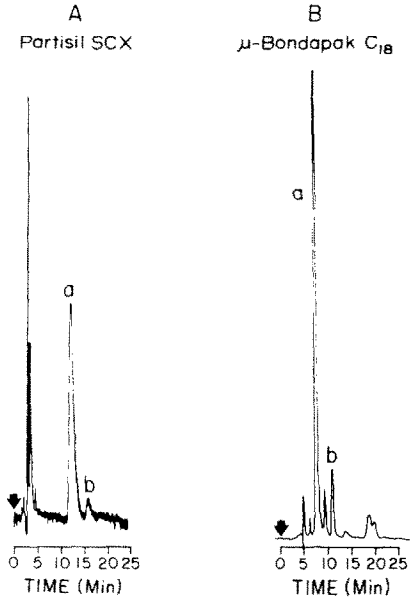

Fig. 3. High pressure liquid chromatogram showing the formation of 7-fluoroethylguanosine from guanosine and BFNU. Guanosine and BFNU were incubated together (see text), the mixture was partially purified on SP-Sephadex C-25, and the derivative-containing fraction was analyzed as in Table 1. Peak a: 7-hydroxyethylguanosine; Peak b: 7-fluoroethylguanosine.

Table 2. Antitumor activity of fluorethylguanosine in BDF_1 mice bearing P-388 ascites leukemia*

Group	Mean survival ± S. D.	T/C†	P‡
Control	8.8 ± 0.8		
50 mg/kg/day	10.3 ± 0.8	1.17	< 0.01
65 mg/kg/day	11.3 ± 0.8	1.28	< 0.01
80 mg/kg/day	11.7 ± 0.8	1.32	< 0.01
120 mg/kg/alternate days	11.2 ± 2.1	1.27	< 0.05

* Mice received 10^6 tumor cells i.p. Drug treatment was started 24 hr later by the i.p. route, and was continued until death.

† T/C is the ratio of the mean survival of the treated group to the mean survival of the control group.

‡ P is calculated according to Student's '*t*' test.

Two mg guanosine was incubated with 10 mg BFNU for 18 hr at 37° in 1 ml of 0.05 M sodium cacodylate buffer, pH 7. A preliminary separation was carried out on an SP-Sephadex C-25 column, 0.9 × 20 cm, eluted at 1 ml/min with 0.05 M sodium formate buffer, pH 5. The last peak to appear, which contained most of the derivatives, was lyophilized to dryness and redissolved in 0.5 ml water. An aliquot was then analyzed on our two high pressure liquid chromatography systems.

These results are shown in Fig. 3. The major derivative demonstrated on both columns is 7-hydroxyethylguanosine as described previously [2], but a small amount of 7-fluoroethylguanosine is also clearly visible. Calculations from the areas of these peaks indicate that approximately 0.2 per cent of the guanosine was converted to β-fluoroethylguanosine by BFNU.

Although we thought that 7-hydroxyethylguanosine might arise from 7-fluoroethylguanosine by hydrolysis, extensive incubation of this compound at 37° in neutral aqueous solution did not produce any of the hydroxyethyl derivative. Thus, as with 3-hydroxyethylcytidine [3], the hydroxyethyl derivative must be formed at the same time as the haloethyl derivatives by hydrolysis of an activated intermediate, or by a different mechanism altogether.

Fluoroethylguanosine as an antitumor agent. The antitumor effect of 7-fluoroethylguanosine was evaluated against murine P-388 leukemia cells. These results are shown in Table 2; increased survival is evident at all doses tested. At extremely high dose levels, acute neurotoxicity with generalized seizures was noted, indicating that the compound may enter the central nervous system. However, it has not yet been tested against intracerebrally implanted cells.

DISCUSSION

The nitrosoureas used clinically contain one or more haloethyl groups and undergo a wide range of reactions with cellular constituents. Cheng *et al.* [9], using chloroethyl cyclohexl nitrosourea labelled in the chloroethyl group, first demonstrated that these

compounds react with nucleic acids. Subsequently, we identified some of the nucleoside modifications which are produced in these reactions [1–3].

A clue to the mechanism by which they are produced developed when we and others showed that the nitrosoureas decompose in neutral aqueous solution to generate haloethyl carbonium ions [4–6]. It is apparent that haloethyl substituents could be added to nucleosides, and that these analog nucleosides might have important biological significance.

In an earlier paper, we successfully identified the first such haloethyl nucleoside, 3-β-fluoroethylcytidine, in a BFNU–cytidine reaction mixture. That compound was characterized by its tendency to undergo an intramolecular condensation similar to an intramolecular cross-linking reaction. This suggested that haloethyl nucleosides might be involved in other cross-linking reactions.

The discovery of 7-β-fluoroethylguanosine introduces another aspect into the pharmacology of the nitrosoureas. This derivative has antitumor activity of its own and there is the possibility that this and other such derivatives could play a role in either the therapeutic or side effects of the nitrosoureas.

The data presented above indicate that 7-β-fluoroethylguanosine is formed in relatively small amounts when BFNU reacts with guanosine. Therefore, a relatively small fraction of the antitumor activity of BFNU is probably attributable to its formation. However, the intraperitoneal administration of such a derivative may grossly underestimate the significance of its intracellular formation. Further studies are in progress to determine the relative importance of this and other nucleoside derivatives formed by the nitrosoureas.

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