

A Mass Balance and Disposition Study of the DNA Methyltransferase Inhibitor Zebularine (NSC 309132) and Three of Its Metabolites in Mice

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Abstract Purpose: To elucidate the *in vivo* metabolic fate of zebularine (NSC 309132), a DNA methyltransferase inhibitor proposed for clinical evaluation in the treatment of cancer.

Experimental Design: Male, CD₂F₁ mice were dosed i.v. with 100 mg/kg 2-[¹⁴C]zebularine. At specified times between 5 and 1,440 minutes, mice were euthanized. Plasma, organs, carcass, urine, and feces were collected and assayed for total radioactivity. Plasma and urine were also analyzed for zebularine and its metabolites with a previously validated high-pressure liquid chromatography assay. A similar experiment was done with 2-[¹⁴C]uridine, the proposed primary metabolite of zebularine.

Results: Maximum plasma concentrations were 462, 306, 33.6, 21.7, and 11.5 μmol/L for total radioactivity, zebularine, uridine, uracil (each at 5 minutes), and dihydrouracil (at 15 minutes), respectively. Total radioactivity, zebularine, uridine, uracil, and dihydrouracil were rapidly eliminated from plasma, and after 45 minutes, none of the individual compounds could be quantitated by high-pressure liquid chromatography. Plasma data were consistent with sequential conversion of zebularine to uridine, uracil, and dihydrouracil. 2-Pyrimidinone was not observed. Prolonged retention of radioactivity, at concentrations higher than in plasma, was observed in tissues. Recovery of given radioactivity in urine (30.3% of dose), feces (0.4% of dose), cage wash (7.9% of dose), and tissues and carcass (6.1% of dose) after 24 hours implied that up to 55% of radioactivity was expired as ¹⁴CO₂. Comparison of zebularine and uridine pharmacokinetic data indicated that ~40% of the zebularine dose was converted to uridine.

Conclusions: Zebularine is extensively and rapidly metabolized into endogenous compounds that are unlikely to have effects at the concentrations observed.

The pyrimidine analogue zebularine (NSC 309132; Fig. 1) is a novel anticancer agent that was originally investigated for its inhibitory effect on cytidine deaminase (1). More recently, it is a potent inhibitor of DNA methyltransferases (2), a class of

enzymes involved in the epigenetic silencing of tumor suppressor genes (3, 4). Furthermore, zebularine enhances the activity of decitabine, a clinically used DNA methyltransferase inhibitor, against human and murine leukemia cell lines (5). Zebularine potentiates decitabine most likely by inhibition of cytidine deaminase-facilitated degradation of decitabine and through direct inhibition of DNA methyltransferases (5). Based on its *in vitro* and *in vivo* activity (6), zebularine has been proposed for clinical evaluation. In preparation for such clinical studies, the plasma pharmacokinetics of zebularine have been characterized previously in mice, rats, and rhesus monkeys (7). Recent *in vitro* studies indicate that zebularine is metabolized to uridine by aldehyde oxidase (EC 1.2.3.1; ref. 8). However, the *in vivo* metabolic fate of zebularine remains to be elucidated. The potential metabolic scheme for zebularine is shown in Fig. 1. It includes oxidation of zebularine to uridine by aldehyde oxidase; removal of the ribose moiety from zebularine and uridine by uridine phosphorylase (EC 2.4.2.3) to produce 2-pyrimidinone and uracil, respectively; reduction of uracil to dihydrouracil by dihydropyrimidine dehydrogenase (EC 1.3.1.2); and subsequent hydrolysis of dihydrouracil to carbon dioxide, ammonia, and β-alanine (9). Because zebularine is most likely metabolized to endogenous compounds, the use of radiolabeled parent compound is required to allow

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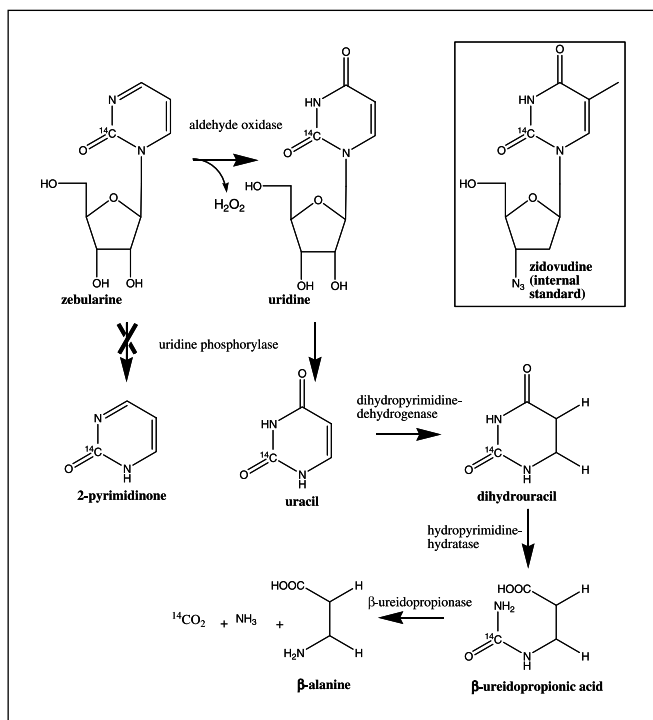


Fig. 1. Chemical structures of zebularine and its main murine metabolites uridine, uracil, and dihydrouracil, as well as zidovudine, which was used as internal standard. We did not detect 2-pyrimidinone, the product of zebularine after loss of the ribose moiety (X). Eventually, the ¹⁴C label is metabolically released as ¹⁴CO₂.

detection and quantitation of zebularine-derived uridine, uracil, and dihydrouracil in the presence of their endogenous counterparts.

The present investigation was designed to characterize the disposition of zebularine and its metabolites in mice. The distribution of zebularine and metabolites in plasma and organs was assessed by liquid scintillation counting, whereas concentrations of zebularine and its metabolites in murine plasma and urine were quantitated with a previously developed hydrophilic interaction high-pressure liquid chromatography assay (10).

Materials and Methods

Chemicals and reagents. 2-[¹⁴C]Zebularine (purity 99%, 18.3 mCi/mmol) and zebularine were provided by the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). 2-[¹⁴C]Uridine (purity 99.9%, 52 mCi/mmol), 2-[¹⁴C]uracil (purity 99.5%, 52 mCi/mmol), 2-[¹⁴C]dihydrouracil (purity 98.2%, 53 mCi/mmol), and 2-[¹⁴C]zidovudine (purity 99.8%, 53 mCi/mmol) were obtained as aqueous solutions from Moravak Biochemicals (Brea, CA). Uridine, uracil, dihydrouracil, 2-pyrimidinone, formic acid, and EDTA were obtained from Sigma-Aldrich (St. Louis, MO). Zidovudine was a gift from Burroughs Wellcome (Research Triangle Park, NC). Anhydrous sodium sulphate and ammonium formate were purchased from Fluka (Buchs, Germany). Isopropanol and acetonitrile were obtained from Fisher Chemicals (FairLawn, NJ). All reagents were of analytical grade. PBS (pH 7.4) was obtained from Biosource International (Camarillo, CA). Hydrogen peroxide (30%) was obtained from Mallinckrodt Baker (Phillipsburg, NJ). Solvable tissue solubilizer was purchased from Perkin-Elmer (Boston, MA). Ready

Safe liquid scintillation mixture was obtained from Beckman Coulter (Fullerton, CA). Water was purified using a Q-gard 1 Gradient Milli-Q system (18.2 MΩ·cm; Millipore, Billerica, MA). Control murine plasma for calibration standards was obtained from Lampire Biological Laboratories (Pipersville, PA). Murine plasma for preparation of the quality control samples was obtained from control CD₂F₁ mice.

Animals. Specific pathogen-free, adult, male CD₂F₁ mice were purchased from Taconic (Germantown, NY). Mice were allowed to acclimate to the University of Pittsburgh Cancer Institute Animal Facility (Pittsburgh, PA) for 1 week before the start of a study. To minimize infection, mice were maintained in microisolator cages in a separate room and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and on a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Ventilation and airflow were set to 12 changes/hour. Room temperatures were regulated at 22 ± 1 °C, and the rooms were kept on automatic 12-hour light/dark cycles. Mice received Prolab ISOPRO RMH 3000 Irradiated Lab Diet (PMI Nutrition International, St. Louis, MO) and water *ad libitum*, except on the evening before dosing, when all food was removed. Mice were 6 to 8 weeks old at the time of dosing. Sentinel animals were maintained in the rooms housing mice and assayed at 3-month intervals for specific murine pathogens by mouse antibody profile testing (Charles River, Boston, MA). Sentinel animals remained free from specific pathogens, indicating that the study mice were pathogen free.

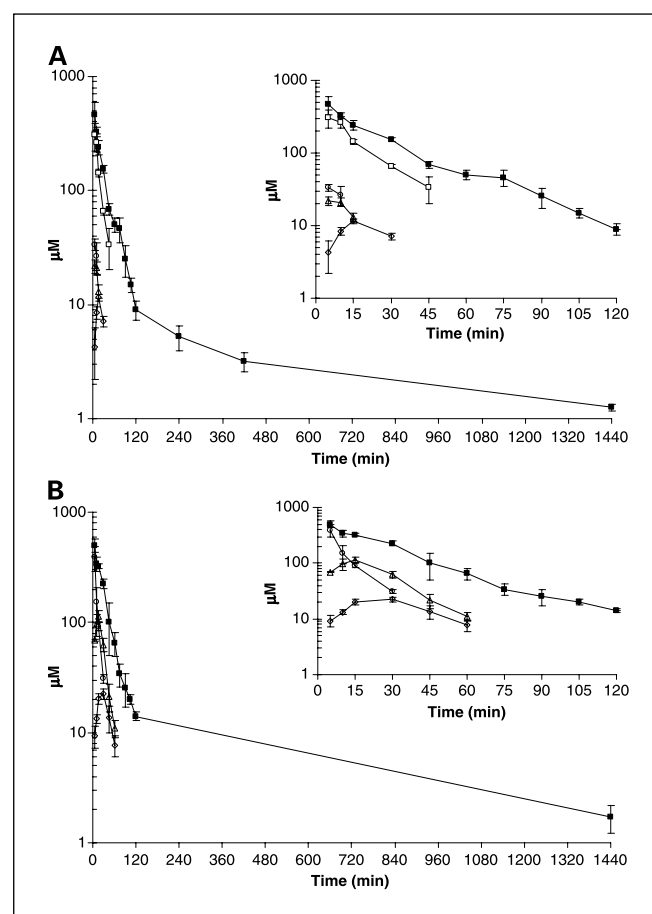


Fig. 2. Points, mean plasma concentration ($n = 3$) versus time curves of total radioactivity (■), zebularine (□), uridine (○), uracil (△), and dihydrouracil (◇) after i.v. administration of 100 mg/kg 2-[¹⁴C]zebularine (A) or 100 mg/kg 2-[¹⁴C]uridine (B) to male, CD₂F₁ mice; bars, SD.

Table 1. Mean total radioactivity, AUC, and percentage of dose accounted for in male, CD₂F₁ mice after i.v. administration of 100 mg zebularine/kg (~15 µCi/mg zebularine)

Time (min)	Plasma	RBC	Liver	Kidney	Spleen	Heart	Lung	Fat	Muscle
5	462	333	333	1031	206	305	240	73.6	245
10	329	239	292	756	212	225	222	76.3	246
15	241	166	289	632	211	131	196	39.1	192
30	154	97.3	297	516	178	95.0	156	27.3	96.8
45	68.6	59.3	280	403	119	80.3	99.4	31.2	62.7
60	50.1	37.9	320	389	103	61.4	91.6	15.6	48.9
75	46.1	36.0	223	402	106	73.7	69.7	17.0	36.8
90	25.2	22.3	301	382	91.7	62.4	57.1	12.9	32.6
105	15.0	15.7	302	340	76.5	52.3	55.6	10.0	22.4
120	8.98	12.2	301	339	88.0	61.7	56.8	10.6	23.2
240	5.22	5.60	247	275	68.5	39.2	41.5	13.0	18.7
420	3.20	3.56	189	169	69.3	29.9	36.3	5.88	16.5
1,440	1.25	2.12	126	77.9	54.0	14.8	22.1	5.33	14.9
AUC	0.29	0.24	4.45	4.28	1.66	0.76	0.92	0.20	0.51

NOTE: All values are means of measurements of three individual organs. Mean total radioactivity is expressed as nmol equivalents zebularine/g tissue. AUC is expressed as µmol-equivalents/g tissue × h. Except for cecum at 30 minutes (86%), coefficients of variation in organ levels ranged from 1.7% to 46%. Coefficients of variation in organ AUC levels ranged from 4.2% to 13%. Coefficients of variation in percentage of dose ranged from 6.0% to 24%.

*0- to 7-hour excreta in the 24-hour mice contained 26.90% of dose (26.54% in urine and 0.36% in feces). Analytes found in urine consisted of dihydrouracil (0.55% of dose), uracil (0.62% of dose), uridine (2.09% of dose), and zebularine (13.89% of dose). 0- to 24-hour excreta contained 38.6% of dose (30.32% in urine, 0.36% in feces, and 7.89% in cage wash).

Plasma and tissue disposition. To investigate the tissue disposition of 2-[¹⁴C]zebularine, specific pathogen-free, adult, male CD₂F₁ mice of ~20 g body weight were dosed i.v. with 100 mg/kg 2-[¹⁴C]zebularine (specific activity, 15 µCi/mg, ~30 µCi/mouse). Similarly, specific pathogen-free, adult, male CD₂F₁ mice of ~20 g body weight were dosed i.v. with 100 mg/kg 2-[¹⁴C]uridine (specific radioactivity, 12 µCi/mg, ~24 µCi/mouse).

Mice were dosed by lateral tail vein injection. Mice ($n = 3$ /time point) were euthanized with CO₂ at 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, 240, 420, and 1,440 minutes after dosing with zebularine and at 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, and 1,440 minutes after dosing with uridine. Blood was collected by cardiac puncture into heparinized syringes and centrifuged at 13,000 × g to obtain plasma. The RBC pellet and the aspirated plasma were stored at -70°C until analysis. Mice were rapidly dissected, and brain, heart, lungs, liver, kidneys, spleen, stomach, cecum, jejunum, ileum, colon, skeletal muscle, fat, testes, and the remaining carcass were weighed, snap frozen in liquid nitrogen, and stored at -70°C until further processing.

Organs were thawed and homogenized in three parts (v/w) of PBS (pH 7.4). The carcass was homogenized in four parts (v/w) of water followed by the addition of 5 mL of 1 mol/L sodium hydroxide and allowed to stand for a week at room temperature.

Zebularine, uridine, uracil, and dihydrouracil in plasma were quantitated with a previously developed hydrophilic interaction high-pressure liquid chromatography assay that was validated based on Food and Drug Administration guidelines for bioanalytical method validation (10). The assay was accurate and precise in the concentration ranges of 5.0 to 100 µg/mL for zebularine, 2.5 to 50 µg/mL for uridine, 1.0 to 10 µg/mL for uracil, and 0.5 to 5.0 µg/mL for dihydrouracil.

Total radioactivity was assessed by liquid scintillation counting on a Wallac 1411 liquid scintillation counter (Wallac Oy, Turku, Finland) using a 10-minute ¹⁴C counting protocol with automatic quench correction. Plasma aliquots of 50 µL were counted after addition of 5 mL Ready Safe scintillant. Aliquots of tissue homogenate (100 µL) were mixed with 200 µL Solvable tissue solubilizer and allowed to stand for 24 hours at room temperature before addition of 5 mL Ready Safe scintillant. Approximately 150 mg of the RBC pellet was accurately weighed into a 20-mL scintillation vial. After addition of

500 µL Solvable tissue solubilizer and 500 µL water, the sample was vortexed and incubated for 10 minutes at 50°C. Next, 100 µL of 0.1 mol/L EDTA and 200 µL of 30% hydrogen peroxide were added followed by vortexing of the sample and incubation at 50 °C for 10 minutes. Finally, 20 mL Ready Safe liquid scintillant was added.

Excretion. To assess urinary and fecal excretion of radioactivity after administration of 2-[¹⁴C]zebularine or 2-[¹⁴C]uridine, mice scheduled for euthanasia at 24 hours were kept in metabolic cages after dosing. Urine and feces were kept on ice and collected separately at 7 and 24 hours after dosing. At the end of the collection period, cages were washed with 15 mL water. Radioactivity was determined by counting a 100 µL aliquot of urine, 1 mL cage wash, or ~100 mg fecal homogenate (three parts of water, v/w) in 20 mL Ready Safe scintillant. Before counting, the fecal aliquot was decolorized by adding 500 µL Solvable tissue solubilizer, 100 µL of 0.1 mol/L EDTA, and 200 µL of 30% hydrogen peroxide and incubating at 50°C for 20 minutes. Quantitation of zebularine, uridine, uracil, and dihydrouracil in urine was accomplished by preparing a 20-fold dilution of urine samples in plasma and analyzing that diluted sample with the hydrophilic interaction high-pressure liquid chromatography assay used for plasma samples. By diluting the urine 20-fold in plasma, the sample is made suitable (<5% of foreign matrix) for quantitative analysis with an assay primarily developed for plasma samples.

Pharmacokinetic analysis. The area under the tissue concentration versus time curve (AUC) of radioactivity in all organs was calculated noncompartmentally using WinNonlin Professional, version 4.1 (Pharsight Corp., Mountain View, CA) and the linear trapezoidal rule. Pharmacokinetic variables for zebularine, uridine, uracil, and dihydrouracil after i.v. dosing of zebularine and uridine, respectively, were calculated. For plasma and RBC, the model for an i.v. bolus input was used (which assumes the highest concentration to occur at time zero minute). For all other organs, the model for an extravascular input was used (which assumes the absence of any radioactivity at time zero minute). The averaged concentrations for each time point were used for these calculations.

The concentration data were also analyzed compartmentally using the ADAPT II software for pharmacokinetic/pharmacodynamic systems analysis (11). We did a pooled analysis using all the

Table 1. Mean total radioactivity, AUC, and percentage of dose accounted for in male, CD₂F₁ mice after i.v. administration of 100 mg zebularine/kg (~15 µCi/mg zebularine) (Cont'd)

Brain	Testes	Stomach	Duodenum	Jejunum	Ileum	Cecum	Colon	Carcass	% Dose
25.5	65.6	145	388	245	211	120	282	278	63.3
27.5	85.3	132	298	182	290	140	220	225	50.8
34.7	96.9	131	242	145	165	131	152	243	50.4
41.1	98.4	124	226	113	174	189	120	170	37.3
39.3	90.9	103	189	103	140	94.2	140	94.5	23.3
42.8	70.2	102	241	116	140	147.1	81.9	78.0	21.1
35.3	45.0	72.7	117	67.3	118	110.0	83.9	90.5	21.3
39.5	35.4	74.6	171	96.6	113	76.0	96.7	53.4	15.9
35.3	30.0	61.3	134	83.6	108	87.7	87.3	43.8	13.6
34.6	26.5	80.3	140	94.4	125	102.2	75.0	48.2	14.3
30.2	18.1	52.0	123	51.5	92.4	101.2	66.9	26.7	9.5
24.1	13.6	43.4	92.7	58.5	62.9	67.6	61.0	28.3	8.6
19.7	8.55	35.0	91.8	42.6	52.4	28.4	39.0	20.6	6.1*
0.59	0.40	1.13	2.54	1.39	1.71	1.51	1.41	0.79	—

individual measurements. Separate models were constructed to describe the data obtained following i.v. administration of zebularine and the data from the i.v. uridine study. In addition, a single, composite compartmental model was developed to describe the pharmacokinetics of zebularine, uridine, uracil, and dihydrouracil following i.v. administration of zebularine and uridine. In all cases, the plasma measurements obtained during the first 60 minutes following the i.v. administrations were used in the analyses (these were the concentrations above the lower limit of quantitation of the assay used). The maximum likelihood option in ADAPT II was used for all estimations and model selection was based on Akaike's Information Criterion (12).

Results

The plasma profiles of total radioactivity and the individually quantitated analytes after administration of zebularine or uridine are shown in Fig. 2A and B, respectively. The plasma profiles of total radioactivity and parent compounds reflect the i.v. dosing with peak concentrations observed at the first time point (5 minutes). The peak plasma concentrations of the metabolites and the time to reach those peak concentrations are compatible with the sequential, metabolic conversion of zebularine to uridine, uracil, and dihydrouracil. The maximum plasma concentrations of total radioactivity (462 µmol/L), zebularine (306 µmol/L), uridine (33.6 µmol/L), and uracil (21.7 µmol/L) were observed at 5 minutes, whereas the maximum plasma concentration of dihydrouracil (11.5 µmol/L) was observed at 15 minutes after dosing of zebularine. After administration of zebularine, zebularine concentrations in plasma declined monoexponentially with a half-life ($t_{1/2}$) of ~12 minutes, a clearance of 1.3 mL/min (~63 mL/min/kg), and a volume of distribution of 22 mL (~1,100 mL/kg). After 45 minutes, zebularine concentration fell below the lower limit of quantitation. Zebularine-derived uridine peaked at approximately 4- to 10-fold the endogenous concentration but returned to endogenous concentrations (3-8 µmol/L; refs. 13, 14) by 15 minutes after dosing. Zebularine-derived uracil peaked at ~3-fold the reported endogenous concentration (7 µmol/L; ref. 15). Although the plasma concentrations of zebularine and its metabolites fell below the lower limit of quantitation by 60 minutes after dosing, total radioactivity in plasma could be detected up to 1,440 minutes.

After i.v. administration of uridine, plasma uridine concentrations declined monoexponentially with a $t_{1/2}$ of ~8.9 minutes, a clearance of 1.4 mL/min (~67 mL/min/kg), and a volume of distribution of 17 mL (~860 mL/kg). After 30 minutes, uridine concentrations in plasma fell below the lower limit of quantitation. The plasma concentration profiles of uracil (peaking at 15 minutes after dosing, apparent $t_{1/2}$ of 12 minutes) and dihydrouracil (peaking at 30 minutes after dosing, apparent $t_{1/2}$ of 19 minutes) were consistent with their sequential metabolic generation from uridine. Because of the short plasma concentration versus time profiles, apparent $t_{1/2}$ for uridine, uracil, and dihydrouracil could not be calculated.

Total radioactivity in plasma, organs, carcass, and excreta after administration of 2-[¹⁴C]zebularine or 2-[¹⁴C]uridine are displayed in Table 1 and 2, respectively. Plasma and tissue radioactivity are expressed as nmol equivalents of zebularine (Table 1) or uridine (Table 2) per gram of tissue. The tables also display AUC (expressed as µmol-equivalents/g tissue × h) and the percentage of the dose accounted for at each individual time point. The radioactivity was widely distributed, and radioactivity levels in most tissues, after the initial distribution phase, were much higher than concomitant plasma radioactivity. Notable exceptions were RBC and fat, which displayed a relatively low accumulation of radioactivity. After both zebularine and uridine administration, the radioactivity in organs versus time profiles were similar.

Both uridine- and zebularine-derived radioactivity were excreted more in urine (33.0% and 30.3% of dose by 24 hours, respectively) than in feces (0.15% and 0.36% of dose by 24 hours, respectively). Because the amount of an analyte excreted in urine reflects the plasma exposure to that analyte (i.e., the recovery has a linear relation with the AUC, provided renal excretion is a linear function of plasma concentration), it was possible to calculate the percentage of the dose of zebularine that was converted to uridine. This was done by dividing the urinary excretion (as a percentage of the dose given) of uridine after administration of zebularine (2.09%) by the urinary excretion of uridine after administration of uridine (5.47%). Based on this calculation, 38% of the zebularine dose given i.v. was converted to uridine.

In the compartmental analyses, several models were considered, including one and two compartment disposition models

Table 2. Mean total radioactivity, AUC, and percentage of dose accounted for in male, CD₂F₁ mice after i.v. administration of 100 mg uridine/kg (~12 µCi/mg uridine)

Time (min)	Plasma	RBC	Liver	Kidney	Spleen	Heart	Lung	Fat	Muscle
5	501	370	316	1062	321	388	295	58.1	249
10	338	254	209	703	450	256	188	71.8	217
15	322	189	241	824	473	237	180	49.5	190
30	225	135	245	730	441	193	153	27.6	107
45	101	78.4	284	585	225	114	94.6	19.5	67.0
60	65.1	56.6	254	390	224	104	79.1	13.5	49.2
75	34.0	31.2	250	466	178	95.6	61.9	11.7	41.9
90	25.6	24.5	272	362	165	85.8	48.4	13.8	31.2
105	20.1	20.4	280	252	149	66.6	61.7	11.3	30.5
120	14.0	17.6	231	229	145	75.7	52.3	9.09	30.7
1,440	1.71	5.59	150	109	88.8	27.9	35.7	5.19	16.6
AUC	0.45	0.45	4.69	4.73	3.08	1.41	1.17	0.20	0.68

NOTE: All values are means of measurements of three individual organs. All values are means of three individual measurements. Mean total radioactivity is expressed as nmol equivalents uridine/g tissue. AUC is expressed as µmol-equivalents/g tissue × h. Coefficients of variation in organ levels ranged from 0.9% to 53%. Coefficients of variation in organ AUC levels ranged from 2.6% to 23%. Coefficients of variation in percentage of dose ranged from 3.5% to 35%.

*0- to 7-hour excreta in the 24-hour mice contained 21.67% of dose (21.76% in urine and 0.09% in feces). Analytes found in urine consisted of dihydrouracil (0.69% of dose), uracil (1.81% of dose), and uridine (5.47% of dose). 0- to 24-hour excreta contained 34.83% of dose (33.02% in urine, 0.15% in feces, and 1.66% in cage wash).

for each analyte and models with linear and saturable metabolism. The compartmental models shown in Fig. 3A and B, provided the best fit to the plasma data following the uridine and zebularine i.v. administration, respectively. The resulting estimated variable values are given in Table 3 for each model. The two models, however, predict different uridine turnover rates ($t_{1/2}$ of 0.48 minutes following i.v. zebularine versus 8.3 minutes following i.v. uridine) as well as different uracil turnover rates ($t_{1/2}$ of 1.7 minutes following i.v. zebularine versus 8.6 minutes following i.v. uridine). This led us to develop a single, composite compartmental model to describe the pharmacokinetics of zebularine, uridine, uracil, and dihydrouracil following i.v. administration of zebularine and uridine (Fig. 3C). In this model, the turnover rates for uridine, uracil, and dihydrouracil are allowed to be stimulated in the presence of zebularine relative to their values following direct administration of uridine. Table 3 lists the estimates for the composite compartmental model variables, where S1, S2, and S2 are the factors by which the turnover rate constants of uridine (KUD), uracil (KUC), and dihydrouracil (KDi) are stimulated in the presence of zebularine. The composite model adequately predicted the concentrations measured as shown in Fig. 3D.

Discussion

The present investigation was designed to characterize the disposition of zebularine and its metabolites in mice. Use of radioactively labeled zebularine enabled us to assess the disposition of zebularine and its endogenous metabolites and discuss the implications of those data for the safety assessment of zebularine.

Our results show that uridine, uracil, and dihydrouracil are the primary metabolic products of zebularine. In plasma, a sharp decline of total radioactivity, parent compound, and metabolites was observed after administration of zebularine and uridine, respectively. As can be inferred from the largely overlapping plasma radioactivity profiles, the distribution

characteristics of zebularine and uridine appear to be qualitatively similar. Although the elimination of zebularine from plasma is very rapid, activity has been shown *in vivo* (6), indicating sufficient exposure to zebularine to exert effects. This may also be attributed to presumed uptake, phosphorylation, and accumulation of zebularine in tissues (see below).

The conversion of zebularine to uridine is catalyzed by aldehyde oxidase (EC 1.2.3.1; ref. 8), a class I oxidoreductase (16). In addition to pyrimidines, aldehyde oxidase also catalyzes the oxidation of pyrroles, pyridines, purines, pterins, and aldehydes (16). In humans, aldehyde oxidase is present in liver, lung, adrenal, testis, and prostate tissue. In rodents, aldehyde oxidase is mainly present in liver, lung, and esophagus (17). For all species, the highest levels are found in the liver (16). The *in vivo* conversion of zebularine to uridine is not surprising, given the literature on zebularine (8) and on close analogues of zebularine that have been investigated as aldehyde oxidase-activated prodrugs for their uracil equivalents. Specifically, 5-ethynyl-2(1H)-pyrimidinone was investigated as a prodrug of ethynyluracil, a dihydropyrimidine dehydrogenase inhibitor and, consequently, a modulator of 5-fluorouracil metabolism. Similarly, 5-fluoro-2-pyrimidinone was synthesized as a 5-fluorouracil prodrug and 5-iodo-pyrimidinone-2'-deoxyribose as a prodrug for 5-iodo-2'-deoxyuridine, a radiosensitizer (16, 18).

It was calculated that ~38% of zebularine dose given i.v. was converted to uridine. The calculation of the 38% conversion only holds if there is no conversion of zebularine to uridine in the kidneys. The presence of aldehyde oxidase in murine kidney is unknown but is reported to be very low in rat kidney (16, 17). Because uridine was found to be the primary metabolite of zebularine, we did a second mass balance study using uridine to determine better the relative importance of the uridine catabolic pathway in the metabolic fate of zebularine.

Endogenous uridine concentrations in human, rat, and mouse plasma range from 3 to 8 µmol/L. These concentrations

Table 2. Mean total radioactivity, AUC, and percentage of dose accounted for in male, CD₂F₁ mice after i.v. administration of 100 mg uridine/kg (~ 12 µCi/mg uridine) (Cont'd)

Brain	Testes	Stomach	Duodenum	Jejunum	Ileum	Cecum	Colon	Carcass	% Dose
24.7	63.7	184	377	238	340	113	168	299	70.0
31.7	83.1	207	324	229	238	76.1	204	290	63.9
41.3	108.5	194	332	270	189	137	182	296	64.8
51.5	113.2	197	319	194	214	88.8	146	190	45.1
53.5	97.4	153	283	142	216	62.0	115	137	33.7
51.3	77.5	187	265	138	163	64.1	128	129	30.8
48.7	66.0	128	265	115	139	69.9	107	135	31.7
41.0	53.0	252	301	107	225	71.6	94.5	134	31.7
43.0	57.7	134	241	123	146	83.6	96.0	82.5	21.6
40.3	50.0	126	233	165	141	81.0	93.3	100	24.3
20.0	20.5	68.2	141	103	78.6	33.0	63.8	32.6	9.8*
0.75	0.93	2.48	4.68	3.27	2.79	1.41	1.97	1.77	—

are tightly regulated, and after administration of exogenous uridine, concentrations are rapidly restored to their normal values (13, 14), which agrees with our observations after administration of uridine. In addition, the distribution volume calculated in our studies (~ 860 mL/kg) agrees with literature values (700 mL/kg) for various mouse strains (19). The clearance calculated in our studies was ~ 300-fold higher than that reported in the literature, which is likely due to higher doses used in the latter studies (3,500 mg/kg), saturating uridine catabolic pathways.

Because uridine is a major metabolite of zebularine, the pharmacologic effects of uridine at supraendogenous levels are relevant to the pharmacologic profile of zebularine. There are various reports of uridine affecting body temperature of both animals and humans. However, those reports are not consistent. In three different mouse strains, high-dose uridine (3,500 mg/kg i.p.) caused severe hypothermia (6-10 °C). A low dose of uridine (100 mg/kg) resulted in a slight increase in temperature (19). In contrast, the 100 mg/kg i.v. dose was also reported to induce hypothermia in rats and mice (13). In rabbits, an animal model known to be sensitive to pyrogenic drugs, uridine administration caused fever (20). In humans, prolonged administration of uridine (1-3 g/m²/h, on and off every 3 hours, for 72 hours) resulted in hyperthermia (20), whereas shivering has also been reported with short infusions of higher doses (21). In conclusion, there are conflicting reports about the effect of uridine on body temperature in animals, but in humans, such effects only occur at very high doses. In addition to effects on body temperature, there is evidence of uridine affecting dopamine, neuropeptide, and amino acid transmitter metabolism (13). I.v. infusion (1 hour) of 10 to 12 g/m² doses of uridine to humans resulted in transient shivering, and this symptom was not related to fever or neurologic abnormalities (21). Scaling the 100 mg/kg dose of zebularine given to mice up to its human equivalent (based on body surface area) would give a dose of 8 mg/kg. Based on the ~ 38% conversion of zebularine to uridine, the anticipated human exposure to uridine would be about 3 mg/kg. Currently, uridine is used at oral doses of 150 to 1,000 mg/kg/d to treat certain developmental disorders and pyrimidine-deficient genetic diseases, such as orotic aciduria (14, 22-24). This is a much larger exposure to uridine than expected to result from zebularine treatment, even if corrected for the approximately 6% to 10% oral bioavailability

of uridine in humans (25). Thus, the exposure of humans to supraendogenous concentrations of uridine resulting from zebularine administration will not likely result in safety issues. Hydrogen peroxide, which is a by-product of the conversion of zebularine to uridine by aldehyde oxidase, may be more relevant to toxicity. The conversion of zebularine to uridine is analogous to the metabolism of 2-pyrimidinone to uracil,⁷ which produces hydrogen peroxide as a by-product in equimolar amounts. Exactly how extensive the hepatocellular redox balance is disturbed by zebularine treatment deserves further investigation.

The metabolic production of uridine from zebularine may also be important when combining zebularine with other cytotoxic therapies, irrespective of whether zebularine is used as a DNA methyltransferase inhibitor or a cytidine deaminase inhibitor. Clinical and preclinical studies indicate that fluoropyrimidine-induced RNA damage can be reversed by administration of uridine (14). Thus, before considering the combination of zebularine and fluoropyrimidines, this possible interaction should be addressed.

We did not observe 2-pyrimidinone as a metabolite of zebularine in either plasma or urine. Apparently, zebularine is not a substrate for uridine phosphorylase, and like cytidine, it must first be converted to uridine before the N-glycosidic bond with ribose can be cleaved. Thus, zebularine appears to mimic the behavior of cytidine both in not being a substrate for uridine phosphorylase and in substituting for the latter during DNA replication, which results in its intended inhibition of DNA methyltransferases. Previously, 2-pyrimidinone has been assessed for *in vivo* antitumor activity against L1210 leukemia in BDF₁ mice.⁸ Treatment of mice on days 1, 5, and 9 with an i.p. dose of 100 or 200 mg/kg 2-pyrimidinone hydrochloride did not result in antitumor activity, but a dose of 400 mg/kg proved toxic. Thus, even if it is metabolically produced in tissues at low levels, 2-pyrimidinone is not likely to have any pharmacologic relevance and, as a substrate for aldehyde oxidase (26), would quickly be converted to uracil.

About 90 minutes after administration, the levels of radioactivity observed in most tissues were much higher than

⁷ BRENDA The Comprehensive Enzyme Information System, <http://www.brenda.uni-koeln.de/index.php4>.

⁸ National Cancer Institute *In vivo* Screening Data on 2-pyrimidinone hydrochloride, <http://www.dtp.nci.nih.gov/index.html>.

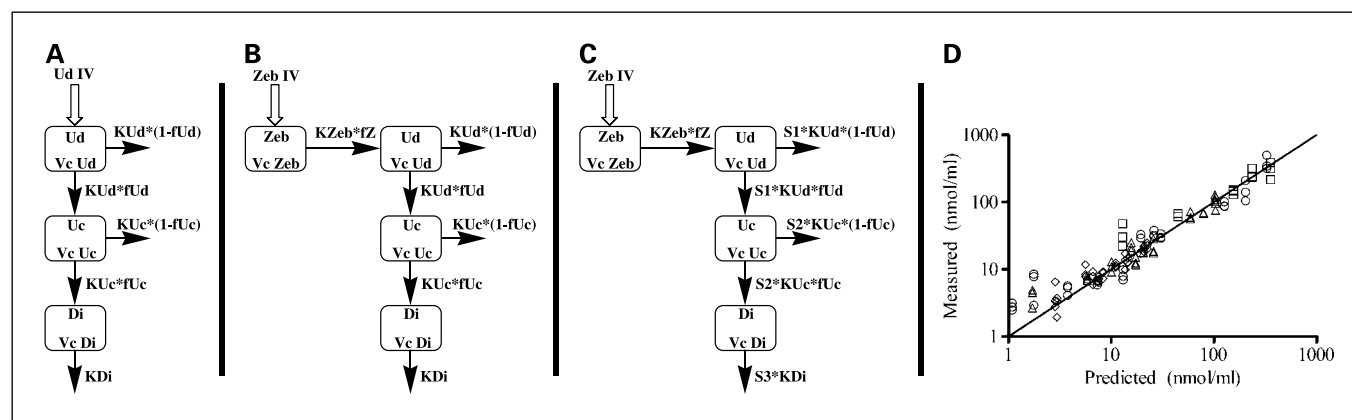


Fig. 3. Compartmental model with linear kinetics fit to the plasma concentration versus time data of zebularine, uridine, uracil, and dihydrouracil after i.v. administration of uridine (A), zebularine (B), zebularine with the factors S1, S2, and S3, representing the increase in turnover rate of uridine, uracil, and dihydrouracil in the presence of zebularine (100 mg/kg; C) to male CD₂F₁ mice. D, predicted versus measured concentrations of zebularine (□), uridine (○), uracil (△), and dihydrouracil (◇). The resulting composite *r*² is 0.90. Empty arrows, administration of the compound; solid arrows, elimination. The fraction of zebularine metabolized (fZ) was set to 0.38 as estimated from the urinary recovery. *Zeb*, zebularine; *Ud*, uridine; *Uc*, uracil; *Di*, dihydrouracil; *fZ*, fraction of zebularine converted to uridine; *fUd*, fraction of uridine converted to uracil; *fUc*, fraction of uracil converted to dihydrouracil; *Vc*, volume of the compartment; *K*, rate constant.

concomitant plasma levels for both zebularine and uridine. This is most likely due to an active transport system taking up nucleosides from plasma (19). Thereafter, intracellular phosphorylation by kinases would result in trapping and accumulation of zebularine and uridine nucleotide species in tissues. RBC do not have active DNA or RNA synthesis and, predictably, contained less radioactivity than plasma. Because zebularine and its metabolites are hydrophilic compounds, it is not surprising that fat, a less well-perfused and lipid-containing tissue, also showed a relatively low accumulation of radioactivity. The relatively low levels, but late time to maximum concentrations of radioactivity, in brain and testis suggests the existence of a blood-brain and blood-testis barrier, respectively, with saturable nucleoside carriers. The radioactivity remaining in various organs is likely to consist of zebularine and uridine nucleotides. It is also conceivable that part of the metabolically produced ¹⁴CO₂ is used in the *de novo* synthesis of endogenous compounds.

The high urinary relative to fecal excretion of radioactivity for both uridine and zebularine is consistent with literature describing the radioactivity excretion pattern of other pyrimidine analogues, such as 5-fluorouracil, capecitabine, and gemcitabine (27–29), which are rapidly excreted in urine with, in general, near-complete recovery of the dose. In the current study, the <100% recovery of zebularine- and uridine-related radioactivity in urine is due to the 2-position of the ¹⁴C label in these compounds. When the 6-position of the pyrimidine ring is used for labeling or when the fluorine is traced by nuclear magnetic resonance, recovery of pyrimidine-related material in urine is nearly complete (27, 28). However, because labeling of pyrimidine analogues in the 2-position results in substantial loss of the ¹⁴C label as ¹⁴CO₂ through expiration, the remaining portion of the structure cannot be traced after that. Our results show that, after 24 hours, only 44.7% of the given 2-[¹⁴C]zebularine dose is accounted for in organs, carcass, and excreta. Thus, approximately half of the

Table 3. Estimated values for the variables of the models shown in Fig. 3

Model	Uridine	Zebularine	Composite
Variable	Estimate (SE)	Estimate (SE)	Estimate (SE)
VcZeb (mL/kg)	—	1,041 (80)	816 (75)
KZeb (min ⁻¹)	—	0.0602 (0.0033)	0.0830 (0.0059)
VcUd (mL/kg)	802 (110)	725 (1,289)	780 (97)
KUd (min ⁻¹)	0.989 (0.0080)	1.03 (1.74)	0.0950 (0.0065)
VcUc/fUd (mL/kg)	1,770 (162)	1,989 (1,414)	1,623 (139)
KUc (min ⁻¹)	0.0684 (0.0054)	0.426 (0.315)	0.0729 (0.0059)
VcDi/fUc (mL/kg)	4,032 (421)	10,070 (1,201)	4,063 (361)
KDi (min ⁻¹)	0.0976 (0.0116)	0.118 (0.017)	0.0974 (0.0096)
S1	—	—	5.22 (0.66)
S2	—	—	3.20 (0.29)
S3	—	—	1.27 (0.13)

NOTE: The fraction of zebularine metabolized (fZ in Fig. 3) was set to 0.38 as estimated from the noncompartmental analysis. Abbreviations: *Zeb*, zebularine; *Ud*, uridine; *Uc*, uracil; *Di*, dihydrouracil; *fUc*, fraction of uracil converted to dihydrouracil; *Vc*, volume of the compartment; *K*, rate constant.

given radioactivity is presumed to be lost as $^{14}\text{CO}_2$ through expiration. Although labeling of pyrimidines in the 2-position is undesirable as a rule, for zebularine, it is less of an issue. The label is part of the metabolic pathway long enough to characterize it. The metabolites resulting after loss of $^{14}\text{CO}_2$ are endogenous (NH_3 and β -alanine), their role in biochemistry is well known; therefore they are less important to trace (9).

The models shown in Fig. 3A and B, with the corresponding variable estimates given in Table 3, best described the concentration versus time data following i.v. uridine and i.v. zebularine, respectively. The volumes of distribution and elimination $t_{1/2}$ for zebularine and uridine derived from the noncompartmental analysis and the compartmental models are similar. However, based on the compartmental model results, the metabolism of uridine and uracil was more rapid in the zebularine study compared with the uridine study. Factors that are otherwise likely to explain differences between the studies, such as animal sex, weight, age, supplier, method of analysis, etc., were constant in the zebularine and uridine studies. This led us to hypothesize an effect of zebularine on the kinetics of its downstream metabolism, as reflected by the factors S1, S2,

and S3 in the composite compartmental model. Enzymes, such as uridine phosphorylase, which are involved in nucleotide metabolism are regulated by their substrates, products, and other nucleotide pools (9, 30–32). The hypothesis represented by the proposed composite model requires further experimental investigation.

In conclusion, all the observed metabolites of zebularine were endogenous substances that are unlikely to contribute to toxicity or activity at the concentrations observed in this study. A human mass balance study of zebularine would provide data to enable interspecies extrapolation and to determine the relevance of murine toxicokinetic data. Because uridine may reduce the cytotoxicity of fluoropyrimidines, combinations of zebularine with fluoropyrimidine therapy in a clinical setting should be preceded by preclinical assessment of this effect.

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