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SPECIES-DEPENDENT DIFFERENCES IN THE BIOCHEMICAL EFFECTS AND METABOLISM OF 5-BENZYLACYCLOURIDINE

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Abstract—The pharmacokinetics and biochemical effects of the uridine phosphorylase (UrdPase) inhibitor 5-benzylacyclouridine (BAU) were investigated in the mouse, rat and monkey. Following i.p. administration of BAU (30 mg/kg) in the mouse and i.v. administration in the rat and monkey, initial BAU plasma half-life values were 36, 36 and 25 min, and the areas under the plasma BAU concentration versus time curves (AUC) were 127, 80 and 76 μM·hr, respectively. Rats were also dosed p.o. and i.v. with BAU at 90 mg/kg, and a comparison of the AUC values showed an oral bioavailability of 70%. Analyses of plasma samples by HPLC indicated that the metabolism of BAU differed in these species. A major BAU metabolite was observed in monkeys. Its concentration was greater than or equal to that of BAU in almost every plasma sample, and its elimination paralleled that of BAU. Urinary recovery of the metabolite was 10-fold higher than the recovery of unchanged drug. The compound was identified as the ether glucuronide of BAU by its UV absorption spectrum, its co-elution with BAU after incubation with β-glucuronidase, and liquid chromatography/mass spectrum analysis. A different metabolite was detected in rat plasma; its maximum concentration was 15% of the BAU level, and its elution position on the HPLC chromatogram was not affected by the action of β-glucuronidase. BAU had equivalent potency against UrdPase in liver extracts from the three species, with K_i values of about 0.17 μM. However, the *in vivo* effects of BAU on plasma uridine concentrations were species dependent. In mice, a 30 mg/kg i.p. dose of BAU increased the plasma uridine concentration to 11 μM from a control level of 1.8 μM. In the rat, a 30 mg/kg i.v. dose of BAU increased plasma uridine to 2.1 from 1.1 μM control levels, and a 300 mg/kg oral dose resulted in a peak plasma uridine concentration of only 6 μM. In the monkey, BAU (30 mg/kg, i.v.) had no effect on plasma uridine despite the presence of 10–100 μM BAU levels in plasma for 1.5 hr. These data show that there are significant differences in the biochemical effects and metabolism of BAU in CD-1 mice, CD rats and cynomolgus monkeys.

Uridine phosphorylase (UrdPase§, EC 2.4.2.3) catalyzes the reversible phosphorolysis of uridine to ribose-1-phosphate plus uracil. This activity is important in the degradation of uridine and other pyrimidine nucleosides as well as in the salvage of pyrimidine bases. Although UrdPase primarily cleaves ribosides, the enzyme is nonspecific and can use pyrimidine 2′- and 5′-deoxyribosides and pyrimidine nucleoside analogs as alternative substrates [1, 2].

UrdPase inhibitors have several pharmacological activities. For example, benzylacyclouridine (BAU) and 5-benzyloxybenzylacyclouridine (BBAU) are two inhibitors discovered by Niedzwicki *et al.* [3] that inhibit the breakdown of 5-fluoro-2′-deoxyuridine (5-FdUrd) and increase its cytotoxicity *in vitro* [4]. BBAU also potentiates the antitumor effect of 5-FdUrd against a human pancreatic tumor xenograft [4]. In addition, inhibition of UrdPase increases plasma and, to a lesser extent, intracellular uridine pools in mice. These pharmacological effects are indirect. Steady-state concentrations of circulating uridine are maintained by a balance between synthesis and degradation [5, 6]. When catabolism is inhibited by BAU, continued biosynthesis in the liver results in a time-dependent increase in plasma and, ultimately, tissue uridine levels. For example, a single 240 mg/kg dose of BAU elevates plasma uridine 15-fold in C57BL/6 mice and increases the intracellular concentration of uridine 2- to 5-fold in colon tumor 38, gut, spleen, kidney and liver over a 4-hr time period [7]. These *in vivo* effects on tissue uridine concentrations are potentially important because high levels of uridine (50 μM) reverse the toxicity of 3′-azido-3′-deoxythymidine (AZT, zidovudine) against human granulocyte-macrophage progenitor cells *in vitro* without impairing antiretroviral activity [8]. In mice, high-dose BAU therapy raises plasma uridine to a peak level of about 50 μM and partially reverses AZT toxicity without affecting its antiviral activity against Rauscher leukemia virus [9, 10]. In addition, the therapeutic index of 5-fluorouracil (5-FU) against advanced solid tumors in mice is increased by delayed administration of uridine and/or BAU [11–14].

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§ Abbreviations: UrdPase, uridine phosphorylase; BAU, 5-benzylacyclouridine, [5-benzyl-1-(2′-hydroxyethoxymethyl)uracil]; BBAU, 5-benzyloxybenzylacyclouridine, [5-(*m*-benzyloxybenzyl)-1-(2′-hydroxyethoxymethyl)uracil]; 5-FdUrd, 5-fluoro-2′-deoxyuridine; AZT, 3′-azido-3′-deoxythymidine (zidovudine); 5-FU, 5-fluorouracil; RT, retention time; LC/MS, liquid chromatography/mass spectrum; and $T_{1/2}$, half-life.

The pharmacokinetics, metabolism and biochemical effects of BAU have been well-characterized in the mouse [13, 15]; plasma BAU levels >10 μM are sufficient to inhibit UrdPase *in vivo* and increase plasma uridine concentrations. In the dog, a dose of 120 mg/kg, i.v., results in plasma BAU concentrations >100 μM that are sustained for at least 6 hr, but the elevation in plasma concentrations of uridine is not as dramatic as seen in mice [16]. In the present report, we show that there are considerable differences in the biochemical effects and metabolism of BAU in CD-1 mice, CD rats and cynomolgus monkeys. Plasma concentrations of BAU that resulted in significant elevations of plasma uridine levels in mice had modest effects in the rat and had no effect on plasma uridine concentration in the monkey, even though the UrdPase isolated from each of these species was equally sensitive to inhibition by BAU *in vitro*. Since the pharmacological effects of BAU on AZT toxicity result from modulation of uridine salvage, these data suggest that the mouse may not be an accurate model for predicting the therapeutic efficacy of BAU in other species. A preliminary report has been presented [17].

MATERIALS AND METHODS

Animals and drugs. CD-1 female mice (18–20 g) and CD male rats (225–250 g) were obtained from Charles River Laboratories (Wilmington, MA). Female cynomolgus monkeys (2–3 kg) were maintained at the White Sands Research Center, Alamogordo, NM. The pharmacokinetic experiments in monkeys were conducted at the White Sands Research Center in accordance with the requirements of the Animal Welfare Act.

BAU was synthesized by F. Orr, D. Musso and J. Kelley of the Division of Organic Chemistry, and *Escherichia coli* UrdPase was prepared by P. Ray of the Division of Molecular Genetics and Microbiology at the Wellcome Research Laboratories. Uridine, *E. coli* β-glucuronidase and D-saccharic acid 1,4-lactone were obtained from the Sigma Chemical Co. (St. Louis, MO). [2-^{14}C]Uridine (56 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA) and was found to be >99% pure. All other reagents were purchased from Sigma.

Animal dosing and blood collection. Pharmacokinetic studies were conducted in mice, rats and monkeys. For i.p. dosing in the mouse, BAU was dissolved in saline (pH 8.5) and administered in a volume of 10 mL/kg. Mice were usually dosed in groups of eighteen to twenty-one animals, and blood was collected from three mice at each time point. Whole blood was obtained by cardiac puncture of CO_2 anesthetized mice with a syringe containing 50 μL of 5% EDTA, and plasma was isolated by centrifugation (3000 *g* for 10 min). For the rat studies, a catheter tube (i.d. 0.025 in. × o.d. 0.047 in.) was inserted into the right jugular vein [18]. The animals were then placed in individual metabolic cages and fed chow and water overnight. Food was removed 6 hr before the start of the experiment. BAU was dissolved in saline (pH 8.5) and administered via the cannulated jugular vein in a volume equivalent to 10 mL/kg. Oral dosing at 90 mg/kg was performed with the same BAU solution via gastric gavage (10 mL/kg). BAU was suspended in 1% methylcellulose for oral dosing at 300 mg/kg and administered by gavage at 10 mL/kg. Different animals (three per group) were used for the i.v. and p.o. studies. Whole blood samples (0.35 mL) were removed from the catheter using a 1-cc syringe containing 50 μL of 5% EDTA. This volume of blood was replaced by the injection of 0.35 mL of saline at each time point. The ability to take sequential blood samples from the same rat resulted in a standard error of approximately 10% between replicates compared to 35% in the mouse studies where the pharmacokinetic curves were constructed from single blood samples taken from twenty mice. For the pharmacokinetics of oral BAU in the monkey, drug was dissolved in 20% propanediol and administered via gavage at 10 mL/kg to two animals (identified as PRO302 and PRO306) that had been fasted overnight. When BAU was given i.v., the compound was dissolved in saline (pH 8.5) and administered via i.v. injection (5 mL/kg) to two animals (PRO302 and PRO237). There was a 2-month time interval between the oral and i.v. studies. In each experiment, blood samples (2.0 mL) were collected from the femoral vein of unanesthetized animals into EDTA tubes and centrifuged as above. Plasma was frozen at −20° and stored for HPLC analyses.

BAU analysis. Plasma concentrations of BAU were determined using reverse-phase HPLC. Plasma samples were thawed and diluted 2-fold with water, then protein was removed from the samples by ultrafiltration using the Centrifree micropartition system (Amicon Division, W. R. Grace & Co., Beverly, MA). HPLC was performed on a reverse-phase Microsorb C18 column (250 mm × 4.6 mm i.d.; Rainin Instrument Co., Woburn, MA) with a Dynamax Axial Compression guard column. A Waters model 712 WISP automated sample injector was used for sample injection, and microcomputer-controlled LKB Bromma 2150 HPLC pumps delivered the mobile phases: 50 mM ammonium acetate buffer, pH 4.8, 0.5% acetonitrile (buffer A), and 50 mM ammonium acetate buffer, pH 4.8, 60% acetonitrile (buffer B). Typically 100 μL of sample was analyzed with a multi-step elution protocol. A 23-min isocratic elution in buffer A was followed by a 10-min linear gradient to 60% buffer B, then a 12-min isocratic elution in 60% buffer B was followed by a re-equilibration wash of 100% buffer A. Flow rates were 1 mL/min, except for two 0.5 mL/min segments (8–23 min and 33–45 min) where the compounds of interest eluted. The effluent was monitored by UV absorption at 265 nm using a Waters model 991 photodiode array detector. In this system, BAU eluted at 38.7 min. For data analysis, the BAU peak area was determined and compared to a standard curve prepared from known concentrations of BAU added to plasma and processed as the sample. The recovery of BAU after ultrafiltration of plasma was greater than 85%, indicating that less than 15% of the BAU in plasma was bound to the protein fraction. A plot of UV

peak area versus BAU concentration was linear between 0.5 and 200 μM.

β-Glucuronidase peak shifts. The effect of β-glucuronidase (EC 3.2.1.31) on the HPLC elution profile of plasma and urine samples was determined by incubating (overnight at 37°) 20 μL of sample (about 0.02 μmol of metabolite) with 1000 enzyme units with or without 8 μmol of saccharic acid 1,4-lactone in 25 mM potassium phosphate buffer, pH 6.8 (400 μL final volume). As defined by the manufacturer, 1 unit of β-glucuronidase will liberate 1 μg (3.2 nmol) of phenolphthalein from phenolphthalein glucuronide per hr at 37° at pH 6.8. After incubation, protein was removed from the samples by ultrafiltration using the Centrifree micropartition system, and the extracts were subjected to HPLC analyses as described above. Enzyme activity was determined in separate 30-min incubations using diluted enzyme and 0.5 mM phenolphthalein as the substrate.

Uridine analysis. Uridine elutes as a distinct peak with a retention time (RT) of 14.5 min under the same HPLC conditions used for the analysis of BAU. The identity of the uridine peak in plasma was determined by: (1) a UV spectrum identical to an authentic sample of uridine; (2) co-elution with a spike of uridine added to the plasma before HPLC; (3) pretreatment of plasma with purified *E. coli* UrdPase (0.4 U/mL for 10 min at room temperature) which eliminated the uridine HPLC peak and generated a peak with the retention time of uracil. The recovery of uridine added to whole blood (mouse or rat) was greater than 98%. For data analysis, the plasma uridine peak area was determined and compared to a standard curve prepared in water. Plots of UV peak area versus uridine concentration were linear between 0.1 and 50 μM. Control (t = 0) plasma uridine concentrations were 1.8 ± 0.5 μM (N = 9) in CD1 mice, 0.8 ± 0.3 μM (N = 29) in CD rats and 3.9 ± 0.9 μM (N = 8) in cynomolgus monkeys. For individual rat experiments, control plasma uridine concentrations were 1.1 ± 0.1 μM for the three rats used in the 30 mg/kg i.v. BAU experiment and 0.6 ± 0.3 μM for the six rats used in the 90 mg/kg i.v. and p.o. BAU experiment.

UrdPase assays. Liver UrdPase was prepared and assayed using minor modifications of the methods previously described by Naguib *et al.* [19]. Mouse livers (N = 5) and rat livers (N = 7) were placed on ice and processed within 1 hr after collection; monkey liver samples (N = 5) were either frozen after collection or processed without freezing. All steps were performed at 4°. Samples (3–6 g) were rinsed in saline, weighed and homogenized (1:3, w:v) in ice-cold 20 mM potassium phosphate buffer (pH 8.0), 1 mM EDTA, 1 mM mercaptoethanol using a Brinkmann model PT3000 polytron (3 cycles at 30,000 rpm for 20 sec). The homogenates were centrifuged at 27,000 *g* for 30 min to remove cell debris then clarified by ultracentrifugation (100,000 *g* for 1 hr). The cytosol fraction was stored at −70° until used. UrdPase activity was determined by monitoring the conversion of [2-^{14}C]uridine to [2-^{14}C]uracil in 1.5-mL Eppendorf tubes containing 20 mM potassium phosphate buffer, pH 8.0, 1 mM EDTA, 170 μM [2-^{14}C]uridine (sp. act. 7.12 μCi/μmol), 1 mM dithiothreitol and enzyme ± inhibitor in a final volume of 60 μL. Reaction mixtures were incubated at 37° for 30 min, and the assay was terminated by heating the Eppendorf tubes in a 100° waterbath for 1 min. Samples were cooled (usually frozen), and protein was removed by centrifugation for 1 min (Fisher Micro-Centrifuge model 235B). A portion of the supernatant fluid (5 μL) was spotted onto plastic silica gel TLC sheets (Macherey-Nagel, Polygram Sil G/UV254) that had been prespotted with 5 μL of a mixture of 10 mM each of uridine and uracil. The plates were developed in chloroform: methanol:acetic acid (90:5:5). Uridine ($R_f = 0.1$) and uracil ($R_f = 0.4$) were identified by UV quenching, cut out and counted in 5 mL of Ultima Gold scintillation fluid (Packard). Typically, each 5-μL sample contained 10,000 cpm total, and the amount of enzyme used in the assay was chosen to catalyze 10% conversion of uridine to uracil in the 30-min uninhibited reaction. The assay was linear with respect to enzyme and time until 20% substrate was converted to product, and high concentrations of BAU (>100 μM) resulted in essentially complete (>95%) inhibition of enzymic activity. One unit is defined as the amount of enzyme that catalyzes the conversion of 1 nmol of uridine to uracil per min under standard assay conditions. Specific activities were calculated from protein concentrations determined by the method of Lowry *et al.* [20].

Apparent K_m values for uridine were determined in assays using approximately 50, 75, 125 and 230 μM uridine and 20 mM phosphate. BAU (150 and 300 μM) was tested as a competitive inhibitor at uridine concentrations of 70, 120 and 210 μM. Apparent K_i values for BAU were calculated using the following equation for competitive inhibitors [21]:

$$K_i = I_{50}/(1 + S/K_m)$$

where I_{50} is the concentration of inhibitor that results in a 50% decrease in reaction velocity, S is the assay concentration of uridine (170 μM), and K_m is the uridine Michaelis constant.

Liquid chromatography/mass spectrum (LC/MS) analyses. Thermospray LC/MS experiments were carried out using a Nermag/Vestec 740C thermospray interface to a Nermag R10-10C quadrupole mass spectrometer. Data acquisition and processing were carried out using the SIDAR data system operating on a DEC PDP 11/73 computer. Samples were separated via HPLC on a NovaPak C18 column using a solvent system composed of (A) 5% acetonitrile and 0.1% trifluoroacetic acid in aqueous ammonium acetate (0.05 M) and (B) 100% acetonitrile. The HPLC was operated with a gradient from 100% A to 70% A in 15 min with a 1 mL/min flow rate, and sample peaks were detected by UV absorbance at 265 nm. The thermospray interface on the Nermag R10-10C mass spectrometer was operated at a source temperature of 240° and a vaporizer temperature of 218°.

RESULTS

BAU pharmacokinetics and uridine perturbation

Table 1. Summary of BAU pharmacokinetic and plasma uridine perturbation data

Species	BAU* Dose (mg/kg)	$T_{\frac{1}{2}}$ (min)	AUC (μM·hr)	Maximum uridine level (μM)	(-fold)†	Effective BAU concentration‡ (μM)
Mouse	30 (i.p.)	36 ± 1	127 ± 11	11	6	10
	30 (i.v.)§	37	100	9	3	10
	120 (i.v.)§	35	475	22	7	10
Rat	30 (i.v.)	36 ± 1	80 ± 3	2.1	2	75
	90 (i.v.)	39 ± 5	450 ± 70	1.5	2.5	100
	90 (p.o.)		316 ± 1	1.9	3.3	80
	300 (p.o.)		944 ± 18	6	6	~200
Monkey	30 (i.v.)	25 ± 1	76 ± 1	6	1.1	>100

* Each value is the mean ± range of 2–4 determinations.

† Fold increase in peak plasma uridine concentration compared to control (t = 0) level.

‡ The BAU concentration corresponding to the peak plasma uridine level in plots of plasma concentration versus time. It is an estimation of the minimum plasma BAU concentration needed to sustain elevated uridine levels.

§ Data from Darnowski and Handschumacher [15].

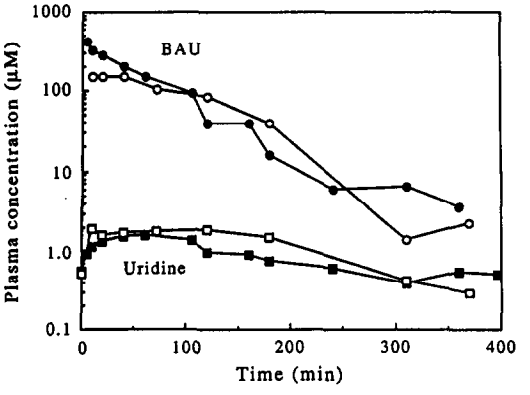

Fig. 1. BAU and uridine plasma concentration versus time curve in CD male rats following administration of 90 mg/kg BAU i.v. (filled symbols) or 90 mg/kg BAU p.o. (open symbols). Blood samples were collected at the times indicated and assayed for plasma BAU and uridine concentration. (Each point represents the average of three separate cannulated rat experiments.) The maximum SEM in these replicates was 10%.

in mice and rats. The administration of BAU (30 mg/kg, i.p.) to female CD-1 mice produced plasma BAU concentrations >100 μM within 5 min after dosing. BAU was eliminated in a first-order process with a half-life ($T_{1/2}$) of 36 min, and plasma uridine concentrations rose 6-fold from a control level of 1.8 μM to a maximum of 11 μM at ~1.5 hr after dosing. These results and additional pharmacokinetic data are summarized in Table 1.

The plasma BAU concentration versus time profiles in rats following i.v. and p.o. administration of BAU (90 mg/kg) are shown in Fig. 1. The i.v. dose resulted in high (~400 μM) BAU concentrations that decreased to 10 μM over a 4-hr time span. The orally administered drug maintained plasma levels at ~100 μM for 2 hr, and then its concentration decreased roughly in parallel with the i.v. dose. A comparison of the AUC of these two doses indicates an oral bioavailability of 70%.

Figure 1 also shows that the i.v. or p.o. administered BAU had similar effects on rat plasma uridine levels. Predose uridine concentrations (0.6 ± 0.3 μM in this experiment) increased and reached a plateau of 1.5 to 2 μM for a 2-hr time period before returning to control levels by 5 hr. An examination of the relationship between the plasma concentrations of BAU and uridine suggests that BAU levels of 80–100 μM were required to sustain the elevated plasma uridine concentrations (Table 1). In other experiments, a 30 mg/kg i.v. dose of BAU perturbed plasma uridine concentration from a control level of 1.1 ± 0.1 μM to a maximum of 2.1 μM at 5–10 min postdose, and a 300 mg/kg oral dose of BAU resulted in a peak plasma uridine concentration of 6 μM at 90 min postdose.

BAU pharmacokinetics and uridine perturbation in monkeys. Administration of BAU (30 mg/kg, i.v.) to the monkey resulted in a concentration versus time curve for plasma BAU that appeared to be biphasic (Fig. 2). However, the BAU concentrations at the latter time points were near our limit of detection due to interfering UV absorbing peaks in monkey plasma. The initial distributive phase (through t = 2 hr) had a $T_{1/2}$ of 25 min. Despite the high initial plasma levels of BAU (~100 μM), plasma uridine concentrations were not perturbed in the monkey (Table 1). The predose uridine concentration was 4.6 ± 0.14 μM; the mean uridine concentration during the 7 hr after BAU administration was 5.1 ± 1.1 μM. In another experiment, monkeys were given an oral dose of BAU (30 mg/kg), and blood samples were collected every hour for 6 hr. The peak plasma concentration of BAU (16.4 ± 9.4 μM) occurred 1 hr after dosing, and the compound was not detectable (<0.4 μM) 4 hr postdose. As with the

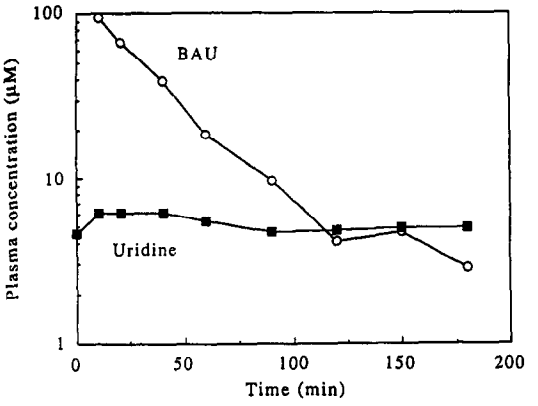

Fig. 2. BAU and uridine plasma concentration versus time curves in female cynomolgus monkeys following the intravenous administration of 30 mg/kg BAU. Blood samples were collected at the times indicated and assayed for plasma uridine and BAU concentrations. Each point represents the average of two experiments. The maximum SEM in these duplicates was 10%.

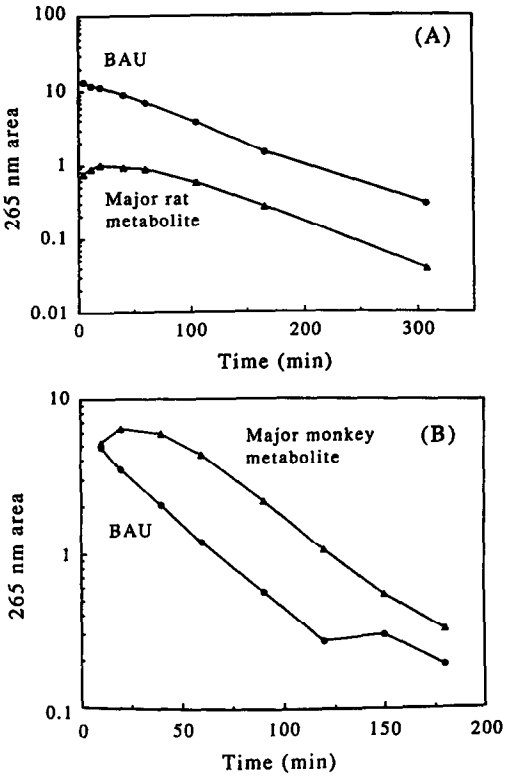

Fig. 3. Plasma concentration versus time curve of BAU and its metabolites in the rat and monkey. (A) Rats were administered 90 mg/kg BAU i.v., and (B) monkeys were administered 30 mg/kg BAU i.v. Plasma samples were analyzed by HPLC. The areas (265 nm absorbance) of the BAU peak (●) and the metabolite peak (▲) were monitored as a function of the time after dosing.

i.v. dose, plasma uridine levels were not elevated by orally administered BAU. In this case, the average predose plasma uridine concentration was $3.8 \pm 0.6\ \mu M$, and the mean uridine concentration during the 4 hr after BAU administration was $3.2 \pm 0.4\ \mu M$.

Metabolism of BAU in the rat and monkey. The HPLC system used in the quantitative analysis of plasma samples resulted in well-resolved elution positions for uridine and BAU. In addition, several new UV absorbing peaks (presumed BAU metabolites) were detected after the administration of BAU to rats and monkeys. The relative amount of BAU metabolites varied considerably. After i.v. administration of BAU to the rat, only small amounts of a metabolite were found in the early plasma samples, and its relative concentration increased to 15% of the BAU level over 5 hr (Fig. 3A). Elimination of the metabolite from rat plasma paralleled that of BAU. In contrast, i.v. administration of BAU to the monkey resulted in high concentrations of metabolite (Fig. 3B). By 1 hr postdose, the concentration of metabolite was 4-fold greater than that of BAU. The elimination of the metabolite from plasma was roughly parallel to that of BAU.

Rat and monkey urine samples were also assayed for BAU and its metabolites. In both cases, the major metabolites in urine were identical to their plasma counterparts with respect to HPLC elution time and UV absorption spectra. In the rat, 13 μmol of metabolite were collected in 24-hr urine compared to 26 μmol of BAU (assuming similar extinction coefficients), with both compounds accounting for 50% of the 90 mg/kg i.v. dose. In the monkey, about 45% of the 30 mg/kg p.o. dose was recovered as BAU or its major metabolite in the 96-hr urine. However, in this case, 146 μmol of metabolite were collected compared to 12 μmol of BAU.

Identification of BAU glucuronide. The UV spectra of the major rat and monkey metabolites in plasma and urine were identical to that of BAU with an absorption peak at 265 nm (in the ammonium acetate/acetonitrile, pH 4.8, HPLC elution buffer). However, the two metabolites differed from each other in their HPLC retention times and in their response to β-glucuronidase. Treatment of monkey plasma with β-glucuronidase resulted in the disappearance of the metabolite (RT = 33.7 min) with a concurrent increase in the area of the BAU peak (Fig. 4, A and B). When saccharic acid (a specific inhibitor of β-glucuronidase) was included in the incubation mixture, the peak shift was prevented (Fig. 4C). An identical result was observed with the major urine metabolite in the monkey. However, β-glucuronidase treatment did not affect the elution position of the rat metabolite (RT = 34.3 min), in either plasma or urine (data not shown).

LC/MS analysis of monkey urine showed two major peaks on the total ion chromatogram with retention times corresponding to BAU (13.5 min) and its metabolite (10.5 min). Thermospray mass spectrum analysis of BAU gave the base peak of the spectrum as the protonated molecular ion at 277 Da;

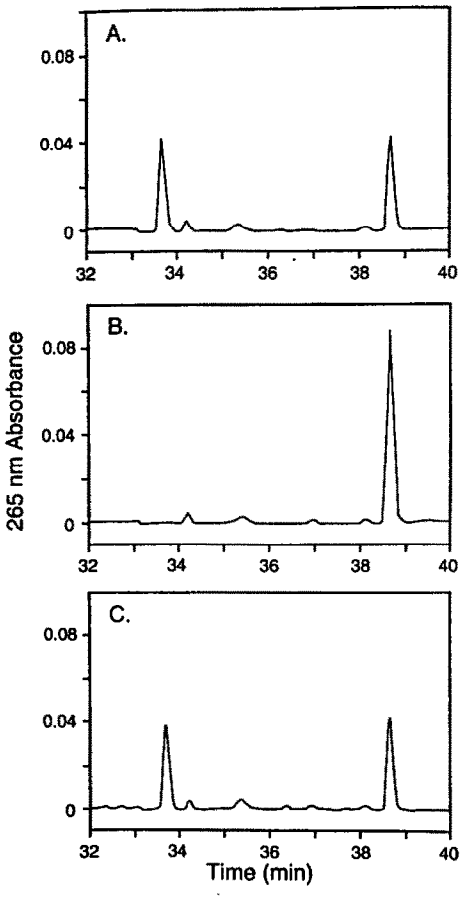

Fig. 4. Effect of β-glucuronidase on the HPLC chromatogram of monkey plasma. (A) Portion of the chromatogram showing the major BAU metabolite (RT = 33.7 min, area = 0.4) and BAU (RT = 38.7 min, area = 0.37) collected 10 min after the 30 mg/kg i.v. dose of BAU. (B) The same sample after treatment with β-glucuronidase as described in Materials and Methods (RT = 33.7 min, area = 0; RT = 38.7 min, area = 0.79). (C) Plasma after treatment with β-glucuronidase and saccharic acid (RT = 33.7 min, area = 0.37; RT = 38.7 min, area = 0.39).

the spectrum of the metabolite peak (Fig. 5) gave a protonated molecular ion at 453 Da which is consistent with a glucuronic conjugate of the parent drug molecule (shown as the inset in Fig. 5). Its expected fragmentation pattern also yielded the protonated molecular ion species of BAU at 277 Da and the ion for the sugar portion of the molecule at 194 Da. Ammonium acetate buffer was used during the thermospray introduction of HPLC effluents into the mass spectrometer, and an ammonium adduct ion of the metabolite molecule was formed at 470 Da. Ammonium adducts were not observed for the fragment ions.

UrdPase from mouse, rat and monkey liver. One possible explanation for the wide variation in uridine response to BAU could be differences in the sensitivity of mouse, rat and monkey UrdPase to inhibitors. Therefore, the kinetic properties of UrdPase were determined in liver extracts from these species. As shown in Table 2, the enzymes were similar with respect to uridine K_m values. In addition, BAU was competitive with uridine in plots of reciprocal velocity versus reciprocal substrate concentration (data not shown), and the K_i value of BAU (0.16 to 0.19 μM) was similar for each enzyme (Table 2). The only difference detected in the preparations was their relative amounts of enzymic activity. The specific activity of the rat liver enzyme was 3- and 4.5-fold greater than that of the monkey and the mouse, respectively.

DISCUSSION

The purpose of this study was to compare the pharmacokinetics and biochemical effects of BAU in several species. These data are important because BAU has pharmacological activities in murine models [9–14] which indicate that the drug may be useful in rescuing cancer patients from 5-FU toxicity and in reversing the hematological toxicity sometimes associated with AZT therapy.

BAU (30 mg/kg, i.p.) was eliminated from mouse plasma in a first-order process with a $T_{1/2}$ of 36 min. These data are similar to the plasma concentration versus time profile reported by Darnowski and Handschumacher [15] for an i.v. dose of 30 mg/kg BAU ($T_{1/2}$ = 36.9 min) and are consistent with the rapid distribution of drug throughout murine tissues. The BAU elimination profiles in the rat and monkey were also first-order processes with $T_{1/2}$ values of 36 and 25 min, respectively. When these three species were administered BAU at 30 mg/kg, AUC values of the plasma drug concentration profiles differed less than 2-fold.

The metabolism of BAU differed considerably in each species. Distinct BAU metabolite peaks were not detected on HPLC chromatograms of mouse plasma or urine. However, radiolabeled BAU was not used, and metabolites could have co-eluted with other UV absorbing peaks. For instance in mouse studies performed with [^{3}H]BAU, Darnowski and Handschumacher [15] observed that 27% of the radiolabel in a 24-hr urine sample eluted as a polar metabolite in an area of the HPLC chromatogram with interfering UV absorbance. In contrast, we observed evidence of BAU metabolism in the rat using unlabeled drug because a novel HPLC peak appeared in plasma samples and increased in a time-dependent fashion. The same presumed metabolite was detected in rat urine. Approximately half of the administered BAU dose was recovered as either BAU or its metabolite over 24 hr, with a majority of this material being parent compound.

BAU was subject to considerably more metabolism in the monkey. A major new plasma peak was evident in all plasma samples, and the same metabolite was found in urine. As with the rat, approximately half of the administered BAU dose was recovered in the urine as either BAU or its metabolite, but in this case the metabolite was the predominate species. Enzymatic characterization indicated that the metabolite was a glucuronide with a β-sugar configuration and that BAU was not modified before glucuronidation (since BAU was

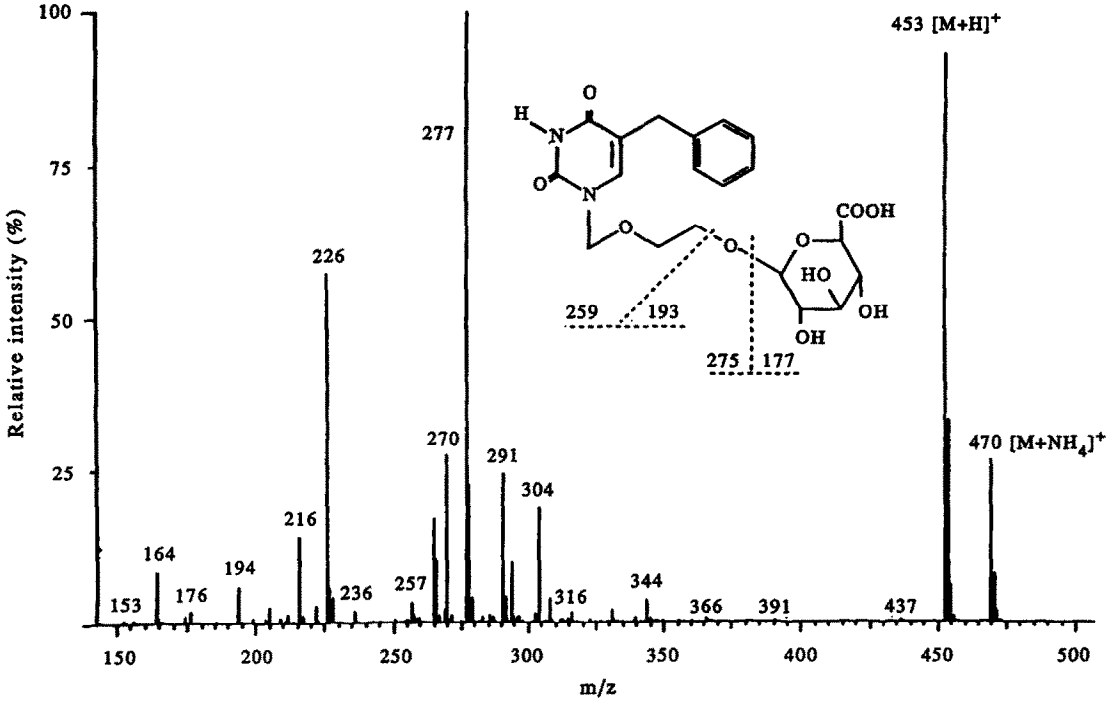

Fig. 5. LC/MS mass spectrum analysis of the major metabolite peak in monkey urine. A 0- to 24-hr urine sample from a monkey dosed with BAU (30 mg/kg, p.o.) was subjected to LC/MS analysis.

Table 2. Properties of UrdPase from the cytosol of mouse, rat and monkey liver

Species	UrdPase activity (U/mg)	Uridine K_m (μM)	BAU K_i (μM)
Mouse	0.58 ± 0.07 (10)	77 ± 18 (4)	0.17 ± 0.03 (11)
Rat	2.6 ± 1.0 (14)	83 ± 17 (6)	0.16 ± 0.01 (4)
Monkey	0.85 ± 0.45 (10)	58 ± 15 (5)	0.19 ± 0.03 (3)

Values are means ± SEM; the number of determinations is given in parentheses.

regenerated by glucuronidase). This latter conclusion is supported by LC/MS analysis. The molecular ions of BAU and glucuronic acid were generated upon fragmentation. The fact that the ammonium ion adduct of the metabolite was observed at 470 Da indicates that the carboxylic acid of the glucuronate is free and that the linkage to BAU is via an ether linkage through the 6′-hydroxy. The glucuronide was a weak inhibitor of UrdPase, resulting in only 25% inhibition of the mouse liver enzyme at 150 μM (data not shown).

The identification of a major glucuronide of BAU was unanticipated since this mechanism of metabolism was not observed with acyclovir or ganciclovir, two compounds with similar acyclic sugar sidechains. However, AZT 5′-*O*-β-glucopyranosylthymidine is formed *in vitro* and *in vivo* [22–24]. As was suggested for AZT [24, 25], the increased lipophilicity of BAU (octanol/pH 7 phosphate buffer partition coefficient = 2.4)* compared to acyclovir and ganciclovir (partition coefficients < 0. 1)* may enhance its association with the microsomal glucuronidation system.

In mice, BAU (30 mg/kg, i.p.) resulted in plasma BAU concentrations that were >10 μM for ~1.5 hr. During this time, a steady rise in plasma uridine concentrations occurred, resulting in a 6-fold elevation (to 11 μM). This perturbation in CD-1 mice was similar to data reported by Darnowski and Handschumacher [13] in C57BL/6 mice. Other mouse studies showed that a linear relationship exists between the BAU dose and peak plasma uridine and that plasma uridine levels of ~50 μM can be achieved by 240–300 mg/kg doses [9, 10, 13, 15]. Approximately 10 μM BAU is the minimum plasma

* Douglas Minick, Burroughs Wellcome Co, personal communication. Cited with permission.

concentration needed to sustain elevated uridine levels in C57BL/6 mice [15]. Because of these studies, the modest effects of BAU on uridine levels in the rat were unexpected. A 90 mg/kg i.v. or p.o. dose of BAU elevated plasma uridine levels only 3.3-fold even though plasma BAU levels were greater than 10 μM for 4 hr (Fig. 1). At the highest dose tested (300 mg/kg, p.o.), BAU only raised plasma uridine concentrations to a peak level of 6 μM. These studies show that plasma BAU levels >75 μM were needed to sustain elevated plasma uridine concentrations in the rat (Table 1). In the monkey, initial plasma BAU levels of 100 μM and concentrations above 10 μM for 90 min had no effect on plasma uridine concentrations. The only dose tested in the monkey was 30 mg/kg, and it may be possible to perturb plasma uridine levels with higher doses. However, when the 30 mg/kg dose is recalculated on a mg/m^2 basis, the monkey (345 mg/m^2) received 2.2-fold more drug than the rat (156 mg/m^2) and 3.8-fold more than the mouse (90 mg/m^2). Little is known about the perturbation of plasma uridine in other species, except for the report of Pizzorno *et al.* [16] noting that the elevation of plasma uridine in dogs treated with BAU (120 mg/kg, i.v.) is not as dramatic as that in mice.

The reason for the species-dependent response to BAU is not known. The kinetic data show that the UrdPase from mouse, rat and monkey have similar binding affinities for uridine ($K_m \sim 70$ μM) and BAU ($K_i \sim 0.17$ μM). These values are in agreement with the uridine K_m (100 μM) and BAU K_i (0.1 μM) reported for the murine enzyme by Niedzwicki *et al.* [3]. In fact, the only difference found among the preparations was their relative amounts of enzymatic activity, with the mouse liver preparations having the lowest UrdPase specific activity. Since the liver is a major site of uridine catabolism [5, 6], the rat may have a higher capacity to degrade uridine and may require greater inhibition of UrdPase before the enzyme becomes rate limiting. The monkey liver samples were more variable with specific activities ranging from 0.53 to 1.47 U/mg. Since liver biopsies were not obtained from the animals used for the pharmacokinetic studies, we cannot speculate on the role of UrdPase specific activity on the poor *in vivo* response to BAU observed in the monkey. Additional mechanisms for the species-dependent effects in BAU response could include differences in the cellular uptake of BAU or regulation of plasma uridine levels. In mice, a 2-fold circadian change in plasma uridine concentration takes place over a 12-hr period [26]. However, BAU-induced increases in monkey uridine levels were expected to begin within an hour after dosing, and it is unlikely that this increase would be negated by circadian-related changes during such a short time period.

The rationale for using BAU to reverse the hematological toxicity of AZT is based on *in vitro* studies by Sommadossi *et al.* [8] in which uridine (50 μM) and, to a lesser extent, cytidine were reported to rescue human granulocyte-macrophage progenitor cells from AZT toxicity in a clonogenic assay. In a separate experiment, uridine did not affect the antiretroviral activity of AZT in human peripheral blood mononuclear cells even at a molar ratio (uridine/AZT) as high as 10,000. Unfortunately, uridine has a low oral bioavailability (<10%) in humans [27], and i.v. dosed uridine causes hyperthermia and phlebitis [28, 29]. Modulation of plasma uridine levels by BAU has the potential of overcoming these side-effects. A key preclinical study performed by Calabresi *et al.* [9, 10] showed that orally administered BAU reverses AZT-induced anemia and leukopenia in Balb/c mice without affecting the activity of AZT against a Rauscher leukemia virus infection. The reduction in AZT toxicity by BAU was dose dependent up to a maximal effect at 300 mg/kg/day. This dose increased plasma uridine from 3 to 50 μM in 3 hr, and the uridine level did not drop below 10 μM until 10 hr after dosing. If sustained high plasma concentrations of uridine are needed to reverse AZT toxicity, the present study indicates that this may not be achieved in the rat (and perhaps the monkey) by the administration of moderate doses of BAU alone.

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