Plasma Pharmacokinetics, Oral Bioavailability, and Interspecies Scaling of the DNA Methyltransferase Inhibitor, Zebularine

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Abstract

Purpose: Zebularine is a DNA methyltransferase inhibitor proposed for clinical evaluation.

Experimental Design: We developed a liquid chromatography/mass spectrometry assay and did i.v. and oral studies in mice, rats, and rhesus monkeys.

Results: In mice, plasma zebularine concentrations declined with terminal half-lives ($t_{1/2}$) of 40 and 91 minutes after 100 mg/kg i.v. and 1,000 mg/kg given orally, respectively. Zebularine plasma concentration versus time curves (area under the curve) after 100 mg/kg i.v. and 1,000 mg/kg given orally were 7,323 and 4,935 μg/mL min, respectively, corresponding to a total body clearance (CL_{tb}) of 13.65 mL/min/kg, apparent total body clearance (CL_{app}) of 203 mL/min/kg, and oral bioavailability of 6.7%. In rats, plasma zebularine concentrations declined with $t_{1/2}$ of 363, 110, and 126 minutes after 50 mg/kg i.v., 250 mg/kg given orally, and 500 mg/kg given orally, respectively. Zebularine areas under the curve after 50 mg/kg i.v., 250 mg/kg given orally, and 500 mg/kg given orally were 12,526, 1,969, and 7,612 μg/mL min, respectively, corresponding to a CL_{tb} of 3.99 mL/min/kg for 50 mg/kg i.v. and CL_{app} of 127 and 66 mL/min/kg for 250 and 500 mg/kg given orally, respectively. Bioavailabilities of 3.1% and 6.1% were calculated for the 250 and 500 mg/kg oral doses, respectively. In monkeys, zebularine $t_{1/2}$ was 70 and 150 minutes, CL_{tb} was 3.55 and 10.85 mL/min/kg after i.v. administration, and CL_{app} was 886 and 39,572 mL/min/kg after oral administration of 500 and 1,000 mg/kg, respectively. Zebularine oral bioavailability was <1% in monkeys. Interspecies scaling produced the following relationship: $CL_{tb} = 6.46 \text{(weight}^{0.9})$.

Conclusions: Zebularine has limited oral bioavailability. Interspecies scaling projects a CL_{tb} of 296 mL/min in humans.

In addition to primary DNA sequence mutations, epigenetic changes, such as aberrant methylation of CpG islands in the promoter region of genes, alter the pattern of gene expression and may play an important role in the progression of cancer (1, 2). As a result, using DNA methylation inhibitors to reactivate silenced tumor suppressor genes has become an attractive strategy in antineoplastic drug development (3–5). Zebularine, an acid-stable ribonucleoside that inhibits DNA methyltransferase (6–8), has modest *in vivo* antitumor activity against murine B16 melanoma and P388 and L1210 leukemias (9). Furthermore, oral administration of zebularine is associated with reactivation of p16 tumor suppressor gene expression in, and inhibition of growth of, s.c. EJ6 bladder carcinoma xenografts (6).

In support of planned clinical evaluation of zebularine, we developed and validated a liquid chromatography/mass spectrometry (LC/MS) method for quantitating zebularine in plasma and then conducted studies in CD_2F_1 mice, Fischer 344 rats, and rhesus monkeys (*Macaca mulatta*) to characterize the plasma pharmacokinetics of zebularine after i.v. and oral dosing. Those pharmacokinetic data were used to calculate the oral bioavailability of zebularine and for interspecies scaling to predict the clearance (CL_{tb}) of zebularine in humans.

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Materials and Methods

Drugs and reagents

Zebularine (NSC 309132) was supplied by the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD) and was stored in the dark at 4°C to 8°C until use. Guanosine (Sigma grade) and formic acid (minimum 95%) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Acetonitrile (optima grade), ammonium acetate (enzyme grade), and methanol (optima grade) were purchased from Fisher Scientific (Fairlawn, NJ). Untreated control mouse, rat, dog, and rhesus monkey plasma were purchased from Lampire Biological Laboratories (Pipersville, PA). Control human plasma was

prepared by centrifugation of outdated, citrate-anticoagulated blood obtained from the Central Blood Bank (Pittsburgh, PA). Medical-grade nitrogen and liquid nitrogen were purchased from Valley National Gases (Pittsburgh, PA).

Animals

Rodents. Specific pathogen-free adult CD_2F_1 male mice (5-6 weeks old) were purchased from Taconic (Germantown, NY). Specific pathogen-free Fischer 344 male rats (7-8 weeks old) were purchased from Hilltop Lab Animals (Scottsdale, PA). Mice and rats were allowed to acclimate to the University of Pittsburgh Cancer Institute Animal Facility (Pittsburgh, PA) for 1 week before being used. To minimize exogenous infection, mice and rats were maintained in microisolator cages in separate rooms 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 in the animal facility were set to 12 changes per hour. Room temperatures were regulated at $72 \pm 2°F$ and the rooms were kept on automatic 12-hour light/dark cycles. Mice and rats 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 and withheld until 4 hours after dosing. Sentinel animals (CD-1 mice or Sprague-Dawley rats in cages with bedding that contained 20% bedding removed from the study animal cages at cage change) were maintained in the rooms housing mice and rats, respectively, and assayed at 3-month intervals for specific murine pathogens by mouse or rat antibody profile testing (Charles River, Boston, MA). Sentinel animals remained free of specific pathogens, indicating that the study mice and rats were pathogen free.

Nonhuman primates. Male and female rhesus monkeys (~ 6-9 years old and 5.8-12 kg in weight) were obtained from Three Springs Scientific (Perkaise, PA) or the California National Primate Research Center (Davis, CA). Monkeys were housed in the University of Illinois at Chicago Animal Facility (Chicago, IL) and treated on a protocol approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago. All monkeys were quarantined for a minimum of 28 days before the start of the study and received at least one complete physical examination before the study. Animal care and housing were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the U.S. Department of Agriculture through the Animal Welfare Act (Public Law 99-198). Monkeys were individually housed in stainless steel cages during quarantine and study. Environmental conditions were set at 12-hour light/dark cycles, temperature between 65°F and 84°F, relative humidity between 30% and 70%, and 10 to 15 fresh air changes per hour. One week before the study and throughout the study, monkeys received 15 to 25 Certified Primate Chow biscuits (PMI Nutrition International), nuts, fruits, or foraging items daily and water *ad libitum*.

In vitro studies

To determine the *in vitro* stability of zebularine in various plasmas, mouse, rat, dog, monkey, and human plasma were equilibrated for 10 minutes either at ambient temperature (~ 22°C) or at 37°C in a water bath (Thelco, Precision Scientific, Chicago, IL). Zebularine was added to produce final concentrations of 1, 20, or 30 μg/mL. Samples were mixed briefly on a Vortex Genie 2 (Model G-560, Scientific Industries, Bohemia, NY) and returned to incubation at either 22°C or 37°C. Triplicate 0.2 mL aliquots were removed immediately and at 1, 2, 4, 5.5, and 24 hours after adding zebularine. Zebularine concentrations were assessed with the LC/MS system described below.

To assess protein binding of zebularine, triplicate samples of zebularine at concentrations of 1, 10, and 100 μg/mL were prepared in PBS (Cambrex BioScience, Walkersville, MD), mouse, rat, dog, monkey, and human plasma, and 1 mL of each was placed into the upper chamber of an Amicon Centrifree YM-30 (regenerated cellulose, 30,000 molecular weight cutoff) ultrafiltration device (Millipore Corp.,

Bedford, MA). After centrifugation of the ultrafiltration devices for 20 minutes at 4°C and $1,500 \times g$, zebularine concentrations in the resulting ultrafiltrates and in the initial solutions were determined with the LC/MS method described below.

Pharmacokinetic studies

Dosing. Dosing solutions for mouse i.v. and oral studies were prepared at 10 mg/mL in sterile 0.154 mol/L NaCl and 100 mg/mL in sterile water, respectively. Dosing solutions for rat i.v. and oral studies were prepared at 50 mg/mL in sterile 0.154 mol/L NaCl and 125 mg/mL in sterile water, respectively. Dosing solutions for rhesus monkey i.v. and oral studies were prepared at 100 or 200 mg/mL in sterile 0.154 mol/L NaCl, respectively. Dosing solutions were freshly prepared on the morning of each study.

Mice were treated with a 100 mg/kg i.v. or 1,000 mg/kg oral dose of zebularine. The i.v. dose was delivered as a bolus in a volume of 0.01 mL/g fasted body weight through a 27-gauge needle and via a lateral tail vein. The oral dose was delivered as a bolus in a volume of 0.01 mL/g fasted body weight through a 1.5-inch, 20-gauge curved gavage needle.

Rats were treated with one of three doses of zebularine. The 50 mg/kg i.v. dose was delivered as a bolus in a volume of 0.001 mL/g fasted body weight through a 27-gauge needle and via a lateral tail vein. Oral doses of either 250 or 500 mg/kg were given as boluses in a volume of 0.002 or 0.004 mL/g fasted body weight, respectively, and were delivered through a 3-inch, 16-gauge curved gavage needle.

Four rhesus monkeys (one male and one female per dose group) were randomly assigned to receive either 500 or 1,000 mg/kg zebularine. On study day 1, each monkey received a single dose of zebularine by slow i.v. bolus via an antecubital vein. The bolus lasted at least 1 minute, and the infusion line was then flushed with ~ 1 mL of sterile 0.154 mol/L NaCl. On study day 16, monkeys received a single oral dose of zebularine at the same dose level as on day 1. The volume in which i.v. and oral doses were delivered was 5 mL/kg of body weight, and doses were based on each monkey's individual body weight on the day of treatment. Each animal was fasted for ~ 16 to 20 hours before dosing.

Sampling. Groups of three mice were euthanized by CO_2 inhalation, and blood was collected by cardiac puncture using heparinized syringes at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 360, 420, 960, and 1,440 minutes after dosing with zebularine or 5 minutes after administration of vehicle. Blood was transferred to microcentrifuge tubes and stored on ice until centrifuged at $13,000 \times g$ for 4 minutes to obtain plasma. Plasma was transferred to clean tubes and stored at −70°C until analyzed.

In the rat study, groups of three rats were sampled at staggered times so that blood was obtained before drug delivery and at the following times after drug administration: 5, 15, 30, 45, 60, 90, 120, 240, 360, 420, 960, 1,440, and 2,880 minutes. Three blood samples were obtained from each rat. The first two blood samples were 300 μL each and were collected into heparinized syringes by tail vein bleeds from the vein opposite the injection site. At the time of the third and final blood sample, rats were euthanized by CO_2 inhalation, and blood was collected into heparinized syringes by cardiac puncture. Terminal sampling was carried out at 15, 120, 420, 1,440, and 2,880 minutes. Blood was transferred to microcentrifuge tubes and stored on ice until centrifuged at $13,000 \times g$ for 4 minutes to obtain plasma. Plasma was transferred to clean tubes and stored at −70°C until analyzed.

In all monkey studies, heparinized blood samples (2 mL) were collected from an indwelling femoral vein catheter before and at 15, 30, 60, 90, 120, 240, 480, and 1,440 minutes after zebularine dosing. In addition, samples were obtained at 5 and 10 minutes after i.v. drug administration. Blood samples were centrifuged at $3,000 \times g$ for 15 minutes, and the resulting plasma was transferred to labeled vials and stored at −80°C until analyzed.

Sample preparation. Rat plasma (100 μL) or mouse or monkey plasma (200 μL) were added to a 1.5-mL microcentrifuge tube and mixed with 10 μL of 100 μg/mL guanosine internal standard in methanol/water (50:50, v/v) and 1 mL acetonitrile. After mixing for 1 minute on a Vortex Genie 2 set at 10, samples were centrifuged for

Table 1. Percentage of bound zebularine in plasma from various species

Zebularine (µg/mL)	Species				
	Mouse	**Rat**	**Beagle**	**Monkey**	**Human**
1	43 ± 2.3*[,†]	73 ± 0.1	34 ± 8.4	42 ± 2.5	57 ± 2.5
10	6 ± 10.3	43 ± 4.2	34 ± 2.1	12 ± 10.9	10 ± 8.2
100	18 ± 2.6	42 ± 1.7	12 ± 6.1	4 ± 3.3	5 ± 13.5

* $n = 3$ for each concentration for each species.
† Mean ± SD.

6 minutes at $16,000 \times g$. The resulting supernatants were removed, transferred to clean 12×75 mm glass culture tubes, and evaporated to dryness under a stream of nitrogen. The dried residues were reconstituted in 100 µL of 10 mmol/L ammonium acetate, and 10 µL were injected by autosampler into the LC/MS system.

Liquid chromatography/mass spectrometry system

The LC/MS system included an Agilent Technologies, Inc. (Palo Alto, CA) model 1100 autosampler with a 100-µL sample loop, an Agilent 1100 binary pump, and a Synergi Hydro-RP 80A (4 µm, 100 × 2 mm) analytic column (Phenomenex, Torrance, CA). The mobile phase was a gradient of 0.2% formic acid in 10 mmol/L ammonium acetate (solvent A) and 0.2% formic acid in methanol (solvent B). The initial mobile phase was 100% solvent A. Between 0 and 10 minutes, the flow rate was maintained at 0.2 mL/min, whereas the percentage of solvent B was increased linearly from 0% to 30%. Between 10 and 12 minutes, the composition and the flow rate were maintained at 30% solvent B and 0.2 mL/min, respectively. Between 12 and 13 minutes, the percentage of solvent B was decreased from 30% to 0%, and the flow rate was increased to 0.4 mL/min. Between 13 and 19.1 minutes, the mobile phase was maintained at 100% of solvent A with a flow rate of 0.4 mL/min. Between 19.1 and 19.8 minutes, the composition of the mobile phase remained at 100% solvent A, but the flow rate was returned to 0.2 mL/min and held at that rate until 20 minutes, when the next sample was injected. Column eluate was analyzed with a Thermofinnigan Surveyor MSQ Single Quadrupole Mass Spectrometer (ThermoQuest, San Jose, CA) operating in electrospray, positive single-ion mode to monitor m/z 229.0 for zebularine and m/z 284.0 for guanosine. The insert probe temperature was set at 400°C with 3 kV applied as the ion spray voltage and 30 V as the orifice voltage. The system was operated with Thermofinnigan Excalibur Software.

The internal standard ratio was calculated for each standard by dividing the analyte peak area by the peak area of the internal standard. Standard curves of zebularine were constructed by plotting the internal standard ratio versus the known concentration of zebularine in the sample. Duplicate standard curves containing zebularine concentrations of 0, 0.03, 0.05, 0.1, 0.3, 0.5, 1, and 3 µg/mL were included with each analytic run. Duplicate quality control samples of 0.04, 0.2, and 0.7 µg/mL were also included with each analytic run. Standard curves were fit by linear regression with weighting by $1/Y^2$ followed by back calculation of concentrations. Samples containing concentrations above the upper limit of the standard curve were reassayed after dilution in the appropriate matrix to a degree calculated to produce a concentration within the linear range of the standard curve.

Pharmacokinetic analyses

The time courses of plasma concentrations of zebularine were analyzed by noncompartmental and compartmental methods. The area under the curve from zero to infinity (AUC) and the terminal half-life ($t_{1/2}$) were estimated using the LaGrange function (10) as implemented by the computer program LAGRAN (11). CL_{tb} was calculated from the equation: CL_{tb} = dose / AUC.

Compartmental modeling was done with the program ADAPT II (12) using maximum likelihood estimation. One-compartment and two-compartment models were fit to the data from i.v. pharmacokinetic studies, and model discrimination was based on Akaike's Information Criterion (13). Compartmental modeling of orally given zebularine used a strategy wherein the variable values for the volume of the central compartment (V_c) and transfer constants k_{pc}, k_{cp}, and k_e were fixed at the values obtained from the i.v. study in the appropriate species, and the values for variables related to the rate of absorption and bioavailability (F) were estimated. The rate of absorption was modeled as both a first-order and a saturable process, so that values for k_a as well as for K_{mabs} and V_{maxabs} were estimated. As with modeling of studies using i.v. zebularine, model discrimination for studies of orally given zebularine was based on Akaike's Information Criterion.

Interspecies scaling of pharmacokinetic data from i.v. studies of zebularine used standard methods (14). Specifically, CL_{tb} for each species was plotted as the dependent variable, weight of each species was plotted as the independent variable, and the relationship was described by the following equation: $CL_{tb} = a(weight^b)$, where a was a calculated constant and b was a power to which weight was raised.

Results

Performance of liquid chromatography/mass spectrometry system

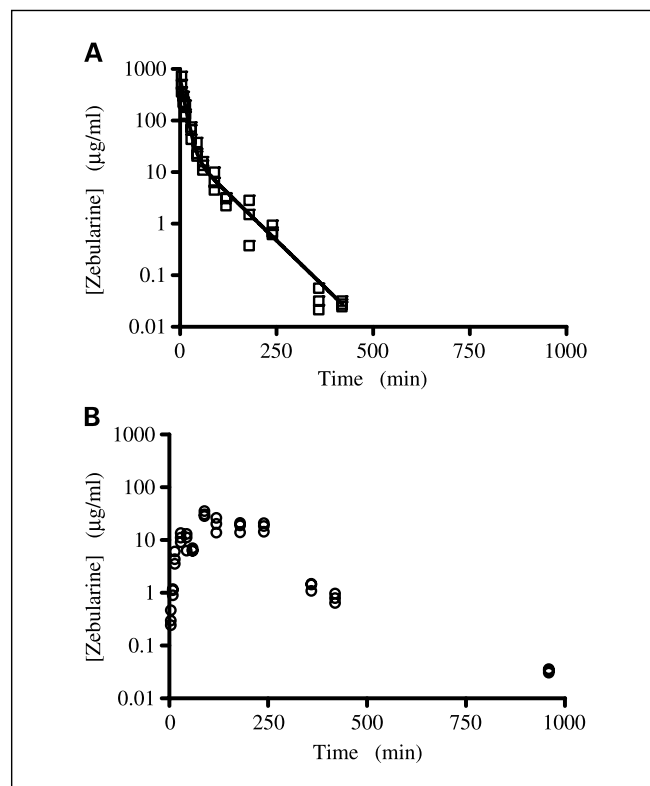Under the LC/MS conditions described above, zebularine and guanosine internal standard eluted at ∼4.7 and 7.0

Fig. 1. Plasma zebularine concentration versus time data after administration of (A) 100 mg/kg i.v. or (B) 1,000 mg/kg given orally to mice. Points, samples from individual mice. The line in (A) represents the concentration versus time profile resulting from a two-compartment, open, linear model fit to the data. Because a suitable compartmental model could not be fit to the concentration versus time data after oral dosing, no line is included in (B).

minutes, respectively. The lower limit of quantitation (15) was 0.03 µg/mL and the standard curve of zebularine was linear between 0.03 and 3.0 µg/mL. No endogenous materials in plasma from any species studied interfered with the quantitation of zebularine or internal standard, and the assay was validated according to suggested guidelines (15).

In vitro studies

After 24-hour incubation at 37°C, there was >50% decomposition of zebularine in human plasma and >35% decomposition in rat plasma. However, when incubated for up to 24 hours at concentrations of 1 or 30 µg/mL in plasma at 22°C, there was <5% decomposition of zebularine in mouse, rat, dog, monkey, or human plasma.

Initial experiments showed that 0.1, 1, 10, and 100 µg/mL solutions of zebularine in PBS did not bind to the membranes present in Amicon Centrifree devices. Subsequent studies showed that zebularine, at concentrations of 1, 10, and 100 µg/mL, was low to moderately protein bound in mouse, rat, dog, monkey, and human plasma, and in each species, there was a tendency for higher concentrations of zebularine to be associated with a smaller percentage of bound drug (Table 1).

In vivo studies

Mice. I.v. delivery of a 100 mg/kg dose of zebularine to mice produced peak zebularine concentrations between 354 and 702 µg/mL (Fig. 1A). Plasma zebularine concentrations declined in a biphasic manner so that after 420 minutes they were less than the lower limit of quantitation of the LC/MS assay (Fig. 1A). Noncompartmental modeling estimated a $t_{1/2}$ of 40 minutes and calculated an AUC of 7,323 µg/mL min and CL_{tb} of 13.65 mL/min/kg (Table 2). Compartmental modeling showed the decrease in plasma zebularine concentrations to be best fit by a two-compartment, open, linear model described by the following variables: V_c, 150 mL/kg; k_{cp}, 0.0137 min^{-1}; k_{pc}, 0.0204 min^{-1}; and k_e, 0.079 min^{-1} (Table 2).

Oral delivery of a 1,000 mg/kg dose of zebularine to mice produced the plasma zebularine concentration versus time profile displayed in Fig. 1B. Peak zebularine concentrations were between 28 and 35 µg/mL and occurred at 90 minutes after dosing. However, plasma zebularine concentrations between 13 and 20 µg/mL were maintained between 120 and 240 minutes and, at 960 minutes after dosing, were still greater than the lower limit of quantitation of the LC/MS assay (Fig. 1B). Noncompartmental modeling calculated an AUC of 4,935 µg/mL min and CL_{app} of 203 mL/min/kg, which indicated an oral bioavailability of 6.7% (Table 2).

Table 2. Pharmacokinetic variables

Results of noncompartmental modeling of plasma zebularine concentration versus time data in mice, rats, and rhesus monkeys given i.v. or oral doses of zebularine

Species	Sex	Dose (mg/kg)	Route	$t_{1/2}$ (min)	AUC (g/mL min)	CL_{tb} (mL/min/kg)	CL_{app} (mL/min/kg)	Bioavailability (%)
Mouse	Male	100	i.v.	40	7,323	13.65	—	—
	Male	1,000	oral	—	4,935	—	203	6.7
Rat	Male	50	i.v.	363	12,526	3.99	—	—
	Male	250	oral	—	1,969	—	127	3.1
	Male	500	oral	—	7,612	—	66	6.1
Monkey	Male	500	i.v.	70	88,020	5.68	—	—
		500	oral	—	84	—	6,021	0.1
	Female	500	i.v.	76	46,080	10.85	—	—
		500	oral	—	12	—	39,572	0.026
	Male	1,000	i.v.	150	104,520	9.57	—	—
		1,000	oral	—	42	—	23,988	0.04
	Female	1,000	i.v.	147	281,220	3.55	—	—
		1,000	oral	—	1,128	—	886	0.4

Pharmacokinetic variables describing compartmental models fit to plasma zebularine concentration versus time data in mice, rats, and rhesus monkeys

Species	Sex	Dose (mg/kg)	Route	V_c (mL/kg)	k_{cp} (min^{-1})	k_{pc} (min^{-1})	k_e (min^{-1})	K_{mabs} (g/kg)	V_{maxabs} (g/min/kg)	Bioavailability (%)
Mouse	Male	100	i.v.	150	0.0137	0.0204	0.079	—	—	—
Rat	Male	50	i.v.	342	0.0034	0.0062	0.0205	—	—	—
	Male	250	oral	342	0.0034	0.0062	0.0205	2,754	71	4.8
	Male	500	oral	342	0.0034	0.0062	0.0205	6,784	220	9.2
Monkey	Male	500	i.v.	444	0.0911	0.794	0.7373	—	—	—
	Female	500	i.v.	738	1.114	2.246	0.9783	—	—	—
	Male	1,000	i.v.	638	0.4648	0.7058	0.5889	—	—	—
	Female	1,000	i.v.	392	0.0968	0.3937	0.5368	—	—	—

A compartmental model that adequately described the absorption process could not be fit to the concentration versus time data associated with the 1,000 mg/kg oral dose given to mice.

Rats. Intravenous delivery of a 50 mg/kg dose of zebularine to rats produced peak zebularine concentrations between 165 and 238 µg/mL (Fig. 2A). Plasma zebularine concentrations decreased in a biphasic manner and were greater than the lower limit of quantitation of the LC/MS assay until 960 minutes after dosing (Fig. 2A). Noncompartmental modeling estimated a $t_{1/2}$ of 363 minutes and calculated an AUC of 12,526 µg/mL min and a CL_{tb} of 3.99 mL/min/kg (Table 2). Compartmental modeling showed the decrease in plasma zebularine concentrations to be best fit by two-compartment, open, linear model described by the following variables: V_c, 342 mL/kg; k_{cp}, 0.0034 min^{-1}; k_{pc}, 0.0062 min^{-1}; and k_e, 0.0205 min^{-1} (Table 2).

Oral delivery of a 250 mg/kg dose of zebularine produced the plasma zebularine concentration versus time profile displayed in Fig. 2B. Peak zebularine concentrations were between 10 and 11 µg/mL and occurred at 120 minutes after delivery. Noncompartmental modeling calculated an AUC of 1,969 µg/mL min and CL_{app} of 127 mL/min/kg, which indicated an oral bioavailability of 3.1% (Table 2). Oral delivery of a 500 mg/kg dose of zebularine produced the concentration versus time profile displayed in Fig. 2C. Peak zebularine concentrations were between 31 and 32 µg/mL and occurred at 120 minutes. However, plasma zebularine concentrations between 18 and 27 µg/mL were maintained between 45 and 240 minutes after dosing. Noncompartmental modeling calculated an AUC of 7,612 µg/mL min and a CL_{app} of 66 mL/min/kg, which indicated an oral bioavailability 6.1%. A two-compartment, open, linear model with an absorption process could be fit to the data from both studies in which zebularine was given orally to rats (Fig. 2B and C). In both studies, a saturable absorption process, described by K_{mabs} and V_{maxabs}, provided a superior fit when compared with a first-order absorption process. The values estimated for K_{mabs} and V_{maxabs} in the 250 mg/kg oral study were 2,754 µg/kg and 71 µg/min/kg, respectively, whereas the values for K_{mabs} and V_{maxabs} in the 500 mg/kg oral study were estimated as 6,784 µg/kg and 220 µg/min/kg, respectively (Table 2). Compartmental modeling estimated bioavailability of 4.8% for the 250 mg/kg oral dose and 9.2% for the 500 mg/kg oral dose.

Monkeys. I.v. delivery of 500 mg/kg zebularine to monkeys produced "peak" zebularine concentrations of 1,094 (male) and 537 (female) µg/mL at 5 minutes after dosing, which was the first blood sample taken (Fig. 3A and B). I.v. delivery of 1,000 mg/kg zebularine produced "peak" zebularine concentrations of 1,656 (male) and 2,853 (female) µg/mL at 5 minutes after dosing (Fig. 3C and D). In each monkey, plasma zebularine concentrations decreased in a biphasic manner after i.v. zebularine administration. In the two monkeys given 500 mg/kg doses of zebularine i.v., plasma zebularine concentrations decreased below the lower limit of quantitation of the LC/MS assay after 480 minutes (male) in one and after 240 minutes in the other (female; Fig. 3A and B). In both monkeys given 1,000 mg/kg doses of zebularine i.v., plasma zebularine concentrations remained above the lower limit of quantitation of the LC/MS assay throughout the 24-hour period in which plasma samples were obtained. Noncompartmental modeling of data from the two monkeys given 500 mg/kg zebularine estimated a $t_{1/2}$ of 70 minutes in one and 76 minutes in the other. The AUCs calculated for these two monkeys were 88,020
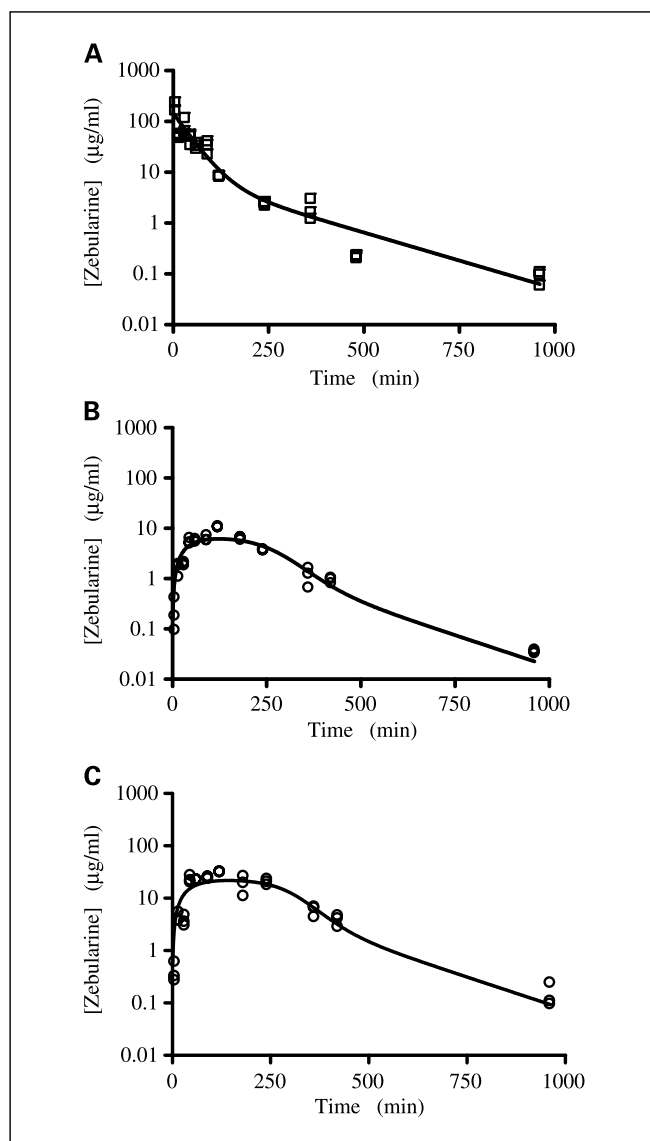

Fig. 2. Plasma zebularine concentration versus time data after administration of (*A*) 50 mg/kg i.v., (*B*) 250 mg/kg given orally, or (*C*) 500 mg/kg oral doses to rats. Points, individual determinations on plasma samples obtained from rats, each of which was sampled at three specified times during the first 24 hours after drug administration. Lines, concentration versus time profiles resulting from the fit of the two-compartment, open, linear model to the data, with oral studies including a saturable absorption component.

and 46,080 µg/mL min, which corresponded to CL_{tb} of 5.68 mL/min/kg and 10.85 mL/min/kg, respectively (Table 2). Noncompartmental modeling of data from the two monkeys given 1,000 mg/kg zebularine estimated $t_{1/2}$ of 150 minutes in the male and 147 minutes in the female. The AUCs calculated for these two monkeys were 104,520 and 281,220 µg/mL min, which corresponded to CL_{tb} of 9.57 and 3.55 mL/min/kg, respectively (Table 2). Compartmental modeling showed the plasma concentration versus time profiles for all four monkeys after i.v. zebularine administration were best fit by a two-compartment, open, linear model. The values for V_c, k_{cp}, k_{pc}, and k_e for each monkey are indicated in Table 2.

Oral delivery of zebularine to monkeys produced the concentration versus time profiles displayed in Fig. 3A to D. Peak plasma zebularine concentrations associated with the
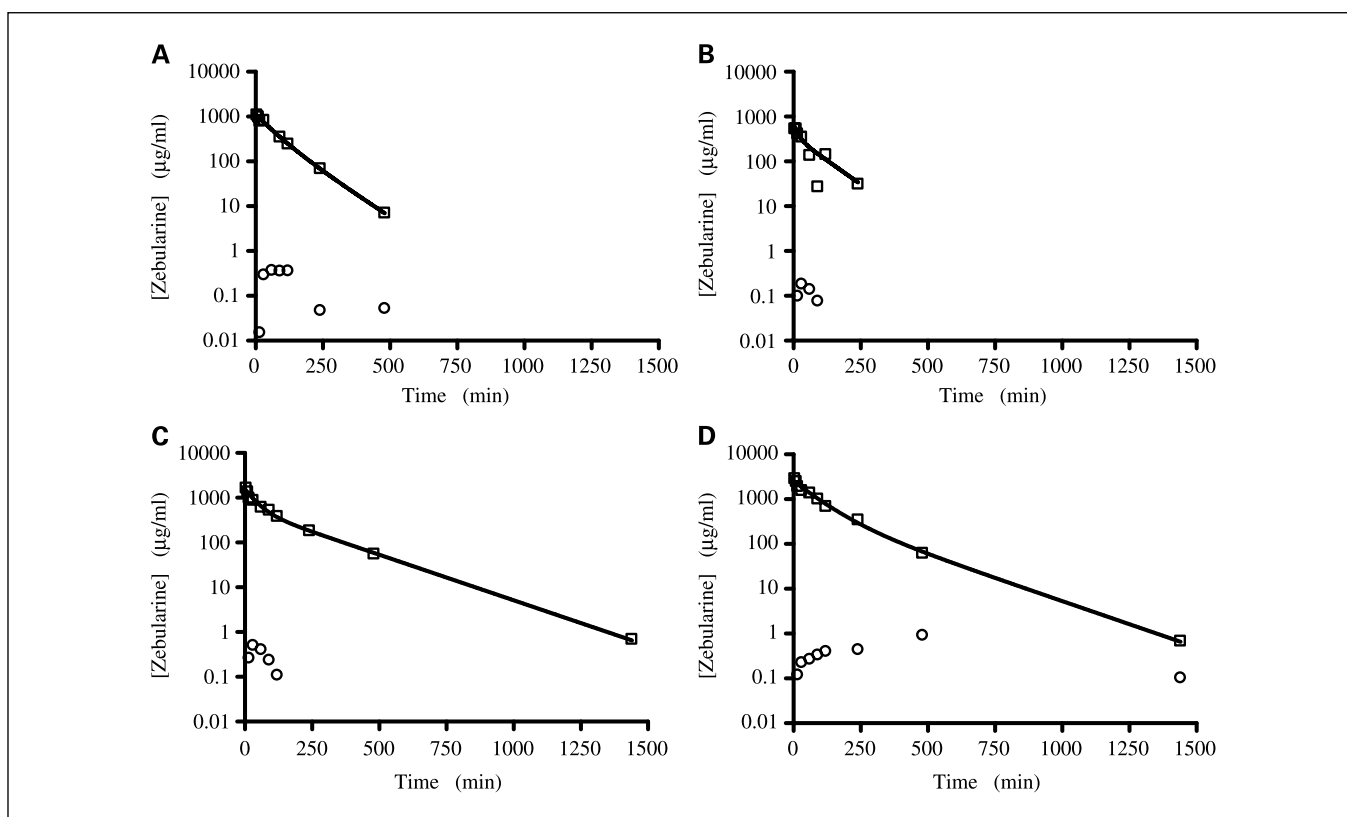
Fig. 3. Plasma zebularine concentrations versus time data in four monkeys, two of which were given 500 mg/kg zebularine doses i.v. (□) and orally [○; (*A*) male and (*B*) female] and two of which were given 1,000 mg/kg doses i.v. (□) and orally [○; (*C*) male and (*D*) female]. Lines in (*A*-*D*), concentration versus time profile resulting from the fit of a two-compartment, open, linear model to the data resulting from i.v. administration of zebularine. Because a suitable compartmental model could not be fit to the concentration versus time data after oral dosing, no lines are included for the oral data displayed in (*A*-*D*).

500 mg/kg oral dose were 0.37 μg/mL in one monkey and 0.18 μg/mL in the other and occurred at 1 and 0.5 hour after dosing. The peak plasma zebularine concentrations associated with a 1,000 mg/kg oral dose were 0.5 μg/mL in one monkey and 0.92 μg/mL in the other and occurred 0.5 and 8 hours after dosing, respectively. Noncompartmental modeling calculated AUCs of 84 and 12 μg/mL min for the monkeys given 500 mg/kg zebularine orally and 42 and 1,128 μg/mL min for the monkeys given 1,000 mg/kg zebularine orally, respectively. These AUCs corresponded to oral bioavailabilities of 0.1% and 0.026% for the animals treated with 500 mg/kg and 0.04% and 0.4% for the animals treated with 1,000 mg/kg (Table 2). A suitable compartmental model capable of describing the absorption of zebularine could not be fit to the plasma zebularine concentration versus time data from any of the four monkeys given oral doses of zebularine.

The availability of i.v. pharmacokinetic data from three species allowed interspecies scaling of zebularine to be done (Fig. 4). When the data displayed in Fig. 4 were fit to the equation: $CL_{tb} = a(\text{weight}^b)$, values of 6.46 for a and 0.9 for b were calculated. When the 70 kg weight of an average human being was used with the equation, a CL_{tb} of 296 mL/min was predicted for the average human.

Discussion

Modulation of reversible genetic alterations associated with malignant phenotypes (1, 2) is a therapeutic strategy that has attracted and continues to attract substantial interest (3–5). Although this strategy has been validated by the recent approval of the DNA methyltransferase inhibitor, 5-azacytidine, as a treatment for myelodysplastic syndrome, there remains considerable interest in developing DNA methyltransferase
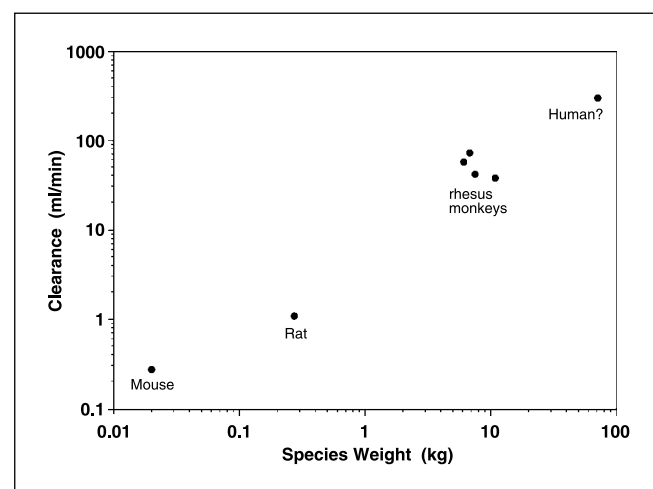

Fig. 4. Relationship between species weight and zebularine clearance. Data for mouse, rat, and rhesus monkeys are those from the current studies and are described by the relationship: clearance = 6.46(weight^0.9). The clearance for humans is predicted based on the relationship calculated from mouse, rat, and rhesus monkey data.

inhibitors that have more desirable chemical properties, less clinical toxicities, or a broader spectrum of activity than those associated with 5-azacytidine (3–5).

Preclinical studies have already shown the ribonucleoside, zebularine, to have several characteristics that make it a viable candidate for clinical evaluation (5–8). Unlike 5-azacytidine, which is unstable in neutral and basic media, zebularine is chemically stable (3, 4). In addition, zebularine has shown activity *in vitro* and *in vivo* in more than one tumor model. Of interest is the fact that, in at least one tumor model, inhibition of tumor growth was accompanied by hypomethylation of the P16 tumor suppressor gene and the appearance of the P16 protein, which implies that the antitumor activity of zebularine might be related to inhibition of DNA methyltransferase (6). Finally, zebularine has proven much less myelosuppressive than 5-azacytidine, which might facilitate evaluation of zebularine as a treatment for solid tumors.

The studies in the current article define several pharmacokinetic characteristics of zebularine that should assist in its clinical development. Preclinical models have shown the reversibility of zebularine's DNA methyltransferase inhibition and the requirement of prolonged exposure of tumors to zebularine for inhibition of tumor growth. Based on the $t_{1/2}$ of zebularine after i.v. dosing of mice, rats, and monkeys, it is likely that frequent dosing or continuous i.v. infusion of zebularine will be necessary to maintain prolonged inhibition of DNA methyltransferase. Although oral administration of zebularine has been shown to have *in vivo* activity in preclinical murine tumor models and oral administration would be the most logistically expedient means of delivering regular and relatively frequent doses of zebularine, the low bioavailability of zebularine in rodents and the extremely low bioavailability of zebularine in rhesus monkeys are a cause for concern. The cause for the low bioavailability of zebularine is unclear, as is the explanation for why the bioavailability of zebularine is so much less in rhesus monkeys than in mice and rats. Potential causes for the low bioavailability include saturation of an absorption process, metabolism of zebularine in the gut wall, and first-pass metabolism in the liver. Based on its structure, zebularine should be a substrate for aldehyde oxidase, which would convert zebularine to uridine, and for kinases, which would convert zebularine to monophosphates, diphosphates, and triphosphates. The LC/MS assay used in our studies did not quantitate phosphorylated zebularine metabolites and would not have been able to differentiate uridine produced from zebularine from the large pool of endogenous uridine. Although modification of the LC/MS method might make quantitation of phosphorylated zebularine metabolites possible, use of stable-labeled zebularine would be required to measure zebularine-derived uridine.

Even with the low bioavailability of zebularine, oral therapy could be viable if (*a*) therapeutically active concentrations of zebularine could be obtained, (*b*) the bioavailability in each patient is consistent so that therapeutic concentrations of zebularine could be maintained, and (*c*) zebularine proves as nontoxic clinically as it has to date in preclinical studies so that an intermittent or unanticipated increase in bioavailability would not result in unexpected, unacceptable toxicity. In addition, it is possible that suitable pharmaceutical formulation modifications could overcome any limitations in bioavailability that might be related to the administration of zebularine in distilled water or 0.154 mol/L NaCl vehicles used in the pharmacokinetic studies in the current article.

Alternatively, the continuous infusion schedule might prove the most suitable way to administer zebularine. Should that be the case, the data in the current article can aid in designing those clinical studies. The availability of i.v. pharmacokinetic data on three species allowed interspecies scaling and a prediction of the zebularine clearance expected in humans. Although this prediction requires validation, the rate of infusion to produce a target plasma steady-state concentration of zebularine can be predicted from the following equation: steady-state concentration = rate of infusion/total body clearance, where a total body clearance of ~300 mL/min can be anticipated in humans.

One final aspect of the current pharmacokinetic studies that should facilitate clinical evaluation of zebularine is the LC/MS assay developed to quantitate zebularine. The assay, which involves small sample volumes, a readily accessible and inexpensive internal standard, and simple sample preparation, is sensitive, robust, and linear over a wide range of zebularine concentrations. Although the assay requires LC/MS instrumentation, it should be implementable at most institutions involved in early-phase clinical trials of antineoplastic agents.

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