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# Decreased host toxicity in vivo during chronic treatment with 5-flourouracil

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***Summary.*** *Chronic weekly administration of FUra to $CD8F_1$ female mice bearing spontaneous mammary tumors produced body weight loss during the first 2 weeks of treatment, which became less severe during subsequent weeks of therapy. To our knowledge, the development of such a decrease in FUra toxicity in vivo during chronic treatment with the drug has not been described previously, and a study of this phenomenon was therefore undertaken in tumor-free $CD8F_1$ female mice. Weekly administration of FUra at 85 mg/kg resulted in toxicity expressed in body weight loss and in depressed peripheral WBC levels; however, the magnitude of these toxic effects decreased significantly by the 5th week of treatment. Pretreatment of normal mice with FUra for 7 weeks resulted in a dose-related shift in the $LD_{50}$ of FUra administered as a subsequent challenge. Compared with an $LD_{50}$ of 240 mg/kg for FUra in normal mice, the $LD_{50}$ in mice pretreated with FUra at 50 or 85 mg/kg per week was found to be significantly elevated to 370 and 460 mg/kg, respectively. Pretreatment with FUra at 85 mg/kg for 7 weeks did not alter the activity of the enzymes responsible for the activation of FUra, namely uridine kinase or orotate phosphoribosyltransferase, in the intestinal epithelium or bone marrow, but it did decrease the 24-h urinary excretion of intact [$^3$H]FUra by almost 40% ($P < 0.01$). In addition, the FUra pretreatment schedule resulted in a 31% ($P = 0.14$) increase in the activity of dihydrouracil dehydrogenase in the liver. These results suggest that increased degradation of FUra can be induced by chronic treatment with the drug. Finally, knowledge of the development of increased drug catabolism was used to increase the therapeutic effectiveness of FUra by its incorporation into an increasing-dose regimen. Mice bearing 24-h transplants of the murine breast tumor were treated with a constant dose of FUra for 12 weeks or with a dose that was increased, after 7 weeks, to a dose normally causing a high degree of drug-related mortality. The group receiving the incremented FUra dose had a significantly slower tumor growth rate without an increase in drug-related toxicity. These results are discussed in light of their obvious clinical implications.*

## Introduction

Since its introduction into clinical medicine over 25 years ago, 5-flourouracil (FUra) has remained an effective and widely used antineoplastic agent. The action of FUra as an anticancer drug stems from its metabolism to the nucleotide level [13] forming either 5-flourodeoxyuridine monophosphate (FdUMP), which is a potent inhibitor of thymidylate synthetase and thus of DNA synthesis [3, 16, 35], or 5-flourouridine triphosphate (FUTP), which can be incorporated directly into RNA, leading to disruptions in RNA processing and function [7, 10, 38]. Activation to the nucleotide form proceeds either by a two-step mechanism utilizing uridine phosphorylase (EC 2.4.2.3 uracil : ribose-1-phosphate phosphotransferase) and uridine kinase (EC 2.7.1.48 ATP : uridine phosphotransferase) or by a single step through orotate phosphoribosyltransferase (EC 2.4.2.10 orotidine-5′-phosphate : pyrophosphate phosphoribosyltransferase) utilizing PRPP [13, 29]. The enzymes needed for these transformations are present in all rapidly dividing cells [5, 13, 14]. Catabolic degradation of FUra follows the same pathway as for normal pyrimidines. Inactivation of FUra begins with the saturation of its C5-C6 double bond to form dihydroflourouracil. This reaction is mediated by dihydrouracil dehydrogenase (EC 1.3.1.2 4,5-dihydrouracil : NADP oxidoreductase), which is found primarily in the liver [2, 4, 6, 13, 25, 30, 34].

In early therapy studies it was observed that FUra lost effectiveness over a period of time despite continued treatment [15, 32]. Reichard and co-workers demonstrated that tumor cells, when cultured in a medium containing FUra, developed resistance to this drug, which was found to be related to the loss of uridine kinase activity [28]. The mechanism of resistance of tumor cells to FUra has since been studied further, and resistance to FUra is presently attributed to differences in the activities of the enzymes responsible for its conversion to the nucleotide form, namely uridine kinase, uridine phosphorylase, and orotate phosphoribosyltransferase, or to differences in the activity of its target enzyme thymidylate synthetase [11, 17, 26, 27, 31].

Because of its potent cytotoxic action against all rapidly growing cells [12], FUra therapy often results in toxic side-effects particularly manifest in bone marrow depression [37] and disruption of the epithelial lining of the gut [16]. In the treatment of spontaneous breast tumors in $CD8F_1$ mice we observed that, in addition to the gradual loss of antitumor activity, these toxic effects became less profound after several weekly courses of FUra treatment. The present study, initial results of which have been presented elsewhere [8], was undertaken to determine whether resistance analogous to that demonstrated in tumor cells can develop in vivo in normal tissues over a prolonged period of FUra treatment.

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## Material and methods

*Mice.* Initial studies on the therapeutic effectiveness of FUra involved $CD8F_1$ female mice bearing spontaneously arising mammary adenocarcinomas. Subsequent toxicity and enzyme studies were carried out in normal (tumor-free) 3-month-old $CD8F_1$ female mice. In later therapy experiments normal $CD8F_1$ female mice bearing first-generation transplants of spontaneous $CD8F_1$ mammary adenocarcinomas were used. The transplant procedure involved injection of 0.3 ml of a 5% tumor brei suspension into the right axillary region of the experimental mice. Therapy studies were begun 24 h after the transplant injection. The breeding, care, and incidence of spontaneous tumor development in $CD8F_1$ mice have been described previously [22, 36].

*Tumor measurements.* The length and width of tumors were determined by external caliper measurements, and the tumor weight was calculated according to the formula: weight (mg) = $a \times b^2/2$, where a = length (mm) and b = width (mm), based on the assumption that the tumors are prolate spheroids with a density of 1.0 [37].

*Toxicity measurements.* Mice were weighed before and weekly during treatment to determine changes in body weight. Peripheral WBC levels were measured electronically in tail vein blood with a Royco Cell Counter before and at timed intervals after initiation of treatment.

*Drug administration.* FUra (Sigma, St. Louis, Mo) was dissolved in 0.9% saline and administered IP at the desired concentration in 0.1 ml/10 g body weight. Due to its limited solubility, when doses of FUra greater than 100 mg/kg were needed they were obtained by giving multiple injections of 10.0 mg/ml solution. [6-$^3$H]FUra (Moravek Biochemicals, Brea, Calif), initial specific activity 20 Ci/mmol, was supplied as samples containing 25 mCi/5 ml sterile water. For urinary clearance studies, [6-$^3$H]FUra was made up to a final concentration of 10.0 mg/ml and 0.01 mCi/ml.

*Determination of $LD_{50}$ of FUra.* Mice were challenged with FUra at doses ranging from 140 to 560 mg/kg. Following the challenge dose the mice were weighed daily and mortality data collected over a 4-week period. The $LD_{50}$ of FUra was subsequently determined from these data by the method of Litchfield and Wilcoxon [20].

*Measurement of uridine kinase and orotate phosphoribosyltransferase activities*

*Tissue preparation.* Approximately 15 cm of the gut was removed, starting at the pyloric sphincter, flushed with ice-cold saline, and slit open lengthwise with scissors. The epithelial cells were scraped off with a clean microscope slide and kept in a homogenizing tube on ice until needed. Bone marrow cells were obtained from the same animals by removing the femur and flushing the marrow cavity with ice-cold saline. Bone marrow cells obtained from two animals were pooled in a single homogenizing tube and kept on ice until needed. Gut and pooled bone marrow cells were homogenized in 1.0 ml of 0.25 *M* sucrose − 0.1 *M* Tris HCl (pH 7.5) and centrifuged at 100,000 *g* in a Spinco Model L refrigerated centrifuge with a Ti50 fixed-angle rotor for 60 min. The supernatant served as the source of enzyme for the following assays.

*Uridine kinase activity.* Uridine kinase was assayed by a modification of the methods of Krystal and Webb [19] and Ahmed [1]. To 400 µl of the supernatant obtained above was added 50 µl of a solution containing 25 m*M* $MgCl_2$, 2 m*M* NaF, and 50 m*M* ATP and 50 µl 5 m*M* [5-$^3$H]Urd (0.1 mCi/50 µl) (Moravek Biochemicals). This mixture was then placed in a 37° C water bath and 75-µl samples removed at various times over the next 30 min. The 75-µl aliquots were placed in a boiling water bath for 2 min to precipitate protein. The precipitate was removed by centrifugation and 25 µl of the resulting supernatant was spotted on DEAE disks and allowed to dry. The disks then were washed a total of six times, twice in 5-m*M* ammonium formate and four times in water. After washing, the filters were placed in scintillation vials and 0.5 ml 0.5 *M* NaCl−1 *N* HCl was added. After 30 min, 5 ml scintillation cocktail (ACS, Amersham, Arlington Heights, Ill) was added and filter-bound radioactivity was measured.

*Orotate phosphoribosyltransferase activity.* Orotate phosphoribosyltransferase was assayed by a modification of the methods of Reyes and Ahmed [1, 29]. To 400 µl of the supernatant was added 50 µl of a solution containing 50 m*M* PRPP (5-phosphoribosyl 1-pyrophosphate)−50 m*M* $MgCl_2$ and 50 µl 5.0 m*M* [6-$^3$H]FUra (0.1 mCi/50 µl). These samples were then processed in the same way as those for the uridine kinase assay mentioned above. The protein content of the 100,000 *g* supernatant was determined by the method of Lowry [21]. Results of both assays were expressed as nmol UMP produced/mg protein/60 min.

*Determination of the amount of FUra excreted in the urine.* Mice were challenged with a single 100-mg/kg injection of FUra containing [6-$^3$H]FUra. Immediately thereafter each mouse was placed in a modified metabolic cage to collect urine over a 24-h period. After the collection period the urine was processed as follows: First, the urine was suspended in 10 ml distilled water and centrifuged at 5,000 *g* for 2 min. A 0.1-ml aliquot of the supernatant was counted for total tritium-related radioactivity. After deproteinization of the remainder of the urine sample with percholic acid, intact FUra was determined by high-pressure liquid chromatography (HPLC). HPLC analysis was carried out on a DuPont 850 HPLC equipped with a Zorbax C-8 column (4.6 mm × 25 cm); the detection wavelength was 254 nm. The buffer used was 0.01 *M* $KH_2PO_4$ in 0.5 m*M* tetrabutylammonium hydrogen sulfate (pH 5) at a flow rate of 2 ml/min at 45° C. Under these conditions the retention time of FUra was 2.50 min.

*Determination of the activity of dihydrouracil dehydrogenase in the liver.* Dihydrouracil dehydrogenase activity was assayed by a modification of the method of Smith and Yamada [9, 33]. Livers were homogenized in 8 volumes of 0.25 *M* sucrose-3 m*M* $MgCl_2$ − 5 m*M* β-ME (β-mercaptoethanol). The liver homogenates were centrifuged at 100,000 *g* for 60 min at 4° C in a Spinco Model L refrigerated ultracentrifuge with a Ti50 rotor. The supernatant, serving as a source of enzyme, was then diluted 1 : 10 with 0.05 *M* potassium phosphate buffer (pH 7.5). To 3.75-ml aliquots of each supernatant/buffer mixture was added 6.75 ml 417 m*M* potassium phosphate buffer (pH 7.45) − 12.5 m*M* β-ME − 0.42 m*M* NADPH. The assay reaction was initiated by the addition of 0.75 ml 3.0 m*M* uracil. The flask containing the reaction mixture was incubated at 37° C and at various times over the next 30 min 1.5 ml was removed and placed in a test tube containing 0.45 ml of

2.12 *N* percholic acid and then placed on ice. The denatured reaction mixtures then were centrifuged at 10,000 *g* for 4 min and to a 1.5 ml aliquot of supernatant was added 0.11 ml 10 *N* NaOH. The absorbance of the neutralized solution was read spectrophotometrically at 290 nm against its control solution in a Beckman Model 35 spectrophotometer. The extinction coefficient for uracil in this reaction was 14.94 $cm^{-1} \cdot \mu M^{-1}$. The protein content of the supernatant/buffer mixtures was determined by the method of Lowry [21].

*Measure of the therapeutic effectiveness of weekly injections of FUra.* Experiments involving 100 $CD8F_1$ female mice bearing 24-h transplants of primary murine breast carcinomas were begun by distributing the mice into four groups according to body weight. Thereafter, the mice in each group received weekly IP injections of FUra according to the following protocol: Group 1, no treatment; group 2, FUra (85 mg/kg per week); group 3, FUra (100 mg/kg per week); and group 4, FUra (85 mg/kg per week) for 7 weeks then FUra (100 mg/kg per week) for the remainder of the experiment. On the day of injection, all mice were weighed and their tumors measured as described previously.

## Results

*Effect of weekly injections of FUra on the growth of spontaneously occurring mammary carcinomas in $CD8F_1$ female mice*

$CD8F_1$ mice bearing spontaneously occurring breast tumors (averaging 250 mg) were treated with either saline or FUra at 100 mg/kg each week for 8 weeks. Pooled results from three experiments (45 mice per treatment group) are presented in Fig. 1. At each observation point FUra caused a statistically significant inhibition of tumor growth in comparison with saline-treated controls. However, although the mean tumor mass was kept relatively constant in the FUra-treated groups during the first 3–4 weeks of therapy, tumor growth inhibition was substantially decreased in spite of continued exposure to FUra during the remainder of the experiment (Fig. 1).

*Effect of weekly injections of FUra on the body weight of $CD8F_1$ female mice bearing spontaneously occurring mammary carcinomas:*

The mice in the experiments described above were weighed at the time of initiation of treatment and again at weekly intervals for 8 weeks. Body weights were corrected by subtracting the calculated tumor weights for each mouse and the percent change in body weight versus time is plotted in Fig. 2. Nadir body weight loss (−8%) occurred in FUra-treated mice on day 14 and was statistically significant ($P < 0.01$) compared with saline-treated controls. Thereafter, it can be seen that in spite of continued drug administration, mice in the FUra group gained weight and by the 5th week of therapy had body weights similar to those at the start of the experiment. It should be noted that the decrease in body weight experienced by control animals after day 35 is a reflection of the overall poor health of these animals, which is a consequence of their large tumors.

*Effect of weekly treatment with FUra on the peripheral white blood cell levels of normal $CD8F_1$ female mice*

To preclude any possible complicating influence of tumor on bone marrow activity, the effect of chronic treatment with FUra on peripheral white blood cell levels was studied in tumor-free, 3-month-old $CD8F_1$ female mice. Peripheral WBC levels were measured in these mice and they were distributed into two groups of 10 mice each, so that mice with approximately equal WBC counts were represented in each group. The control group received weekly injections of saline for 15 weeks and the experimental group was treated with FUra at 85 mg/kg per week for 15 weeks. Peripheral WBC counts were performed on all mice in both groups 4 days after each weekly course of treatment. (This time point was selected on the basis of preliminary experiments, which indicated the WBC nadirs occur 4 days after this dose of FUra.) As may be seen in Fig. 3, WBC levels in normal mice ranged from 11,000 to 19,000 cells/$mm^3$, whereas the WBC levels in FUra-treated

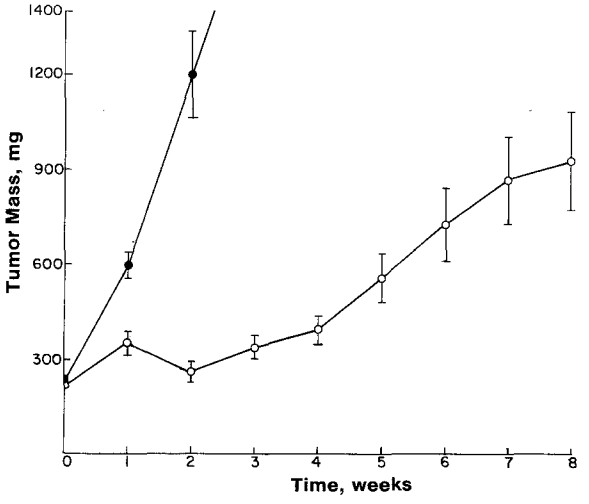


**Fig. 1.** Effect of FUra, 100 mg/kg weekly, on the growth of spontaneously occuring mammary carcinomas in adult female $CD8F_1$ mice. Each *point* represents the data pooled from 45 animals and the *cross bars* represent the SEM. Control animals (●) received weekly injections of saline

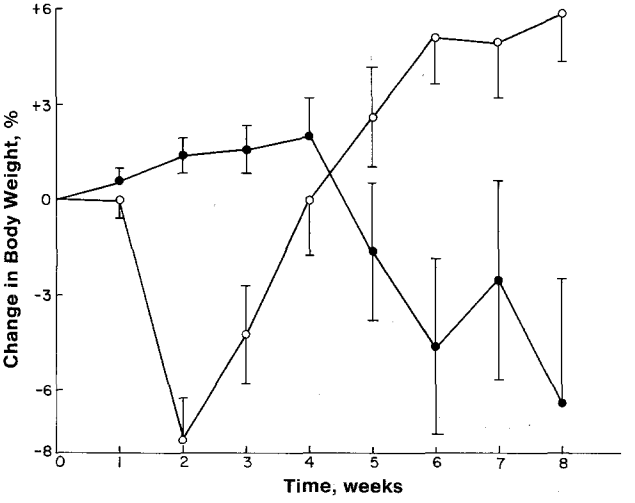


**Fig. 2.** Effect of FUra, 100 mg/kg weekly, on the body weight of adult female $CD8F_1$ mice with spontaneously occuring mammary carcinomas. Each *point* represents the pooled data from 45 animals and the *cross bars* represent the SEM. Control animals (●) received weekly injections of saline

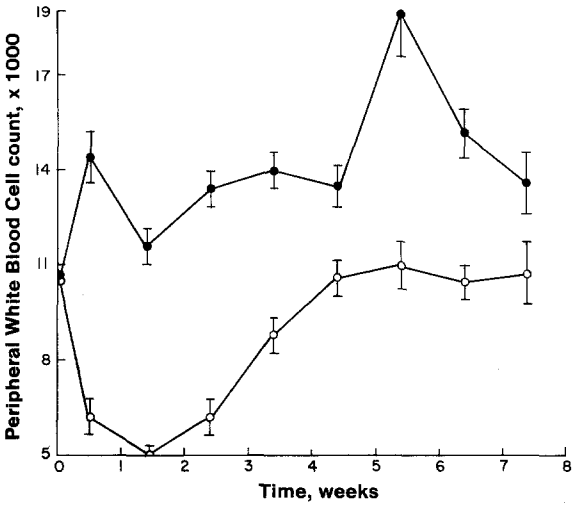


**Fig. 3.** Effect of weekly injections of FUra, 85 mg/kg, on the peripheral white blood cell levels in normal, 3-month-old, $CD8F_1$ female mice. Each *point* represents the pooled data from 10 animals and the *cross bars* represent the SEM. Control animals (●) received no treatment

**Table 1.** Calculated $LD_{50}$ of FUra in control mice and mice pretreated for 7 weeks with one of two dose regimens of FUra

| Pretreatment,[a] drug dose | $LD_{50}$[b] (mg/kg) | 95% Cinfidence limits[b] (mg/kg) |
|---|---|---|
| None | 240 | 200–288 |
| FUra, 50 mg/kg | 370 | 330–414 |
| FUra, 85 mg/kg | 460 | 410–515 |

[a] Consisted of one injection weekly for 7 weeks: challenge dose of FUra administered 7 days after the last pretreatment injection
[b] As determined by the method of Litchfield and Wilcoxon [20]

**Table 2.** Results of bone marrow and gut assay for uridine kinase and orotate phosphoribosyltransferase activity

| Tissue | Pretreatment,[a] drug dose | Enzyme activity (nmol UMP formed/mg protein/60 min) mean ± SEM | |
|---|---|---|---|
| | | Uridine kinase | Orotate phospho-ribosyl transferase |
| Bone marrow | None | 16.8 ± 3.2 | 5.4 ± 1.2 |
| | | $P > 0.5$[b] | $P > 0.5$ |
| | FUra, 85 mg/kg | 19.6 ± 4.0 | 5.0 ± 2.2 |
| Gut | None | 1.80 ± 0.32 | 0.40 ± 0.04 |
| | | $P > 0.05$ | $P > 0.5$ |
| | FUra, 85 mg/kg | 1.95 ± 0.21 | 0.44 ± 0.04 |

[a] Consisted of one injection weekly for 7 weeks: assays carried out 7 days after the last pretreatment injection
[b] As determined by Student's *t*-test

mice were depressed from an average of approximately 11,000 cells/$mm^3$ whole blood to 6,200, 4,500, and 6,200 after the first, second, and third doses of FUra, respectively. Following subsequent weekly doses of FUra, WBC levels remained significantly lower than those of the saline-treated control mice but they were never dangerously low and they were not depressed below the WBC level recorded at the start of the experiment.

*Determination of $LD_{50}$ of FUra in mice pretreated with seven weekly injections of FUra:*

Table 1 presents data obtained from the determination of the $LD_{50}$ of FUra in normal $CD8F_1$ female mice that had been pretreated weekly for 7 weeks with one of two doses of FUra (50 mg/kg or 85 mg/kg). Seven days after the last pretreatment injection the mice were challenged with varying doses of FUra to determine the $LD_{50}$. As can be seen from the results presented in Table 1, the $LD_{50}$ of FUra was approximately 240 mg/kg in control animals, while the $LD_{50}$ of FUra in mice pretreated with this drug was considerably higher. Specifically, mice pretreated for 7 weeks with weekly injections of FUra at 50 or 85 mg/kg had $LD_{50}$ values of 370 or 460 mg/kg, respectively.

*Determination of uridine kinase and orotate phosphoribosyltransferase activity in mice pretreated with seven weekly injections of FUra*

Normal $CD8F_1$ female mice were distributed into two groups. The control group received no treatment and the second group was treated with FUra, at 85 mg/kg per week for 7 weeks. Seven days after the last pretreatment injection enzyme preparations were obtained from the intestinal mucosa and bone marrow of mice from each group and analyzed for uridine kinase and orotate phosphoribosyltransferase activities. The results of these assays, as presented in Table 2, indicate that although the activity of both uridine kinase and orotate phosphoribosyltransferase in the gut is substantially lower than in the bone marrow, there was no significant difference in these activities between control mice and mice pretreated with FUra.

*Determination of the amount of FUra in the urine of mice pretreated with seven weekly injections of FUra*

Seven days after the last pretreatment injection and immediately before the 24 h urine collection started both normal mice and mice pretreated for 7 weeks with weekly injections of FUra at 85 mg/kg received [6-$^3$H]FUra at a dose of 100 mg/kg (100 mg/kg (100 uCi/kg). Results obtained from the analysis of the collected urines both for intact FUra and the presence of tritium label are presented in Table 3. From Table 3 it is clear that there is essentially no difference in the recovery of the radioactive label (representing both intact FUra and its degradation products) between the two groups. In contrast, there is a 37% decrease ($P = 0.00006$) in the amount of intact FUra in the urine of the FUra-pretreated mice.

*Determination of the activity of dihydrouracil dehydrogenase in the livers of mice pretreated with seven weekly injections of FUra*

The activity of dihydrouracil dehydrogenase in livers obtained from either normal $CD8F_1$ mice or mice pretreated with FUra over a 7-week period is presented in Table 4. Normal mouse liver contained unit 0.086 units (1 unit converts 1 μmol uracil to dihydrouracil/h [33]) enzyme activity/mg protein, whereas the activity of this enzyme was increased by 31% to 0.113 units/mg protein in liver from FUra-pretreated mice ($P = 0.14$).

**Table 3.** Concentration of FUra in the urine

| Pretreatment,[a] drug dose | Total urine radioactivity, (24 h) CPM | % Recovery of ($^3$H) label in urine | FUra concentration in reconstituted urine ($\mu M$) mean ± SEM | Significance vs control[b] |
|---|---|---|---|---|
| None | $2.0 \times 10^6$ | 91 | 8.00 ± 0.33 | – |
| FUra, 85 mg/kg | $2.1 \times 10^6$ | 94 | 5.02 ± 0.26 | 0.00006 |

[a] Consisted of one injection weekly at the dose indicated for 7 weeks: challenge dose of (6-H$^3$)-FUra administered 7 days after the last pretreatment injection and consisted of a FUra dose of 100 mg/kg and 100 μCi/kg (~ 3 μCi/mouse)
[b] As determined by Student's *t*-test

**Table 4.** Activity of dihydrouracil dehydrogenase

| Pretreatment,[a] drug dose | Enzyme activity (units[b]/ mg liver protein), mean ± SEM | Activity relative to control mice (%)[c] | Significance compared with control mice[d] |
|---|---|---|---|
| None | 0.086 ± 0.011 | – | |
| FUra, 85 mg/kg | 0.113 ± 0.013 | 131 | $P = 0.14$ |

[a] Consisted of one injection weekly at the dose indicated for 7 weeks: assays were carried out at 7 days after the last pretreatment injection
[b] One unit of activity converts 1 μmol uracil to dihydrouracil/h
[c] Activity for control mice represents 100%
[d] As determined by Student's *t*-test

*In vivo increase in the therapeutic effectiveness of FUra by its incorporation into a dose-increasing schedule*

Groups of 100 CD8F$_1$ female mice received 0.3 ml of a 5% tumor brei by injection and were distributed into four groups. The first group served as control and received weekly injections of saline. Group 2 received weekly injections of FUra at a dose of 85 mg/kg. Group 3 received weekly injections of FUra at a dose of 100 mg/kg. Group 4 received weekly injections of FUra at 85 mg/kg for the first 7 weeks and thereafter received weekly injections of the drug at 100 mg/kg. Examination of Figs. 4 and 5 indicates that by day 28 the control group had tumors of approximately 1.5 g and by the 7th week one-half of the animals in this group were dead. Tumor growth in groups 2 and 3 was similar, animals in both groups having a mean tumor weight of 1.5 g at week 10. The drug-related mortality caused by weekly injections of FUra in group 2 was 20% by week 7 and almost 30% by week 12. This is markedly different than that seen in group 3, with 50% dead by week 4 and 80% by week 12. Group 4 exhibited an identical tumor growth and mortality pattern with group 2 through the first 7 weeks. After week 7, the point at which the FUra dose was increased in group 4, the mortality pattern still mirrored that of group 2 but with significantly improved effect on tumor growth.

## Discussion

As demonstrated by results presented in Fig. 1, chronic, weekly treatment of CD8F$_1$ mice bearing spontaneous breast tumors with a therapeutic dose of FUra produced tumor stasis during the first few weeks of therapy, but the effectiveness of

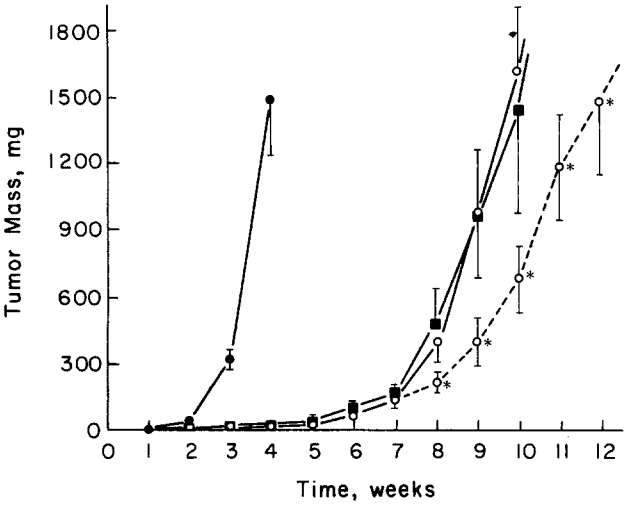


**Fig. 4.** Effect of weekly injections of FUra on the growth of a 24-h transplant of a murine mammary adenocarcinoma into normal CD8 female mice. Each group represents data from 50 animals and the *cross bars* represent the SEM. Control animals (●) received no treatment; the three remaining groups received weekly injections of FUra at 85 mg/kg (○———○), FUra at 100 mg/kg (■), or FUra at 85 mg/kg for 7 weeks followed by weekly injections of FUra at 100 mg/kg (○ – – – ○). * *P*-value (as determined by Student's *t*-test) was ≤ 0.05 between groups 2 and 4

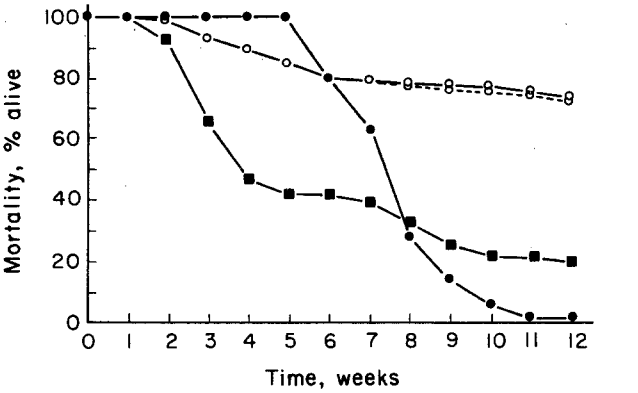


**Fig. 5.** Effect of weekly injections of FUra on the survival rate of CD8F$_1$ female mice with 24-h transplants of a murine mammary adenocarcinoma. Each group represents data from 50 animals (●) received no treatment; the three remaining groups received weekly injections of FUra at 85 mg/kg (○———○), FUra at 100 mg/kg (■), or FUra at 85 mg/kg for 7 weeks followed by weekly injections of FUra at 100 mg/kg (○ – – – ○)

the drug gradually diminished so that by the 4th or 5th week the tumors began to increase in size despite continued treatment with the same dose of FUra. This is not a novel finding. In fact, the development of resistance to FUra has been attributed to the development, or selection, of tumor cell variants with decreased levels of either uridine kinase or orotate phosphoribosyltransferase [18, 24, 27–29] leading to a decreased level of phosphorylation of the drug at any given dose. While tumor resistance is a well-documented finding, the development of refractoriness in normal host target tissues during chronic treatment with FUra has not been described. Therefore, the decreased level of host toxicity (as manifested in body weight loss following each dose of the drug) in tumor-bearing mice after several weeks of FUra therapy (Fig. 2) was a surprising finding.

In addition to its toxic effect on intestinal epithelium, FUra is known to be cytotoxic for bone marrow cells in the mouse [37]. As shown in Fig. 3, peripheral WBC levels in normal, tumor-free mice were severely depressed to just above levels considered to be lethal [37] after the first and second courses of treatment with FUra at 85 mg/kg. However, after continued weekly treatment with the same dose of FUra the effect on WBC levels was sharply diminished, so that WBC levels rebounded and remained at the low end of the normal range of values after the 5th or 6th week of treatment.

Since mortality associated with FUra in mice is believed to be due to its effect on intestinal mucosa and bone marrow [13], it seemed likely from these results that after several weeks of chronic treatment with FUra, the lethality of subsequently administered FUra would be reduced. The results shown in Table 1 confirm this hypothesis. After weekly pretreatment with FUra at a dose of either 50 or 85 mg/kg for 7 weeks, the $LD_{50}$ for FUra, as determined by a challenge dose of the drug 7 days after the last pretreatment injection, was 370 or 460 mg/kg, respectively, as compared to 240 mg/kg in age- and sex-matched mice that had not received FUra pretreatment. This doubling in the $LD_{50}$ from 240 mg/kg to 460 mg/kg represents a profound increase for a drug with as steep a dose-response ratio as FUra [13].

The degree of cytotoxicity produced by a given dose of FUra in vivo is the net result of two opposing metabolic processes, namely metabolic activation of the drug to the nucleotide level and the catabolic degradation of the drug to $\alpha$-fluoro-$\beta$-alanine, ammonia, and $CO_2$ [2, 13, 23, 34]. Since in the whole animal both processes occur simultaneously, it was possible that the development of refractoriness to FUra-induced cytotoxicity in normal target tissues in the mouse during chronic exposure to the drug could have been the result of either a decrease in the activity of the enzymes responsible for activating FUra (uridine kinase and/or orotate phosphoribosyltransferase), or an increase in the activity of the catabolic enzymes (most probably dihydrouracil dehydrogenase, the first and rate-limiting enzyme in catabolic pathway [13, 34]). Since resistance, mediated by decreased levels of activating enzymes, has been documented to occur in many types of tumors [17, 28, 32], we measured the levels of uridine kinase and orotate phosphoribosyltransferase in the gut and bone marrow of normal mice that had been treated with seven weekly doses of FUra at 85 mg/kg and in untreated controls. Results shown in Table 2 indicate that the level of these enzymes was not altered in either the gut or bone marrow by pretreatment with FUra, and therefore, it appears that the in vivo ability to activate FUra was not altered in these target tissues by FUra treatment.

Subsequent experiments evaluated the ability of the mouse to degrade FUra. This was accomplished by first challenging either normal mice or mice pretreated with FUra for 7 weeks with a high dose of [$^3$H]FUra and then assaying urine collected from these mice over the next 24-h period. These results indicate that there was no difference in the recovery of the $^3$H label between normal and FUra-pretreated mice (Table 3). However, when the urine was assayed specifically for intact FUra, the group that was pretreated with FUra had approximately 40% less intact drug than did the control group (Table 3). This result suggests that the ability to degrade FUra had been increased significantly above normal levels in these mice during the 7-week pretreatment with FUra.

Since FUra is primarily degraded in the liver [34], more direct evidence for the biochemical changes induced by chronic FUra treatment was provided by measurements of the activity of dihydrouracil dehydrogenase in livers from normal and FUra-pretreated mice. The results, presented in Table 4, indicate that pretreatment with FUra for 7 weeks resulted in a 31% increase in the activity of this enzyme. While this represents a relatively modest increase in the capacity of the host to degrade FUra, the steep dose-response curve for FUra translates this into a large difference in drug-related toxicity.

The final experiment was designed to determine whether the information gathered in this study could have possible clinical relevance. To this end, mice bearing a 24-h breast tumor transplant were started on treatment regimens consisting of either a level weekly dose of FUra or a dose that was increased by 35% after 7 weeks. From the results presented in Figs. 4 and 5 it is clear that in a therapeutic situation mice are better able to tolerate a higher dose of FUra after the 7-week pretreatment period. This increase in dose to a normally toxic level leads to a statistically significant improvement in drug effectiveness, which extends the period of tumor stasis without increasing the drug-related mortality.

From the evidence presented here it is clear that the initial observation of a decrease in host toxicity during the course of prolonged FUra therapy is not a consequence of resistance developing to FUra in normal target tissues, gut, and bone marrow, but rather is the reflection of a tolerance-like phenomenon related to an induced increase in catabolic enzymes in the liver. To our knowledge this phenomenon has not been previously reported for FUra, nor for any other antimetabolite used in chemotherapy. Although we plan to, we have not yet examined tumor cells in this system for the development of classic resistance (depressed levels of FUra activating enzymes), nor have we correlated the increased catabolism of FUra with a decreased incorporation of FUra into RNA. Nevertheless, it is obvious that the gradual loss of therapeutic effectiveness, observed during chronic treatment of tumor-bearing mice (Fig. 1) with FUra, is attributable at least in part to the induced increase in FUra catabolism in the host.

The clinical implications of these findings are clear, and indicate that studies should be considered to determine whether in fact pyrimidine-catabolizing enzymes in human tissues can be induced in a similar fashion. If their activities can be so induced it seems that therapy with a constant dose of FUra might be improved by its conversion to a dose-increasing regimen which compensates for increased host catabolism.

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