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# *In Vivo* Effect of 5-Ethynyluracil on 5-Fluorouracil Metabolism Determined by $^{19}$F Nuclear Magnetic Resonance Spectroscopy

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## ABSTRACT

**Biochemical modulation of 5-fluorouracil (5-FU) has been used over the past 20 years to improve the therapeutic efficacy of this antineoplastic agent. Recently, modulation of the catabolic pathway of this fluoropyrimidine has been the focus of extensive preclinical and clinical investigation. Dihydropyrimidine dehydrogenase catalyzes the rate-limiting step in the catabolism of 5-FU and rapidly degrades 60–90% of the drug. An irreversible inactivating inhibitor of this enzyme, 5-ethynyluracil (EU), markedly improves the antitumor effect of 5-FU in animal models. Early clinical studies have shown a substantial alteration of the systemic disposition of 5-FU with an increase in 5-FU terminal half-life and have also indicated that EU allows safe oral administration of 5-FU by improving the oral bioavailability of the fluoropyrimidine, which is otherwise too erratic and unpredictable for a drug with such a limited therapeutic window.**

**We evaluated the effect of EU on the metabolism of 5-FU in mice bearing colon 38 tumors using $^{19}$F nuclear magnetic resonance spectroscopy. *Ex vivo* measurements of tissue extracts from liver, kidney, and tumor indicated a >95% elimination of α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine signals in the tissues of mice that received 2 mg/kg of EU before administration of 5-FU. The spectra also showed an increased formation of fluoronucleotides in both normal and tumor tissues, a prolonged presence of 5-FU, and the accumulation of 5-fluorouridine that otherwise is undetectable, particularly in normal tissues. The *in vivo* NMR experiments on colon 38 tumors confirmed these findings, showing a complete elimination of the α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine signals in tumors treated with EU and a dramatic formation and accumulation of 5-fluorouridine mono-, di-, and triphosphates and 5-fluorouridine. Thus, by inactivating dihydropyrimidine dehydrogenase, EU prolonged the half-life for 5-FU, almost completely eliminated its catabolism for 4–6 h, which led to an increased accumulation of 5-fluorouridine mono-, di-, and triphosphates in both normal and tumor tissues.**

## INTRODUCTION

The biochemical modulation of 5-FU[3] by several agents, including methotrexate, leucovorin, *N*-(phosphonoacetyl)-L-aspartic acid, and levamisole has been, for the past 15–20 years, a major element of clinical investigation, particularly for the treatment of gastrointestinal malignancies (1–3). These drug regimens were either based on stabilizing the ternary complex with thymidylate synthase, therefore affecting its inhibition, or in favoring the formation of fluoronucleotides with subsequent increased incorporation into RNA. Despite all of these attempts, the response rate remains in the 20–30% range, and the survival in patients with metastatic disease is only modestly affected by any of these drug combinations (4).

Recently, the attention has shifted from the anabolic pathway of 5-FU. New approaches have been taken by modulating the catabolism of the fluoropyrimidine. Through the catabolic pathway, 5-FU is first reduced to 5-fluoro-5,6-dihydrouracil, which was discovered recently to impair the antitumor effect of the parent compound (5). 5-Fluoro-5,6-dihydrouracil is then later converted to FβAL, an end product that has been found neurotoxic in animal models (6–8). More than 70% of a given dose of 5-FU is rapidly catabolized; DPD is the limiting enzyme of the drug elimination, and <10% of the drug is excreted unchanged in the urine (9). DPD catalyzes the NADPH-dependent reduction of 5-FU as well as that of its two natural substrates thymine and uracil, which are converted to the corresponding 5,6-dihydropyrimidines (10). DPD activity is found in most of the tissues, particularly liver, intestinal mucosa, kidney, and several tumors (11). An inverse correlation has been shown in certain tumors between enzymatic activity and 5-FU sensitivity (12). A patient population with a deficiency in this critical enzyme has been identified. The administration of standard doses of 5-FU to these patients results in severe toxicity associated with minimal catabolism and a prolonged 5-FU half-life, up to 10-fold longer (13). An irreversible inhibitor of DPD, EU (Eniluracil, GW776) has been introduced recently at the preclinical and clinical level (14–17). EU is a mechanism-based inactivator of this enzyme. Preclinical studies to modulate the antitumor activity of 5-FU in rats bearing Ward colorectal carcinoma showed improved therapy and therapeutic index for 5-FU, even compared with other regimens containing more established modulators such as leucovorin and *N*-(phosphonoacetyl)-L-aspartic acid (16). Clinical studies have indicated significant changes in the systemic disposition of 5-FU, with a dramatic change to its terminal half-life that increased to 4.5 h after a 10-mg/m$^2$ oral dose with 2 mg/m$^2$ of EU (17). Variability of DPD activity, particularly at the level of the intestinal mucosa, with consequent changes in clearance, has been associated with the erratic bioavailability of 5-FU after oral administration (18). The administration of EU significantly increased and stabilized the bioavailability of the fluoropyrimidine (72–128%), enabling a safe oral administration of the drug in cancer patients (17).

This study characterizes the effect of EU on 5-FU metabolism in normal and neoplastic tissues using $^{19}$F-NMR spectroscopy measurements *in vivo* on mice bearing colon 38 tumors and *ex vivo* on liver, kidney, and tumor tissue extracts.

## MATERIALS AND METHODS

**Drugs and Chemicals.** 5-FU, 50 mg/ml solution formulated for clinical use, was purchased from Pharmacia (Kalamazoo, MI). EU was a generous gift from Dr. T. Spector (Glaxo Wellcome), and sodium pentobarbital was obtained from Butler Co. (Columbus, OH). All other chemicals were purchased from Aldrich (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO).

**$^{19}$F-NMR Experiments.** C57/BL6 mice, 6–8 weeks of age, were obtained from the National Cancer Institute (Bethesda, MD) and received s.c. injections in both flanks with a brei of colon 38 tumor. At the estimated tumor weight of 200–500 mg, the mice received 5-FU-based chemotherapy treatment followed by $^{19}$F-NMR measurements (19).

Mice with tumor implants were divided into two treatment groups. Both treatment groups received a single dose of 200 mg/kg of 5-FU in normal saline injected i.p. This dosage of 200 mg/kg of 5-FU compared with 100 mg/kg used

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[3] The abbreviations used are: 5-FU, 5-fluorouracil; FβAL, α-fluoro-β-alanine; DPD, dihydropyrimidine dehydrogenase; NMR, nuclear magnetic resonance; EU, 5-ethynyluracil; FUPA, α-fluoro-β-ureidopropionic acid; FUrd, 5-fluorouridine; FNuct, 5-fluorouridine mono-, di- and triphosphates; TCA, trichloroacetic acid.

for the pharmacokinetic studies was purposely selected to achieve a greater sensitivity, particularly in the *in vivo* determinations, and to follow more in detail changes in the metabolism and in the accumulation of the generated fluorinated species. The first treatment group received only 5-FU, whereas a second treatment group also received an i.p. dose of 2 mg/kg of EU diluted in saline 30 min before 5-FU administration.

For the *ex vivo* measurements, tumor, kidney, and liver tissues were harvested at 30, 60, 120, 240, and 360 min after 5-FU administration and then frozen immediately in liquid nitrogen. Tissues were then homogenized in two volumes of 15% TCA and centrifuged at 1000 × *g* for 15 min, and supernatant was removed. To neutralize the tissue samples, TCA supernatants were extracted with a 45:55 trioctylamine:freon solution. The aqueous layer was then removed, lyophilized to near dryness, and dissolved in 50 mM Tris buffer; the pH was adjusted to 7.0 with 1 mM NaOH. The samples were then placed in 5-mm NMR tubes, and $D_2O$ was added to achieve a final concentration of 90% solute and 10% $D_2O$ (19).

$^{19}$F-NMR spectroscopy was performed on the tissue extracts using the Yale-490 and Bruker AM-500 MHz spectrometers operating at 460 and 470 MHz, respectively, for fluorine. A 5-mm $^{19}$F/$^{1}$H/$^{2}$H NMR probe, manufactured by Cryomagnet Systems, Inc. (Indianapolis, IN), was used for all of the $^{19}$F *ex vivo* NMR measurements. Broadband decoupling was used for all experiments to eliminate H-F couplings and to improve resolution and signal:noise (S:N) in the spectra.

Spectral parameters for the $^{19}$F-NMR *ex vivo* tissue extract samples were as follows: probe temperature, 25°C; sweep width, 30 kHz; number of scans, 4096; repetitive delay of 1.0 s; pulse width, 14 $\mu$s; and 4096 time domain points for each Fourier Induction Decay.

*In vivo* $^{19}$F-NMR experiments were conducted on a 4.7 T, 30 cm-wide bore Bruker spectrometer operating at 188.55 MHz for fluorine. To optimize *in vivo* $^{19}$F-NMR measurements, the $T_1$ of 5-FU was measured at a concentration of 20 mM in whole blood using an inversion recovery experiment at 300 MHz. A value of $T_1$ of 1.2 s was obtained for 5-FU; based on this value, a repetition rate of 1.0 s was selected for multiple average $^{19}$F-NMR measurements in mice (19).

For the *in vivo* experiments, a probe was specifically designed to accommodate a mouse with its tumor positioned in the active region of a 1 cm-diameter coil placed in the center of the 30 cm-bore magnet. The coil can be tuned and matched to the fluorine ($^{19}$F) resonance and ($^{1}$H) at 188.55 and 200.41 MHz, respectively. A pulse width of 15 $\mu$s was used, which translated to a 45° pulse width and which was determined on a 5-FU phantom whereby a 30-$\mu$s pulse translated to a flip angle of 90°. All *in vivo* $^{19}$F-NMR spectra were acquired with 1024 transients, repetition time of 1.0 s, 2048 time points, and a sweep width of 25 kHz.

For every *in vivo* study, acquisition parameters were set for $^{19}$F on a vial containing 20 ml of 5 mM 5-FU in aqueous solution. The phantom was used to reference 5-FU at 0 ppm. After determining the resonance frequency of 5-FU, the transmitter frequency was offset −10 ppm from this value to place the transmitter in the center of interest between 5-FU (0 ppm) and its catabolite F$\beta$AL (-19 ppm). Initial shimming on the phantom was accomplished by using the $^{19}$F coil as an $^{1}$H receiver coil, thereby using the $^{1}$H signal.

Mice were anesthetized with sodium pentobarbital (35 mg/kg i.p.) for the 2–2.5 h acquisition period (20) and then received injections with a 200-mg/kg i.p. dose of 5-FU in the presence or in the absence of EU 2 mg/kg administered 30 min before the fluoropyrimidine. The animals were then positioned in the probe with the tumor on the flank of the mouse placed consistently in the active region of the coil. The probe with the mouse was then immediately placed in the magnet and tuned to the frequency of $^{1}$H, and then shimming was performed until a linewidth of 50–60 Hz was achieved. After the probe was tuned and matched to the $^{19}$F frequency, NMR acquisition was begun, and spectra were collected every 20 min for up to 2–2.5 h.

*In vivo* and *ex vivo* $^{19}$F-NMR spectra were processed using the NMR software Felix 95 (MSI, San Diego, CA) on a SGI Indy workstation. *In vivo* and *ex vivo* $^{19}$F-NMR spectra were zero filled to 4096 and 8192 data points, respectively, and phased manually with zero order phase placed on the $^{19}$F resonance of 5-FU and first order phase on the outer resonant peaks of the spectra. Baseline points for a third order polynomial baseline correction were empirically chosen and ranged over the entire sweep width of the spectra. The phase and baseline correction values were then applied to the subsequent sequential Fourier Induction Decays.

NMR identification of 5-FU metabolites FUPA, F$\beta$AL, FUrd, and 5-fluoro-UMP were determined on standard solutions of these chemical species prepared at concentrations of 5 mM and pH 7.0 (19).

The 5-FU peak in the *in vivo* and *ex vivo* $^{19}$F-NMR measurements was referenced at 0 ppm. The following spectral ranges were used for the integration of peaks in the *ex vivo* measurements: +6.6 > FNuct > +4.0, +4.0 > FUrd > +3.0 +1.3 > 5-FU > −1.7, −18.5 > FUPA > −19.2 and −19.2 > FUPA > −20.6. Spectra were phased, baseline corrected, and integrated over the spectral ranges defined above using the processing software Felix95. The integrals were used to calculate the relative ratios of metabolites and fluoronucleotides in mice treated with 5-FU and the combination 5-FU plus EU.

**Plasma Pharmacokinetics of 5-FU.** The pharmacokinetics of 5-FU in the presence or absence of EU was determined in C57/BL6 mice. 5-FU was administered at 100 mg/kg i.p. in the presence of [5-$^{3}$H]FU (20 $\mu$Ci/mouse). EU at the dose of 2 mg/kg preceded the administration of 5-FU by 30 min. At 2, 5, 10, 20, 30, 60, 120, 240, and 360 min after the administration of the radiolabeled dose of 5-FU, 300 $\mu$l of blood were collected from two animals for each group using heparinized Natelson pipettes. The plasma was separated by centrifugation at 1000 × *g* for 10 min, deproteinized with two volumes of 15% TCA, and extracted with trioctylamine:freon as described previously. The concentration of 5-FU was determined after separation from the other radioactive species present in the extracted samples using an HPLC method described previously (21). Briefly, plasma was mixed with 20 $\mu$l of 1 M sodium acetate (pH 4.8) and 200 $\mu$l of a 200 mg/ml solution of sodium sulfate. Five ml of *n*-propanol:ether solution (16:84 v/v) was added, and after the extraction, the aqueous phase was adjusted to neutral pH by adding 1 M $H_2SO_4$. The samples were separated on a C18 Microsorb column eluted with 50 mM $K_2HPO_4$ buffer (pH 3.0). The collected peaks monitored by UV absorbance at 254 nm were then measured for radioactivity.

The pharmacokinetic analysis has been conducted using a software package for the analysis of nonlinear models PCNONLIN 4.2 (ClinTrials, Lexington, KY) and standard pharmacokinetic equations.

## RESULTS

**5-FU Pharmacokinetics.** The pharmacokinetics of 5-FU in C57/BL6 mice was evaluated in the absence of EU or after the administration of 2 mg/kg of EU 30 min before the i.p. injection of 100 mg/kg of 5-FU. As shown in Fig. 1 and Table 1, EU caused a dramatic change in 5-FU systemic disposition. The $T_{1/2}$ was prolonged by 4-fold to 42 min, the area under the curve increased almost 7-fold, and the total clearance declined more than 6-fold in mice pretreated with 2 mg/kg of EU. All of these pharmacokinetic parameters are in agreement with an earlier preclinical report (15).

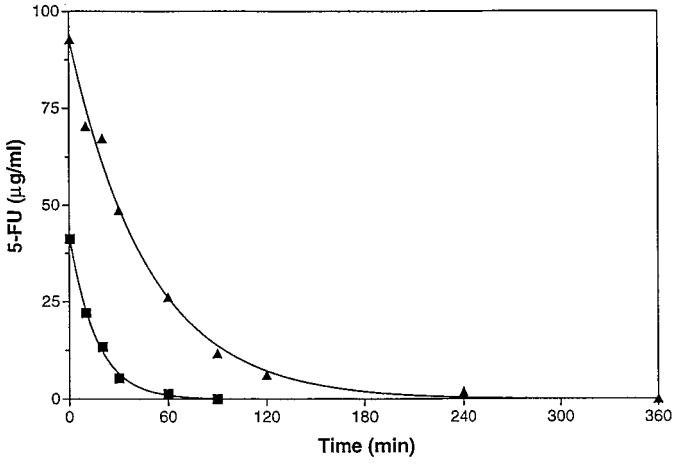


Fig. 1. Plasma concentration *versus* time profile of 5-FU in mice after 5-FU (100 mg/kg) administration in the absence or presence of EU (2 mg/kg). Each time point represents the mean value from three to four mice. ▲, 5-FU alone; ■, 5-FU+EU.

Table 1 *Pharmacokinetic parameters of 5-FU (100 mg/kg) after EU administration (2 mg/kg) in C57/BL6 mice*

Concentration of 5-FU was determined after HPLC separation from the other radioactive species present in the extracted plasma samples as described in "Materials and Methods." Determination of the pharmacokinetic parameters was conducted using PCNONLIN 4.2 software (ClinTrials, Lexington, KY) and standard pharmacokinetic equations.

| | 5-FU | 5-FU/EU |
|---|---|---|
| AUC 0 → 24 (μgml/h) | 11 ± 1 | 72 ± 8 |
| $T_{1/2}$ (min) | 11 ± 1 | 42 ± 6 |
| $CL^a$ (ml/h) | 89 ± 8 | 14 ± 1 |
| *Vd* (ml) | 24 ± 3 | 11 ± 2 |

[a] *CL*, clearance.

***Ex Vivo* $^{19}$F-NMR Experiments.** $^{19}$F-NMR measurements were made on the tissue and tumor extracts from C57/BL6 mice bearing colon 38 tumor treated with 5-FU and the combination 5-FU/EU. The samples represent only the acid-soluble fraction obtained after TCA precipitation and do not include the fluorinated species incorporated into nucleic acids.

Fig. 2*a* shows the $^{19}$F-NMR spectrum of liver tissue extract obtained 60 min after i.p. administration of 5-FU. In mice treated with 5-FU alone, the only species present, besides 5-FU at 0 ppm, are the two main catabolites FβAL and FUPA that represent >80% of the total fluorinated derivatives. No signal for (*R*)-5-fluoro-5,6-dihydrouracil at −32 ppm was detected in this spectrum as well as in the spectra of other tissue extracts, clearly indicating that the opening of the ring with the formation of FUPA is faster than the hydrogenation of 5-FU. At 120 min (Table 2), we observed the expected decrease in the level of 5-FU. The catabolites represent close to 95% of the total fluorinated forms. When 5-FU is combined with EU, the spectrum (Fig. 2*b*) indicated a substantial presence of fluoronucleotides (FNuct), which are below the limit of detection in mice treated with 5-FU alone, and also a signal at 3.5 ppm corresponding to FUrd. The relative low concentration 5-fluorodeoxynucleotides and the likely position of their signal in the region of fluoronucleotides preclude a distinct determination of these fluorinated derivatives. The other noticeable difference between the two regimens is the absence of FUPA and FβAL when EU is preadministered with the 5-FU. At 120 min (Table 2), we observed a reduction in the level of 5-FU, a modest increase in FUrd, and a doubling in the relative amount of FNuct. Later time points reveal the progressive disappearance of 5-FU and FUrd, leaving the FNuct as the only fluorinated derivative present in liver.

A similar phenomenon is seen in kidney extracts (Table 3), where FβAL and FUPA account for >85% of the fluorinated species at 60 and 120 min after the administration of 5-FU alone. With the regimen containing EU, the two catabolites are not detected, and we observed the formation and accumulation of FUrd and FNuct that represent almost 50% of the total after 60 min and increase to 80% 6 h after 5-FU administration. The remarkably high levels of 5-FU, at this late time point, confirm the substantial renal elimination of unchanged 5-FU. This is shown in patients to be ~60% of the administered 5-FU dose compared with <5% in the absence of EU (22).

Another significant point is the difference between the two regimens seen in plasma extracts (data not shown). In the case of 5-FU alone, we recorded the presence of 5-FU, FβAL, and FUPA rapidly decreasing within 60 min below the limit of detection of this methodology (10–15 μM). With the combination regimen, we observed only 5-FU signal, which was present for up to 4 h after drug administration.

The analysis of colon 38 tumor tissue extracts is summarized in Fig. 3 and Table 4. 5-FU alone resulted in the almost equal accumulation of 5-FU, FUPA, FβAL, and FNuct, 60 min after the administration of the drug (Fig. 3*a*), followed at 120 min (Table 4) by an increased

Table 2 *Effect of EU on 5-FU metabolism in liver tissue extracts*

The values are expressed as percentages (±SD) of fluorine signal derived from integrals of peaks obtained from $^{19}$FNMR spectra of liver extracts from mice treated with 200 mg/kg of 5-FU alone and EU (2 mg/kg) preceding by 30 min 5-FU (200 mg/kg). Each time point represents the mean of four to seven determinations conducted on different tissue extracts. Pulse parameters are reported in "Materials and Methods."

| | FNuct | FUrd | FU | FUPA | FβAL |
|---|---|---|---|---|---|
| 1 h 5-FU | ND[a] | ND | 11 ± 5 | 54 ± 17 | 35 ± 13 |
| 2 h 5-FU | ND | ND | 7 ± 4 | 35 ± 5 | 58 ± 12 |
| 1 h 5-FU+EU | 27 ± 11 | 9 ± 4 | 64 ± 15 | ND | ND |
| 2 h 5-FU+EU | 52 ± 10 | 15 ± 6 | 33 ± 6 | ND | ND |
| 4 h 5-FU+EU | 79 ± 8 | 10 ± 4 | 11 ± 3 | ND | ND |
| 6 h 5-FU+EU | 97 ± 2 | ND | 3 ± 2 | ND | ND |

[a] ND, not detectable.

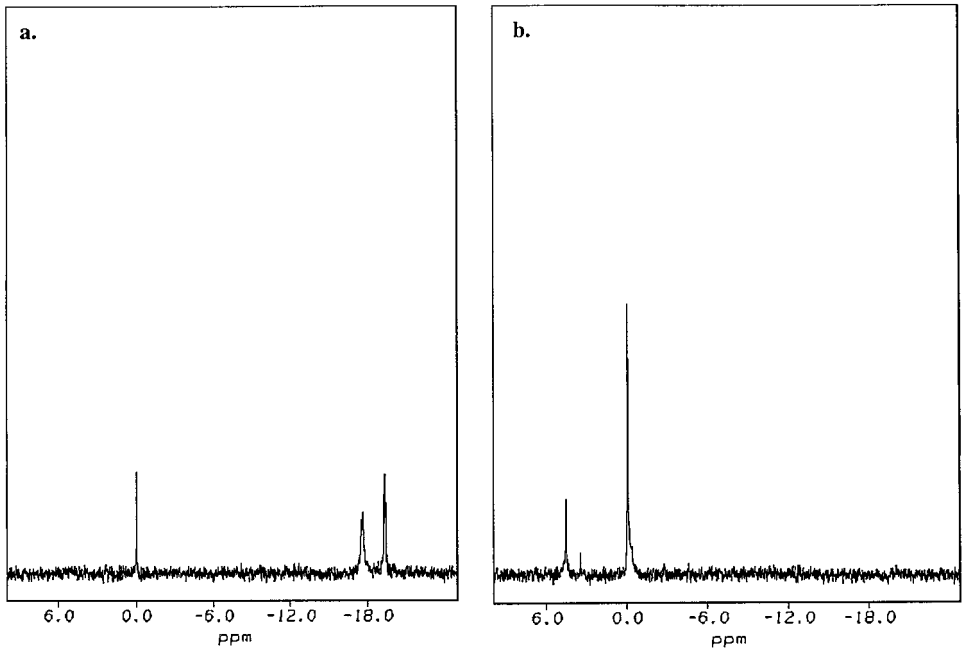


Fig. 2. *Ex vivo* $^{19}$F-NMR spectra of liver tissue extract at 60 min after 5-FU (200 mg/kg) administration in the absence or presence of EU (2 mg/kg) modulation. *a*, 5-FU alone; *b*, 5-FU+EU. 5-FU and other fluorinated metabolite resonances are identified as follows: 0 ppm, 5-FU; −17.5 ppm, FUPA; −19 ppm, FβAL; 3.6 ppm, FUrd; and multiplet at 5.1 ppm, FNuct. Spectra were taken on a Bruker AM-500 NMR with the following spectral parameters: transmitter frequency, 470 MHz; sweep width, 30 kHz; pulse width, 14 μs; relaxation delay, 1.0 s; and number of scans, 4096.

Table 3 *Modulation of EU on 5-FU metabolism in kidney*

The values are expressed as percentages (±SD) of fluorine signal derived from integrals of peaks obtained from $^{19}$FNMR spectra of kidney extracts from mice treated with 200 mg/kg of 5-FU alone and EU (2 mg/kg) preceding by 30 min 5-FU (200 mg/kg). Each time point represents the mean of four to seven determinations conducted on different tissue extracts. Pulse parameters are reported in "Materials and Methods."

| | FNuct | FUrd | FU | FUPA | FβAL |
|---|---|---|---|---|---|
| 1 h 5-FU | ND[a] | ND | 11 ± 7 | 46 ± 7 | 43 ± 12 |
| 2 h 5-FU | ND | ND | 14 ± 5 | 35 ± 8 | 51 ± 17 |
| 1 h 5-FU+EU | 29 ± 12 | 16 ± 5 | 55 ± 18 | ND | ND |
| 2 h 5-FU+EU | 56 ± 8 | 7 ± 3 | 37 ± 8 | ND | ND |
| 4 h 5-FU+EU | 66 ± 8 | ND | 34 ± 7 | ND | ND |
| 6 h 5-FU+EU | 80 ± 11 | ND | 20 ± 5 | ND | ND |

[a] ND, not detectable.

formation of FNuct that represents more than one-third of the fluorinated derivatives and only a slight decrease in 5-FU level because of tumor trapping (23, 24).

When 5-FU was injected i.p. after administration of EU, we observed a dramatic decrease of the signals for the catabolic species, a significant accumulation of FNuct, and a substantial amount of FUrd, ~25% of the fluorinated species. Because all tissues were treated and extracted similarly, even if an exact quantitation is not possible with this analytical approach, we can unequivocally affirm that the administration of EU results in a 2–3-fold increase in the accumulation of 5-FU and in the formation of FNuct in colon 38 tumors compared with 5-FU as a single agent. The presence of 5-FU and FUrd progressively decreases over time, but both species are still detectable after 6 h. FNuct become the predominant fluorinated derivatives after 2 h from 5-FU administration and represent 80–90% of the total after 4–6 h.

***In Vivo* $^{19}$F-NMR Experiments.** $^{19}$F NMR spectroscopy was performed *in vivo* on C57/BL6 mice bearing colon 38 tumors using the same doses of 5-FU and EU used for the *in vitro* experiments.

Fig. 4 shows the *in vivo* $^{19}$F NMR stack plots of murine tumors treated with 5-FU and 5-FU/EU. Each stack plot shows seven sequential spectra acquired at the $^{19}$F resonance frequency of 188.55 MHz at intervals of 20 min beginning 20 min after 5-FU administration. Variations have been observed in the accumulation of fluorinated derivatives among different animals, and we found it mostly related to the size of the tumor. Animals with particularly large tumors (750–1000 mg) resulted in weaker spectra, likely because of a larger necrotic portion of the tumor, which lowers drug accumulation and metabolism.

In the $^{19}$F-NMR stack plot of 5-FU alone (Fig. 4*a*), initially we noticed a single peak at 0 ppm, representing 5-FU that continues to increase in amplitude up to 60 min after 5-FU injection, followed by a steady decrease. The signal corresponding to FNuct (4–5 ppm) starts to be visible at 40 min and continues to increase up to 120 min from the time of the 5-FU injection. The peaks representing the catabolites FUPA (−17.5 ppm) and FβAL (−19 ppm) can be detected after 40 min and maximize in intensity at the final time point of 140 min.

For 5-FU/EU combination regimen (Fig. 4*b*), the peak representing 5-FU reaches its maximum after 20 min and remains almost constant over time up to the final time point. The formation of FNuct is rapidly observed at the first time point (20 min) and progressively increases in amplitude, reaching its maximum at the last time point recorded. A small signal for FUrd is present at 60 and 80 min, and its presence at later points is likely to be masked by a wide FNuct signal. No signal

Table 4 *Changes in 5-FU metabolism in colon 38 tumors after EU administration*

The values are expressed as percentages (±SD) of fluorine signal derived from integrals of peaks obtained from $^{19}$FNMR spectra of colon 38 tumor extracts from mice treated with 200 mg/kg of 5-FU alone and EU (2 mg/kg) preceding by 30 min 5-FU (200 mg/kg). Each time point represents the mean of four to seven determinations conducted on different tissue extracts. Pulse parameters are reported in "Materials and Methods."

| | FNuct | FUrd | FU | FUPA | FβAL |
|---|---|---|---|---|---|
| 1 h 5-FU | 17 ± 7 | ND[a] | 29 ± 10 | 23 ± 8 | 31 ± 9 |
| 2 h 5-FU | 36 ± 10 | ND | 24 ± 9 | 19 ± 6 | 22 ± 7 |
| 1 h 5-FU+EU | 33 ± 11 | 26 ± 7 | 42 ± 15 | ND | ND |
| 2 h 5-FU+EU | 44 ± 6 | 28 ± 8 | 32 ± 11 | ND | ND |
| 4 h 5-FU+EU | 78 ± 6 | 11 ± 4 | 11 ± 3 | ND | ND |
| 6 h 5-FU+EU | 90 ± 7 | 7 ± 3 | 3 ± 2 | ND | ND |

[a] ND, not detectable.

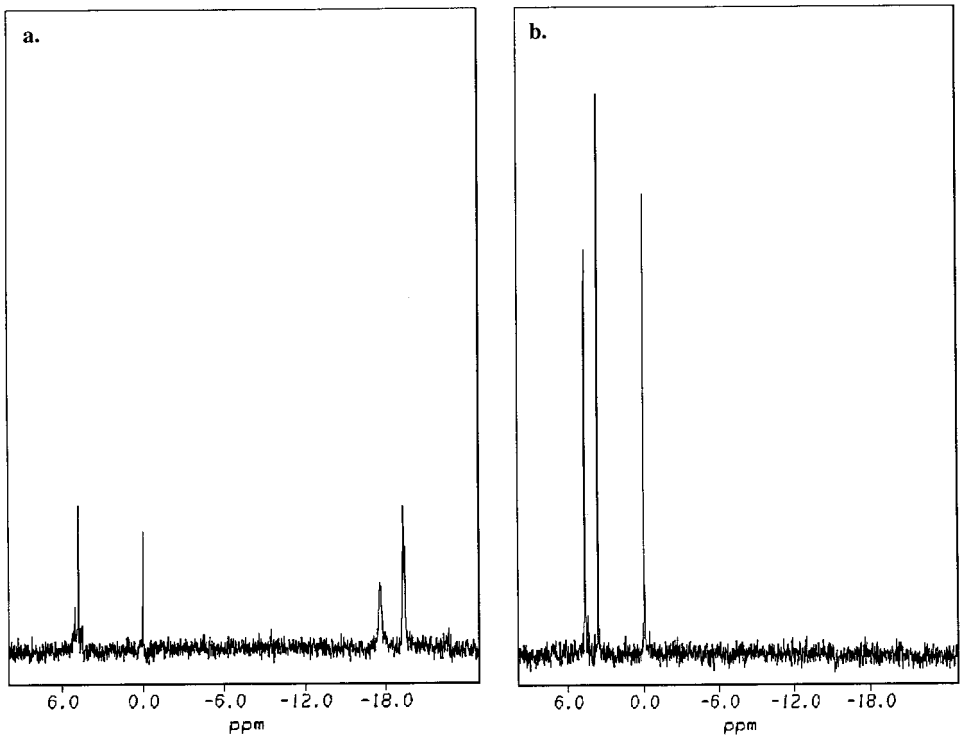


Fig. 3. *Ex vivo* $^{19}$F-NMR spectra of colon 38 tumor tissue extracts at 60 min after 5-FU (200 mg/kg) administration in the absence or presence of EU (2 mg/kg) modulation. *a*, 5-FU alone; *b*, 5-FU+EU. 5-FU and other fluorinated metabolite resonances are identified as follows: 0 ppm, 5-FU; −17.5 ppm, FUPA; −19 ppm, FβAL; 3.6 ppm, FUrd; and a multiplet at 5.1 ppm, FNuct. Spectra were taken on a Bruker AM-500 NMR with spectral parameters: transmitter frequency, 470 MHz; sweep width, 30 kHz; pulse width, 14 μs; relaxation delay, 1.0 s; and number of scans, 4096.

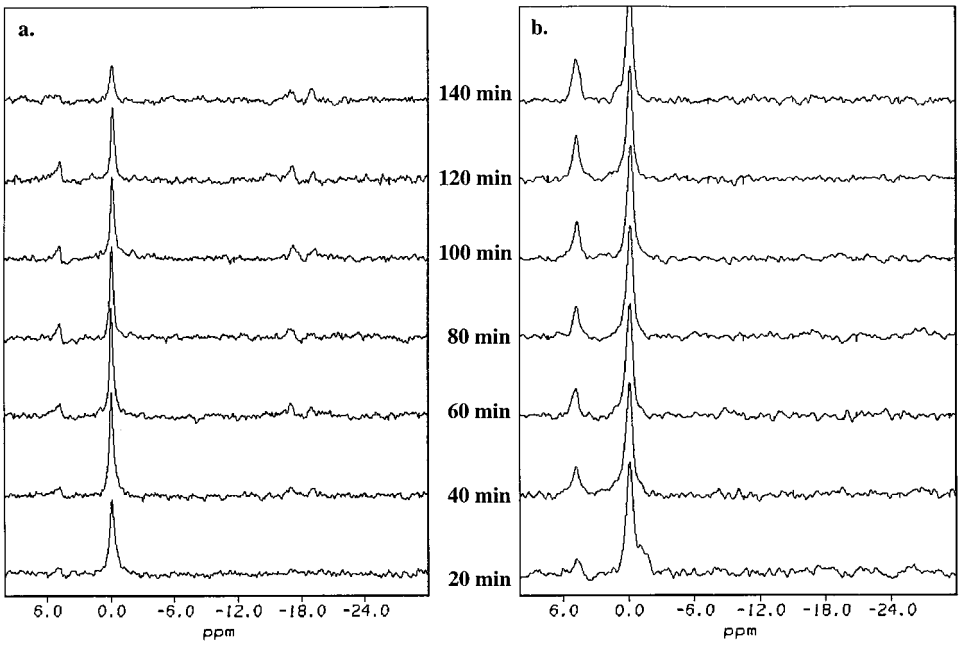


Fig. 4. Sequential *in vivo* $^{19}$F-NMR spectra acquired from colon 38 tumors implanted in the flank of C57/BL6 mice at 20-min intervals after 5-FU (200 mg/kg) administration in the absence or presence of EU (2 mg/kg) modulation. Stack plots: *a*, 5-FU alone; and *b*, 5-FU+EU. Spectra were taken on a Bruker 4.7 T, 30 cm-bore NMR with the following spectral parameters: transmitter frequency, 188.55 MHz; sweep width, 25 kHz; pulse width 15 $\mu$s; relaxation delay, 1.0 s; and number of scans, 1024.

was detected in the catabolite region from −17.5 to −19 ppm, representing FUPA and F$\beta$AL, respectively.

## DISCUSSION

The metabolism of 5-FU after administration of the irreversible inhibitor of DPD, EU, is greatly affected both at the catabolic as well as at the anabolic level, resulting in an increase of the systemic disposition of the fluoropyrimidine. EU suppressed the catabolism of 5-FU in normal and tumor tissues with consequent elevation in the formation of FNuct and FUrd. The same effect was observed in a similar $^{19}$F-NMR study on isolated rat liver (25). In liver and kidney, the administration of 5-FU alone results only in the presence of the signals for the parent compound and its main catabolites; however, the anabolic derivatives remain undetected. The effect of EU modulation on the activation and accumulation of 5-FU anabolites appears to be prevalent in tumor tissue to confirm an increased therapeutic index observed in different animal models, mice and rats, used during the preclinical development of the combination regimen (15, 16, 26). The prolonged presence of 5-FU in kidney, up to 6 h after the administration of the drug with EU, confirms the shift from mainly metabolism mediated by DPD to renal elimination of the unchanged fluoropyrimidine. A recent clinical study of the combination has indicated a correlation between systemic clearance of 5-FU and creatinine clearance after both i.v. and oral administration (17).

Because of a limited analytical sensitivity, most of the *in vivo* NMR experiments presented here were performed at relatively high doses of 5-FU, 200 mg/kg, which correspond to the maximum tolerated dose for 5-FU alone. However, lower doses of 25 and 50 mg/kg resulted in a very similar drug distribution and metabolism in experiments conducted on tissue extracts. With the lower dosages of 5-FU, similar to the ones used for preclinical studies, the higher metabolic activation in the presence of EU results in a sufficient FNuct accumulation in tumor to maintain the antimetabolic effect but results in a limited exposure and accumulation in normal tissues, ensuring safety of administration and reduced host toxicity (data not shown).

Both our *ex vivo* and *in vivo* data show a prolonged and elevated presence of free 5-FU in tumor tissue confirming the "trapping effect." This phenomenon is present in mice treated with 5-FU alone as well as with the combination regimen. The time necessary for 5-FU to reach its maximum concentration in tumors is variable among animals and ranges from 50 to 70 min after administration of the drug alone and increased to 80–100 min with the combination EU/5-FU.

Because of the delayed peak of 5-FU in tumors and the relatively short acquisition time (2–2.5 h) for the *in vivo* procedure, an accurate determination of the $T_{1/2}$ for 5-FU was not possible. However, it can be estimated to be ~45 min for the single-agent regimen and over 2 h for the drug combination. Both values represent a 3–4-fold increase over the plasma pharmacokinetic parameter, confirming a drug concentrating effect in tumors (19, 23, 24).

This intratumoral trapping of 5-FU has been described in other experimental tumor models and clinical studies in patients, indicating a correlation with a higher rate of response to the chemotherapeutic regimen (23, 24). This phenomenon, together with an active anabolism of the drug, becomes even more critical at low concentrations. Reduced catabolism with increased FNuct formation and tumor trapping could partially explain the more favorable therapeutic index of the modulation.

The reduced formation of (*R*)-5-fluoro-5,6-dihydrouracil appears to be another reason for the improved therapeutic index of this new biochemical modulation regimen. The addition of this early catabolite to a 5-FU/EU regimen significantly reduced the therapeutic efficacy and caused slightly increased gastrointestinal toxicity in a rat model (5). It has been postulated that by inactivating DPD, the elevation in uracil and thymine concentrations could also contribute to an improved therapeutic index (5). A study that reports the effect of EU on the antitumor activity of tegafur, an oral prodrug of 5-FU, and UFT, where the same drug is combined with uracil in a 4:1 molar ratio, indicates that the combination of tegafur with EU has a better therapeutic activity and less toxicity than EU plus UFT, suggesting a prejudicial effect of very high levels of uracil to 5-FU antineoplastic activity (27).

Finally, the reduced formation of 5-FU catabolites, particularly F$\beta$AL, has been linked to a decreased neurotoxic effect of 5-FU in dogs and resulted only in a minor gastrointestinal effect in animals treated with EU (8). F$\beta$AL and apparently other by-products of its catabolism have been associated with severe neurotoxicity in dogs and cats (6, 7, 28). This manifestation of 5-FU toxicity also represents a

serious side effect in humans, particularly associated with a prolonged infusion of the drug with consequent accumulation of its catabolites (29–31). A recent study has indicated that both FβAL and β-alanine, the final catabolite of uracil degradation, interfere with γ-aminobutyric acid re-uptake at the level of the GAT-2 transporter in rats, possibly elucidating an important mechanism responsible for the neurotoxic effect of 5-FU (32).

In summary, EU inhibition of 5-FU catabolism results in the almost complete elimination of FUPA and FβAL in normal and tumor tissues. Consequently, accumulation of FNuct and FUrd occurs in liver, kidney, and colon 38 tumor tissues, and prolongation of 5-FU half-life is observed in plasma and tumor tissues.

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