Effect of Uridine on the Metabolism of 5-Fluorouracil in the CD_8F_1 Murine Mammary Carcinoma System[5]

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Abstract: The effect of uridine on the incorporation of 5-fluorouracil into RNA and the inhibition of DNA synthesis by the FdUMP block of thymidylate synthetase was studied in the CD8F1 murine mammary carcinoma system. The administration of exogenous uridine resulted in about a one third reduction of 5-fluorouracil in RNA of tumor and normal tissues. However, unlike thymidine, uridine was unable to reverse the *early,* partial inhibition of DNA synthesis. The amount of fluorouridine nucleotides and (5-fluorouracil)RNA formed in various tissues correlates with the level of orotate phosphoribosyl transferase activity suggesting that the major pathway for activation of 5-fluorouracil to nucleotide form in these tissues is via phosphoribosyl transferase. Enzyme preparations from three different murine tumors convert about 15 times as much 5-fluorouracil to FUMP as they do uracil to UMP. In contrast, the ratio of FUMP to UMP formed in enzyme preparations from gut and bone marrow is lower, 2–6 fold. However, in none of these tissues was the *in vitro* conversion of 5-fluorouracil to FUMP or incorporation into RNA substantially inhibited by uracil. Examination of tumor, gut and bone marrow uridine nucleotide pools showed that the thymidine-uridine-5-fluorouracil schedule does increase uridine nucleotide pools. Thus, the reduction in 5-fluorouracil in RNA is probably not due to inhibition of the conversion of 5-fluorouracil to FUMP by uracil (derived from phosphorylase cleavage of uridine) but, rather, is probably due to the elevated levels of UTP. We conclude that the protection from 5-fluorouracil toxicity afforded by the addition of uridine is due to the reduction in 5-fluorouracil in RNA rather than by reversal of the FdUMP block on thymidylate synthetase.

The fluorinated pyrimidine, 5-fluorouracil (FUra), is widely used in the treatment of a variety of human cancers. Activation of the base to the nucleotide forms, FdUMP[6] and FUTP, is a prerequisite for biological activity (1). FdUMP inhibits the enzyme thymidylate synthetase, thereby blocking the *de novo* synthesis of dTTP. FUTP is readily incorporated into RNA where it results in disruption of RNA synthesis, processing and function (2).

We have used the CD_8F_1 murine mammary carcinoma system in extensive studies designed to elucidate the relative importance of (FUra)RNA versus FdUMP inhibition of thymidylate synthesis (3–9). This tumor-host system is of particular interest because it has been shown to exhibit the best known chemotherapeutic correlation with human breast cancer (8, 10). These studies have suggested that the incorporation into RNA is the primary lesion leading to the ultimate biological activity of FUra (3–7). Cytotoxicity correlates well with the level of FUra in RNA and is not reversed by the administration of thymidine (5–7).

Having identified the incorporation into RNA as the major determinant of FUra activity in the CD_8F_1 tumor-host system, uridine was selected as a potential modulating agent for attempts to selectively reduce host toxicity. The studies presented in this paper were designed to examine the effects of uridine on FUra metabolism. Specifically, we wished to determine the effect of uridine on: 1) the incorporation of FUra into tumor and host tissue RNA; 2) the activation of FUra to FUMP; 3) the size of uridine nucleotide pools in tumor and normal tissues; 4) renal clearance of FUra.

Materials and Methods

Source of Drugs and Radiolabelled Compounds

Pyrimidine nucleosides and bases were obtained from Sigma, St. Louis, MO; 5-fluorouracil from Hoffman-La Roche, Nutley, NJ; (6-^{3}H)FUra from Moravek Biochemicals, Brea, CA; ^{32}P (as carrier-free H_3PO_4) from New England Nuclear, Boston, MA; (6-^{3}H)Ura and (5-^{3}H)UR from Amersham, Arlington Heights, IL.

Animals

The experiments detailed in this paper utilized CD_8F_1 mice bearing first-generation transplants of the CD_8F_1 spontaneous murine mammary carcinoma, male CD_2F_1 mice with transplanted colon carcinoma 26 and male BDF_1 mice with transplanted colon carcinoma 38 (8, 10, 11, 12). They were allowed food and water ad lib. All drugs were made up in 0.85 % NaCl solution and were injected intraperitoneally.

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[6] Abbreviations used: FUra, 5-fluorouracil; FdUMP, 5-fluorodeoxyuridine monophosphate; FUTP, 5-fluorouridine triphosphate; (FUra)RNA, 5-fluorouracil-containing RNA; TdR, thymidine; UR, uridine; Ura, uracil; UdR, deoxyuridine; PRtase, phosphoribosyl transferase; PRPP, 5'phosphoribosyl-l'-pyrophosphate; HPLC, high pressure liquid chromatography.

Incorporation of Precursors into RNA and DNA

Radioactive precursors were injected intraperitoneally in saline. After a 1 or 2 hr labelling period, the animals were sacrificed by cervical dislocation. Bone marrow was collected by flushing the marrow cavity of the femur with ice-cold saline. All tissues were first frozen in dry ice/alcohol so that all samples from a given experiment could be processed at one time.

Tumor and intestinal tissues were homogenized in TNE buffer containing 1 % Triton-X 100 (TNE: 0.01 M Tris-HCl, pH 7.6; 0.15 M NaCl; 0.001 M EDTA). The homogenate was treated with sodium dodecyl sulfate, sonicated, digested with Pronase for 60 min at 37° C (0.2 mg/ml, predigested for 2 hr at 37° C), and extracted with chloroform/isoamyl alcohol (24:1 vol/vol). Bone marrow was processed the same way except processing began with resuspending the bone marrow pellet in TNE followed by sonication. In some experiments, samples were also extracted with phenol/cresol (7:1 vol/vol).

Samples of this material were precipitated with trichloroacetic acid to determine total radioactivity. Other samples were first treated with alkali (0.4 M NaOH, for 90 min at 37° C) to determine alkali-stable, trichloroacetic acid-precipitable radioactivity. DNA content was measured by the Burton modification of the diphenylamine color reaction (13). The difference between the total and alkali-stable radioactivity was assumed to represent radioactivity in RNA.

In Vitro Phosphoribosyl Transferase Activity

Samples of tumor, intestine and pelleted bone marrow were homogenized in 0.1 M Tris:HCl buffer (pH 7.8) containing 0.25 M sucrose. Intestinal mucosa was prepared by flushing a 10–15 cm section of small intestine with ice-cold saline, slitting it open and removing the lining cells by scraping with a glass microscope slide. The homogenate was centrifuged at 100,000 × g for 30 min and the resulting supernatant was used as the source of enzyme. Protein content was determined by the Lowry procedure (14). The reaction mixture contained 10 µl (6-^{3}H) FUra or (6-^{3}H) Ura (10 mM, 25 µCi per µmol), 10 µl 50 mM PRPP and 80 µl of enzyme. After a 30 min incubation at 37° the reaction was stopped by placing the tubes in boiling water for 90 sec, the precipitated protein was pelleted and aliquots of the supernatant were spotted on DEAE discs. The discs were washed six times, twice with 5 mM ammonium formate and four times with water. After being placed in scintillation vials, 0.5 ml of 1.5 N HCl, 0.5 M NaCl was added. After 30 min, aqueous scintillation cocktail was added and radioactivity determined.

In Vitro Uridine Kinase Activity

Enzymes samples were prepared as above. The reaction mixture contained 10 µl (5-^{3}H)UR (0.1 mM, 0.5 mCi/mMol), 10 µl ATP (50 mM in 0.5 M Tris:HCl, pH 7.8, 25 mM $MgCl_2$ and 2 mM NaF) and 80 µl enzyme. Conversion of (5-^{3}H)UR to (5-^{3}H)UMP was determined as outlined in the PRtase assay.

Uridine Nucleotide Pool Measurements

Tumor bearing animals were anesthetized with sodium pentobarbital or ether. As soon as the animal lost consciousness the tumor was removed and immediately homogenized in 2 ml of ice-cold 1.2 N perchloric acid. Intestinal mucosa and bone marrow were collected as described above. The pelleted intestinal mucosa and bone marrow were homogenized in PCA. After centrifugation of the homogenate, the supernatant containing the acid-soluble molecules was placed in a boiling water bath for 15 min, chilled, then neutralized with $KHCO_3$ to remove perchlorate. After centrifugation, the supernatant was filtered through a 0.22 micron Millipore filter prior to analysis by high pressure liquid chromatography. Heating the acid extracts converts pyrimidine ribonucleotides to UMP and CMP (and FUTP to FUMP). Purine nucleotides are cleaved to the free bases adenine and guanine (15). HPLC analysis of nucleotides was done with a Dupont 850 system using a Spectra-Physics calculating intergrator. Nucleotides were separated by isocratic ion-pairing using a Dupont C_8 column and a buffer consisting of 25 mM KH_2PO_4 in 5 mM tetrabutylammonium hydrogen sulfate (Aldrich Chemicals), 2 ml per min at 35° C. This system allows the quantitation of FUra, fluorouridine, fluorodeoxyuridine, FUMP, FdUMP and UMP in a single run. The precipitate pellet was washed with cold 0.6 N perchloric acid, dissolved in 1.0 N NaOH and protein content was determined by the Lowry procedure (14). Results were calculated as nMol UMP or FUMP per mg of protein in the perchloric acid precipitate. In some experiments tumor UTP was measured by HPLC analysis of unboiled perchloric acid extracts.

Urine Collection and Analysis

Animals received (^{3}H)FUra at 50 mg/kg either alone or with pyrimidine pretreatment. The animals were placed in homemade metabolic cages in which urine and feces were collected in a 10 cm petri dish. The animals had access to food and water at all times. After 7 hrs the animals were removed and the urine was dissolved in 10 ml of water. Aliquots were counted directly to determine total recovery of radioactivity (representing intact FUra, FUR and FUdR as well as degradation products). Other aliquots were treated with perchloric acid and $KHCO_3$ for analysis by HPLC with a Dupont C_8 column using a buffer of 20 mM KH_2PO_4, 5 mM tetrabutyl-ammonium hydrogen sulfate, pH 6.0. Fractions were collected to determine the amount of radioactivity co-chromatographing with FUra, FUR and FUdR.

Results

We observed that the addition of uridine to TdR plus FUra provided a therapeutic gain over TdR plus FUra alone (16). Therefore, we examined the effect of thymidine (TdR) and uridine (UR) on the initial incorporation of FUra into tumor RNA, and into the RNA of two host tissues, intestinal mucosa and bone marrow, that are the targets of FUra toxicity. The level of FUra in RNA in each of these 3 tissues was measured at 2 hours after the administration of FUra alone (group 1), FUra plus TdR (group 2), FUra plus TdR and UR (group 3), or FUra plus TdR and uracil (Ura) (Group 4, discussed below). The pooled data from four such experiments are presented in Table I. Thymidine at 500 mg/kg essentially eliminates catabolic degradation of FUra making more FUra available for activation to the nucleotide form (5). The addition of TdR resulted in an average 3-fold increase in the amount of FUra incorporated into RNA (calculated on a per mg of DNA basis) in all three tissues. Since uridine itself is also able to inhibit the degradation of FUra, thereby increasing the incorporation into RNA (5–7), the effect of the addition of uridine was compared in the TdR-FUra combination to eliminate this effect. The second dose of UR is given in order to maintain elevated blood levels of UR during the labelling period. The addition of UR resulted

in an approximate one third reduction of the incorporation of FUra into the RNA of each of the three tissues although in each tissue it was still elevated above that achieved with FUra alone. Similar results are obtained when the incorporation of FUra is calculated at *equivalent* RNA synthesis using ^{32}P as a measure of RNA synthesis.

There were two possible mechanisms that may account for the observed decrease in the generation of (FUra) RNA in the presence of uridine. First, uracil, derived from phosphorylytic cleavage of UR, may compete with FUra for activation to the nucleotide form. Second, elevated levels of UTP may compete with FUTP for utilization by RNA polymerase.

We examined the possibility of competition of uracil at the activation step. Consistent with the observations of others (17, 18), the major pathway for FUra activation in these tissues appears to be via the orotate phoshoribosyl transferase (OPR-tase) pathway, rather than by conversion by phosphorylase to fluorouridine and subsequent phosphorylation by UR kinase. In support of this conclusion, we detect no fluorouridine in plasma, urine, tumor, gut and bone marrow by HPLC analysis after FUra administration without also giving uridine. Further, the level of *in vitro* fluorouracil-phosphoribosyl transferase activity (FUra-PRtase) correlates with the observed incorporation of FUra into RNA and the level of (^3H) FUMP formed in the three tissues at 30 min after administration of (^3H) FUra. For example, Table II compares the *in vivo* incorporation of FUra into RNA of tumor and normal tissues and the level of fluorouridine nucleotides *in vivo* with *in vitro* determinations of PRtase activity calculated from a separate group of animals.

When we measured the relative ability of tumor and normal host tissue to convert FUra and uracil to nucleotides we observed that in three different murine tumor systems, the CD_8F_1 mammary tumor, the CD_2F_1 Colon 26 and the BDF_1 Colon 38, enzyme from tumor tissue possessed a much greater capacity to convert FUra to nucleotides as compared with uracil than did the normal tissues. (Table III, compare the ratio of the generation of FUMP to UMP, last column.) The difference appears to reflect the tissue specific levels of FUra-PRtase activity. In all three animal models the tumor possesses considerable higher levels of FUra-PRPPtase than does intestine or bone marrow, whereas all three tissues contain about the same level of uracil-PRtase activity.

The lower FUMP/UMP ratio of normal tissues suggested the possibility that preferential protection of normal tissues might be achieved with uracil rather than UR. The utilization of uracil would circumvent the generation of fluorouridine which can be formed from FUra and ribose-1-phosphate

Table I. Effect of Thymidine and Uridine on the in Vivo Incorporation of 5-Fluorouracil into RNA from the CD_8F_1 Murine Mammary Tumor, Intestine and Bone Marrow.
CD_8F_1 female mice bearing the advanced transplant mammary carcinoma were given the indicated courses of drugs. Injections were spaced 30 min apart. After a 2 hr labelling period the animals were sacrificed by cervical dislocation and the incorporation of FUra into RNA was determined. Subscripts indicate dose in mg/kg.

Treatment	Tumor	pmol FUra in RNA per mg DNA intestine	Bone marrow
1. $FUra_{50}$	781 ± 78	74 ± 15	137 ± 23
2. TdR_{500} ½hr $FUra_{50}$	1946 ± 295 (2.5)*	203 ± 54 (2.7)	453 ± 151 (3.3)
3. TdR_{500} + UR_{500} ½hr $FUra_{50}$ ½hr UR_{500}	1378 ± 309 (1.8)	118 ± 29 (1.6)	308 ± 126 (2.3)
4. TdR_{500} + Ura_{250} ½hr $FUra_{50}$ ½hr Ura_{520}	1647 ± 380 (2.1)	166 ± 42 (2.2)	606 ± 318 (4.8)

*( ) = fold increase in (FUra)RNA compared to FUra alone.

Table II. Comparison of 5-Fluorouracil-Phosphoribosyl Transferase Activity with the Incorporation of 5-Fluorouracil into RNA.

Tissue	PRtase Activity: nmol FUMP formed/30 min/mg protein *(in vitro)*[1]	nmol FUMP/mg protein *(in vivo)*[2]	nmol FUra in RNA per mg DNA *(in vivo)*[3]
CD_8F_1 Mammary carcinoma	10.5 ± 2.1	1.31 ± 0.06	634 ± 127
CD_8F_1 Intestine	2.4 ± 0.8	0.37 ± 0.09	91 ± 14
CD_8F_1 Bone marrow	3.3 ± 1.1	0.44 ± 0.06	186 ± 61

[1] Tissues were homogenized in 0.01 M Tris:HCl (pH 7.8) containing 0.25 M sucrose. The supernatant obtained after centrifugation of the homogenates at 100,000 × g for 30 min served as the source of enzyme. The reaction mixture contained 5 mM PRPP and 1 mM (6-^3H) FUra (25 μCi/umol). Incubation was for 30 min at 37°. The reaction was stopped by boiling. After removing the precipitated protein aliquots were spotted on DEAE discs. Data are the means ± the S.E.M. for two experiments for a total of 16 animals.

[2] *In vivo* formation of fluorouridine nucleotides. Animals received (^3H)FUra (50 mg/kg). After 30 min the animals were sacrificed and perchloric acid extracts prepared. FUTP and FUDP were converted to FUMP by heating and FUMP was measured by HPLC. Data are the means ± the S.E.M. for two experiments of 4 animals each.

[3] *In vivo* incorporation of (^3H) FUra into RNA as described for Table I. Animals received (^3H) FUra at 50 mg/kg. Data are the means ± the S.E.M. for eight experiments of 3 or 4 animals each.

Table III. Conversion of Uracil and Fluorouracil to Nucleotides by Phosphoribosyl Transferase from Normal and Malignant Murine Tissues.
Tissues were homogenized in 0.01 M Tris: HCl, pH 7.8, 0.25 M sucrose. The supernatant obtained after centrifuging the homogenates for 30 min at 100,000 × g was used as the source of enzyme. The reaction mixture contained 5 mM PRPP and 1 mM base. The data are the mean ± the S.E.M. for 16 animals each for CD_8 and 4 animals for BDF_1 (bone marrow was pooled as a single sample).

Source of enzyme	(FUra)PR-tase activity nmol FUMP/30 min/mg protein	(Ura)PR-tase activity nmol UMP/30 min/mg protein	FUMP/UMP
CD_8F_1			
Mammary carcinoma	10.5 ± 2.1	0.5 ± 0.1	21.0
Intestine	2.4 ± 0.8	0.3 ± 0.1	8.0
Bone marrow	3.3 ± 1.1	1.1 ± 0.1	3.0
CD_2F_1			
Colon 26	9.3 ± 1.6	0.7 ± 0.1	13.3
Intestine	1.1 ± 0.3	0.5 ± 0.1	2.2
Bone marrow	3.5 ± 0.8	1.1 ± 0.1	3.2
BDF_1			
Colon 38	11.6 ± 0.9	0.8 ± 0.2	14.5
Intestine	1.2 ± 0.1	1.2 ± 0.1	1.0
Bone marrow	5.8	1.6	3.6

Table IV. Effect of Uracil and Orotic Acid on the Conversion of 5-Fluorouracil to Nucleotide by PR-Transferases of Normal and Malignant Murine Tissues.
In vitro PRtase assays were performed as described in Materials and Methods and Table II, except that 70 µl of enzyme was used with 10 µl of H_2O or 10 µl 40 mM Ura or 10 µl 40 mM orotic acid. Data are the means ± the S.E.M. for a total of 9 animals.

Tissue	Labelled base	Addition	Mol FUMP formed/30 min/ mg protein	%Inhibition
CD_8F_1 Tumor	2 mM FUra	–	18.9 ± 1.7	–
		4 mM uracil	17.2 ± 1.5	9
		4 mM orotic acid	1.7 ± 0.3	91
CD_8F_1 Intestine	2 mM FUra	–	1.8 ± 0.4	–
		4 mM uracil	1.5 ± 0.3	16
		4 mM orotic acid	1.4 ± 0.3	24
CD_8F_1 Bone marrow	2 mM FUra	–	7.3 ± 2.3	–
		4 mM uracil	7.0 ± 2.1	4
		4 mM orotic acid	3.5 ± 0.9	52

(derived from phosphorylase cleavage of UR). However, the addition of a uracil schedule to the TdR-FU combination resulted in little or no reduction in (FUra)RNA in normal tissues (group 4, Table I)

The reason for this became apparent when we compared the ability of orotic acid and uracil to compete in an *in vitro* FUra-PRtase reaction (Table IV). The addition of 4 mM unlabelled uracil to 2 mM (6-^{3}H) FUra resulted in no significant inhibition of the conversion of FUra to FUMP, whereas 4 mM orotic acid gave almost complete inhibition (91%) with enzyme prepared from tumor tissue. Orotic acid was less effective at inhibiting the reaction with enzyme from the two normal tissues. In other experiments we have determined that up to 10 mM uracil (with 2 mM FUra) does not inhibit the conversion of FUra to FUMP (data not shown).

That uracil did not compete with FUra for activation via PRtase suggested that the site of UR action is not at the level of FUra activation, rather it is at the level of UTP competition. To determine the effect of this UR schedule on uridine nucleotide pools, CD_8F_1 mammary tumor-bearing mice received the (TdR+UR)---FUra---UR schedule and were sacrificed at 30 min intervals after beginning the injection schedule. Because the length of time required to collect and process intestine and bone marrow results in considerable breakdown of nucleoside triphosphates, we first converted the uridine nucleotides to monophosphate form by heating the acid extracts. Changes in the "total acid-soluble uridine nucleotide pool" are assumed to reflect changes in the UTP pool (15). Measurement of UMP was done by high pressure liquid chromatography. The data presented in Fig. 1 were calculated as nmol UMP per mg of protein in the perchloric acid precipitate and are expressed as the per cent change from control values. The figure shows that UMP pools are expanded at the time of FUra administration (30 min after TdR+UR). By 90 min after FUra administration, UMP pools are returning toward normal levels in all three tissues. As a check, measurement of tumor *UTP* following TdR+UR gave the same result as measurement of UMP (data not shown).

In order to assess the relative abilities of CD_8F_1 breast tumor and normal tissues to convert UR to UMP we compared the levels of UR kinase in these tissues (Table V). Setting the level observed in tumor homogenates as 1.0 for comparison, we observed higher levels of UR kinase in the bone marrow and brain and lower levels in intestinal mucosa and liver. These results agree with those presented in Fig. 1 in which the greatest increase in uridine nucleotides was obtained in bone marrow.

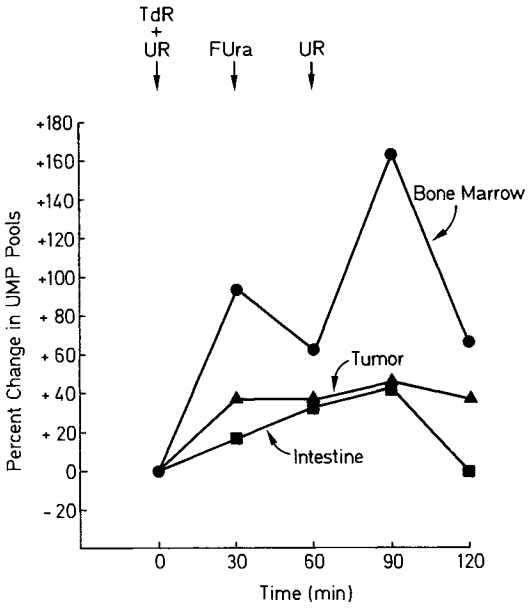

Fig. 1 Effect of uridine on the level of uridine ribonucleotides in tumor, intestine and bone marrow. CD_8F_1 tumor bearing mice received the following schedule of drugs: TdR + UR---30 min---FUra---30 min---UR. TdR and UR were administered at 500 mg/kg, FUra at 50 mg/kg I.P. At 30 min intervals after the initial injection, some of the animals were sacrificed, and perchloric acid extracts were prepared from tumor, intestine and bone marrow. The extracts were heated to convert UTP to UMP, and the UMP was measured by HPLC. Results were calculated as nmol UMP per mg of protein in the perchloric acid precipitate. Data are the per cent change values in untreated mice averaged for three experiments, each experiment containing 4 animals per time point. Control values, expressed as nmol UMP per mg of protein were: tumor, 14.9 ± 1.6; intestine, 16.7 ± 0.9; bone marrow, 6.9 ± 1.3.

Table V. Uridine Kinase Levels in Normal and Malignant Tissues of the CD_8 Mouse.
Tissues were homogenized in 0.01 M Tris: HCl, pH 7.8, 0.25 M sucrose. The supernatant obtained after centrifuging the homogenates for 30 min at 100,000 × g was used as the source of enzyme. The reaction mixture contained 5 mM ATP and 0.01 mM (5-^{3}H)-uridine. Data are the average ± S.E.M., n = number of individual tissue preparations. Each marrow preparation represents at least four mice.

Tissue	n	pmol UMP formed/ 30 min/mg protein	Relative to tumor
Mammary tumor	17	457 ± 68	1.00
Intestinal mucosa	15	109 ± 36	0.24
Bone marrow	9	2914 ± 947	6.38
Liver	5	258 ± 45	0.56
Brain	6	510 ± 48	1.12

The relatively higher bone marrow uridine kinase level might be expected to result in a greater increase in FUMP formation by the phosphorylase-kinase pathway in this tissue with the administration of uridine. Therefore, we measured the effect of this uridine schedule on uridine and fluorouridine nucleotide pools in CD_8 tumor, gut and bone marrow (Table VI). Animals received either TdR---30 min---(^{3}H)FUra or (TdR+UR)---30 min---(^{3}H)FUra---30 min---UR and were sacrificed at 30 min and 60 min after receiving the FUra. TdR and UR were administered at 500 mg/kg each, and the (^{3}H) FUra was 50 mg/kg. Perchloric acid extracts were prepared and pyrimidine nucleotides converted to the monophosphate form as described for Fig. 1. UMP was calculated from the integrated area of the UMP peak on the HPLC chromatogram, and FUMP was estimated from recovery of tritium radioactivity co-chromatographing with an FUMP marker. The data in Table VI, averaged from three separate experiments, show that at 30 min after FUra (60 min after the first dose of UR in group 2) UMP pools in the TdR+UR tissues are slightly elevated while FUMP pools are slightly depressed. At 60 min after FUra (30 min after the second dose of UR) UMP pools are further expanded. In contrast to the 30 min time point, FUMP pools are elevated above those in the TdR-FUra tissues. Readily measurable levels of fluorouridine (and fluorodeoxyuridine) are found in the plasma and tissues of the TdR+UR animals. However, any additional formation of FUMP via uridine kinase is offset by the expansion of the UMP pools to maintain the UMP/FUMP ratio at or above the level attained in the absence of uridine.

Since 500 mg/kg of TdR is sufficient to give > 90 % inhibition of FUra catabolism (5), the addition of UR or Ura would not greatly increase this effect. But we wished to know if the added pyrimidines could further increase FUra availability by decreasing renal clearance. Animals received (3H)FUra at 50 mg/kg, either alone or with pyrimidines as outlined in Table VII. Urine was collected for 7 hr, and then aliquots were measured for the total per cent of input radioactivity (representing intact FUra plus degradation products) recovered in the urine. Other aliquots were analysed by HPLC to determine the fraction of the urine radioactivity in the form of FUra, FUR and FUdR. The data in Table VII show that pretreatment with TdR, or the TdR plus UR, or TdR plus Ura regimens, had little effect on the total amount of radioactivity excreted. While there is little change in the total excretion, representing the sum of degradation products plus intact drug, pretreatment with TdR did increase the fraction in the urine represented by intact drug. The slight further increase with the addition of UR or Ura suggests that these compounds may provide a small additional block on catabolism. Note that "intact drug" here includes FUR and FUdR since there is considerable phosphorylase conversion of FUra to nucleoside following administration of TdR and UR. Overall, however, the addition of UR or Ura to TdR does not seem to significantly affect either the degradation or renal clearance of FUra. Similar results were obtained with a 24 hr collection while the variability of urine output prevented the use of a 2 hr collection period to correspond to the 2 hr (FUra)RNA experiments.

Finally, we asked whether protection from FUra toxicity with UR might be due to expanded dUMP pools displacing FdUMP from thymidylate synthetase, thereby, preventing or

Table VI. In vivo Effect of Uridine on the Ratio of Uridine to Fluorouridine Nucleotides in CD_8F_1 Mammary Tumor, Intestine and Bone Marrow.

Schedule and tissue[1]	30 min after FUra			60 min after FUra		
	nmol FUMP[2]	nmol UMP	UMP/FUMP	nmol FUMP	nmol UMP	UMP/FUMP
CD_8 Tumor						
1. TdR − FUra	0.70 ± 0.08	14.0 ± 1.5	20.0	0.69 ± 1.10	14.4 ± 1.2	20.9
2. TdR + UR − FUra − UR	0.57 ± 0.07	17.4 ± 1.7	30.5 (1.53x)*	0.99 ± 0.13	22.4 ± 1.8	22.6 (1.08x)
Intestine						
1. TdR − FUra	0.40 ± 0.03	19.6 ± 2.1	49.0	0.36 ± 0.07	15.5 ± 1.4	43.1
2. TdR + UR − FUra − UR	0.28 ± 0.03	21.8 ± 2.1	77.9 (1.59x)	0.43 ± 0.03	23.5 ± 2.0	54.7 (1.27x)
Bone Marrow						
1. TdR − FUra	0.52 ± 0.08	8.3 ± 1.9	16.0	0.26 ± 0.05	5.0 ± 0.6	19.2
2. TDR + UR − FUra − UR	0.38 ± 0.08	11.1 ± 2.8	29.2 (1.83x)	0.51 ± 0.14	18.1 ± 4.5	35.5 (1.85x)

[1] CD_8F_1 tumor bearing mice received either thymidine or thymidine plus uridine (500 mg/kg each) 30 min prior to (^{3}H) FUra (50 mg/kg). In addition, group 2 mice that were sacrificed 60 min after the FUra received a second dose of UR 30 min after FUra. Animals were sacrificed at 30 min and 60 min after administration of FUra.

[2] Perchloric acid extracts were prepared, heated to convert pyrimidine ribonucleotides to the monophosphate form, and analaysed by HPLC. UMP was measured by the integrated area of the UMP peak on the chromatogram. FUMP was estimated by tritium radioactivity cochromatographing with an FUMP marker. Data was calculated as nmol FUMP or UMP per mg of protein in the perchloric acid precipitate and is an average for three experiments ± S.E.M., four animals per group, per time point in each experiment.

*() = fold increase in UMP/FUMP ratio with the addition of uridine.

Table VII. Effect of Thymidine, Uridine and Uracil on Renal Clearance of 5-Fluorouracil.

Treatment[1]	Per cent of input radioactivity recovered in urine[2]	Per cent of urinary radioactivity as *"intact"* FUra[3]
$FUra_{50}$	53.0 ± 2.3 %	64.9 ± 1.2 %
$TdR_{500} \xrightarrow{30\ min.} FUra_{50}$	60.5 ± 1.8 %	92.7 ± 0.9 %
$TdR_{500} + UR_{500} \xrightarrow{30\ min} FUra_{50}$	55.6 ± 2.2 %	96.3 ± 1.5 %
$\xrightarrow{30\ min} UR_{500}$		
$TdR_{500} + URa_{250} \xrightarrow{30\ min} FUra_{50}$	55.2 ± 1.3 %	95.3 ± 1.2 %
$\xrightarrow{30\ min} Ura_{250}$		

[1] Animals received (^{3}H) FUra, 50 mg/kg, and TdR, 500 mg/kg, UR, 500 mg/kg or Ura, 250 mg/kg as indicated. Urine was collected for 7 hr after FUra administration.

[2] Urine was diluted in 10 ml of water. Aliquots were counted to determine total recovery of radioactivity in the urine as a per cent of the amount of radioactivity injected into the animal.

[3] Aliquots of diluted urine were treated with perchloric acid and $KHCO_3$, then analysed by HPLC for radioactivity chromatographing as FUra, FUR and FUdR, the sum of which represents "intact" FUra.

Table VIII. Effect of Thymidine, Uridine and Deoxyuridine on the Early, Partial Inhibition of DNA Synthesis by 5-Fluorouracil in the CD_8 Mammary Tumor.
Tumor-bearing CD_8F_1 mice received unlabelled 5-fluorouracil (FUra, 100 mg/kg) at zero time. Some groups then received thymidine (TdR), uridine (UR) or deoxyuridine (UdR) supplementation at the indicated time. All groups received ^{32}P (carrier-free $H_3{}^{32}PO_4$) 6 hr after FUra. After a 1 hr labeling period, the animals were sacrificed and the incorporation of ^{32}P into pronase- and alkali-stable trichloroacetic acid-precipitable material was determined. Data represent the average of 2 experiments ± the S.E.M., 4 animals per group in each experiment.

Treatment	Alkali-stable ^{32}P cpm per mg DNA	
1. Control $\xrightarrow{}{}^{32}P$	23507 ± 930	(1.00)
2. $FUra_{100} \xrightarrow{6hr} {}^{32}P$	14840 ± 1044	(0.63)
3. $FUra_{100} \xrightarrow{5½hr} TdR_{500} \xrightarrow{½hr} {}^{32}P$	21810 ± 1543	(0.93)
4. $FUra_{100} \xrightarrow{5½hr} UR_{500} \xrightarrow{½hr} {}^{32}P$	11600 ± 1778	(0.49)
5. $FUra_{100} \xrightarrow{5½hr} UR_{1000} \xrightarrow{½hr} {}^{32}P$	13265 ± 1169	(0.56)
6. $FUra_{100} \xrightarrow{5½hr} UR_{1000} \xrightarrow{½hr} {}^{32}P$	13781 ± 9778	(0.59)

reducing FUra-induced inhibition of DNA synthesis (19). The time interval of 6 hrs after FUra was chosen because we had previously shown that at 6 hrs the inhibition of DNA synthesis was only partial and was reversible by thymidine, whereas at 24 hrs inhibition was complete and no longer affected by administering thymidine (5–7). The results are detailed in Table VIII and show that thymidine, but not uridine, can reverse the inhibition of DNA synthesis. Neither a larger dose of uridine nor deoxyuridine were able to reserve the inhibition (Group 5 and 6).

In these last two groups the interval between pyrimidine administration and ^{32}P was increased to 5½ hr which presumably would allow for greater accumulation of dUMP. Like thymidine, uridine had no effect on the complete inhibition of DNA synthesis observed after 24 hr exposure to FUra (data not shown).

Discussion

Uridine has been shown to protect from the toxic effects of FUra (3, 4, 16, 20, 21 and R. Johnson, pers. communic.). This protection can occur in two ways. First, UR may effectively compete with FUra for incorporation into RNA. Second, elevated dUMP pools might compete with FdUMP to overcome the inhibition of thymidylate synthetase (19). We believe the evidence presented in this paper argues against the second possibility, since uridine and deoxyuridine were not able to reverse the inhibition of DNA synthesis (Table VIII), indicating that at the doses used (500 and 1000 mg/kg) insufficient dUMP accumulates to relieve the FdUMP block on thymidylate synthetase.

Relative to FUra incorporation into RNA, UR has two competing effects. First, as a source of uracil, it is able to inhibit the catabolic degradation of FUra, making more FUra available for activation to the nucleotide form resulting in increased incorporation into RNA (5). Secondly, it can increase the level of competing UTP pools thereby inhibiting the use of FUTP by RNA polymerase. Thymidine at 500 mg/kg is sufficient, by itself, to prevent catabolism of FUra (5). Thus by adding UR to the TdR-FUra combination, the UR effects on FUra catabolism are effectively minimized allowing us to evaluate the UTP pool effect. We also determined that the addition of UR (or Ura) did not significantly affect renal clearance of FUra (Table VII). However, it was unexpected that a ten-fold molar excess of uridine (500 mg/kg × 2), as compared to FUra (50 mg/kg), would result in only about a one third reduction in (FUra) RNA (Table I). This may be due to the fact that 500 mg/kg UR causes only a limited expansion of uridine nucleotide pools (Fig. 1). A 2 hr labelling period was designed to measure the effect of UR (or Ura) on the initial incorporation of FUra into RNA (Table I). The effect of UR on uridine nucleotide pools would be constantly changing as the UR is absorbed and then eliminated. A 2 hr endpoint for incorporation represents the average affect of UR over 2 hrs. Thus, determination of the constantly changing UMP/FUMP ratio at any one time point may not reflect the final (FUra) RNA level. Additionally, Piper has suggested that UR is incorporated into at least two different UTP pools, only one of which serves as the primary source of UTP for RNA synthesis (22). Therefore, the changes we measure in total uridine nucleotides may reflect a greater change than occurs in the major precursor pool. Even much larger doses of uridine (3500 mg/kg) result in only about

a three-fold increase in uridine nucleotide pools, though the increase lasts much longer, up to 8 hrs (R.C.S., unpublished observations).

The decrease in FUra incorporation into RNA which results from the addition of uridine appears to be caused largely by competition with expanded uridine nucleotide pools rather than by competition with FUra for activation to the nucleotide form. In mammalian systems, a single enzyme, orotate PRtase, appears to be responsible for both the *de novo* pathway conversion of orotate to OMP as well as the salvage of pyrimidine bases (23). However, because of the very high pH optimum for uracil, this base is a poor substrate at physiological pH (24). This would explain why uracil was not effective in inhibiting the conversion of FUra to FUMP. These results are consistent with those of Kessel et al. (24) who found that orotate and FUra were good substrates for a PRtase from murine leukemia cells, whereas uracil was not. The pH optimum for FUra was a full pH unit below that for Ura, and the Km for FUra was 50-fold lower (24). Similarly, uracil did not inhibit the FUra-PRtase reaction using homogenates of Yoshida Sarcoma or rat liver (25). These results explain our observation that uracil, unlike uridine, does not inhibit the incorporation of FUra into RNA (Table I). Other workers have also reported that uracil does not reverse the growth inhibition of FUra in mammalian cells, rather it increased the antitumor activity of FUra and its prodrug Ftorafur (presumably through its ability to competitively inhibit the catabolic degradation of the pyrimidine analogues) (26, 27).

Taken together, the evidence presented here leads us to conclude that protection from FUra toxicity afforded by uridine administration occurs through a reduction of the incorporation of FUra into RNA. It should be noted that tissues can have differing sensitivities to FUra in RNA (1, 28). Glazer and Lloyd have demonstrated that in a human colon carcinoma cell line, cell survival correlated well with the absolute level of FUra in RNA rather than with inhibition of DNA synthesis (28). After reaching a threshold level of incorporation, cell viability decreased sharply with small increases in (FUra)RNA. Conversely, a small *decrease* in (FUra)RNA can produce a large *increase* in survival if the decrease reduces the (FUra)RNA level below the threshold level for that particular tissue. Thus an equal percentage reduction can produce a different result in different tissues if one tissue is reduced below its threshold level while the other remains above it.

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