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Use of oral uridine as a substitute for parenteral uridine rescue of 5-fluorouracil therapy, with and without the uridine phosphorylase inhibitor 5-benzylacyclouridine*

Daniel S. Martin[1, 2], Robert L. Stolfi[2], and Robert C. Sawyer[2]

[1] Developmental Chemotherapy, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA
[2] Cancer Research Laboratories, Department of Surgery, Catholic Medical Center, New York, NY 11421, USA

Summary. Initial clinical trials have demonstrated that uridine (Urd) rescue given i.v. over at least 3 days can ameliorate 5-fluorouracil (FUra) toxicity; to avoid Urd-induced phlebitis in the peripheral veins of patients, a central vein is used. The latter necessity, along with the need for 3 days of i.v. administration, makes Urd rescue by parenteral means a cumbersome and complicated clinical procedure. It would appear preferable to use oral Urd; however, the oral Urd dose in the clinic is limited, as high doses cause diarrhea. Therefore, using a tumor-bearing murine model we investigated as to whether low doses of oral Urd coupled with a Urd phosphorylase inhibitor benzylacyclouridine (BAU), would effect safe rescue of FUra toxicity with preservation of antitumor activity. A high-dose FUra-containing drug combination that included parenteral Urd rescue was used as a control; other groups of tumor-bearing mice received the same drug combination, except that p.o. Urd was substituted for i.p. Urd. In the absence of BAU, p.o. Urd could effect rescue while maintaining an antitumor effect comparable to that obtained with i.p. Urd. When given concomitantly with BAU, a 50% reduction in the oral Urd dose (i.e., from 4,000 to 2,000 mg/kg) enabled the achievement of a comparable therapeutic index. Intraperitoneal Urd produces very high (6–8 m*M*) plasma and tissue Urd levels, which remain above 100 μ*M* for at least 6 h. In contrast, neither oral Urd nor oral BAU alone raised plasma Urd concentrations above about 50 μ*M*. However, the combination of oral Urd plus oral BAU gave a peak plasma Urd level of about 300 μ*M*, and the level was maintained above 100 μ*M* for 6 h. Following oral Urd administration, gut tissue levels of Urd were in the m*M* range and those of BAU were in the range of 10–20 μg/g tissue, a level sufficient to result in substantial inhibition of Urd phosphorylase. Oral Urd plus oral BAU appears to be a promising clinical alternative to parenteral administration of Urd for selective rescue of FUra toxicity.

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Offprint requests to: 89-15 Woodhaven Blvd., New York, NY 11421, USA

Abbreviations used: BAU, benzylacyclouridine; FUra, 5-fluorouracil; (FUra)RNA, RNA containing incorporated FUra; LV, leucovorin; MTX, methotrexate; PALA, *N*-phosphonacetyl-L-aspartate; Urd, uridine

Introduction

The delayed administration of uridine (Urd) can improve the therapeutic index of 5-fluorouracil (FUra) in tumor-bearing mice. Beginning 2–24 h after FUra administration and given for 2–5 days as either a continuous infusion or intermittent i.p. injections, Urd has been demonstrated to be capable of rescuing normal tissues in tumor-bearing mice from high, potentially lethal doses of FUra, with a resulting improvement of FUra's antitumor effect [8, 9, 16–19]. The interval between the administration of FUra and that of Urd is necessary to prevent the latter from interfering with the initial anabolism or catabolism of FUra. In such a delayed rescue schedule, Urd has proven to be a useful biochemical modulator of FUra, preferentially rescuing normal tissues from FUra toxicity and thereby enabling the safe escalation of the maximum tolerated dose of FUra, with a consequent enhancement of the antitumor activity of FUra and FUra-containing drug combinations.

The above preclinical findings have stimulated several reported [11, 24–26], and ongoing [7, 21] phase I clinical trials of parenteral Urd. The initial clinical studies were encouraging, in that prolonged infusion of Urd was found to rescue the white blood cells and platelets in humans even with continued weekly exposure to FUra [20, 25]. However, these clinical trials have been complicated to conduct. Parenteral administration of Urd for several days following FUra necessitates the insertion of a central vein catheter to avoid phlebitis [11, 24–26]; oral administration of Urd would circumvent these problems and greatly simplify clinical protocols.

In a preliminary clinical study, Van Groeningen et al. [26] found that although oral Urd could elevate plasma Urd levels for significant periods, diarrhea was a dose-limiting toxicity. In a preclinical study in mice, Klubes et al. [10] noted that whereas the bioavailability of oral Urd was low, oral administration of the drug resulted in a much lower but more prolonged and relatively constant plasma level of Urd than did parenteral administeration. Urd is catabolized by Urd phosphorylase in normal tissues, and benzylacyclouridine (BAU) is known to be a specific inhibitor of this enzyme [2, 5]. Both the ability of BAU to increase the plasma concentration of Urd and the equal effectiveness of oral vs. i.v. BAU have been established [4a]. Moreover, BAU has been reported to increase the antitumor activity of fluoropyrimidines [2, 3], and its potential to augment Urd rescue regimens has been suggested [3].

We evaluated the combination of oral Urd plus oral BAU as a substitute for parenteral Urd rescue of toxicity following a previously described, modulated FUra-based drug combination [17], in which *N*-phosphonacetyl-L-aspartate (PALA) plus methotrexate (MTX) preceded high-dose FUra, which was then followed by leucovorin (LV) rescue for MTX and parenteral Urd rescue for FUra. This regimen, in which PALA and MTX were used as metabolic modulators of FUra anabolism and to increase the incorporation of FUra into RNA [14], has yielded impressive therapeutic results in advanced-tumor-bearing mice [17].

Materials and methods

Murine tumor model. All experiments were carried out in 3- to 4-month-old CD_8F_1 mice bearing first-passage transplants of CD_8F_1 spontaneous mammary carcinomas [15, 23]. Ten mice bearing single tumors of approximately equal weight were distributed in each group. Treatment was begun 3 to 4 weeks after tumor transplantation, when the tumors weighed an average of 100–200 mg for the chemotherapy experiments or 300–500 mg for the biochemical studies. The mice were weighed before each of three weekly courses of the indicated treatment (see legend to Table 1).

Drugs. FUra, LV, MTX, and Urd were obtained from Sigma Chemical Co. (St. Louis, Mo) and dissolved immediately before use in 0.85% NaCl solution. BAU was synthesized by Dr. K. Watanabe (Sloan Kettering Memorial Cancer Institute) and diluted in saline. PALA (obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, Md) was dissolved in 0.85% NaCl solution, and the pH was adjusted to 7.2 with 1 *N* NaOH before adjustment to the final volume. Unless otherwise noted, all injections were i.p., and all drugs were given such that the desired dose was contained in 0.1 ml/10 g body weight. Oral doses of Urd and BAU were given by gavage.

Plasma and tissue preparations for high-performance liquid chromatography. Mice received either Urd, BAU, or Urd plus BAU by oral or i.p. administration. At various time points, animals were anesthetized with pentobarbital and blood or blood plus a section of small intestine were collected. Plasma was collected by centrifuging whole blood. The intestine was flushed with ice-cold saline before being quick-frozen in either liquid nitrogen or dry ice-acetone. Plasma was deproteinized with trichloroacetic acid (TCA). Intestinal sections were homogenized in 1.2 *N* perchloric acid. After centrifugation, the supernatant containing acid-soluble material was neutralized by extraction with a 1:2 mixture of tri-N-octyl amine in freon (1,1,2-trichloro-trifluoroethane). The neutralized supernatant was then filtered through a Milipore filter prior to high-performance liquid chromatographic (HPLC) analysis. Recoveries of Urd and BAU from the intestine were normalized to the frozen wet weight of the tissue.

HPLC analysis. HPLC measurements of Urd and BAU levels in plasma and tissue were done on a Waters 840 HPLC system using a Waters Radial-PAK C18 cartridge or a Waters steel-jacketed C18 column. For plasma Urd, the buffer was 0.05 *M* KH_2PO_4 (pH 6.75) and the Radial-PAK cartridge or a Waters steel-jacketed C18 column. For plasma Urd, the buffer was 0.05 *M* KH_2PO_4 (pH 6.75) and the Radial-PAK cartridge was used. For tissue Urd, the steel C18 column was used with a buffer of 15 m*M* KH_2PO_4 in 3 m*M* heptane sulfonic acid (pH 6.75) at 8° C. Plasma and tissue BAU measurements were done with the Radial-PAK cartridge and a mobile phase of 20% MeOH.

Urd phosphorylase. Enzyme Preparation: Red blood cells (RBCs), plasma samples, and sections of small intestine were collected as described above; other tissues were also quick-frozen in liquid nitrogen or dry ice-acetone. Bone marrow was collected by flushing the marrow cavity of the femur with ice-cold saline; marrow from 12 mice were pooled into a single sample. All tissues except plasma but including packed RBCs were homogenized n 5 volumes of 100 m*M* KH_2PO_4 (pH 7.4). After centrifugation for 30 min at 100,000 *g* and 4° C, the resulting supernatants and plasma samples were passed through a Pharmacia DNA-25 Sephadex column equilibrated with the KH_2PO_4 buffer to remove endogenous nucleosides, bases, and nucleotides. The protein content was determined by the Lowry procedure [12]. Enzyme assay: The reaction in a total volume of 250 µl contained either 1 m*M* Urd (intestine) or 100 µ*M* Urd (all other tissues) plus water or BAU and 200 µl enzyme. The reaction tubes were incubated at 37° C. The reactions were stopped by the addition of 250 µl 20% TCA after 15 min (intestine) or 30 min (all other tissues). After the removal of acid-insoluble material by centrifugation, the supernatant was neutralized by extraction with a 1:2 mixture of tri-*N*-octyl amine in freon. The Urd and uracil content was measured by HPLC.

Results

In each of six separate experiments, a group of CD_8F_1 mice bearing advanced first-passage breast tumors were treated with a previously described [17] high-dose, FUra-containing drug combination followed by a Urd rescue regimen given i.p., identified as "standard" Urd rescue i.p. (column 1) in Table 1. The results confirm our previous report that this drug combination produces a significant level of tumor regression [in this series of experiments, a 34% partial regression (PR) rate], with an acceptable average body-weight change (−11%) and an average drug mortality of only 3%. In some of the present experiments as well as in previous studies, the same FUra-containing drug combination without the Urd rescue resulted in virtually 100% mortality (data not shown).

In each of the experiments listed in Table 1, additional groups of mice were treated with the same FUra-containing drug combination, except that in place of the "standard" Urd rescue, i.p. they received Urd orally at one of various doses, BAU orally at 240 mg/kg, or the combination of oral Urd at one of various doses plus oral BAU at 240 mg/kg. Not all doses of oral Urd could be included in each experiment. However, with one exception (experiment 1974), in all experiments one group received the "standard" FUra-containing combination drug regimen with a parenteral (i.p.) Urd rescue regimen ([17]; see legend to Table 1); therefore, this group was the reference control (column 1) in each experiment.

In comparison with the six groups that received the "standard" i.p. Urd rescue regimen (column 1; average mortality of 3%, average weight change of −11%, and a

Table 1. Equivalence of p.o. and i.p. Urd rescue of combination chemotherapy with a well-modulated high-dose FUra regimen[a]

Experiment number[b]	Dead/total								
	"Standard" Urd rescue i.p.	Urd_{5000} p.o.	Urd_{4000} p.o.	Urd_{3000} p.o.	BAU_{240} p.o.[c] + Urd_{3000} p.o.	BAU_{240} p.o.[c] + Urd_{2000} p.o.	BAU_{240} p.o.[c] + Urd_{1000} p.o.	BAU_{240} p.o.[c] + Urd_{500} p.o.	BAU_{240} p.o.
Columns	1	2	3	4	5	6	7	8	9
1934 (F)	1/9	1/10							
1944 (F)	0/10	1/10	1/10	5/10	0/10				
1953 (F)	0/10	1/10	0/10	1/10	0/10	0/10			
1956 (F)	1/10			5/10	2/10				
1965 (M)	0/10			2/10		0/10	0/10		8/10
1974 (F)							3/9	6/10	
1976 (M)	0/10			3/10	1/10	2/10	3/10	6/10	6/10
Average mortality	3%	10%	5%	32%	8%	7%	21%	60%	70%
Average weight change	−11%	−9%	−15%	−16%	−13%	−14%	−17%	−25%	−32%
% PR[d]	34%	20%	40%	46%	38%	27%	28%	(15%)	(20%)

[a] Excepting the substitution of oral for i.p. Urd, all mice received a previously published [17] "standard" i.p. regimen once a week × 3, as follows: $PALA_{100}$ − 17 h → MTX_{300} − 2.5 h → $FUra_{150}$ − 2 h → LV_{300} + Urd_{1500} − 2.5 h → Urd_{3500} − 17 h → LV_{300} + Urd_{3500} − 7 h → Urd_{3500}. Subscripts = dose in mg/kg; observations 7 days after the third course of treatment

[b] Advanced first-passage CD_8F_1 breast tumor transplants in CD_8F_1 mice; tumors were almost 1 month old and weighed 100–200 mg when treatment was initiated

[c] Oral rescue: Urd, BAU, or BAU + Urd was given p.o. 2 h after FUra and again 8, 17, 25, and 32 h later

[d] PR = partial regressions (≤ 50% of initial tumor size)

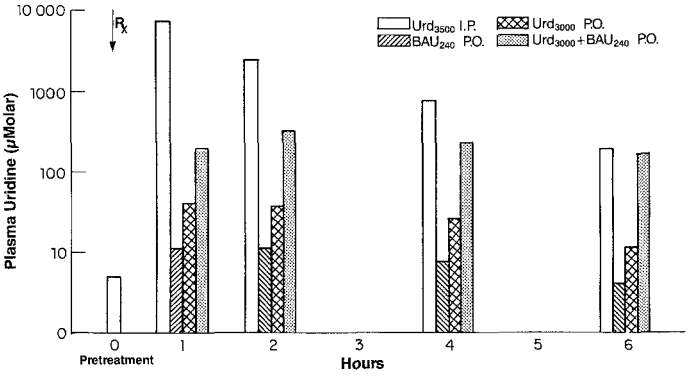

Fig. 1. Comparison of the plasma pharmacokinetics of Urd following parenteral or oral administration

PR rate of 34%), the three groups that received Urd_{5000} p.o. (column 2) had a similar average mortality rate and weight loss but a somewhat lower PR rate (20%), suggesting that the Urd dose was likely too high and had caused some reversal of the antitumor effect. The p.o. dose of Urd_{4000} (column 3) yielded results that were clearly similar to those in the control group (column 1), whereas p.o. Urd_{3000} provided inadequate rescue (average mortality, 32%). In contrast, the addition of p.o. BAU to either p.o. Urd_{3000} (column 5) or p.o. Urd_{2000} (column 6) clearly protected the host (average mortality rates of only 8% and 7%, respectively), with average weight loss and antitumor effects similar to those in the control group (column 1). However, despite the presence of BAU, further reduction of the oral dose of Urd (i.e., 1,000 or 500 mg/kg; columns 7 and 8, respectively) failed to provide adequate host protection. The substitution of parenteral Urd by p.o. BAU_{240} alone (column 9) was not useful (average mortality, 70%).

A comparison of the plasma pharmacokinetics of Urd following parenteral or oral administration is presented in Fig. 1. Note that the Urd concentration is plotted on a log scale. At 3,500 mg/kg, i.p. Urd produced a very high plasma concentration of the drug, on the order of 6–8 m*M* at 1 h; thereafter, the level fell, reaching approximately 100 μ*M* by 6 h. In contrast, nearly the same dose of Urd (3,000 mg/kg) given orally produced much lower peak plasma Urd levels of about 50 μ*M*, which fell to 11 μ*M* at 6 h. At an oral dose of 240 mg/kg, BAU alone raised plasma Urd from about 5 μ*M* to 20–25 μ*M*; Darnowski and Handschumacher [4a] obtained similar results, with oral BAU at 120 mg/kg producing 20 μM plasma Urd. However, combining oral Urd with oral BAU markedly increased plasma Urd to between 200 and 300 μ*M*.

Because oral Urd plus BAU was given every 8 h, plasma samples were analyzed for Urd following a second dose of Urd rescue. Despite the fact that the plasma Urd level was 75 μ*M* at 8 h, the time when the second dose of Urd plus BAU was given, there was no evidence that a subsequent dose of Urd plus BAU resulted in higher levels than the first dose (Fig. 2). The solid bars in Fig. 2 indicate the plasma level of BAU in μg/ml; a peak level of 50 μg/ml was obtained at 1 h, falling to 7 μg/ml by 8 h.

A comparison of tissue Urd levels with those found in plasma at the corresponding time following the parenteral or oral administration of Urd is presented in Table 2. The CD_8F_1 tumor has basal levels of Urd that greatly exceed plasma Urd concentrations; in this particular first-generation, CD_8F_1 tumor transplant, the basal level was 29 nmol/g tissue. We found great variation in tumor Urd levels in both spontaneous CD_8F_1 tumors and the first-generation transplants prepared from them, ranging from a low of 8 to a peak of 80 nmol/g tissue (data not shown). At 2 h after the administration of high-dose i.p. Urd (group

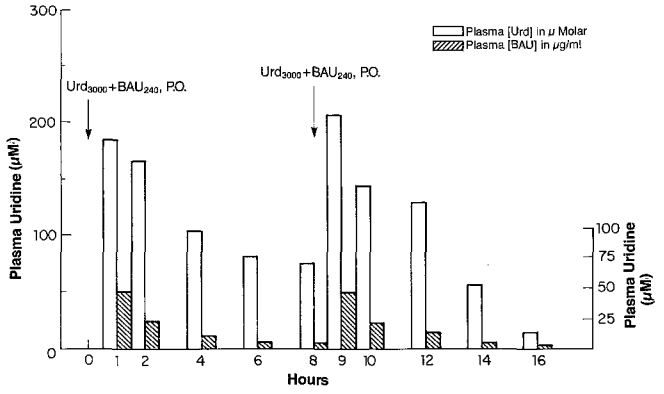

Fig. 2. Comparison of the plasma pharmacokinetics of Urd following oral administration of Urd + BAU and after a second dose of Urd + BAU rescue

Table 2. A comparison of plasma and tissue levels of Urd following its i.p. or p.o. administration

	Urd levels:		
	Pretreatment	2 h post-Urd	4 h post-Urd
A. 3,500 mg/kg Urd, i.p.			
1. Plasma	5 μ*M*	2.9 m*M*	0.6 m*M*
2. Tumor	29 nmol/g	2.8 μmol/g	1.0 μmol/g
3. Small intestine	12 nmol/g	2.4 μmol/g	0.6 μmol/g
4. Bone marrow[a]	40 nmol/g	2.9 μmol/g	0.3 μmol/g
B. 3,000 mg/kg Urd + 240 mg/kg BAU, p.o.			
1. Plasma		313.0 μ*M*	242.0 μ*M*
2. Tumor		386.0 nmol/g	326.0 nmol/g
3. Small intestine		4.0 μmol/g	2.4 μmol/g
4. Bone marrow		1056.0 nmol/g	456.0 nmol/g

Tumor-bearing CD_8F_1 male mice received 3,500 mg/kg Urd i.p. (group A) or 3,000 mg/kg Urd plus 240 mg/kg BAU p.o. At 2 and 4 h after Urd administration, animals were sacrificed for the determination of plasma and tissue Urd concentrations

[a] Because the small amount of the bone marrow obtained precluded the determination of a "wet weight," recoveries were calculated per milligram protein. Since the CD_8F_1 tumor, whole gut, and liver have an average of 8% wet weight as protein, the same percentage was assumed for bone marrow in conversions of nmol/mg protein to nmol/g tissue for this table

Table 3. A comparison of Urd phosphorylase levels in several CD_8F_1 murine tissues

Tissue	nmol Urd degraded/ min per mg protein
Tumor	0.4
Liver	0.2
Intestine	23.0
Bone marrow	0.3
Plasma	<0.1
RBC	<0.1

Partially purified Urd phosphorylase was prepared by homogenizing tissue (except plasma), centrifuging at 100,000 g, and desalting by Sephadex G 25 chromatography. The conversion of Urd to uracil was determined by HPLC

Table 4. Effect of BAU on intestinal Urd phosphorylase activity *in vitro*

BAU (μg/ml)	nmol Urd degraded/ min per mg protein	% Inhibition
0	23.0 ± 0.8	
1	14.0 ± 1.9	39%
10	1.6 ± 1.0	93%
100	0.2 ± 0.2	99%

A partially purified preparation of murine intestinal Urd phosphorylase was prepared by scraping the intestinal epithelium, homogenizing the tissue in phosphate buffer, centrifuging at 100,000 g for 30 min and desalting with a Sephadex G 25 column. The reaction mixture contained 200 μl enzyme, 25 μl 10 m*M* Urd, and 25 μl water or 10 × BAU solution. After a 15-min reaction at 37° C, protein was removed with TCA and the amount of Urd converted to uracil was determined by HPLC

Table 5. A comparison of plasma and intestinal BAU levels

Time (h)[a]	Plasma (μg/ml)	Intestine (μg/g tissue)
2	25 ± 2	21 ± 2
4	16 ± 1	13 ± 2
6	9 ± 1	10 ± 1

[a] Normal CD_8F_1 male mice received 3,000 mg/kg Urd plus 240 mg/kg BAU p.o. At 2, 4, and 6 h after determination, four animals were sacrificed, and the plasma and intestine were analyzed by HPLC for BAU content 1 h after Urd plus BAU administration

A, Table 2), plasma Urd averaged 2.9 m*M* and tissue levels were in excess of 2 μmol/g. At 4 h, plasma Urd had fallen to 0.6 m*M* and tissue levels were similarly lower, indicating that these extremely high tissue levels were maintained only in the face of pressure from very high plasma Urd.

The lower plasma Urd levels achieved with oral Urd (plus BAU) produced lower tissue levels of Urd, but, again, tissue levels were similar to the plasma Urd level (group B, Table 2) at 2 and 4 h except in the gut. For the small intestine, the very high levels achieved (well in excess of millimolar Urd) were likely due to the locally high concentration of Urd in the intestine following oral administration.

Of the tissues tested, the small intestine revealed by far the highest levels of Urd phosphorylase (Table 3) [4]. We tested the ability of BAU to inhibit this enzyme in vitro. The conversion of Urd to uracil was inhibited by 93% (Table 4) by BAU at a dose of 10 μg/ml, a level that was achieved in vivo in both plasma (Fig. 2) and intestine (Table 5) at the BAU dose used.

Discussion

The weekly maximum tolerated dose (MTD) of FUra as a single agent is 100 mg/kg in tumor-bearing CD_8F_1 mice; when FUra is given in combination with other anticancer agents, it is usually necessary that the dose be lowered below this level to avoid drug mortality. However, under the protection afforded normal tissues by delayed Urd rescue, weekly FUra can be safely and substantially raised well above its MTD – e.g., to 150 mg/kg in the drug combination reported here, and to 225 mg/kg as a single agent [18]

– with a consequent increase in antitumor efficacy. The ability of Urd to rescue FUra toxicity in human normal tissues has previously been demonstrated [20, 25] and several studies evaluating the potential for the combination of high-dose FUra followed by Urd rescue to improve the antitumor selectivity of FUra at the clinical level are ongoing [7, 21]. It should be noted that the inception of rescue with Urd must be delayed to a time well after FUra administration. If these agents are co-administered, the toxicity of FUra is increased in both tumor and normal tissues without improvement of the therapeutic ratio [1, 6].

The biochemical mechanism by which Urd rescue improves the therapeutic index of FUra has not been established. It has been suggested that increased intracellular concentrations of UTP (derived from exogenous Urd) compete with FUTP at the level of RNA polymerase such that after the initial period of incorporation, further formation of (FUra)RNA is decreased, the enhanced antitumor selectivity presumably being due to differing sensitivities to (FUra)RNA between the FUra-sensitive tumors and normal tissues [13, 16, 22].

Similarly, the plasma levels of Urd necessary to reverse the toxicity of FUra to normal tissues have not been established. Although intraperitoneal Urd produces millimolar plasma and tissue levels of Urd (Fig. 1 and Table 2), it is clear from the data that such extremely elevated levels are not necessary, since oral Urd plus oral BAU produces peak plasma levels of only 200–300 μM and is equally effective as a rescue regimen as high-dose i.p. Urd. It seems likely that the critical factor in rescuing FUra toxicity is the maintenance of elevated plasma Urd levels for an extended period of time (e.g., 2 or more days). As seen in Fig. 1, short exposures to 50 μM Urd appear to be inadequate for rescue purposes, since 3,000 mg/kg oral Urd failed to provide adequate protection against FUra combination chemotherapy (Table 1). However, maintenance of plasma Urd levels above 70 μM for 2 days, as was accomplished with q8h Urd plus BAU (Fig. 2), effectively prevented FUra toxicity to the host.

Of the tissues tested, by far the highest level of Urd phosphorylase was found in the intestinal epithelium (Table 3). The BAU dose used, 240 mg/kg, produced BAU levels of at least 20 μg/g tissue (Table 5), a concentration shown to inhibit this enzyme substantially in vitro (Table 4). Control experiments in which mice received oral Urd plus BAU containing [^{14}C] inulin showed that the saline flush procedure removed about 85% of the ^{14}C radioactivity, suggesting that the intestinal levels of Urd and BAU reported in Tables 2 and 5 were largely contained within the cells rather than on the outside.

The finding that oral Urd can satisfactorily substitute for parenteral Urd in improving the therapeutic index of FUra appears significant in view of the clinical problems involved with the parenteral administration of Urd through a central venous catheter for several days following each course of FUra therapy. Furthermore, since oral Urd can result in diarrhea, which is a dose-limiting side effect in patients [26], the demonstration that the concomitant administration of oral BAU enables the achievement of an optimal rescue effect from a lower oral dose of Urd without compromising FUra's antitumor activity offers additional encouragement that the oral Urd plus BAU approach for selective rescue of FUra toxicity may have clinical advantage.

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