Note

Strain Difference of Oxidative Metabolism of the Sedative-hypnotic Zaleplon by Aldehyde Oxidase and Cytochrome P450 In Vivo and In Vitro in Rats

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Summary: The in vivo and in vitro metabolism of the sedative-hypnotic agent zaleplon (ZAL) to 5-hydroxylated ZAL (5-oxo-ZAL) and N-desethylated ZAL (desethyl-ZAL) was studied in four strains of rats. Incubation of ZAL with liver microsomes afforded desethyl-ZAL via cytochrome P450-catalyzed reaction, with little strain difference. In contrast, incubation of ZAL with liver cytosol afforded 5-oxo-ZAL with marked strain differences. ZAL hydroxylase activity was well correlated with aldehyde oxidase activity in these strains. The highest level of 5-oxo-ZAL and the highest activity of aldehyde oxidase were observed in cytosol from Sea:SD rats, followed by Jcl:SD rats, while Crj:SD and WKA/Sea rats showed low levels. When ZAL was administered to Sea:SD and WKA/Sea rats, both 5-oxo-ZAL and desethyl-ZAL were detected in blood as the major in vivo metabolites. However, the concentration of 5-oxo-ZAL was far higher in Sea:SD rats than in WKA/Sea rats, while that of desethyl-ZAL was far lower in Sea:SD rats. The levels of 5-oxo-ZAL in blood were closely correlated with the strain differences of cytosolic ZAL hydroxylase activity and benzaldehyde oxidase activity. Our results indicate that variability in the formation of 5-oxo-ZAL from ZAL in vivo in various strains of rats is primarily due to strain differences of hepatic aldehyde oxidase activity.

Keywords: zaleplon; 5-oxo-zaleplon; desethyl zaleplon; aldehyde oxidase; in vivo metabolism; rat strain difference

Introduction

Zaleplon (ZAL, N-[3-(3-cyanopyrazolo[1,5-a]pyrimidin-7-yl)-phenyl]-N-ethylacetamide) is a nonbenzodiazepine sedative-hypnotic agent used for the treatment of insomnia.1,2 In humans and various animal species, the major metabolites of ZAL are the 5-hydroxylated ZAL (5-oxo-ZAL) and N-desethylated ZAL (desethyl-ZAL) was studied in four strains of rats. Incubation of ZAL with liver microsomes afforded desethyl-ZAL via cytochrome P450-catalyzed reaction, with little strain difference. In contrast, incubation of ZAL with liver cytosol afforded 5-oxo-ZAL with marked strain differences. ZAL hydroxylase activity was well correlated with aldehyde oxidase activity in these strains. The highest level of 5-oxo-ZAL and the highest activity of aldehyde oxidase were observed in cytosol from Sea:SD rats, followed by Jcl:SD rats, while Crj:SD and WKA/Sea rats showed low levels. When ZAL was administered to Sea:SD and WKA/Sea rats, both 5-oxo-ZAL and desethyl-ZAL were detected in blood as the major in vivo metabolites. However, the concentration of 5-oxo-ZAL was far higher in Sea:SD rats than in WKA/Sea rats, while that of desethyl-ZAL was far lower in Sea:SD rats. The levels of 5-oxo-ZAL in blood were closely correlated with the strain differences of cytosolic ZAL hydroxylase activity and benzaldehyde oxidase activity. Our results indicate that variability in the formation of 5-oxo-ZAL from ZAL in vivo in various strains of rats is primarily due to strain differences of hepatic aldehyde oxidase activity.

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hepatic ZAL-oxidizing activity compared with other animals and humans. For example, Kawashima et al. reported that aldehyde oxidase-mediated 5-oxidation of ZAL in vitro proceeds to a far greater extent in monkeys than in rats. On the other hand, we have found a significant strain difference of aldehyde oxidase activity in rat liver using benzaldehyde, N-1-methylnicotinamide and methotrexate as test substrates. Further, methotrexate 7-hydroxylase activity in liver cytosol from several strains of rats varied considerably, but was closely correlated with in vivo aldehyde oxidase activity. Kawashima et al. studied ZAL metabolism in a strain of rats (Crl:SD) with low aldehyde oxidase activity, and this may be the reason why the metabolizing capacity of rat liver aldehyde oxidase was found to be so low (about 1%) compared with that in monkeys. However, in order to understand the role of aldehyde oxidase in the metabolism of ZAL, which is metabolized by both aldehyde oxidase and cytochrome P450, it is important to study the differences in ZAL disposition in strains of rats expressing different levels of aldehyde oxidase activity.

In the present work, we aimed to evaluate strain differences of ZAL metabolism to 5-oxo-ZAL and desethyl-ZAL in rats and to establish whether these are due to strain differences of metabolizing enzyme activity. Our results indicate that cytochrome P450-mediated formation of desethyl-ZAL showed little strain variation. On the other hand, ZAL hydroxylase-mediated formation of 5-oxo-ZAL showed marked strain variation, and ZAL hydroxylase activity was well correlated with cytosolic aldehyde oxidase activity. We also confirmed that strain difference in the formation of 5-oxo-ZAL in vivo is related to variation of aldehyde oxidase activity, and 5-oxo-ZAL was the predominant metabolite in Sea:SD rats, which have high aldehyde oxidase activity. These findings will be important in assessing the significance of animal model data concerning metabolism of drugs that are substrates of aldehyde oxidase.

Materials and Methods

Chemicals: Materials were obtained from the following sources: benzaldehyde and benzoic acid from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). ZAL was kindly donated by Medical Research Laboratories, Lederle Japan (Tokyo, Japan). Desethyl-ZAL were synthesized according to Radl et al. (20) 5-OH-ZAL was purified by means of HPLC from a large-scale incubation mixture, which consisted of 10 µmol of ZAL and 20 ml of rat liver cytosol in a total volume of 100 ml of 0.1 M phosphate buffer (pH 7.4). The purified metabolite was identified using 'H-NMR and MS.

Animals: Four different strains of male rats (6–7 weeks age) were used, i.e., Crl:SD from Clea Japan Inc. (Tokyo, Japan); Crl:SD from Charles River Japan, Inc. (Yokohama, Japan); WKa/Sea and Sea:SD from Seiwa Experimental Animals, Ltd. (Fukuoka, Japan). Wka/Sea is a Wistar strain, and Sea:SD, Crl:SD and Jcr:SD are Sprague-Dawley strains. The animals were housed in cages at 22°C with a 12-h light/dark cycle, with free access to tap water and a standard pellet diet, MF (Oriental Yeast Co., Ltd., Tokyo, Japan).

Liver preparations: Livers were excised and homogenized in four volumes of 1.15% KCl using a Potter-Elvehjem homogenizer. The microsomal and cytosolic fractions were obtained from the homogenate by successive centrifugation at 9,000 x g for 20 min and 105,000 x g for 60 min. The microsomal pellets were resuspended in the solution to make 1 ml equivalent to 1 g of liver.

Assay of ZAL-metabolizing activity: The incubation mixture consisted of 0.1 µmol of ZAL and microsomes or cytosol equivalent to 50–100 µg of liver in a final volume of 1 ml of 0.1 M K,Na-phosphate buffer (pH 7.4). Incubation was performed at 37°C for 60 min. The microsomal pellets were re-suspended in the solution to make 1 ml equivalent to 1 g of liver.

Assay for benzaldehyde oxidase activity: Benzaldehyde oxidase activity was assayed according to our reported method. Briefly, the decrease in absorption at 249 nm consequent upon oxidation of benzaldehyde to benzoate was monitored in 165 mM K,Na-phosphate buffer (pH 7.4). Incubation was performed for 60 min. The microsomal pellets were re-suspended in the solution to make 1 ml equivalent to 1 g of liver.

Western blot analysis of rat liver aldehyde oxidase: Aldehyde oxidase protein was determined by Western blot analysis of liver cytosol proteins from the four strains of rats. Rat liver cytosolic proteins (5 µg) were separated by SDS-polyacrylamide gel electrophoresis (7.5% gel) and transferred to polyvinylidened fluoride membranes (0.2 mm; Bio-Rad, Hercules, CA, USA) by electroblooding. The membranes were then incubated with 5% skimmed milk in 25 mM Tris-buffered saline (pH 7.6)–0.1% Tween 20 for 1 h and probed with a rabbit anti-rat aldehyde oxidase (1:1,000) for 3 h. The membranes were washed, and antibody binding was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG, followed by development with ECL Plus (GE Healthcare UK Ltd., Buckinghamshire, England). The density of the bands was quantitated using Mac BAS Ver. 2.5 software.
Detection of ZAL and its metabolites in blood of rats: ZAL was given orally to rats at a single dose of 50 mg/kg body weight suspended in 0.5% carboxymethylcellulose. The control rats were given the vehicle only. About 0.1 ml of blood was collected from an ocular vein, and plasma was obtained by centrifugation. The amounts of ZAL, 5-oxo-ZAL and desethyl-ZAL in the plasma were determined by HPLC. Plasma was extracted and the samples were assayed in the same way as described for the in vitro samples.

Statistical analysis: All data were expressed as mean ± S.D. Statistical analyses were performed by paired Student's t-test for comparison of two groups with a significance level of p < 0.05.

Results and Discussion

Strain differences of ZAL hydroxylase activity in rat liver: When the activity for conversion of ZAL to 5-oxo-ZAL was assayed in liver cytosol from several rat strains, each bar represents the mean ± SD of four rats. ZAL-metabolizing activities in liver cytosol and microsomes were assayed by HPLC determination of the amounts of 5-OH-ZAL and desethyl-ZAL formed. For details, see Materials and Methods.

Detection of ZAL and its metabolites in blood of rats: ZAL was given orally to rats at a single dose of 50 mg/kg body weight suspended in 0.5% carboxymethylcellulose. The control rats were given the vehicle only. About 0.1 ml of blood was collected from an ocular vein, and plasma was obtained by centrifugation. The amounts of ZAL, 5-oxo-ZAL and desethyl-ZAL in the plasma were determined by HPLC. Plasma was extracted and the samples were assayed in the same way as described for the in vitro samples.

Statistical analysis: All data were expressed as mean ± S.D. Statistical analyses were performed by paired Student's t-test for comparison of two groups with a significance level of p < 0.05.

Results and Discussion

Strain differences of ZAL hydroxylase activity in rat liver: When the activity for conversion of ZAL to 5-oxo-ZAL was assayed in liver cytosol from the four strains of rats, marked variability was found. The highest activity was observed with Sea:SD rats, followed by Jcl:SD, while Crj:SD and WKA/Sea showed low activity (Fig. 2A). The difference in the activity between Sea:SD and WKA/Sea strains was about 24-fold. Western blot analysis using aldehyde oxidase antibody was performed to estimate the aldehyde oxidase level in liver cytosol. The observed band density of aldehyde oxidase in SDS-PAGE correlated well with ZAL hydroxylase activity, except in the case of WKA/Sea rats (Fig. 3A). This can be explained by the fact that aldehyde oxidase in WKA/Sea rats exists largely in an inactive 150kDa form, as demonstrated by Itoh et al.21,22) (Fig. 3B). The inactivation is associated with 377G>A nucleotide substitution (Sea:SD and Jcl:SD have 377C, while Crj:SD has 377T; data not shown). The level of 300-kDa active form of aldehyde oxidase was well correlated with that of ZAL hydroxylase activity, supporting the idea that ZAL hydroxylase activity is due to aldehyde oxidase. Benzaldehyde oxidase activity (due to aldehyde oxidase) was also well correlated with ZAL hydroxylase activity (Fig. 2C). These results indicate that the strain differences of ZAL hydroxylase activity in rat livers are due to strain differences of aldehyde oxidase present in the liver cytosolic fraction. In contrast, the desethyl-ZAL-generating activity was almost the same in all four strains of rats (Fig. 2B).

Strain differences in levels of ZAL metabolites in blood of rats after ZAL administration: The in vivo metabolism of ZAL in rats was compared in Sea:SD and WKA/Sea strains. When ZAL was administered orally to the two strains at the dose of 50 mg/kg, both 5-oxo-ZAL and desethyl-ZAL were detected in the blood as metabolites, together with intact ZAL. However, 5-oxo-ZAL was detected in only small amounts in WKA/Sea, a low aldehyde oxidase activity strain, compared with Sea:SD, a high aldehyde oxidase activity strain, after ZAL dosing (Fig. 4). The pharmacokinetic parameters for ZAL and its metabolites are summarized in Table 1.

In vivo–in vitro relationship of ZAL metabolism mediated by aldehyde oxidase: Our findings here indicate that the degree of 5-hydroxylation of ZAL in rats in vivo closely reflects the strain differences of ZAL hydroxylase activity, as well as hepatic microsomal aldehyde oxidase activity. Thus, we consider aldehyde oxidase to function as the major enzyme catalyzing this reaction, and the strain differences of 5-oxo-ZAL formation from ZAL in rats in vivo to be attributable primarily to differences of hepatic aldehyde oxidase activity.
Table 1. Pharmacokinetic parameters of ZAL, 5-oxo-ZAL and desethyl-ZAL after p.o. administration of ZAL to Sea:SD and WKA/Sea rats

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<th>ZAL</th>
<th>5-oxo-ZAL</th>
<th>Desethyl-ZAL</th>
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<tr>
<td></td>
<td>Sea:SD</td>
<td>WKA/Sea</td>
<td>Sea:SD</td>
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<tr>
<td>AUC (nmol × h/mL)</td>
<td>133 ± 15.6</td>
<td>142 ± 51.7</td>
<td>82 ± 16</td>
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<tr>
<td>Cmax (nmol/mL)</td>
<td>26.8</td>
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<td>Tmax (h)</td>
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Each bar represents the mean ± S.D., n = 3, *p < 0.05, **p < 0.01 vs. Sea:SD rats.

Fig. 4. Plasma concentration time profiles of ZAL (A), 5-oxo-ZAL (B) and desethyl-ZAL (C) in Sea:SD and WKA/Sea rats after p.o. administration of ZAL.

Amounts of ZAL, 5-oxo-ZAL and desethyl-ZAL detected in blood of Sea:SD and WKA/Sea rats after p.o. administration of ZAL were determined by HPLC as described in Materials and Methods. Each value represents the mean ± SD of four rats.

Roles of aldehyde oxidase and cytochrome P450 in the metabolism of ZAL in vivo: It is interesting that the level of desethyl-ZAL in blood of WKA/Sea strain rats was higher than that in Sea:SD strain, although the in vitro oxidase activities were almost the same. This is presumably because of the lower level of aldehyde oxidase in WKA/Sea strain rats, and consequent reduced formation of 5-oxo-ZAL. In other words, a greater proportion of ZAL was available for the formation of desethyl-ZAL by cytochrome P450 in vivo. Thus, aldehyde oxidase and cytochrome P450 appear to act in a complementary manner.

In this study, marked strain variation of 5-oxo-ZAL formation, mediated by aldehyde oxidase, was found in rats. This is likely to have an important influence on the suitability of rat model experiments for predicting disposition in humans. In humans, aldehyde oxidase activity is to be known higher than in rats, although there is high inter-individual variation.14,23-25 In fact, ZAL is effectively metabolized to 5-oxo-ZAL in humans and the level of desethyl-ZAL is much lower than in other species.5 Thus, consideration of inter-individual variation in aldehyde oxidase activity will be important in clinical application of ZAL. Based on our results, we wish to emphasize that great care is necessary in extrapolating animal data on ZAL to humans.

References


