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EFFECT OF 5-BENZYLACYCLOURIDINE, A POTENT INHIBITOR OF URIDINE PHOSPHORYLASE, ON THE METABOLISM OF CIRCULATING URIDINE BY THE ISOLATED RAT LIVER

ANNE MONKS,* OVELLA AYERS and RICHARD L. CYSYK
Laboratory of Chemical Pharmacology, Division of Cancer Treatment, National Cancer Institute, National Institues of Health, Bethesda, MD 20205, U.S.A.

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Abstract—5-Benzylacyclouridine (BAU) is a specific inhibitor of uridine phosphorylase, the first enzyme in the catabolism of uridine. It was found that 20 and 100 μM BAU dramatically reduced the rapid clearance of trace amounts of either [^{14}C]uridine or hyperphysiologic concentrations of non-labeled uridine by the isolated rat liver perfused with an artificial oxygen carrier. In the absence of exogenously added uridine, non-treated livers maintained circulating concentrations of 1–2 μM uridine. In the presence of 20 μM BAU, these concentrations were increased 2- to 3-fold higher than physiologic levels (1.4 ± 0.6 μM) and remained elevated for the duration of the experiment (120–160 min). In the presence of 100 μM BAU, uridine concentrations rose continuously at rates of between 80 and 150 nmoles per hr per g of liver, and the clearance of a single radioactive spike of uridine was reduced extensively. The half-life of a uridine spike was extended 2-fold in the presence of 20 μM BAU and 5- to 6-fold in the presence of 100 μM BAU. Exogenously added uridine (15 and 40 μM) was cleared rapidly by non-treated livers, with a half-life of approximately 10 min. However, BAU at a concentration of 20 μM increased the half-life of 15 or 40 μM uridine added to the perfusate by approximately 10-fold. A 100 μM concentration of BAU inhibited the removal of 40 μM circulating uridine, but with 15 μM uridine there was a continuous increase in the circulating concentration similar to that seen in the absence of added uridine. We conclude that extensive inhibition of uridine phosphorylase occurs at 100 μM BAU and partial inhibition at 20 μM BAU. These data indicate independent catabolic and excretory functions of the rat liver with respect to uridine.

Uridine phosphorylase (UrdPase) functions as an important enzyme in the hepatic catabolism of uridine [1, 2] and is thought to limit the *in vivo* cytotoxicity of certain pyrimidine nucleoside analogues (e.g. fluorodeoxyuridine, fluorouridine, bromodeoxyuridine and iododeoxyuridine [3, 4]) by cleaving them to the less active bases. We reported recently [5] that the isolated perfused rat liver can maintain circulating uridine concentrations similar to those found in rat plasma by a combination of continual excretion of this nucleoside into the perfusate and rapid removal of circulating uridine entering the liver, so that an apparent steady-state concentration of 1–2 μM is maintained. Other recent studies [6, 7] using different methods have shown that uridine entering the liver is catabolized extensively and replaced in the circulation largely from hepatic pools of acid-soluble uracil nucleotides.

Less than 2% of the uridine removed by the liver is salvaged and recovered in the pyrimidine nucleotide pool; the remainder is catabolized rapidly to products beyond uracil in the pyrimidine catabolic pathway. The first step in this degradation of uridine is the conversion to uracil by UrdPase. Recently, 5-benzylacyclouridine (BAU) has been synthesized and found to be a potent inhibitor of UrdPase isolated from Sarcoma 180 cells and mouse livers (K_i = 98 nM) but had no effect on thymidine phosphorylase, uridine-cytidine kinase, or thymidine kinase [8]. In the present study, we have examined the effect of BAU on the export and removal of uridine by the isolated perfused rat liver. Our data show that the export and removal activities of the liver are separate and independent functions. The implications of these findings on the use of BAU to potentiate the effectiveness of certain pyrimidine nucleoside analogues *in vivo* and to effect post-treatment rescue of normal cells are discussed.

METHODS

The BAU was synthesized by Dr. Shih-Hsi Chu, of Brown University [8]. Rat livers, isolated surgically from natural circulation, were maintained on a MX Ambec 210 perfuser (MX International Inc., Aurora, CO). The perfuser has the facility to collect samples of the liver effluent or samples from the perfusate reservoir. Fluosol-43 (manufactured by the Green Cross Corp., Japan, and distributed by Gibco, New York, NY) was used as an inert oxygen carrier for these perfusion experiments. The use of Fluosol-43 has eliminated artifacts resulting from the

* Author to whom correspondence should be sent: Dr. Anne Monks, National Institutes of Health, Building 37, Room 5A13, Bethesda, MD 20205, U.S.A.

use of erythrocytes which can interact with circulating uridine [5]. Fluosol-43 is an emulsion of perfluorotributylamine (0.86 M) and pluronic F-68 (polyoxypropylene-polyoxyethylene copolymer, 26 mg/ml), NaCl (100 mM), KCl (4.6 mM), $CaCl_2$ (2.5 mM), $MgCl_2$ (2.1 mM), $NaHCO_3$ (25 mM), glucose (1 mM) and hydroxyethyl starch (30 mg/ml).

Liver function was monitored by means of serum glutamyl pyruvic acid transaminase (SGPT) levels measured on an ABA 100 biochromatic analyzer (Abbott Laboratories, Dallas, TX). An Ambec pressure indicator, series 1020 (MX International), monitored resistance to the 15 ml/min flow of perfusate, and results were discarded if the pressure increased by more than 5 mm Hg during the course of the perfusion. Liver appearance was also monitored and, if the liver became swollen and discolored, the perfusion was halted and the experiment terminated. Bile was collected for the duration of the experiment, and rates of excretion (41.2 ± 11.7 μl per hr per g) were found to compare well with published rates of bile production of 30–48 μl per hr per g [9]. The maintenance of normal bile flow is a measure of the viability of the liver.

Male Sprague–Dawley rats (240 ± 20 g) were anesthetized by intraperitoneal sodium pentobarbital, the bile ducts were cannulated, and their livers were surgically isolated from the circulation by the method of Hems *et al.* [10]. The isolated livers were allowed to equilibrate on the perfusion apparatus for 45–60 min prior to the addition of uridine. During this time, control livers were perfused with only 250 ml Fluosol-43, while experimental livers were perfused with 250 ml Fluosol-43 plus 20 or 100 μM BAU. After the equilibration period, either 5 μCi of [^{14}C-U]uridine (529 mCi/mmole) or a 15 or 40 μM concentration of cold uridine was added to the perfusate to begin the experiment.

Samples were prepared for analysis as follows. Aliquots (3 ml) were collected from the perfusate at intervals of between 5 and 15 min for the duration of the experiment (2–3 hr). An extra 1-ml aliquot was collected at 45-min intervals to be analyzed for SPGT as a measure of hepatic cell necrosis [11]. Samples were centrifuged at 25,000 *g* to sediment the fluorocarbon particles. Two milliliters of supernatant fraction was mixed with 10 nmoles of 5-methyl cytidine (internal standard) and placed in boiling water for 2 min to inactivate any enzyme activity which could affect nucleoside concentrations. These samples were then treated with xanthine oxidase to convert hypoxanthine to xanthine, as the former interferes with the uridine peak on the chromatogram [12]. A volume of 400 μl of each sample was injected by a WISP 710B automatic injector (Waters Associates Inc., Milford, MA) onto an Altex model 312 high pressure liquid chromatograph (HPLC) equipped with a Whatman PXS 5/25 ODS column and measured by the method of Karle *et al.* [13]. The samples were eluted with acetate buffer (0.01 M sodium acetate plus 0.01 M acetic acid, pH 4.5) plus 2.5% methanol at 1.5 ml/min. Peak heights were recorded at both 254 nm (Altex model 153 u.v. detector) and 280 nm (Altex model 155-30 variable wavelength u.v. detector). The ratio of corresponding peak heights at each wavelength, when constant, indicated the absence of contaminating substances. The uridine peak was eluted in 6 min and the 5-methyl cytidine in 9 min under these conditions. The ratio of peak heights of uridine:5-methyl cytidine was used to quantitate the uridine concentration from a standard curve. Standard curves were linear from 0 to 25 μM uridine.

For radioactive samples, total radioactivity was measured in 2 × 100 μl aliquots of the supernatant fractions, and, after injection of 400 μl onto the HPLC, fractions of column effluent were collected at 0.4 min intervals by an LKB model 2111 multirac collector (LKB Instruments Inc., Rockville, MD) and counted in a Beckman LS 9000 scintillation counter to determine the distribution of radioactivity in these samples. Radioactive data were expressed as dpm/ml perfusate in the uridine fraction (dpm uridine/ml) and specific activity of uridine (dpm/nmole) which was calculated by:

$$\frac{\text{(dpm collected in uridine peak—background dpm)/ml injected}}{\text{nmole/ml uridine}}$$

Kinetic parameters were calculated from the radioactive clearance data as follows:

$$\text{Elimination rate constant, min}^{-1}\text{ (Kelim)} = \frac{1}{\text{slope of semilog plot}}$$

$$\text{Elimination half-life, min } (T_{1/2}) = \frac{0.693}{\text{Kelim}}$$

At the end of an experiment, the liver was excised rapidly from the carcass and placed immediately in liquid nitrogen. Once frozen, it was weighed and fragmented by shaking, and a 2-g portion (still frozen) was added to 10 ml of 10% trichloroacetic acid (TCA) and 1 μmole of 5-methyl cytidine (internal standard). The tissue was homogenized immediately in the TCA, and the precipitate was removed by centrifugation at 1000 *g* for 15 min. The acid-soluble supernatant fraction was washed in a 1:2 mixture of freon:tri-*n*-octylamine to remove the excess TCA and neutralize the supernatant. Enzymatic degradation of uracil nucleotides to uridine was then effected by the treatment of the supernatant fraction with snake venom and alkaline phosphatase. A 400-μl aliquot was injected on the HPLC, and fractions were collected to determine the radioactivity in the uracil nucleotide pool.

RESULTS

Figure 1 compares uridine concentrations in the perfusate produced by non-treated or BAU (20 and

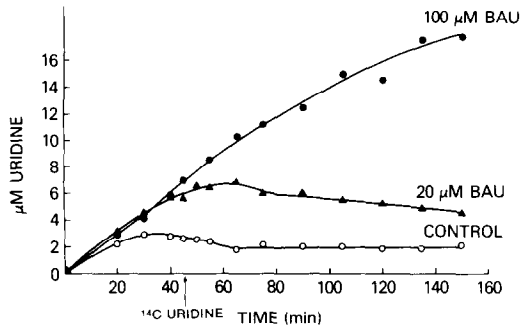

Fig. 1. Uridine concentrations in the circulating perfusate from control (○—○), 20 μM BAU-treated (▲—▲) and 100 μM BAU-treated (●—●) livers. The radioactive uridine (see Figs. 2–4) was added at 45 min.

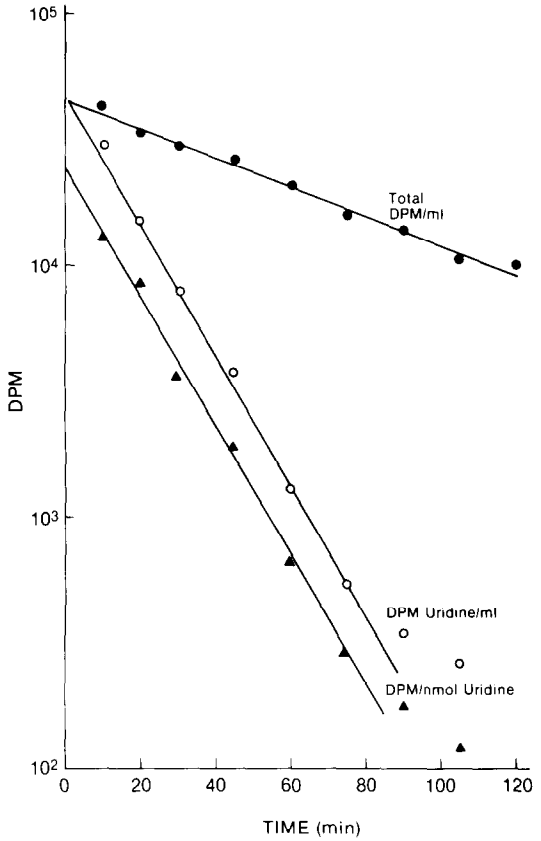

Fig. 2. Fate of a radioactive spike of uridine in the perfusate of a control livers. Key: (●—●) the dpm/ml of total radioactivity, (○—○) the dpm/ml of perfusate which is confined to uridine, and (▲—▲) the specific activity of uridine.

100 μM)-treated livers. The non-treated liver maintained a steady-state concentration in the perfusate of 1–2 μM uridine. We have shown previously [5] that this circulating concentration is a result of continuous excretion of uridine, as there is extensive removal of any uridine entering the liver. This rapid catabolism is illustrated in Fig. 2 by the fate of a single radioactive spike of [^{14}C-U]uridine in an untreated liver. The radioactive uridine was added to the perfusate after 45 min when a steady state of circulating uridine had been reached. The kinetic parameters from these data and all other radioactive perfusion experiments are shown in Table 1. All these data are dependent on the characteristics of the perfusion system, where the volume in which uridine is distributed (250 ml) and the flow rate through the liver (15 ml/min) affect quantitative, but not comparative, data [5]. This volume and perfusion rate are kept constant throughout all experiments.

There was a rapid removal of [^{14}C]uridine from

Table 1. Kinetic parameters of a single [^{14}C]uridine spike in the perfusate of non-treated or BAU-treated isolated rat livers

	Radioactivity (dpm/ml perfusate)				Specific activity (dpm/nmole uridine in perfusate)	
	Total radioactivity		Uridine fraction			
1 (A) Half-life (min)						
Control	55	53.5*	8.3	9.8*	8.2	9.9*
	52†		11.2		11.6	
20 μM BAU	97		23		22	
	60		42		42	
	53†	70.0	20	28.3	21	28.3
100 μM BAU	91		58		35	
	90†	90.5	64	61	42	22.5
1 (B) Kelim (min^{-1})						
Control	0.0126	0.0130*	0.0830	0.0724*	0.0842	0.0719*
	0.0133†		0.0618		0.0596	
20 μM BAU	0.0071		0.0297		0.0317	
	0.0131†	0.0106	0.0345	0.0269	0.0324	0.0269
	0.0116		0.0166		0.0166	
100 μM BAU	0.0076	0.0077	0.0120	0.0114	0.0198	0.0182
	0.0078†		0.0108		0.0166	

* Mean of individual values.
† Illustrated in Figs. 2–4.

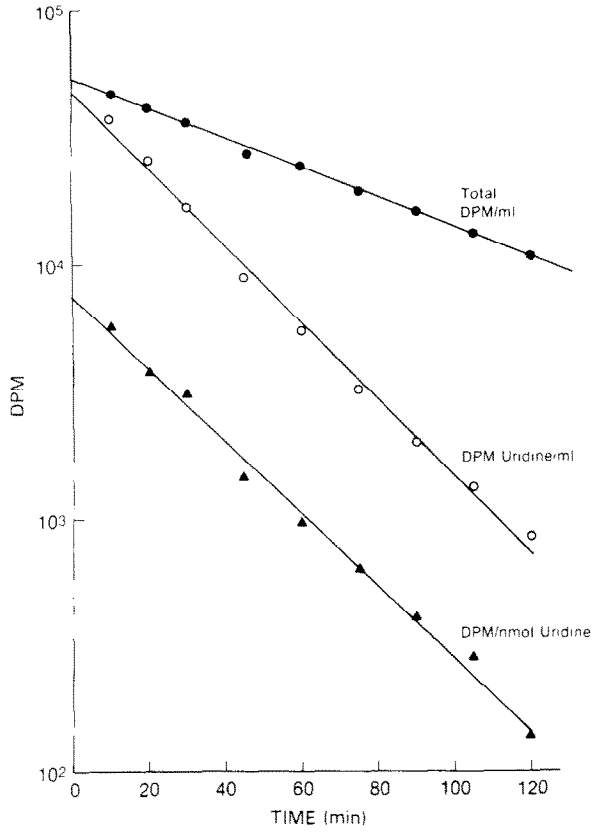

Fig. 3. Fate of a radioactive spike of uridine in the perfusate of a 20 μM BAU-treated liver. Key: (●—●) the dpm/ml of total radioactivity, (○—○) the dpm/ml of perfusate which is confined to uridine, and (▲—▲) the specific activity of uridine.

the perfusate by the non-treated livers with a half-life of approximately 10 min and an elimination constant (Kelim) of approximately 0.07 min^{-1}. The kinetic curves of the dpm uridine/ml and dpm/nmole are similar, indicating the equilibrium state of the circulating uridine (1–2 μM) maintained in the perfusate (Fig. 1). The slow loss of total radioactivity (dpm/ml total, $T_{1/2} \simeq 50$ min) reflects catabolism of uridine by the liver and excretion of radioactive metabolites into the perfusate. As little or no ^{14}C could be detected in the area of uracil elution on the HPLC, these metabolites excreted by the liver are products beyond uracil in the catabolic pathway of uridine. Therefore, with non-treated livers, total levels of circulating uridine similar to those found in rat plasma are maintained. However, from the elimination half-life of non-treated livers ($T_{1/2} = 9.9$ min), it can be calculated that in one complete circulation of the perfusate through the liver (16.7 min for 250 ml perfusate at 15 ml/min), 75% of uridine entering the liver is removed. This was confirmed by direct measurement of the specific activity of the uridine entering and exiting the liver. The specific activity of the uridine immediately exiting the liver was approximately 25% of that entering the liver at that time. When non-treated livers were analyzed for uracil nucleotides at the completion of experiments, 2.3 ± 0.4% of the total administered radioactivity was found in the uracil nucleotide pool. To accommodate this extensive degradation of circulating uridine, the liver must excrete 100–200 nmole per hr per g of liver to maintain the 1–2 μM uridine seen in the perfusate.

Figure 1 illustrates the effects of two concentrations of BAU on the level of circulating uridine. With 20 μM BAU the uridine concentration was higher than control at steady state, ranging from 4 to 8 μM in all experiments. In experiments with this dose of BAU, there were variations in the response of different livers as shown by the kinetic parameters (Table 1) for elimination of the [^{14}C]uridine spike. Figure 3 illustrates one experiment with 20 μM of added BAU. The half-life of dpm/nmole of uridine was increased to 20 or 40 min, and the similarity of the half-life of dpm uridine/ml reflects the essentially unchanging concentration of circulating uridine. The elimination rate constant of uridine was also reduced markedly in the presence of 20 μM BAU.

Circulating uridine concentrations in the perfusate of 100 μM BAU-treated livers increased with time (Fig. 1). This increase was a result of effective inhibition of UrdPase by this concentration of BAU. Figure 4 illustrates the elimination of a radioactive spike of uridine with kinetic parameters shown in Table 1. There was a vastly reduced rate of elimination of the radioactive uridine reflected in the longer half-lives, reduced Kelim of the specific activity (dpm/nmole), and, particularly, the dpm

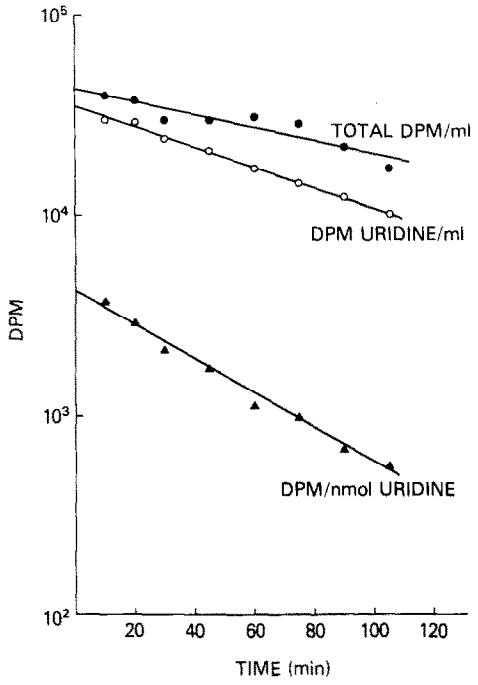

Fig. 4. Fate of a radioactive spike of uridine in the perfusate of a 100 μM BAU-treated liver. Key: (●—●) the dpm/ml of total radioactivity, (○—○) the dpm/ml of perfusate which is confined to uridine, and (▲—▲) the specific activity of uridine.

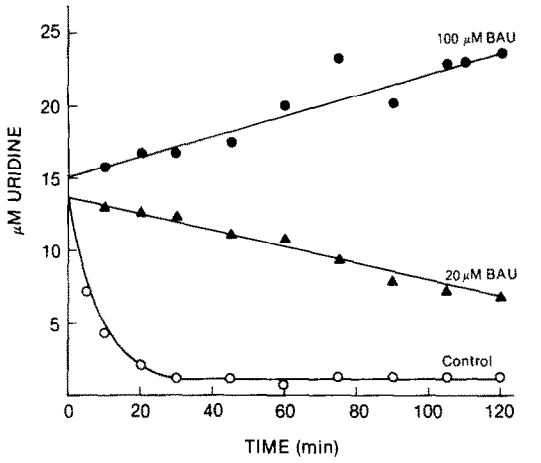

Fig. 5. Elimination of 15 μM uridine from the perfusate by control (○—○), 20 μM BAU-treated (▲—▲) and 100 μM BAU-treated (●—●) livers.

uridine/ml of perfusate. The difference in the kinetic parameters of dpm uridine/ml and dpm/nmole of uridine is a measure of the increasing total uridine in the perfusate resulting from the continued excretion of uridine in the presence of an inhibitor of its degradation. Using the final perfusate concentration of uridine attained by the two 100 μM BAU-treated livers, the minimal rates of uridine excretion were calculated to be 80 and 155 nmoles per hr per g, which compares favorably with the rate determined for non-treated livers (100–200 nmoles per hr per g). The reduction in the rate of uridine elimination in BAU-treated livers is due to the inability of the liver to catabolize the administered radioactive uridine as a result of inhibition of UrdPase. Thus, with 100 μM BAU, catabolism of circulating uridine was reduced so drastically that much of it remained in the perfusate. However, the excretory function of the liver was apparently unaffected, and circulating uridine concentrations continued to rise for the duration of the experiment.

Total radioactivity decreased more slowly from the perfusate of BAU-treated livers, as shown by a longer half-life and a reduced Kelim. This increase in the total circulating radioactivity may be due to the inability of the liver to store the nucleoside uridine as efficiently as it does the uridine metabolites. Measurement of radioactivity incorporated into the uracil nucleotide pool of 20 and 100 μM BAU-treated livers was found to be 10.1 ± 6.8% of the total radioactivity administered, which suggests a more efficient salvage of uridine in the presence of BAU.

Figures 5 and 6 show the clearance by control and BAU-treated livers of hyperphysiological concentrations of uridine (15 or 40 μM). Control livers rapidly reduced the circulating uridine concentration to approximately 1–2 μM and maintained this concentration. The 20 μM BAU-treated livers very slowly reduced the circulating concentration of uridine at a rate of approximately 5 nmoles per ml per hr following both the 15 and 40 μM initial uridine concentrations. With 100 μM BAU-treated livers, there was no net removal of uridine from the circulating perfusate with a starting concentration of 15 μM uridine, but, rather, a gradual increase in circulating levels. However, with 100 μM BAU and 40 μM uridine there was no increase in the circulating concentration, which remained steady for the initial 60 min and then slowly declined. This may be due

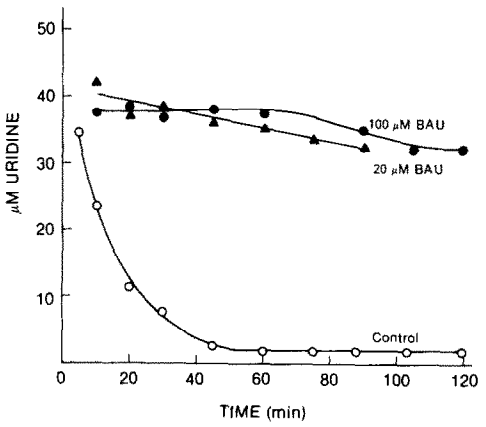

Fig. 6. Elimination of 40 μM uridine from the perfusate by control (○—○), 20 μM BAU-treated (▲—▲) and 100 μM BAU-treated (●—●) livers.

to successful competition for the UrdPase sites in the liver by the higher concentration of uridine.

DISCUSSION

The liver functions as an important organ in the regulation of circulating concentrations of uridine [5–7]. The isolated perfused rat liver excretes uridine into the perfusate at a relatively constant rate but removes virtually all uridine entering the liver in a single pass. Uridine removed by the liver does not re-enter the pyrimidine nucleotide pool but is catabolized to products subsequent to uracil in the pyrimidine catabolic pathway. These studies suggest that the uridine excretory and removal processes of the liver function independently of each other. To fully resolve whether these processes are independent or interdependent functions, we evaluated the effects of BAU, a potent inhibitor of UrdPase, on the catabolism and excretion of uridine by the isolated perfused rat liver.

The results show that micromolar concentrations of BAU dramatically reduced the rapid clearance and catabolism of trace amounts of [^{14}C]uridine and hyperphysiologic concentrations of non-labeled uridine. The half-life of a single [^{14}C]uridine spike was increased 2-fold by 20 μM BAU and 5- to 6-fold by 100 μM BAU. Also, whereas 15 and 40 μM uridine were cleared with a half-life of approximately 10 min, there were only slight reductions in uridine concentrations over 120 min in the presence of 20 μM BAU. With the 100 μM BAU-treated liver, there was no reduction in the 15 μM uridine concentration, but rather a sustained increase. However, an initial 40 μM uridine concentration did not increase, indicating that some effective competition for UrdPase sites may be occurring at this concentration.

BAU appeared to have no effect on the excretion of uridine by the liver. In the presence of 100 μM BAU and no exogenously added uridine, the circulating concentration of uridine rose throughout the experiment. When the same experiment was repeated with 20 μM BAU, the uridine concentration rose until it was 2- to 3-fold higher than physiologic levels and then remained at this elevated level for the remainder of the experiment. Metabolism of BAU was not studied but may have been a factor in the inability of 20 μM BAU to sustain its inhibition of phosphorolysis. Alternatively, there may be a more effective competition for uridine nucleoside phosphorylase by the elevated uridine concentration at 20 μM BAU than at 100 μM BAU.

These data demonstrate the independent nature of the excretory and catabolic functions of the liver. The inhibition of UrdPase had no apparent effect on the continual excretion of uridine by the livers; thus, there appears to be no sensory effect by the liver of circulating uridine. Uridine entering the hepatic capillary beds is extracted and rapidly broken down, with most of the metabolites released into the circulation.

BAU was found to increase the amount of uridine salvaged by the liver and converted to pyrimidine nucleotides. In the normal, non-treated liver, less than 2% of the circulating uridine was salvaged; the remainder was catabolized to products beyond uracil in the pyrimidine catabolic pathway. In the presence of BAU, 5–10% of the circulating uridine removed by the liver was recovered in the pyrimidine nucleotide fraction of the liver. These data indicate that there are active kinases in rat liver, but that circulating uridine may be degraded before it has access to the kinases. Tseng and Gurpide [1] found that a large fraction of exogenous uridine never crossed the hepatic cell membrane into the cell but was degraded to uracil by a membrane bound UrdPase and returned to the medium as uracil. Our data show that, when UrdPase is successfully inhibited, there is still only minimal salvage of uridine. Thus, an efficient *de novo* pyrimidine pathway or an extremely efficient system for reutilizing nucleosides within the liver itself is responsible for maintenance of continual output of uridine.

BAU was synthesized as an inhibitor of UrdPase to potentiate the cytotoxic properties of pyrimidine nucleoside analogues by preventing their cleavage to the less active bases [8]. One aspect to be considered in such a drug combination is the effect of UrdPase inhibition on the metabolism of certain endogenous pyrimidine nucleosides that have the potential of modifying the cytotoxic effects of the nucleoside analogues under study. For example, if UrdPase is inhibited to prolong the tissue exposure of fluorodeoxyuridine (FdUrd), would there be a resultant increase in plasma uridine concentration that could antagonize the FdUrd effect? The results of the current study indicate that this might be an important factor limiting the usefulness of BAU.

BAU could be an important tool with which to modify endogenous levels of circulating uridine. In a recent preliminary report by Levy *et al.* [14], administration of 30 mg/kg BAU to mice resulted in a vastly increased half-life of a tracer dose of uridine and approximately a 20-fold increase in plasma uridine levels. In rats, following this dose of BAU, we have observed a 4-fold increase in the circulating uridine concentration (unpublished observations). The importance of endogenous concentrations of circulating uridine during chemotherapy is unknown but is a subject of speculation [7, 15, 16]. Uridine can reduce the toxicity of certain inhibitors of the *de novo* pyrimidine pathway, e.g. pyrazofurin and *N*-(phosphonacetyl)-L-aspartate (PALA) [17, 18]. Additionally, administration of uridine has been proposed for rescue therapy following treatment of patients with 5-fluorouracil (5-FU). Recently, Klubes *et al.* [19] demonstrated that continuous infusion of high levels of uridine for 5 days could reverse the lethal toxicity of a single dose of 5-FU in mice. Administered uridine has a very short half life (5–10 min) in a laboratory animals ([7], unpublished observations), which can probably be accounted for by the virtual first-pass catabolism of uridine by the liver [5, 6]. Thus, inhibition of UrdPase by BAU to reduce the rate of uridine removal could prove to be a more efficient method for rescue of normal cells following 5-FU treatment. In this manner, a less invasive schedule of uridine administration than continuous infusion could be utilized.

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