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ROLE OF FLUROACETATE IN THE TOXICITY OF 2-FLUROETHYLNITROSOUREAS

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Abstract—The possible role of fluroacetate in the toxicity and antitumour activity of the fluroethylnitrosoureas, BFNU and FCNU has been studied in CBA mice bearing the TLX5 lymphoma either sensitive (TLXS) or resistant (TLXRT) to nitrosoureas. Treatment of mice bearing either TLXS or TLXRT tumours with either BFNU or FCNU caused an elevation in the citrate levels of heart, kidney and tumour, but not the liver, 24 hr after drug administration. Heart citrate levels were maximally elevated 10-fold, while the levels in kidney and tumour were increased 3- to 6-fold. Tissue levels of flurocitrate were determined by glc after conversion to the ethyl ester. This showed maximum levels of fluroacetate production in heart, with lower levels in kidney, tumour and liver. Treatment of K562 human erythroleukaemia cells *in vitro* with BFNU caused an inhibition in the production of $^{14}CO_2$ from ^{14}C palmitate and [U-^{14}C] glucose. These results suggest that some of the effects of the fluroethylnitrosoureas may be related to fluroacetate production and the consequent blocking effect on aconitase. This effect is probably related more to the generalized toxicity of these agents than to their therapeutic efficacy.

The haloethylnitrosoureas are a group of broad spectrum antitumour agents with activity against human lymphomas, melanomas, gliomas and cancers of the gastrointestinal tract [1]. The degradation products of these agents, which are formed during chemical decomposition, are capable of both alkylation and carbamoylation of cellular macromolecules [2]. Alkylation is generally considered to be the principal mechanism of antitumour activity through cross-linking of DNA [3]. This cross-linking is believed to arise as a result of haloethylation of a nucleophilic site on the first strand of DNA, followed by a displacement of the halide ion by a nucleophilic site, either on the same or on an opposing DNA strand, forming an ethyl bridge. There is little difference in the antitumour effectiveness of chloroethyl and fluroethylnitrosoureas, both of which are more active than the corresponding iodo compounds [4]. However, cross-linking with BFNU* might be expected to be much less than with BCNU because F^- is a much weaker leaving group than Cl^-, and in fact no cross-linked product is obtained when FCNU was reacted with DNA [5], suggesting that it is not involved in the cytotoxicity of this agent.

The aqueous decomposition of nitrosoureas in buffered solution gives high yields of the corresponding haloalcohol [6]. When BFNU is heated at 37° in 0.1 M phosphate buffer, pH 7.4, a significant quantity (85%) of 2-fluroethanol is formed [7]. 2-Fluroethanol is a highly toxic product which is converted into fluroacetaldehyde by liver alcohol dehydrogenase and catalase and further oxidation to fluroacetate occurs by liver aldehyde dehydrogenase [8]. The latter acts as a substrate for the lethal synthesis of flurocitrate [9, 10], which has a blocking effect upon aconitase [11]. This leads to an accumulation of citrate in the affected tissue and cell death by immobilization of Ca^{2+}.

This study attempts to determine the role of 2-fluroethanol in the antitumour activity and in the toxicity produced by the fluroethylnitrosoureas.

MATERIALS AND METHODS

BFNU and FCNU were obtained as generous gifts from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD).

Animal experiments. Female CBA mice bearing the TLX5 lymphoma, either sensitive (TLX5) or resistant (TLXRT) to nitrosoureas, were injected i.p. either with 250 μl of the fluroethylnitrosourea in 10% dimethylsulphoxide in arachis oil, or with vehicle alone. After 24 hr the animals were killed by cervical dislocation, the tissues were rapidly removed, frozen in liquid nitrogen and stored at −20° prior to further processing. Ascites tumour cells were removed in a syringe and stored on ice. Red blood cell contamination was removed by washing with 0.016 M Tris–HCl, pH 7.2, containing 7.5 g of ammonium chloride/1 and after a further wash with 0.9% NaCl. The tumour pellet was quick frozen in liquid nitrogen and stored at −20°.

Determination of citrate. To isolate citrate frozen tissue samples were ground in an ice-cold pestle and mortar containing 5% tricholoacetic acid and were left at room temperature for 1 hr with occasional swirling. Protein was removed by centrifugation and

* Abbreviations used: BCNU, *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea; BFNU, *N*,*N*′-bis(2-fluroethyl)-*N*-nitrosourea; FCNU, *N*-(2-fluroethyl)-*N*-cyclohexyl-*N*-nitrosourea.

the supernatant was extracted with three volumes of ether, to remove the trichloroacetic acid, and lyophilized. The lyophilized supernatant was dissolved in 200 μl of $ZnCl_2$/triethanolamine, pH 7.6, and the citrate was determined by the method of Dagley [12]. Control experiments showed that added amounts of citrate were quantitatively recovered in this process.

Detection of fluroacetate by glc. Samples were extracted with ether as described for determination of citrate, dried over anhydrous Na_2SO_4 and the ether was evaporated. The residue was dissolved in an equal volume of ethanol together with 1 μl of concentrated H_2SO_4 and heated at 95–100° for 20 min. Ethyl fluroacetate was detected by glc using a 1 m chromosorb 102 column at 140° and a detector temperature of 250° [13]. A standard calibration curve was constructed each day using authentic ethyl fluroacetate. A typical separation pattern obtained is shown in Fig. 1.

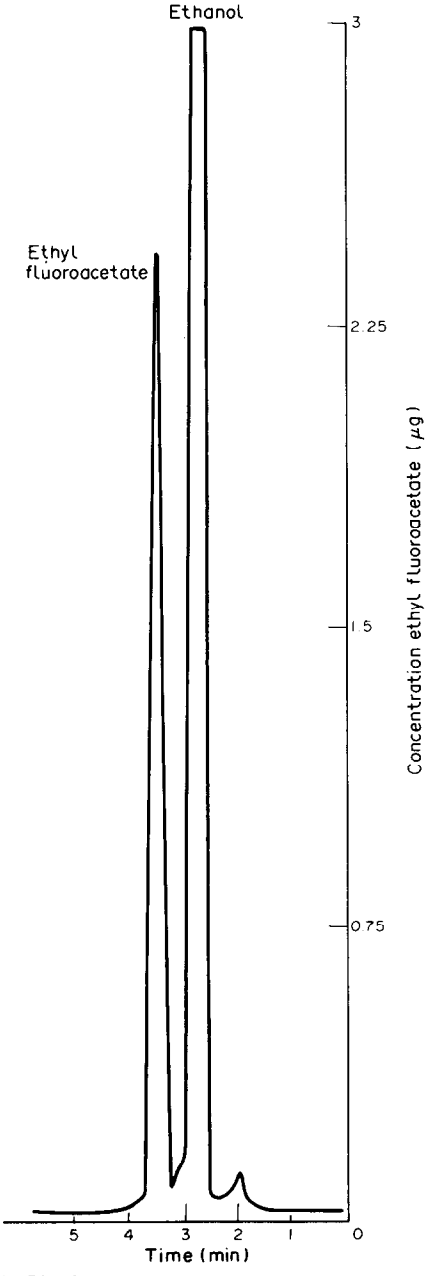

Fig. 1. Glc chromatogram of heart extract after treatment with BFNU.

Effect of fluroethylnitrosoureas on cells in tissue culture. Tumour cells (2.15×10^5/ml; 10 ml) were treated with either BFNU or 2-fluroethanol and incubated in RPMI 1640 medium containing 10% foetal calf serum supplemented either with [U-^{14}C] glucose (2 μCi) or [U-^{14}C] palmitic acid (0.25 μCi; 3.57 μmoles) and were maintained for 24 hr at 37° in scintillation vials equipped with rubber stoppers and a centre well. At the end of the incubation 0.3 ml of 3 N NaOH was injected into the centre well and 0.5 ml of 2 N perchloric acid was injected through the rubber cap to stop the reaction and to release $^{14}CO_2$ from the medium. After a further 60 min of incubation, in order to ensure complete absorption of the released $^{14}CO_2$ into the alkaline solution, the contents of the centre well were combined with 15 ml PCS scintillation fluid (Hopkin and Williams) and the radioactivity determined.

RESULTS

The fluroethylnitrosoureas BFNU and FCNU show antitumour activity against the TLX(S) lymphoma, the percentage increase in survival time at a dose of 40 mg/kg being 50 and >300% respectively [14]. In contrast the TLX(RT) lymphoma is completely resistant to both agents [14]. The effect of BFNU on citrate levels in liver, heart, kidney and TLX(S) cells 24 hr and 48 hr after addition of curative doses of the drug is shown in Table 1. After 24 hr there is a marked (10-fold increase) in the concentration of citrate in the heart, which is highly significant ($P < 0.001$). Citrate levels are also increased 3- to 6-fold in kidney and tumour, but there is no alteration of the level in the liver. Citrate levels are also elevated in heart and kidney of non-tumour-bearing mice. The level of citrate in the heart still remains elevated 48 hr after drug administration, but the levels in liver, kidney and tumour are not significantly different from the controls. In general citrate levels decline markedly 48 hr after drug administration, and for both BFNU and FCNU the peak citrate level is observed earlier with the highest concentration of the drugs.

The effect of FCNU on tissue citrate levels in TLX(S) tumour-bearing mice 24 hr and 48 hr after drug addition is similar to that of BFNU (Table 1). Thus while liver citrate is unaltered by drug addition, heart citrate levels are maximally elevated sevenfold ($P < 0.01$), kidney levels are also maximally elevated sevenfold ($P < 0.01$) and tumour levels are elevated twofold. There is some reduction in citrate levels in heart and kidney 48 hr after drug administration, although they still remain elevated, whilst the level in the tumour does not differ significantly from the control values.

In contract to the fluroethylnitrosoureas neither BCNU (Table 1) or a related drug, mitozolomide (CCRG 81010) have any effect on citrate levels of tissues at concentrations producing similar toxicity.

Table 1. Effects of BFNU, FCNU and BCNU on the citrate content of tissues of mice bearing the TLX(S) tumour 24 hr and (in brackets) 48 hr after treatment

Treatment	Tissue citrate content ± S.E.M.* (μmoles/g fresh wt.) Liver		Heart		Kidney		Tumour (μmoles/10^8 cells)	
Vehicle	0.51 ± 0.1	(0.63 ± 0.17)	1.29 ± 0.29	(2.15 ± 0.59)	1.15 ± 0.24	(1.25 ± 0.64)	0.45 ± 0.10	(0.49 ± 0.23)
BFNU 40 mg/kg	0.28 ± 0.01	(0.21 ± 0.17)	10.0 ± 0.08	(5.12 ± 3.36)	4.59 ± 0.42	(0.93 ± 0.39)	1.04 ± 0.44	(0.44 ± 0.05)
BFNU 80 mg/kg	0.47 ± 0.6	(0.38 ± 0.06)	9.84 ± 1.01	(16.20 ± 5.22)	7.80 ± 0.39	(2.28 ± 0.57)	0.90 ± 0.21	(0.83 ± 0.13)
BFNU 160 mg/kg	0.68 ± 0.18	(0.55 ± 0.16)	12.84 ± 2.37	(5.96 ± 0.92)	4.21 ± 0.24	(3.61 ± 1.63)	1.18 ± 0.18	(1.19 ± 0.57)
FCNU 40 mg/kg	0.47 ± 0.02	(0.70 ± 0.30)	5.32 ± 0.97	(6.34 ± 0.60)	2.09 ± 0.07	(3.02 ± 0.36)	0.60 ± 0.60	(0.42 ± 0.01)
FCNU 80 mg/kg	0.38 ± 0.22	(0.97 ± 0.27)	6.81 ± 0.21	(6.47 ± 0.58)	3.78 ± 0.33	(5.29 ± 0.57)	0.39 ± 0.15	(0.23 ± 0.13)
FCNU 160 mg/kg	1.01 ± 0.59	(0.88 ± 0.28)	9.41 ± 2.81	(5.21 ± 0.38)	7.18 ± 1.79	(1.85 ± 0.15)	0.77 ± 0.16	(0.25 ± 0.12)
BCNU 40 mg/kg	0.49 ± 0.2		2.25 ± 0.8		1.39 ± 0.5		0.51 ± 0.3	
BCNU 80 mg/kg	0.46 ± 0.1		1.81 ± 0.4		1.11 ± 0.4		0.54 ± 0.3	
BCNU 160 mg/kg	0.48 ± 0.2		2.23 ± 0.2		0.56 ± 0.3		0.41 ± 0.3	

* Results are mean of three experiments.

Table 2. Effect of BFNU and (in brackets) FCNU on the citrate content of tissues of mice bearing the TLX5(RT) tumour 24 hr after treatment

Treatment	Tissue citrate content ± S.E.M.* (μmoles/g fresh wt.) Liver		Heart		Kidney		Tumour (μmoles 10^8 cells)	
Vehicle	0.40 ± 0.13	(0.40 ± 0.13)	2.10 ± 0.41	(2.10 ± 0.41)	1.03 ± 0.19	(1.03 ± 0.19)	0.38 ± 0.08	(0.38 ± 0.08)
BFNU 40 mg/kg	0.69 ± 0.41	(0.40 ± 0.30)	8.10 ± 0.08	(6.19 ± 0.54)	1.12 ± 0.56	(2.0 ± 1.38)	0.27 ± 0.19	(0.18 ± 0.05)
BFNU 80 mg/kg	0.56 ± 0.15	(0.35 ± 0.04)	7.50 ± 0.52	(13.38 ± 2.7)	2.64 ± 1.35	(4.19 ± 3.17)	0.68 ± 0.37	(0.95 ± 0.48)
BFNU 160 mg/kg	1.02 ± 0.18	(0.78 ± 0.18)	7.30 ± 1.46	(12.77 ± 2.9)	4.28 ± 1.98	(7.75 ± 2.08)	0.95 ± 0.21	(1.03 ± 0.30)

* Results are mean of three experiments.

Table 3. Concentration of fluroacetate in tissues of mice bearing the TLX(S) tumour 24 hr after administration of either FCNU or BFNU

Treatment	Tissue fluroacetate (±S.E.M.) (μg/g fresh weight) Liver	Heart	Kidney	(μg/10^8 cells) Tumour
FCNU 40 mg/kg	0.034 ± 0.037	2.53 ± 0.14	0.22 ± 0.15	0.16 ± 0.21
FCNU 80 mg/kg	0.085 ± 0.084	3.32 ± 0.11	3.02 ± 2.92	0.26 ± 0.20
FCNU 160 mg/kg	0.142 ± 0.049	7.95 ± 4.58	2.21 ± 1.5	0.21 ± 0.27
BNFU 40 mg/kg	0.058 ± 0.1	2.36 ± 1.03	0.36 ± 0.23	ND
BNFU 80 mg/kg	0.073 ± 0.059	3.36 ± 2.69	1.06 ± 0.61	ND
BFNU 160 mg/kg	0.205 ± 0.133	3.49 ± 1.85	0.90 ± 0.19	ND

Determined as the ethyl ester. Mean of two experiments in duplicate
ND—not detectable in tumours.

This suggests that the citrate elevation is a specific effect of the fluroethylnitrosoureas and is not the result of a general toxic effect of these types of agents.

To determine whether the elevation of tissue citrate levels by the fluroethylnitrosoureas is related to their antitumour activity, or to general toxicity, the effect of BFNU on tissue citrate levels has been determined in mice bearing the TLXRT tumour, which is resistant to triazenes and nitrosoureas [14]. The results for BNFU and FCNU are presented in Table 2. Both drugs again cause an increase (3- to 6-fold; $P = 0.001$) in both heart and kidney citrate levels without a concomitant increase in the citrate content of the liver. However, tumour citrate levels are also raised threefold ($P = 0.007$ and 0.01) by 160 mg/kg BFNU and FCNU. This suggests no differential response between the sensitive and resistant TLX5 lymphoma to drug-induced citrate elevation.

The concentration of fluroacetate, determined by glc as the ethyl ester, in the tissues of mice 24 hr after administration of either BFNU or FCNU is given in Table 3. For both drugs fluoroacetate is present with maximal concentrations being observed in the heart, followed by the kidney. The concentration in the liver is minimal for both drugs. No fluroacetate is detectable in the tumour 24 hr after administration of BFNU.

The effect of incubation of human erythroleukaemia cells, K562, for 24 hr with various concentrations of BFNU on the production of $^{14}CO_2$ from [U-^{14}C] glucose and ^{14}C-palmitate is shown in Fig. 2. The effect of 2-fluroethanol on $^{14}CO_2$ production from ^{14}C-palmitate is also shown for comparison. Both agents cause a dose-related reduction in $^{14}CO_2$ production, although much higher concentrations of 2-fluroethanol have to be used to produce the same effect, probably due to the virtual absence of alcohol dehydrogenase in any of the tumour cell lines. These results suggest that fluroacetate may be formed directly from BFNU, and may not form 2-fluroethanol as an intermediate.

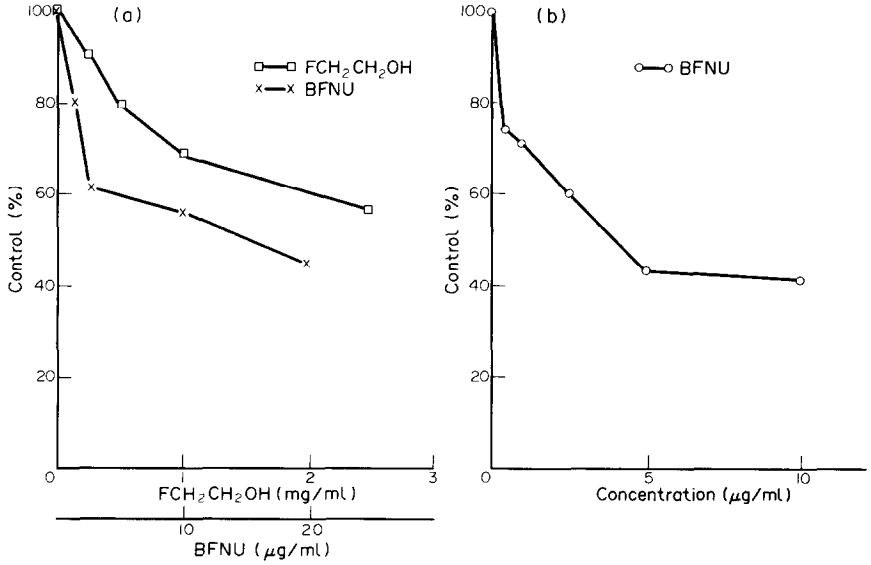

Fig. 2. (a) Effect of 2-fluroethanol (□) and BFNU (×) on $^{14}CO_2$ production from ^{14}C-palmitate after 24 hr drug treatment. (b) Effect of BFNU on $^{14}CO_2$ production from [U-^{14}C] glucose after 24 hr drug treatment. $^{14}CO_2$ was assayed as described in Methods, and is expressed as a percentage of a control culture not exposed to the drug.

DISCUSSION

Treatment of tumour-bearing mice with the fluroethylnitrosoureas BFNU and FCNU caused an appreciable increase in the citrate levels of kidney, heart and tumour, with little effect on liver citrate content. Spencer and Lowenstein [15] also observed that fluroacetate poisoning had relatively little effect on the citrate content of rat liver under a variety of conditions, while kidney levels increased seven to eightfold. A similar differential response of liver and kidney to fluroacetate poisoning has also been reported by Potter and Busch [16]. This lack of accumulation of citrate in liver after fluroacetate may be a function of the different specificites of acetyl-CoA synthetase of liver and kidney [17]. Acetyl-CoA synthetase from pigeon liver has been shown to activate acetate, but not fluroacetate, whereas the enzyme from rabbit kidney activates both substrates [18].

Tissue levels of fluroacetate correlate with those of citrate. Thus 24 hr after treatment with either FCNU or BFNU significant levels are found in the heart and reduced levels in the kidney. Levels of fluroacetate are only increased in tumour tissue after FCNU. This may be due to rapid accumulation of flurocitrate. This suggests that the accumulation of citrate arises from the blocking effect on aconitase.

Teetens *et al.* [19] also provided some evidence that the toxicity of FCNU may be attributed in part to the release of fluroacetate. Monacetin, considered to afford protection against fluroacetate by providing a readily available supply of intracellular acetate ions, also proved efficacious in preventing the lethality of FCNU. Large doses of FCNU administered to Rhesus monkeys cause neurotoxicity, with ataxia spasms in the arm and neck muscles, prostration and severe vomiting [20]. These effects are similar to those produced by fluroacetate poisoning [21].

Both bis(2-fluroethyl)nitrosoureas and bis(2-fluroethyl)triazenes are more toxic than the corresponding bis(2-chloroethyl) derivatives [4, 22]. This increased toxicity probably arises from release of fluroethanol, which can be oxidized to fluroacetate by tissue enzymes, followed by lethal synthesis of flurocitrate. Although fluroethylnitrosoureas appear to inhibit the TCA cycle of tumour cells *in vitro* it seems unlikely that the therapeutic efficacy arises from fluroacetate production since citrate levels are elevated in both sensitive and resistant tumours after treatment with BFNU and FCNU.

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