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BENZYLACYCLOURIDINE

PHARMACOKINETICS, METABOLISM AND BIOCHEMICAL EFFECTS IN MICE*

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Abstract—The pharmacokinetics, tissue distribution and urinary excretion of the uridine (Urd) phosphorylase (EC 2.4.2.3) inhibitor 5-benzylacyclouridine (BAU) were studied in C57BL/6 female mice by reverse-phase HPLC. The plasma clearance of BAU after i.v. administration followed first-order kinetics with a half-life of approximately 36 min. Other pharmacokinetic parameters such as volume of distribution (17 ml), clearance rate (0.3 ml/min) and the elimination rate constant (0.019 hr^{-1}) were relatively constant over a dose range of 5 to 240 mg/kg when based on a first-order clearance model. Following oral administration, BAU was rapidly absorbed from the gut; peak plasma concentrations occurred within 30 min and were approximately 60% of equivalent i.v. doses. The distribution of BAU between plasma and most major organs was rapid and efficient, the exceptions being spleen and brain, which maintained only 40% and 10%, respectively, of the plasma BAU concentration. Approximately 41% of the injected dose of BAU was recovered intact in urine within 24 hr. Another 27% appeared as a more polar metabolite which, at a concentration of 50 μM, did not inhibit murine Urd phosphorylase. A near linear relationship was observed between the injected dose of BAU and its ability to increase the plasma concentration of Urd; i.v. injections of 30, 120 and 240 mg/kg increased plasma Urd 3-, 7- and 15-fold respectively. The utility of these data in the design of combination chemotherapy regimens containing BAU and related compounds is discussed.

Benzylacyclouridine (BAU‡) is one of a series of 5-substituted acyclonucleosides synthesized and studied by Niedzwicki, Chu and co-workers [1, 2]. BAU shares common structural and biochemical features with the 5-benzyluracils [3] and the pyrimidine acyclonucleosides [4]. It is a potent and specific inhibitor of the enzyme uridine (Urd) phosphorylase (EC 2.4.2.3) but does not inhibit thymidine phosphorylase (EC 2.4.2.4) [1].

Initial interest in this compound focused on its use as a biochemical tool to study pyrimidine catabolism and salvage, both *in vitro* and *in vivo*. Studies in our laboratory indicated that, *in vivo*, the selective inhibition of Urd phosphorylase by BAU markedly increased the plasma concentration of Urd [5, 6]. Studies by Monks *et al.* [7] supported this finding and indicated that *in vitro* BAU could inhibit the ability of perfused rat liver to clear Urd from the perfusate. BAU has also been shown to have potential therapeutic utility. Chu *et al.* [8] have shown *in vitro* that the growth inhibitory potential of FdUrd against human lung (LX-1) and human pancreatic carcinoma (DAN) cells is enhanced by the simultaneous addition of BAU. We have shown that, when administered 24 hr after fluorouracil (FUra), BAU can enhance the therapeutic effectiveness of FUra against advanced transplants of colon tumor 38 in mice [9]. More recently, we reported that the increased effectiveness of FUra in this combination may be a reflection of the ability of BAU to increase dramatically, in a tissue-specific manner, tissue Urd pools [10]. Chu *et al.* [11] also have reported that *in vivo* BAU can protect mice from FdUrd-induced toxicity.

These findings suggest that BAU has potential clinical utility in combination with the fluorinated pyrimidines or other agents that disrupt *de novo* pyrimidine nucleotide biosynthesis. To permit evaluation of BAU in Phase I trials, we now report on its plasma clearance, tissue distribution and urinary excretion in mice as well as its biochemical effects. Preliminary aspects of these findings have been reported [6, 12].

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‡ Abbreviations: BAU, 5-benzylacyclouridine, [5-benzyl-1-(2′-hydroxyethoxymethyl)uracil]; Urd, uridine; FdUrd, 5-fluoro-2′-deoxyuridine; FUra, 5-fluorouracil; FUrd, 5-fluorouridine; $T_{\frac{1}{2}}$, plasma half-life; V_d, volume of distribution; K_e, elimination rate constant; and AUC, area under the curve.

MATERIALS AND METHODS

All experiments utilized 3-month-old C57B1/6 female mice (hereafter called C57) obtained from the National Institutes of Health. The tissue distribution of BAU was measured in C57 female mice bearing advanced (0.4 to 1.0 g) subcutaneous transplants of colon tumor 38 [13, 14].

BAU was the gift of Dr. S. Chu of Brown University. Low specific activity [^{3}H]BAU (0.5 mCi/mmol) was prepared by Dr. P. K. Chang at Yale University by isotopic exchange between BAU and tritiated water in the presence of K_2PtCl_4 according to the method developed for labeling phenylalanine [15]. [2-^{14}C]FUrd (56 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). All other reagents were purchased from the Baker Chemical Co. (Phillipsburg, NJ). For injection purposes BAU was dissolved in warm ethanol and diluted 1:5 in saline with the drug concentration adjusted so that 0.1 ml was injected/10 g of body weight. The animals received 0.2 ml/10 g of body weight when the 240 mg/kg dose was administered because of the limited solubility of BAU. Intravenous injections were administered via the tail vein, whereas the oral administration of BAU was accomplished by an intubation needle.

The BAU content of extracts of biological fluids or tissues was quantitated on a modular HPLC system utilizing an Altex model 100 pump, an Altex model 153 UV detector (254 nm) and a Kipp–Zonen model BD-41 chart recorder. The column was a Rainin Microsorb C-18 (25 cm × 4.6 mm) maintained at 20° and eluted at 1.0 ml/min with 25% MeOH (in water). Samples (50–200 μl) were injected with a Waters model WISP 710A automated sample processor. Under these conditions, the retention time for BAU was 37 min with the relationship between peak area and BAU concentration linear over a range of 0.5 to 500 μM.

The plasma clearance of an i.v. bolus of BAU was studied in groups of six C57 mice that received a single injection of BAU at doses ranging from 5 to 240 mg/kg. The upper i.v. dose was limited in these studies by the ethanol content and the limited solubility of BAU. At various times following the injection, 200 μl of whole blood was collected in heparinized Natelson pipettes. The plasma was separated and deproteinized with 15% trichloroacetic acid (TCA) (preliminary equilibrium dialysis experiments indicated that less than 5% of BAU was bound to plasma protein). The TCA was extracted with trioctylamine in freon as previously reported [9], and there was no apparent loss of BAU from the aqueous material after trioctylamine extraction. Mathematical analysis of the plasma kinetics of BAU was carried out by the method of Greenblatt and Koch-Weser [16, 17] and confirmed utilizing the PCNONLIN program [18].

Each plasma sample was also chromatographed to determine its Urd concentration by our previously reported HPLC method [9].

The plasma kinetics of orally administered BAU were determined in the same manner in groups of six C57 mice that received single oral injections at doses of either 30 or 120 mg/kg.

The tissue distribution of BAU was monitored after single i.v. injections at doses of either 30 or 120 mg/kg in groups of four C57 mice. Between 30 and 300 min, 200 μl of whole blood was obtained from each animal and prepared as above for HPLC analysis of its BAU content. The animal was then immediately killed by cervical dislocation; liver, kidneys, spleen, lung, brain, colon tumor 38, and striated muscle were removed, weighed and homogenized in 2 vol. of 15% TCA at 0°. In addition, 15 cm of gut, starting at the pyloric sphincter, was removed, flushed with ice-cold saline, weighed, and homogenized in 2 vol. of 15% TCA. After centrifugation at 1500 *g* for 5 min, the supernatant fraction was removed from each tissue sample and mixed with an equal volume of 1 N trioctylamine in 1,1,2-trichlorotrifluoroethane (freon) to extract the TCA. The aqueous layer was removed, and its BAU content was determined by the HPLC method described above.

To assay the amount of intact BAU and metabolites excreted in urine, groups of three C57 mice received a single i.v. injection of BAU at 15 to 120 mg/kg or a 30 mg/kg injection of [^{3}H]BAU (~1 μCi/mouse). Immediately after drug administration, mice were placed in individual metabolic cages and urine was collected in petri dishes over the next 24 hr. After the collection period, the urine specimen was prepared for analysis by a modification of previously reported methods [19]. The urine was dried, and the residue was suspended in 4 ml of distilled water and centrifuged at 5000 *g* for 5 min to remove particles. To an aliquot of the supernatant fraction was added 2 vol. of 15% TCA at 0°. The acid-soluble material was then prepared and assayed by the isocratic HPLC method described above. In addition to this analysis, the urine collected from mice that received [^{3}H]BAU was assayed by non-isocratic HPLC methods using a methanol gradient to detect radioactive peaks which did not elute with BAU. A Rainin C-18 column was eluted at 1 ml/min and maintained at 20°. The 50-min gradient was generated by an Altex model 110A Controller and was linear, ranging from 0 to 50% MeOH in water. The column effluent was monitored at 254 nm, and the retention time for BAU was approximately 40 min. Fractions (1 ml) of the column eluant were collected with an LKB model 7000 fraction collector. Aliquots (100 μl) of each fraction were added to 5 ml Liquiscint (National Diagnostics, Somerville, NJ) and the ^{3}H-related radioactivity in each sample was quantitated by a Beckman model 7000 LS liquid scintillation counter.

The ability of the unknown radioactive metabolite detected in urine to inhibit Urd phosphorylase was assayed with an enzyme preparation from the cytosol (100,000 *g*) fraction of murine liver as previously described [10]. Activity was assayed by incubating 10–50 μl of the particle-free cytosol at 37° with 50 mM Tris (pH 7.4) containing 1 mM inorganic phosphate, 200 μM [^{14}C]FUrd (3.5 μCi/ml) and various concentrations of BAU or its metabolites in a final volume of 100 μl (the concentration of BAU metabolites was estimated from its ^{3}H-specific activity). Portions (10 μl) of the reaction mixture were removed at various times, spotted directly onto silica gel TLC plates (E. Merck, Darmstadt, West Germany), and dried immediately at 80°. TLC plates were developed and assayed for ^{14}C-related radioactivity in the FUra and FUrd regions as previously described [10]. Protein was determined by the method of Bradford [20], and activity was expressed as nanomoles of [^{14}C]FUrd converted per hour per milligram of protein.

Table 1. Pharmacokinetic parameters of BAU after intravenous injection

Dose (mg/kg)	Pharmacokinetic parameters					
	$T_{\frac{1}{2}}$ (min)	V_d (ml)	K_e (hr^{-1})	AUC	Plasma concn (t_0) (μM)	Clearance rate (ml/min)
30	36.9 ± 2.4	18.4 ± 0.8	0.019 ± 0.001	6,042 ± 297	113 ± 4	0.36 ± 0.10
120	35.1 ± 1.6	16.4 ± 0.4	0.020 ± 0.001	28,520 ± 961	564 ± 14	0.31 ± 0.11

Each value is the mean ± SEM of three to five determinations.

RESULTS

The pharmacokinetics of BAU were determined in mice by utilizing an isocratic reverse-phase HPLC system. This system allows the quantitation of 5 ng to 5 μg of BAU/100 μl of plasma or tissue extract (0.5 μM to 0.5 mM) and affords complete resolution from other tissue or body fluid components (Fig. 1). The retention time of BAU by this method was 37 min, and its recovery was greater than 95%. Plasma clearance of intravenous BAU followed first-order kinetics with a plasma half-life of approximately 36 min (Fig. 2). The calculated volume of distribution, 17 ml, was consistent with the rapid distribution of BAU in the total body water. This was confirmed by examination of the tissue distribution of BAU after administration of various i.v. doses. The concentration of BAU in most of the major organs assayed was equivalent to that in plasma over the period tested (Fig. 3). The notable exceptions were spleen, which maintained approximately 40% of the plasma concentration of BAU, and brain, which contained about 10% of the plasma concentration of BAU at any given time. Over the range of doses tested, other basic pharmacokinetic parameters such as elimination rate constant (K_e) and clearance were also constant (Table 1). These results suggest that first-order processes reponsible for the metabolism, distribution, and elimination of BAU were not saturated even at higher doses.

BAU was effectively absorbed from the gut following its oral administration (Fig. 2). Peak plasma concentrations were achieved within 30 min, and its clearance from plasma was slightly slower than that after an i.v. injection, presumably a reflection of the continued absorption from the gut. Although the maximum plasma concentration obtained after oral dosing was only 50–60% of an equivalent i.v. dose, the longer $T_{\frac{1}{2}}$ achieved almost an equivalent bioavailability by this route (oral bioavailability was 80% compared to i.v. administration over a 6-hr period). Evidence for the functional equivalence of the two routes is seen in their effects on plasma Urd concentrations (Fig. 4).

After the i.v. administration of unlabeled BAU, approximately 41% of the injected dose was recovered intact in urine within 24 hr as measured by HPLC (Table 2). This was confirmed by use of a 30 mg/kg injection of low specific activity [^{3}H]BAU. In these studies approximately 75% of the injected tritium label was recovered in a 24-hr urine sample; again 42% of the injected [^{3}H]-label eluted with authentic BAU upon HPLC analysis. Approximately 5% of the injected label eluted with 5-benzyluracil (BU), while 27% appeared to be a more polar metabolite of BAU (Fig. 5). This metabolite was concentrated and shown not to inhibit Urd phosphorylase at concentrations of approximately 50 μM; BAU caused 50% inhibition at a concentration of between 5 and 10 μM in this system.

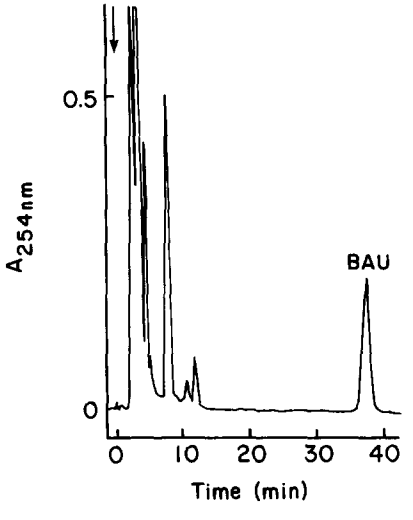

Fig. 1. Reverse-phase HPLC determination of BAU in deproteinized murine plasma. Plasma containing 15 μM BAU was deproteinized with 15% TCA, extracted with trioctylamine in freon, and analyzed isocratically at 20° using a C-18 column eluted with 25% MeOH as described in Materials and Methods.

Table 2. Recovery of intact BAU in urine

Dose (mg/kg)	Percentage of injected dose recovered intact in urine (%)
15	42.5 ± 8.1
30	44.5 ± 6.6
60	32.1 ± 0.8
120	38.1 ± 3.3
	$\bar{X}$ = 41.0 ± 3.5

After administration of BAU at the doses indicated, mice were placed in metabolic cages, and the urine was collected for 24 hr. The resulting specimens were prepared and analyzed to detect BAU as described in Materials and Methods. Each value is the mean ± SEM of three to six determinations.

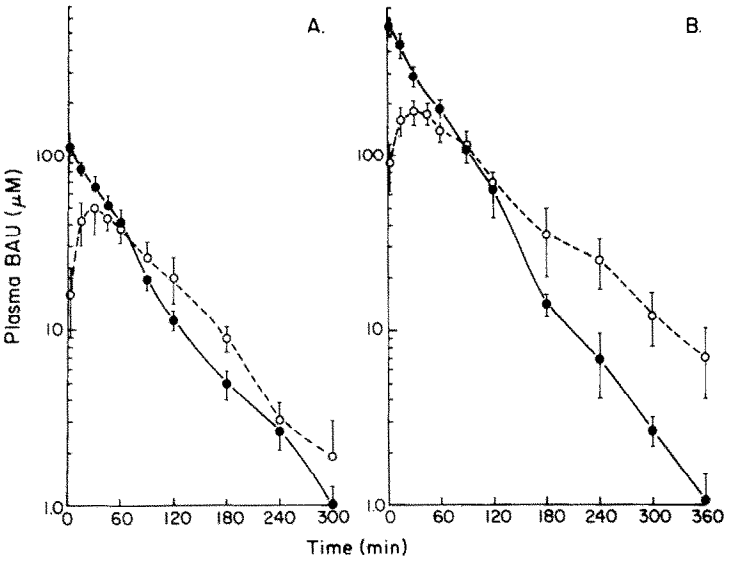

Fig. 2. Plasma concentration vs time profile for BAU following i.v. (●) or oral (○) administration. At the indicated times, whole blood was collected, and the plasma was prepared for HPLC analysis to detect BAU as described in Materials and Methods. Each point represents the mean ± SEM of three to eight determinations. (A) 30 mg BAU/kg; and (B) 120 mg BAU/kg.

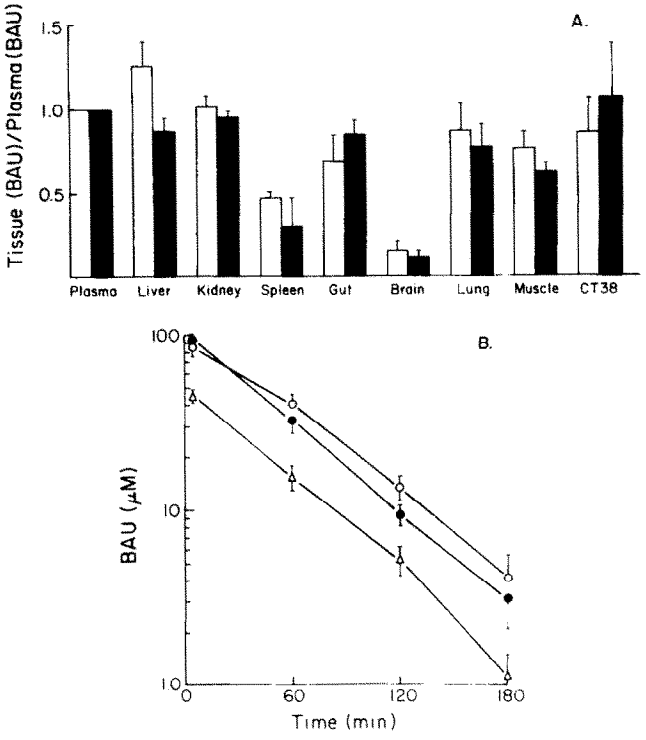

Fig. 3. Tissue/plasma ratios of BAU after i.v. injection. At various times after administration of BAU, plasma and tissues were obtained and processed to determine their BAU content as described in Materials and Methods. (A) Tissue ratios: each bar represents the mean ± SEM of three to ten comparisons between individual tissues and plasma at 30–300 min after i.v. injections of either 30 mg BAU/kg (□) or 120 mg BAU/kg (■). (B) Kinetics of BAU clearance from plasma (●), liver (○) and spleen (△) after a 30 mg/kg i.v. dose. Each point represents the mean ± SEM of three observations.

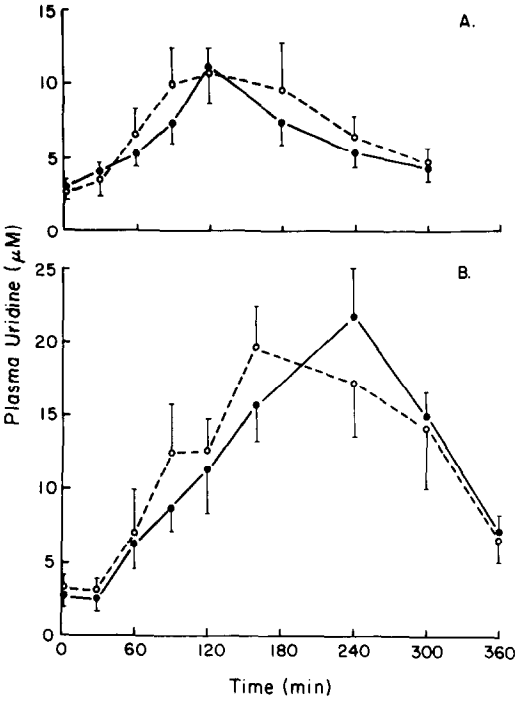

Fig. 4. Effect of i.v. (●) or oral (○) doses of BAU on the plasma Urd concentration. At the indicated times, whole blood was collected and the plasma was prepared for HPLC analysis to detect Urd as described in Materials and Methods. Each point represents the mean ± SEM of three to six determinations. (A) 30 mg BAU/kg; and (B) 120 mg BAU/kg.

A functional consequence of the first-order clearance of BAU over the range of doses analyzed is the apparent direct relationship between the injected dose of BAU and its ability to increase the concentration of Urd in plasma (Fig. 6).

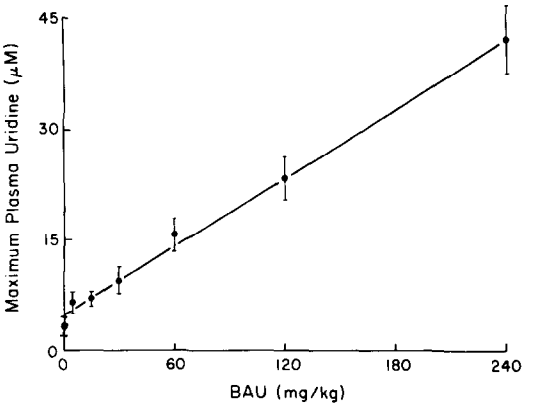

Fig. 6. Dose–response of the plasma concentration of Urd to BAU administration. After i.v. administration of BAU, whole blood was collected, and the plasma was prepared for HPLC analysis to detect Urd as described in Materials and Methods. The maximum concentration of Urd achieved is represented as a function of injected dose. Each point represents the mean ± SEM of three determinations.

DISCUSSION

The apparent first-order kinetics of BAU clearance from plasma as well as the equal effectiveness of oral versus i.v. administration has been established. Using a sensitive reverse-phase HPLC system, BAU has been shown to be rapidly and efficiently distributed throughout most of the major murine organs assayed, including the model colon tumor, CT38. Thus, the *in vivo* ability of BAU to increase the therapeutic effectiveness of FUra and to selectively elevate tissue Urd pools [9, 10] is not a consequence of preferential distribution to target tissues or impaired entry into tumor tissue. Furthermore, the clearance of BAU from the tissues tested paralleled

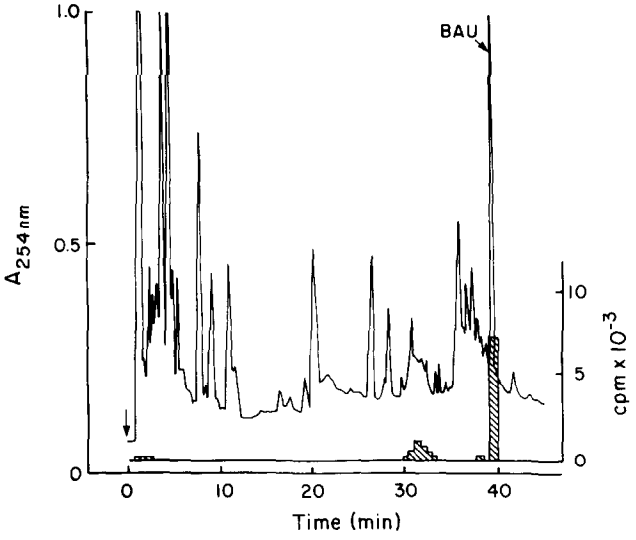

Fig. 5. Radiochromatogram of murine urine after injection of [^{3}H]BAU (30 mg/kg, 1 μCi/mouse). Mice were placed in metabolic cages, and after 24 hr the collected urine was processed and analyzed non-isocratically to detect [^{3}H]BAU as described in Materials and Methods.

its clearance from plasma (Fig. 3B). It would appear that this rapid equilibrium of BAU between plasma and tissue is not the result of it being a substrate for the nucleoside transport systems. Studies by Lee *et al.* [21] indicate that BAU, at 200 μM, causes inhibition of the NBMPR-sensitive protein carrier responsible for the facilitated diffusion of nucleosides but that it is itself not a substrate. We found that, unlike Urd, BAU was not concentrated in splenocytes by the Na^+-dependent process and did not inhibit the active transport of Urd in this model system.* Thus, the efficient tissue distribution of BAU is probably a consequence of its lipophilicity and it may enter cells by simple (non-carrier mediated) diffusion. However, if its hydrophobicity were the only mechanism responsible for BAU distribution, we might expect to see both a β and a γ-phase $T_{\frac{1}{2}}$ as observed with other hydrophobic compounds and higher concentrations of BAU in tissues with high lipid concentrations.

The only significant metabolite of BAU recovered in the urine accounted for approximately one-third of the total injected dose but was not an inhibitor of Urd phosphorylase. This metabolite may be an oxidation product because of its greater polarity as evident from its shorter retention time in the reverse-phase HPLC system. However, the lack of inhibition of Urd phosphorylase discouraged attempts to purify quantities sufficient to determine its structure. As further studies may warrant, [^{3}H]BAU of higher specific activity or [^{14}C]BAU can be prepared to facilitate the characterization of this urinary metabolite as well as the 25% of the injected dose of BAU not recovered in the urine.

Examination of the relationship between the concentrations of Urd and BAU in plasma (Figs. 2 and 4) suggests that a BAU concentration of approximately 10 μM must be maintained in order to sustain the elevated plasma concentration of Urd. Further, this relationship was observed at all BAU doses assayed in this study. This relationship was surprising since the K_i of BAU, determined with purified Urd phosphorylase, has been reported to be approximately 0.1 μM [1]. The low sensitivity is consistent, however, with our previous observation that in the cytosol fraction of murine liver between 5 and 10 μM BAU is needed to inhibit Urd phosphorylase activity by 50%. In addition, in normal murine tissues, the concentration of Urd is 20–30 μM [10], a concentration sufficient to compete with 10 μM BAU.

The profound effect of BAU on the plasma and tissue concentrations of Urd is not limited to mice. We have reported recently on the ability of this agent to increase concentration of Urd in canine plasma [22]. Taken together, these data add support to our interest in the use of this agent to modify the nucleoside environment of normal and neoplastic cells and encourage further exploration of chemotherapeutic regimens containing BAU. The highly tissue specific effects of BAU documented in our earlier report [9] coupled with its low toxicity, rapid clearance and high bioavailability by the oral route suggest a basis for its combination not only with 5-FUra but also other agents that affect the synthesis and availability of pyrimidines. We are presently using these data in the design of 5-FUra rescue regimens which contain BAU.

* J. W. Darnowski and R. E. Handschumacher, unpublished observation.

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