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α-Fluoro-β-alanine: Effects on the Antitumor Activity and Toxicity of 5-Fluorouracil

Shousong Cao,† David P. Baccanari,‡ Youcef M. Rustum,* Stephen T. Davis,‡ Robert L. Tansik,‡ David J. T. Porter‡ and Thomas Spector‡§*

*Department of Pharmacology and Therapeutics, Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, NY 14263; and ‡Glaxo Wellcome Research Laboratories, Research Triangle Park, NC 27709, U.S.A.

ABSTRACT. We have shown previously that (*R*)-5-fluoro-5,6-dihydrouracil ($FUraH_2$) attenuates the antitumor activity of 5-fluorouracil (FUra) in rats bearing advanced colorectal carcinoma. Presently, we found that α-fluoro-β-alanine (FBAL), the predominant catabolite of FUra that is formed rapidly via $FUraH_2$, also decreased the antitumor activity and potentiated the toxicity of FUra. In rats treated with Eniluracil (5-ethynyluracil, GW776), excess FBAL, in a 9:1 ratio to FUra, produced similar effects when administered 1 hr before, simultaneously with, or 2 hr after FUra. FBAL also decreased the antitumor activity of FUra in Eniluracil-treated mice bearing MOPC-315 myeloma at a 9:1 ratio with FUra, but not at a 2:1 ratio. FBAL did not affect the antitumor activity of FUra in mice bearing Colon 38 tumors. We also evaluated the effect of thymidylate synthase (TS) and thymidine kinase (TK) from tumor extracts after FUra ± Eniluracil ± FBAL treatment. The activity of TK was similar among the three groups at both 18 and 120 hr. There was also no difference in TS inhibition (~35%) at 18 hr. However, significantly more TS inhibition was observed in the Eniluracil/FUra group than in the FUra-alone group at 120 hr. FBAL did not alter the effect of Eniluracil/FUra in TS inhibition. Neither $FUraH_2$ nor FBAL affected the IC_{50} of FUra in culture. Thus, the effect of FBAL did not result from direct competition with FUra uptake or immediate anabolism. Either another downstream catabolite that is not formed in cell culture is the active agent, or the effect requires the complexity of a living organism or an established tumor. BIOCHEM PHARMACOL **59**;8:953–960, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. uracil reductase; dihydropyrimidine dehydrogenase; thymidylate synthase; 5-fluorouracil; Eniluracil; α-fluoro-β-alanine

Eniluracil (5-ethynyluracil, GW776) is a potent inhibitor of DPD‖ [1, 2] that prevents the catabolism of FUra in mice and rats [3, 4]. Without Eniluracil, > 80% of a FUra dose is converted rapidly to $FUraH_2$ and eventually to FBAL [5–7] according to the catabolic pathway diagrammed in Fig. 1. Consequently, Eniluracil markedly increases the systemic exposure to FUra in laboratory animals [8] and in cancer patients [9]. For example, Eniluracil increases the plasma half-life of FUra in patients from approximately 10 min [10] to 5 hr [9]. Furthermore, Eniluracil prevents FUra degradation during absorption by inhibiting DPD and thereby enables oral dosing [9]. Most importantly, Eniluracil increases the therapeutic index of FUra by 2- to 4-fold in mice [3] and by 6-fold in rats bearing advanced colorectal tumors [11] and markedly improves the absolute efficacy of FUra in rats [11]. However, the improvements in FUra therapy conferred by Eniluracil exceed those that should result from simply improving the pharmacokinetics of FUra [11].

We speculated that the FUra catabolites produced in the absence of Eniluracil may affect the antitumor activity of FUra. In rats bearing advanced colorectal carcinoma, we demonstrated that excess $FUraH_2$ decreased the antitumor activity of FUra in Eniluracil-treated rats to the level of activity produced by FUra alone [12]. Although this study indicated that Eniluracil improves the efficacy of FUra by preventing the excessive formation of $FUraH_2$, it raised the possibility that a downstream catabolite is the impeding agent.

In this report, we studied FBAL, the predominant FUra catabolite, in the same rat tumor model and in two tumor-bearing mouse models. We evaluated the activity of TS and TK from Ward tumor extracts treated with FUra alone, Eniluracil/FUra, or Eniluracil/FUra/FBAL at 18 and 120 hr post-treatment. We also investigated the timing of the FBAL dose with respect to the FUra dose and the effect of FBAL and $FUraH_2$ on the activity of FUra in cell culture.

† Corresponding author: Shousong Cao, M.D., Department of Pharmacology and Therapeutics, Grace Cancer Drug Center, Roswell Park Cancer Institute, Buffalo, NY 14263. Tel. (716) 845-1638; FAX (716) 845-8857; E-mail: scao@sc3101.med.buffalo.edu

§ Current address: 5216 Old Chapel Hill Rd., Durham, NC 27707.

‖ *Abbreviations*: DPD, dihydropyrimidine dehydrogenase; FUra, 5-fluorouracil; $FUraH_2$, (*R*)-5-fluoro-5,6-dihydrouracil; FBAL, α-fluoro-β-alanine; TK, thymidine kinase; TS, thymidylate synthase; PR, transient partial (at least 50%) tumor regression; CR, complete tumor regression that is sustained for 90 days post-therapy; and DTT, dithiothreitol.

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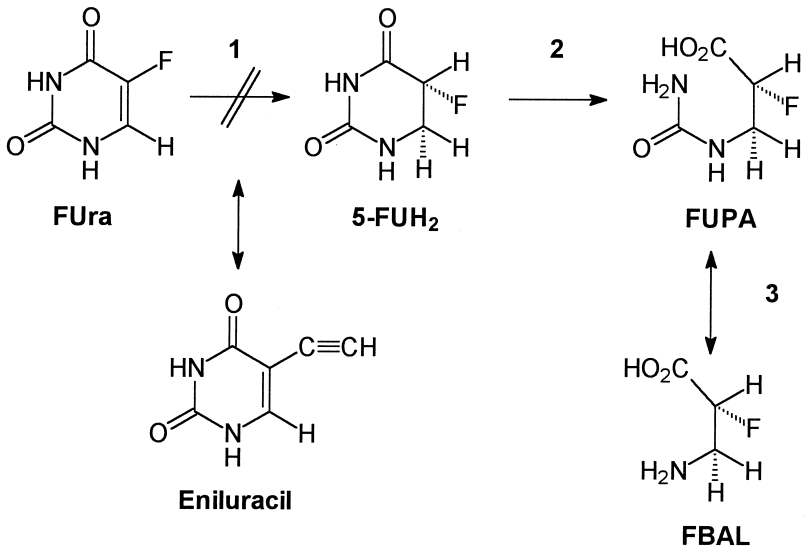

FIG. 1. Catabolic pathway of FUra and its blockade by Eniluracil. The reactions are catalyzed by: (1) dihydropyrimidine dehydrogenase (DPD, uracil reductase, EC 1.3.1.2); (2) dihydropyriminidase (EC 3.5.2.2); and (3) β-ureidopropionase (β-alanine synthase, EC 3.5.1.6).

MATERIALS AND METHODS

Animals and Tumors

Eight- to twelve-week-old female Fisher 344/HSD rats (150–180 g) were obtained from Harlan Sprague Dawley, Inc. Six- to eight-week-old female Balb/C mice (18–20 g) were obtained from Charles River Laboratories. C57BL/6 × C3HF$_1$ (B6C3F$_1$) mice were obtained under contract from various commercial suppliers. Ward colorectal carcinoma tumor fragments (≈100 mg) were transplanted s.c. into rats, and treatment was initiated 12–14 days later when tumor weights were approximately 3.0 g as previously described [11]. Balb/C mice with s.c. murine MOPC-315 myeloma tumors were treated when tumors reached 150–250 mg (mm^3). In experiment 1, 10^6 MOPC-315 tumor cells from cell culture were implanted in mice to establish tumors. In experiment 2, tumors were passaged three times s.c. in mice prior to the initiation of therapy. Briefly, excised tumors were forced through a stainless steel screen, and the extracted cells were passed sequentially through 18-, 20-, and 23-gauge needles. The tumor extract (0.05 mL) was implanted s.c. in the right axillary region. B6C3F$_1$ mice with s.c. murine Colon 38 tumors were treated after the tumor reached approximately 150 mg as previously described [3].

Materials

Eniluracil was synthesized at the Glaxo Wellcome Research Laboratories. FUra was purchased from Hoffmann-La Roche Inc. The biologically formed (*R*)-enantiomers of FUraH$_2$ and FBAL were synthesized and purified as described [13].

Drug Solutions

Eniluracil was dissolved in sterile saline adjusted to pH 10 with NaOH. FBAL was dissolved in sterile saline and adjusted to pH 7 with NaOH immediately before use. FUra was diluted with sterile saline.

Drug Doses and Schedule

For the tumor-bearing rat model, FUra was administered (100 mg/kg/week alone or 10 mg/kg/week with Eniluracil) by i.v. push once weekly for 3 weeks 1 hr after Eniluracil. Eniluracil was administered i.p. at 1 mg/kg. FBAL was administered at 90 mg/kg/week by i.v. push, 1 hr before, simultaneously with, or 2 hr after FUra administration. Each group had four rats per experiment, and each experiment was repeated at least three times. For the MOPC-315 tumor-bearing mouse model, a single dose of FUra was administered i.p. 30 min after pretreatment with 2 mg/kg of Eniluracil i.p. as described earlier [3]. Nine to ten mice were included in each study arm. For the Colon 38 tumor-bearing mouse model, FUra was administered daily for 7 or 9 days, and Eniluracil (2 mg/kg, i.p.) was administered 30 min prior to FUra as described previously [3]. Each treatment group contained eight mice. All studies were in compliance with Institute approved protocols and company policy on the use of animals.

Toxicity Evaluation

Animal body-weight loss, diarrhea, stomatitis (mouth ulceration), alopecia (hair loss), and lethality were determined daily for a minimum of 4 weeks and observed at least twice a week for 3 months after treatment.

Antitumor Activity

Tumor regression was expressed as PR when tumor weight was reduced temporarily by at least 50% (in general, the tumors with PR would subsequently regrow within 2 weeks after cessation of treatment); and as CR when tumors were

resolved completely and were not detectable for at least 90 days after treatment when the animals were killed. Tumor volumes were calculated from the tumor length and width, which were measured with the aid of a Vernier caliper. Tumor weight was calculated as tumor weight = 1/2 (length × width^2). Measurements of Ward tumors were taken once a day during the first week and every 2 days thereafter. Similar measurements were made for MOPC-315 and Colon 38 tumors.

Tumor Sample Collection

For biochemical studies, [^{3}H]FUra was administered to rats (six/group) with advanced (3 g) implants of Ward carcinoma. The doses were 100 mg/kg (3 mCi/kg) of [^{3}H]FUra alone, 15 mg/kg (0.46 mCi/kg) of [^{3}H]FUra with Eniluracil, or 15 mg/kg (0.46 mCi/kg) of [^{3}H]FUra with Eniluracil and FBAL treatment. Eniluracil was administered 1 hr before FUra and every 24 hr for an additional 2 days after FUra therapy. A fourth group of tumor-bearing rats served as controls for untreated levels of TS and TK. Tumor samples were collected 18 or 120 hr after the FUra dose and rapidly frozen between blocks of dry ice and in liquid nitrogen.

Tumor Extraction for Enzyme Assays

Tumor samples, typically 0.25 g, were homogenized (on ice) in 3.75 mL of 250 mM Tris–Cl, pH 7.8, 250 mM NaF, 12.5 mM DTT, and 2.5 mM EDTA containing 100 μM phenylmethylsulfonyl fluoride, 25 μg/mL of α_1-antitrypsin, 1 μg/mL of soybean trypsin inhibitor, 5 μg/mL of aprotinin, and 10 mM benzamidine using a Brinkmann Polytron PT3000, with a small generator (PT-DA3007/2), at 18,000 rpm (two 15-sec cycles with 45 sec between cycles). After centrifugation (100,000 *g*, 1 hr), the supernatant (called S-100) was used directly in TK assays. To prepare the extract for assays of free TS, dUMP and unbound FdUMP were removed from the extract by incubating 250 μL of S-100 with 250 μL of 10% activated charcoal/1% BSA/0.1% dextran at 4° for 15 min. Charcoal was removed by centrifugation (Fisher Micro-Centrifuge, 5 min at 4°), and the supernatant was analyzed for free TS. To prepare the extract for assays of total TS, bound FdUMP was stripped from the enzyme by incubating 250 μL of S-100 with 250 μL of 0.75 M NH_4HCO_3, 15 mM CMP, pH 7.8, plus 25 μL of 1.6 mM dUMP. After 3 hr at 30°, the solution was desalted on a 1-mL Sephadex G-25 column equilibrated with 100 mM Tris–Cl, 5 mM DTT, 100 mM NaF, 15 mM CMP, 1 mM EDTA, pH 7.8, at 4°. The protein eluting in the void volume was analyzed for total TS.

TK Assay

To assay TK, 25 μL of S-100 was added to 25 μL of a reaction mixture containing 100 mM Tris–Cl, pH 7.8, 30 mM NaF, 5 mM $MgCl_2$, 5 mM ATP, 5 mM phosphocreatine, 0.24 U/μL of creatine phosphokinase, and 200 μM [^{14}C]thymidine (0.05 μCi) and incubated at 37°. At 0, 15, and 30 min, 5 μL was removed and spotted onto Whatman DE81 paper. After the reaction mixtures dried, the paper was washed with gentle rocking for 5 min at room temperature with 5 mM ammonium formate, followed by 70% ethanol for an additional 5 min. After drying, the papers were placed into 5 mL of Ultima Gold scintillation fluid (Packard), and the phosphorylated [^{14}C]thymidine was quantitated using a Packard Tri-Carb 1900CA Liquid Scintillation Analyzer.

TS Assay

TS was quantitated by measuring the enzyme-catalyzed release of ^3H_2O from [5-^{3}H]dUMP using a modification of the method of Hashimoto *et al.* [14]. Free and total TS were assayed in extracts prepared via charcoal treatment and Sephadex G-25 filtration, respectively. Thirty microliters of extract (diluted if needed with 100 mM Tris–Cl, pH 7.8, 5 mM DTT, 100 mM NaF, 1 mM EDTA, and 15 mM CMP) was added to 30 μL of a reaction mixture containing 67 mM Tris–Cl, pH 7.8, 67 mM NaF, 3.35 mM DTT, 0.67 mM EDTA, 0.8 mM 5,10-methylenetetrahydrofolate, 10 mM CMP, and 20 μM [5-^{3}H]dUMP (0.6 μCi) and incubated at 37°. At 0, 30, and 60 min, 10-μL samples were removed from the reaction and mixed with ice-cold 4% trichloroacetic acid in a 1.5-mL microcentrifuge tube. The mixture was centrifuged, and three 2.5-μL drops of 100% KOH were placed carefully in the cap of each centrifuge tube. The centrifuge tubes were incubated overnight at 30°, during which time ^3H_2O was absorbed by the KOH. After removing the caps, the KOH was neutralized with 100 μL of glacial acetic acid, and the radioactivity was determined as described above. At least 26 tumor fragments from each treatment group were assayed for TS activity.

Cell Culture

Ward colon carcinoma cells were established in culture from tumors grown s.c. in female Fischer rats. The medium was RPMI 1640 without folic acid containing 25 mM HEPES (Life Technologies) and supplemented with 20 mM glutamine, 1 mM sodium pyruvate, 10 nM calcium leucovorin, 2 μg/mL of transferrin, 0.2 U/mL of bovine insulin, 20 nM sodium selenite, and 10% undialyzed fetal bovine serum (HyClone). MOPC-315 cells were obtained from the American Type Culture Collection and adapted to growth in RPMI 1640 without folic acid containing 25 mM HEPES, 20 mM glutamine, 1 mM sodium pyruvate, 10 nM calcium leucovorin, and 10% dialyzed fetal bovine serum.

Cell Cytotoxicity Assays

Ward tumor cell growth was assayed in 96-well microtiter plates. Cells were seeded at 750 cells per well and allowed to attach for 24 hr at 37° in a humid incubator with 5% CO_2. Modulation medium, which was medium supple-

mented with either 100 μM FBAL or 200 μM $FURaH_2$, was added either 30 min prior to FUra or simultaneously with FUra. After 72 hr at 37° in a humid incubator with 5% CO_2, cell growth was measured using the sulforhodamine B assay [15]. IC_{50} is the concentration of inhibitor required to reduce the growth of cells to 50% of the control value.

For clonogenic assays, 4000 Ward cells were plated into 60-mm tissue culture dishes and incubated for 24 hr at 37° in a humid incubator with 5% CO_2. Unattached cells were aspirated, and the medium was replaced with fresh medium or modulation medium. After 30 min at 37° and 5% CO_2, FUra was added, and the incubation was continued for 5 hr. Medium was then aspirated, the cells were washed twice with PBS, and fresh medium or modulation medium was added. After 48 hr, medium was removed and replaced with fresh plain medium. After 7 days of incubation, the plates were stained with 1% crystal violet in 60% methanol containing 10% formalin and 5% glacial acetic acid. Colonies were counted on an Artek Colony Counter.

FUra inhibition of MOPC-315 growth was determined in a 72-hr continuous exposure assay. Cells in log-phase growth were seeded into 96-well microtiter plates at a density of 3500 cells per well and incubated at 37° in a humid incubator with 5% CO_2. Either 100 μM FBAL or 200 μM $FURaH_2$ was added 30 min prior to the addition of FUra, and the plates were incubated for 3 days. Growth was assayed using a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] procedure [16]. Briefly, 50 μL of MTT (Sigma) prepared in medium was added to each well (to give a final MTT concentration of 1 mg/mL), and the plates were incubated for 2 hr at 37°. Medium was removed, and dimethyl sulfoxide was added to solubilize the formazan product. Absorbance was measured with a microplate reader at 550 nm.

Statistical Analysis

The differences between the mean values were analyzed for significance ($P \leq 0.05$) using Student's *t*-test.

RESULTS

Effect of FBAL on Antitumor Activity and Toxicity in Rats Bearing Advanced Ward Colorectal Carcinoma

We selected a 10 mg/kg dose of FUra and a 90 mg/kg dose of FBAL to approximate the FBAL exposure achieved in rats dosed with 100 mg/kg of FUra in the absence of Eniluracil. It was too toxic when FBAL was given at 108 mg/kg with the same dose of FUra and Eniluracil, which produced diarrhea and lethality in a significant number of animals (data not shown).

The data in Fig. 2A and Table 1 show that FBAL impaired the antitumor activity of FUra when FBAL was administered on three different schedules relative to FUra administration. FBAL produced similar effects when administered 1 hr before, simultaneously with, and 2 hr after FUra administration.

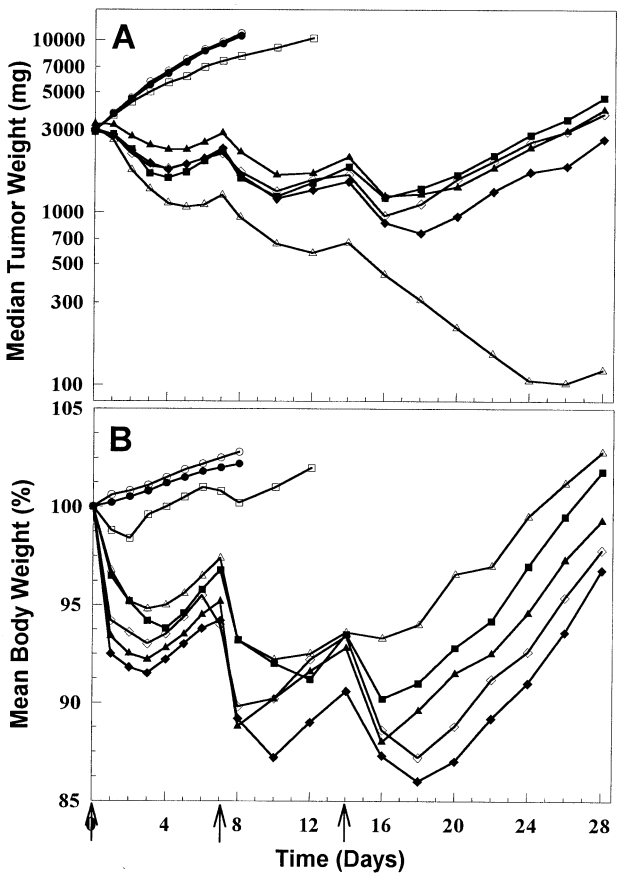

FIG. 2. Antitumor activity (A) and body weight change (B) of the FUra-therapeutic regimens in rats bearing advanced colorectal carcinoma. Time delays between dosing are indicated in parentheses. Key: (○) untreated control; (●) Eniluracil 1 mg/kg; (□) FBAL 90 mg/kg; (■) FUra 100 mg/kg; (△) Eniluracil 1 mg/kg (1 hr) + FUra 10 mg/kg; (▲) Eniluracil 1 mg/kg (1 hr) + FUra 10 mg/kg + FBAL 90 mg/kg; (◇) Eniluracil 1 mg/kg + FBAL 90 mg/kg (1 hr) + FUra 10 mg/kg; and (◆) Eniluracil 1 mg/kg (1 hr) + FUra 10 mg/kg (2 hr) + FBAL 90 mg/kg. Rats were treated on days 0, 7, and 14 (arrows). The body weight of animals was 150–180 g. Tumor weight and toxicity were assessed as described in Materials and Methods. Each experiment with four rats per treatment arm was repeated three to nine times. The data points are the average values for all experiments.

FBAL also enhanced the toxicity of FUra in tumor-bearing rats. FUra and Eniluracil with FBAL increased body weight loss (Fig. 2B) and the incidences of diarrhea, stomatitis, and lethality relative to FUra and Eniluracil alone (Table 2). In addition, alopecia, which was not observed with FUra or FUra and Eniluracil, occurred in almost half of the rats treated with FBAL combined with FUra and Eniluracil (Table 2).

Effect of FBAL on the Antitumor Activity in Mice Bearing MOPC-315 Myeloma Tumor

The effect of FBAL on the antitumor activity of the combination of Eniluracil with FUra was studied in mice bearing MOPC-315 s.c. tumors. Eniluracil was adminis-

TABLE 1. Antitumor efficacy of the FUra-therapeutic regimens

Drug* administered at time:			Antitumor activity (%)	
0 hr	1 hr	3 hr	CR	PR
(1) Eniluracil			0	0
(2) FBAL			0	0
(3) FUra			13	83
(4) Eniluracil	FUra		96	4
(5) Eniluracil	FUra + FBAL		36†	64
(6) Eniluracil + FBAL	FUra		42†	58
(7) Eniluracil	FUra	FBAL	50†	50

Rats were dosed with the indicated regimens once a week for 3 weeks, and tumor volumes were assessed as described in Materials and Methods.
*Doses: FUra alone, 100 mg/kg (maximum tolerated dose); FUra in combination with Eniluracil, 10 mg/kg; Eniluracil, 1 mg/kg; FBAL, 90 mg/kg.
†Significantly different from group 4, $P < 0.01$. Note that Groups 5, 6, and 7 are not statistically different from each other.

tered i.p. at 2 mg/kg 30 min before FUra and for 2 days following FUra administration. Eniluracil alone did not affect the growth of MOPC-315 tumors (Fig. 3, experiment 1, and Ref. 3). FUra at 5 mg/kg (minimal effective dose) was administered as a single i.p. dose, or it was co-administered with 45 mg/kg of FBAL. In two separate experiments, FBAL attenuated the antitumor activity of FUra (Fig. 3). In the first experiment, FBAL decreased tumor growth inhibition (on day 8) from 71 to 17% ($P < 0.01$). In the second experiment, FBAL decreased tumor growth inhibition (on day 7) from 54 to 30% ($P < 0.05$). The effects of FBAL were dose-dependent; it did not affect the antitumor activity of FUra when administered at 10 mg/kg with FUra plus Eniluracil (data not shown).

Effect of FBAL on Antitumor Activity in Mice Bearing Colon 38 Tumors

The effect of FBAL was also studied in mice bearing Colon 38 s.c. tumors. Eniluracil and FUra were administered i.p. for 7 or 9 days with or without FBAL. In the first experiment, treatment was initiated 7 days after tumor implantation when the average tumor size was 134 ± 35 mg. On day 40, none of the untreated mice (average tumor size: 2590 ± 840 mg), 5 of 7 mice treated with Eniluracil and 3 mg/kg of FUra, and 7 of 8 mice treated with Eniluracil and 3 mg/kg of FUra plus 25 mg/kg of FBAL were tumor-free. Clearly, FBAL did not affect the antitumor activity of FUra in this study. The experiment, therefore, was repeated to test the effect of FBAL with more advanced tumors. This time drugs were administered for 7 days beginning 18 days after implantation, when the average tumor size was 515 ± 83 mg. Again, FBAL did not affect the antitumor activity of FUra significantly. For example, on day 27, the average tumor size was 1640 ± 200 mg for the untreated mice, 470 ± 110 mg for mice treated with Eniluracil and 3 mg/kg of FUra, and 480 ± 140 mg for mice treated with Eniluracil and 3 mg/kg of FUra plus 30 mg/kg of FBAL. No groups had tumor-free mice.

Inhibition of TK and TS

We measured the activity of TK and TS (free and total) from tumors of rats treated with FUra alone, Eniluracil/FUra, or Eniluracil/FUra/FBAL. At 18 hr post-treatment, TK was similar in the three treatment groups, in which it was inhibited by 98% (data not shown). TS inhibition was also similar in the three groups (~35%). At 120 hr post-treatment, however, TS in Eniluracil/FUra-treated tumors was inhibited significantly more than in tumors treated with FUra alone; no change in effect was observed

TABLE 2. Toxicity of the FUra-therapeutic regimens

Drug* administered at time:			Incidence (%)				
0 hr	1 hr	3 hr	MWL†	Diarrhea	Stomatitis	Alopecia	Death
(1) Eniluracil			0	0	0	0	0
(2) FBAL			2 ± 1	0	0	0	0
(3) FUra			10 ± 3	15	4	0	0
(4) Eniluracil	FUra		8 ± 2	0	0	0	0
(5) Eniluracil	FUra + FBAL		13 ± 2	17	8	42	8
(6) Eniluracil + FBAL	FUra		12 ± 4	8	0	50	0
(7) Eniliracil	FUra	FBAL	14 ± 4	17	8	50	17

Rats were dosed with the indicated regimens once a week for 3 weeks, and toxicities were assessed as described in Materials and Methods. N = a total of 16 animals for each treatment group; 4 independent experiments were performed.
*Doses: FUra alone, 100 mg/kg; FUra in combination with Eniluracil, 10 mg/kg; Eniluracil, 1 mg/kg; FBAL, 90 mg.kg.
†MWL: maximum weight loss of percent of pretreatment body weight (means ± SD).

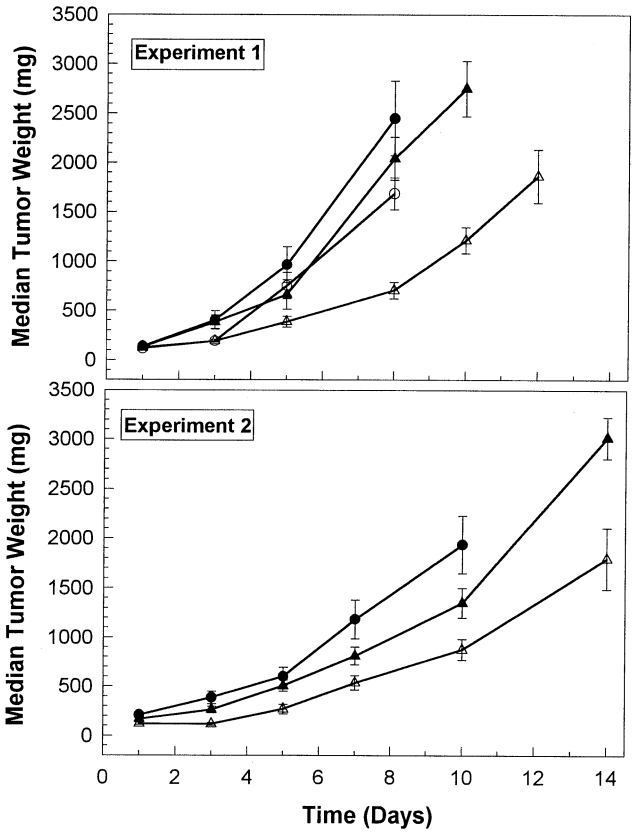

FIG. 3. Antitumor activity of the FUra-therapeutic regimens in mice bearing MOPC-315 myeloma tumors. Key: (○) untreated control; (●) Eniluracil 2 mg/kg; (△) Eniluracil 2 mg/kg + FUra 5 mg/kg; and (▲) Eniluracil 2 mg/kg + FUra 5 mg/kg + FBAL 45 mg/kg. Mice were treated on day 0. Eniluracil was administered 30 min before FUra and for 2 days after FUra therapy. FUra and FBAL were administered together. Nine to ten mice were used in each group. The data points (means ± SD) are the average values for all mice in each group. The tumor volumes between the Eniluracil/FUra group and the Eniluracil/FUra/FBAL group were significantly different ($P < 0.01$ in experiment 1 and $P < 0.05$ in experiment 2).

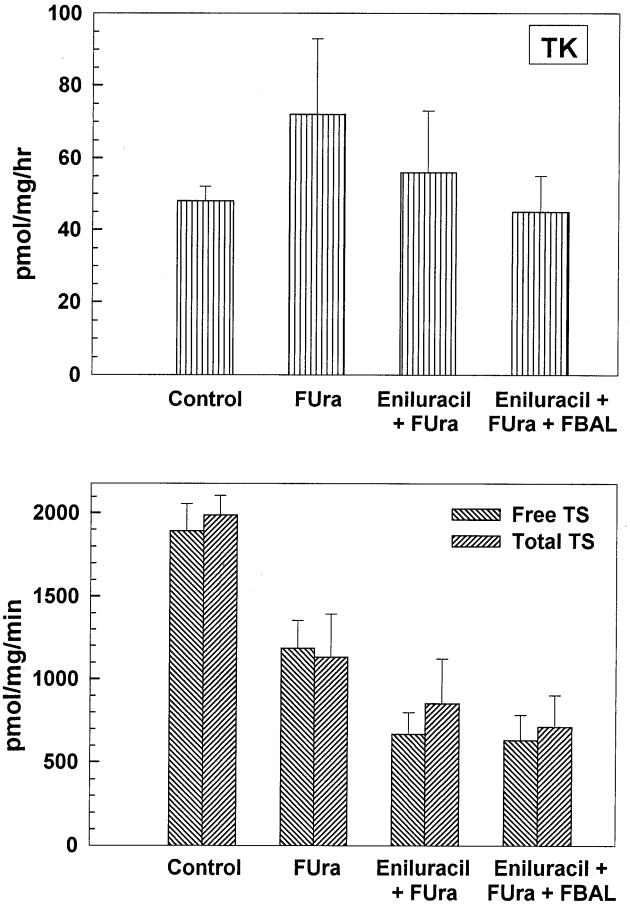

FIG. 4. Specific activities of TK and TS in Ward tumor extracts 120 hr after treatment with FUra, FUra/Eniluracil, or FUra/Eniluracil/FBAL. Values in each column are the means ± SD of 5–6 independent samples (duplicate assays for each sample). The TS activities were significantly different between FUra and Eniluracil/FUra or Eniluracil/FUra/FBAL ($P < 0.01$).

when the addition of FBAL to Eniluracil/FUra was compared with Eniluracil/FUra (Fig. 4). At this time point, the activity of TK had recovered fully in all three treatment groups.

Effect of FBAL and FUraH_2 on the Cell Growth Inhibition of FUra In Vitro

Tumor cells were grown in the presence of FUra alone or in combination with either FBAL or FUraH_2. Neither 100 μM FBAL nor 200 μM FUraH_2 altered FUra inhibition of MOPC-315 cells in continuous exposure experiments (Table 3). In addition, FBAL and FUraH_2 did not affect the IC_{50} of F Ura for the Ward carcinoma cell line in either a 5-hr clonogenic or a 72-hr continuous exposure assay (Table 3). No difference was seen in the FUra inhibition when FBAL and FUraH_2 were added 30 min before or simultaneously with FUra (data not shown).

DISCUSSION

The data of this study with FBAL confirm an earlier observation [12] that catabolites of FUra diminish the antitumor activity of FUra in rats bearing Ward tumors. FBAL is as effective as FUraH_2 [12] in attenuating the antitumor activity of FUra. This observation was not surprising for the following reasons. First, FBAL is the major catabolite of FUra that is formed rapidly via FUraH_2, and it accounts for more than 70% of the total FUra derivatives detected in urine from patients treated with FUra [6, 17]. Second, because FBAL has a terminal half-life of 33 hr, it accumulates in humans [6] and in rats [8]; therefore, it is the likely catabolite that directly or indirectly interferes with antitumor activity of FUra. However, the possibility that another catabolite is involved was not eliminated by our data. For example, FBAL is converted to fluoride and malonic semialdehyde *in vitro* by mitochondrial L-alanine-glyoxylate aminotransferase II [18]. Trace amounts of fluoride are detected in urine and plasma of patients treated with FUra [19] or 5′-deoxy-5-fluorouridine [20]. Moreover, fluoroacetate and 2-fluoro-3-hydroxypropi-

TABLE 3. FUra inhibition of cell growth *in vitro*

Drug	IC_{50} (μM)		
	Ward carcinoma		MOPC-315
	Clonogenic assay* (5-hr exposure)	Growth inhibition† (72-hr exposure)	Growth inhibition‡ (72-hr exposure)
FUra alone	3.8 ± 0.2	0.13 ±0.02	0.58 ±0.03
FUra + FBAL (100 μM)	4.1 ± 0.7	0.12 ±0.01	0.53 ± 0.02
FUra + $FUraH_2$ (200 μM)	4.1 ± 0.6	0.11 ± 0.01	0.61 ± 0.02

Values are means ± SEM.
*Results are the average of 3 separate assays in quadruplicate.
†Results are the average of 3–5 separate assays in triplicate.
‡Results are the average of 2 separate assays in triplicate.

onic acid are produced metabolically from FUra in liver perfusates [21]. *Ex vivo* measurements of tissue extracts from liver, kidney, and Colon 38 tumors showed a > 95% elimination of α-fluoro-β-ureidopropionic acid and FBAL signals in mice treated with Eniluracil/FUra [22]. *In vivo* NMR experiments on Colon 38 tumors found a complete elimination of the α-fluoro-β-ureidopropionic acid and FBAL signals in tumors treated with Eniluracil/FUra and a dramatic formation and accumulation of 5-fluorouridine mono-, di-, and triphosphates and 5-fluorouridine [22].

Our results with mice bearing MOPC-315 myeloma tumors also demonstrated the detrimental effects of FUra catabolites on the antitumor activity of FUra. When FBAL and FUra were administered at a ratio of 9:1, FBAL significantly diminished the antitumor activity of FUra. This ratio reflects the proportions produced in cancer patients treated with FUra, where more than 80% of the dose is catabolized rapidly [5–7]. It is noteworthy that FBAL was without effect when administered at a ratio of 2:1, with respect to FUra.

Eniluracil increases the therapeutic index of FUra in both models [3, 11], and it markedly improves the absolute antitumor efficacy in the Ward tumor model [11]. Thus, suppression of the formation of FUra catabolites by Eniluracil is a reasonable explanation for the therapeutic improvements in these models. However, other factors could also contribute to the observed effect. For example, Ward tumors from rats treated with the maximum tolerated dose (MTD) of FUra (15 mg/kg) and Eniluracil (1 mg/kg) incorporated more anabolites and exhibited a prolonged inhibition of TS compared with tumors treated with the MTD of FUra (100 mg/kg) alone (our unpublished results and Fig. 4). In addition, although Eniluracil also increases the therapeutic index of FUra in mice bearing Colon 38 tumors [3], FBAL did not attenuate the antitumor activity of FUra in this model when it was administered at 25–30 mg/kg. The reason for the discrepancy between the Colon 38 model and the other two models is not known. Perhaps, higher ratios of FBAL to FUra or a larger absolute dose of FBAL are required to produce an effect in the Colon 38 model. The dose of FBAL was 30 mg/kg (with 3 mg/kg of FUra) in the Colon 38 tumor and 45 and 90 mg/kg in the MOPC-315 and Ward tumors, respectively.

Interestingly, the *in vivo* data also demonstrated that the effect of FBAL is not a result of direct competition with FUra uptake or immediate anabolism. FBAL administered 1 hr before or 2 hr after FUra had similar attenuating effects as FBAL administered concomitantly with FUra in the rat tumor model. These dosing schedules were selected to exclude the time frames when the major concentration of FBAL would overlap with that of FUra. The cell culture data further indicated that effects of $FUraH_2$ and FBAL do not involve direct competition. Neither catabolite changed the ability of FUra to inhibit either Ward or MOPC-315 tumor cells in culture (Table 3). Thus, as mentioned above, either another downstream catabolite that is not formed in cell culture is the active agent or the effect requires the complexity of a living organism or an established tumor. In any case, these studies add another bit of intrigue to the FUra saga that investigators have been assembling for the past three decades.

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