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Zebularine metabolism by aldehyde oxidase in hepatic cytosol from humans, monkeys, dogs, rats, and mice: Influence of sex and inhibitors

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Abstract—To aid in the clinical evaluation of zebularine, a potential oral antitumor agent, we initiated studies on the metabolism of zebularine in liver cytosol from humans and other mammals. Metabolism by aldehyde oxidase (AO, EC 1.2.3.1) was the major catabolic route, yielding uridine as the primary metabolite, which was metabolized further to uracil by uridine phosphorylase. The inhibition of zebularine metabolism was studied using raloxifene, a known potent inhibitor of AO, and 5-benzylacyclouridine (BAU), a previously undescribed inhibitor of AO. The Michaelis–Menten kinetics of aldehyde oxidase and its inhibition by raloxifene and BAU were highly variable between species.

1. Introduction

Zebularine is a pyrimidinone ribonucleoside that targets epigenetic modulation of DNA methylation.[1,2] It is being proposed for clinical evaluation as an oral antitumor agent. However, in a recent preclinical pharmacokinetic study of zebularine, the authors found poor oral bioavailability in rhesus monkey, Fisher 344 rat, and CD_2F_1 mouse.[3] In this study, only parent zebularine was measured, leaving open the possibility that metabolism might account for its poor oral bioavailability.

Zebularine is a cytidine analog that lacks the amino group normally found on the 4-position of the cytosine base. The 2-hydroxypyrimidine base of zebularine, which does not have a ribose sugar, has been identified as a substrate for aldehyde oxidase (AO, EC 1.2.3.1).[4,5] Additionally, reports have described the activation by AO of closely related compounds 5-iodo-2-pyrimidinone-2′-deoxyribose (IPdR) and 5-fluoro-2-pyrimidinone to 5-iodo-2′-deoxyuridine (IUdR) and 5-fluorouracil, respectively.[6,7] The activity of AO differs among species, strains of species, and even between male and female of the same strain, and sex differences in AO activity can be regulated by hormone levels.[4,8–10]

Raloxifene (Rlx) was developed as a selective estrogen receptor modulator and is recognized with antiosteoporotic and other beneficial properties.[11,12] Raloxifene is an oral drug, clinically available and a potent inhibitor of AO.[13,14] 5-Benzylacyclouridine (BAU) was first used as a laboratory tool for inhibition of uridine phosphorylase.[15–17] It was studied in animals to reduce 5-fluorouracil toxicity and potentiate the antitumor activity 5-fluoro-2′-deoxyuridine and subsequently Phase I clinical trials were performed.[18–20]

The results of our study, contained in this report, show that zebularine is an excellent substrate for hepatic AO, indicating that the poor oral bioavailability of zebularine may be due to rapid hepatic metabolism of absorbed zebularine to uridine with further hepatic degradation to uracil, Figure 1. No added cofactors are required for these enzyme activities. In a search for inhibitors of AO to enhance oral bioavailability of zebularine clinically, we investigated a known potent inhibitor, raloxifene, and discovered a previously unknown inhibitor, 5-benzylacyclouridine.[11,12] Moreover, results obtained with human liver cytosol were compared to those from

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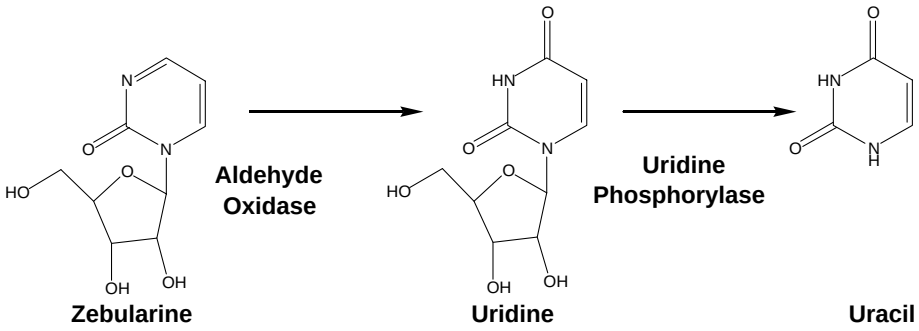

Figure 1. Enzymatic pathway of zebularine. Zebularine is metabolized by AO to uridine in hepatic cytosol. Uridine is metabolized further to uracil by uridine phosphorylase. No added cofactors are required for these enzyme activities.

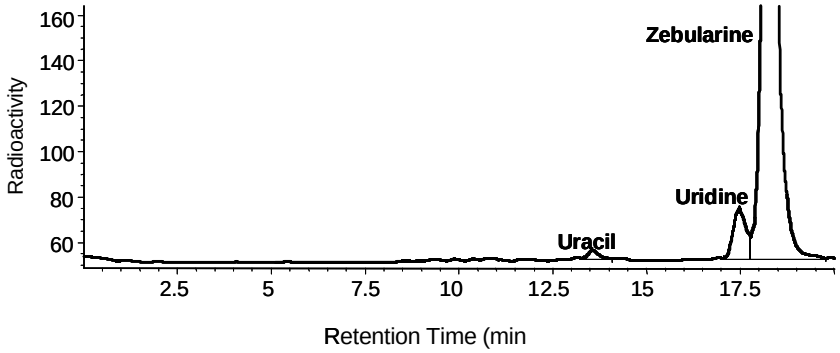

Figure 2. HPLC radio-chromatogram of zebularine and metabolites. Sample was from rat hepatic cytosol, 120 μg protein, and 40 μM [^{14}C]zebularine, and subjected to 15 min incubation. Chromatogram was acquired by on-line radioactivity detection. Zebularine was metabolized by AO to uridine (6.5% of radioactivity) and uridine was metabolized further by uridine phosphorylase to uracil (1.1% of radioactivity).

hepatic cytosolic preparations from mammalian species used in the preclinical development of zebularine.

2. Results

Two HPLC columns in series were required to separate uridine from zebularine when using radioactivity detection with a 0.5 ml flow cell. The amount of cytosolic protein was varied to keep the total metabolism in each sample less than 25% of the parent. Figure 2 shows the chromatogram of a typical separation of zebularine and metabolites. The peaks labeled uracil and uridine eluted with and had the same spectra as uracil and uridine reference standards (data not shown). If zebularine was a substrate for uridine phosphorylase, its product would be the 2-hydroxypyrimidine base. The reference standard for the base elutes near uracil and no evidence was found to suggest that zebularine was a direct substrate for uridine phosphorylase.

The metabolic rate for different concentrations of zebularine in both male and female human liver cytosol is shown in Figure 3 and the Lineweaver–Burk in Figure 4. These analyses were performed for each species and segregated by sex where possible.

The V_{max} and K_m values in the male and female human liver cytosol were similar (Table 1). Likewise, there were no sex differences for these values in the Sprague–Dawley rat. The CD-1 mouse had the greatest differences in values of K_m and V_{max} between the sexes. In fact, the male mouse had the highest K_m and V_{max} values for all the species. The K_m value in the female was 4-fold lower than in the male and the V_{max} value was 50-fold lower than the value in the male mouse. The female mouse had the lowest measurable V_{max} of all the cytosolic fractions, except the Beagle dog. Despite using 3 mg protein in the cytosol incubation, there was no evidence of enzymatic activity in the male Beagle dog.

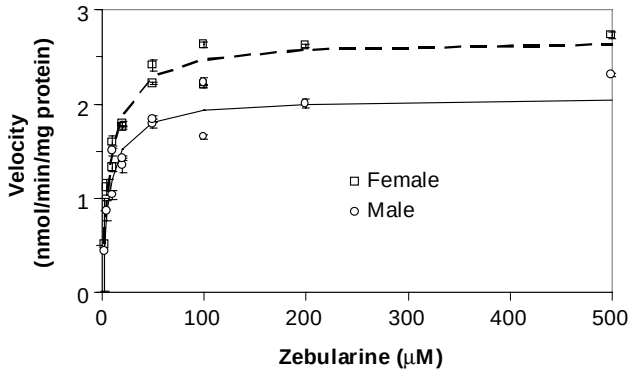

Figure 3. Velocity (nmol/min/mg protein) vs zebularine concentration (μM). Velocity of zebularine metabolism was determined from triplicate samples of pooled hepatic cytosol from male (n = 28) and female (n = 14) humans. The lines are the plots generated using the estimated K_m and V_{max} values from the Lineweaver–Burk analysis. The symbols are the mean and standard deviation of triplicate samples.

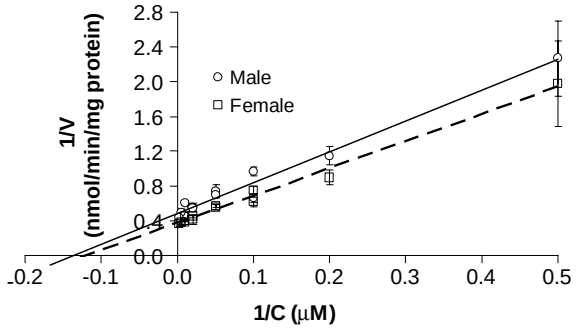

Figure 4. Lineweaver–Burk plot. Lineweaver–Burk analysis, $1/v$ (nmol/min/mg protein) vs $1/C$ (μM zebularine), of the human data presented in Figure 3. The slope and y-intercept were used to determine the K_m and V_{max} for metabolism of zebularine by AO. The lines are the linear regression fit of the data and the symbols are the mean and standard deviation of triplicate samples.

Table 1. K_m (μM) and V_{max} (nmol/min/mg protein) for aldehyde oxidase in hepatic cytosol of different species

	Sex	K_m[a]	V_{max}[a]
Human	Male	7.3	2.1
	Female	8.4	2.7
Cynomolgus monkey	Male	15	4.6
Beagle dog	Male	No activity	
Sprague–Dawley rat	Male	11	0.85
	Female	11	0.90
CD-1 mouse	Male	102	11
	Female	27	0.22

[a] Values are determined from the slope and intercept of double reciprocal plots obtained from triplicate samples ranging over six or more concentrations of zebularine.

Figure 5 compares the velocity versus substrate concentration curves for each species. For human and rat, there was little difference in the curves for the male and female. As previously noted, the greatest difference was observed in the data for the male and female mice. At low concentrations, <15 μM, the velocity of metabolism in the male mouse was less than in the male and female human as well as the male monkey.

Inhibition of zebularine metabolism by raloxifene was most sensitive in human liver cytosol with an IC_{50} value <10 nM (Table 2). The IC_{50} values for monkey, mouse, and rat are greater than 50-fold higher than in human. The second inhibitor, BAU, was discovered while using it as a potential tool to simplify the analysis of metabolites by blocking uracil formation through inhibition of uridine phosphorylase (Fig. 1). In addition, it was noticed that zebularine metabolism was also inhibited. The IC_{50} value for BAU is lowest in the human cytosol (200–300 μM) and only 3- to 4-fold more potent than in monkey and mouse. The IC_{50} value for rat liver cytosol is about the same as for human.

3. Discussion

The amount of AO activity is variable among species, strains, and sex.[4,8–10] Our study is one of the most comprehensive surveys performed at the same time using the same substrate in mammalian species that are used for preclinical testing. We found that the male CD-1 mouse had the highest values of V_{max} and K_m for AO in the cytosol fractions tested. The female CD-1 mouse had a 50-fold lower value of V_{max} but still a higher value for K_m than the other species. No other strains of mice were tested. The Beagle dog had no detectable AO activity using zebularine as a substrate, in agreement with other reports of little or no AO activity in the dog.[21,22] Care must be taken when evaluating chemotherapeutic agents

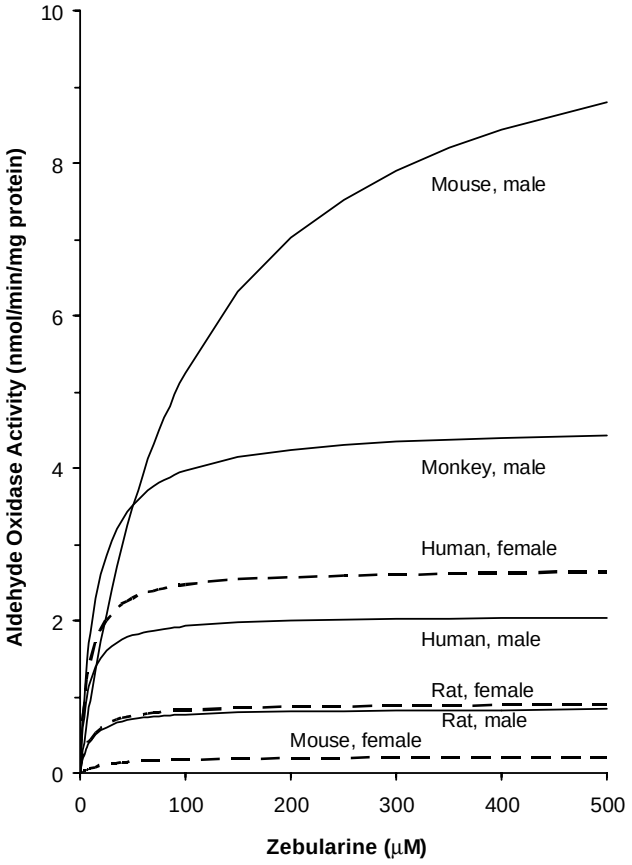

Figure 5. Metabolism of zebularine by AO in hepatic cytosol. Lines show the plots obtained when the estimated K_m and V_{max} values are applied to the Michaelis–Menten equation for enzyme metabolism. Solid lines were used for males and dashed lines for females of each species. Symbols showing the data and standard deviation were omitted to improve the clarity of the plot. A representative goodness of fit can be seen in the male and female human data shown in Figures 3 and 4.

Table 2. Inhibition of zebularine metabolism in male hepatic cytosol of different species

	Approximate IC_{50}[a]	
	BAU (μM)	Rlx (nM)
Human	250	8
Cynomolgus monkey	800	500
Sprague–Dawley rat	300	>1100
CD-1 mouse	>800	500

[a] Approximate IC_{50} value determined from percent inhibition in duplicate samples using two concentrations of zebularine and three concentrations of inhibitor.

with primary elimination routes of enzymatic degradation by AO. There may be differences in activity or toxicity when evaluating these agents in different species, different strains of species, and even different sexes of the same strain, especially in the mouse.

Toxicity studies of chemotherapeutic agents under development are performed in various mammalian species. Due to the variable AO activity among species, it will be more difficult to predict a safe starting dose for humans based on the preclinical testing of zebularine. On the other hand, differences in AO activity in the different sex of the same strain of mouse may be helpful in distinguishing toxicity caused by the parent zebularine molecule.

Yoshihara and Tatsumi described differences in rates of 2-hydroxypyrimidine metabolism by purified hepatic AO from male and female mice.[4] In that study, there were no sex-related differences in inhibition of AO by several inhibitors. Hence, we studied the inhibition of zebularine metabolism in only the males of each species. The Lineweaver–Burk analysis at the different inhibitor concentrations for each species suggested a mixed noncompetitive inhibition for both inhibitors.[23] Obach demonstrated that raloxifene could be an uncompetitive or a noncompetitive inhibitor depending on substrate.[14]

Our data suggest that hepatic AO may be the limiting factor in the oral bioavailability of zebularine. Preclinical studies in monkey, rat, and mouse indeed describe a low oral bioavailability.[3] One means to increase bioavailability may be coadministration of an inhibitor of AO. Raloxifene, an antiosteoporotic, is clinically available and a potent inhibitor of AO.[13,14] The concentration required to inhibit zebularine metabolism by 50% in human liver cytosol is less than 10 nM. In healthy volunteers given a single 185 mg oral dose of raloxifene, plasma concentrations reached 25 nM.[12] Higher concentrations of raloxifene are likely along its initial path of absorption. Thus, modulation of zebularine catabolism in humans is feasible.

BAU was first used as a laboratory tool for inhibition of uridine phosphorylase.[15–17] When given to animals and as an investigational drug in humans, BAU reached sufficient concentrations to inhibit uridine phosphorylase.[18–20] BAU can be given orally and may provide an alternate choice for clinical inhibition of AO activity.

Moreover, inhibition of hepatic AO may be beneficial to the clinical effectiveness of other pyrimidinones. IPdR is a prodrug that is activated by AO to yield IUdR.[6] IUdR is metabolized by hepatic thymidine phosphorylase and much of it may be inactivated to IUra during absorption without reaching the systemic circulation. Despite the fact that AO is required for activation of IPdR, it may be beneficial to initially inhibit this activation until the maximum amount of IPdR can be absorbed. For orally absorbed drugs that are activated by AO and then quickly inactivated in the liver by different enzymes, an inhibitor of AO may increase the absorption of the prodrug, allowing an increased circulation of the desired activated drug to reach its target in the cell.

4. Conclusion

Zebularine is metabolized by aldehyde oxidase in hepatic liver cytosol from most mammals. The amount of activity can vary among species, between sexes of the same species, especially CD-1 mouse, and is absent in at least one species, the Beagle dog. The metabolism of zebularine by aldehyde oxidase can be inhibited by raloxifene and BAU. Both inhibitors might be used clinically to improve the oral bioavailability of zebularine and other pyrimidinone nucleosides, such as IPdR.

5. Experimental

Zebularine and [2-^{14}C]zebularine, 18.3 mCi/mmol, were obtained through the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI, Rockville, MD. Raloxifene (Rlx) was acquired commercially. 5-Benzylacyclouridine (BAU) was a generous gift from Shih Hsi Chu, PhD, Brown University, Providence, RI.

5.1. Kinetic and inhibition studies

The hepatic cytosolic fractions prepared from human, Cynomolgus monkey, Beagle dog, Sprague–Dawley rat, and CD-1 mouse were obtained from CellzDirect, Los Angeles, CA. The human liver cytosol was pooled from 28 male or 14 female individuals. Each 300 μl enzyme assay consisted of 50 μl of 500 mM potassium phosphate, pH 7.5, containing 50 mM EDTA, 150 μl water ± inhibitor, 50 μl diluted cytosol, and 50 μl [^{14}C]zebularine (varying concentrations) to start the incubation. The amount of cytosolic protein used per sample ranged from 0.02 to 0.2 mg for each species, except the male Beagle dog (up to 3 mg protein). Incubation was performed at 37 °C for 15 min and stopped by placing the samples in boiling water for >2 min. Samples were centrifuged for 5 min at ~20,000*g*. The supernatant was injected directly into the HPLC.

Metabolic studies were performed in triplicate at zebularine concentrations that were approximately <0.3 to >10 times the value of K_m for each species. Inhibition studies were performed in duplicate at 10 and 40 μM zebularine. Three inhibitor concentrations were chosen in an attempt to bracket the IC_{50} value for each species and were compared to uninhibited controls.

The samples were analyzed by HPLC with on-line radioactivity detection with a 0.5 ml flow cell and a scintillation cocktail rate of 4 ml/min. The mobile phase consisted of 10 mM sodium acetate, pH 5, at a flow rate of 0.6 ml/min through two Zorbax SB300, C_8, 5μm, 4.6 × 250 mm, columns (Agilent Technologies, Palo Alto, CA) in series.

5.2. Lineweaver–Burk analysis

The values for K_m and V_{max} were determined from the linear regression fit of the double reciprocal plot of 1/velocity (nmol/min/mg protein) vs 1/concentration (μM).

The value of the K_m is $-1/(x\text{-intercept})$ and the value of V_{max} is K_m/slope.

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