Obesity-induced increase of CYP2E1 activity and its effect on disposition kinetics of chlorzoxazone in Zucker rats

| 報告者 | キャミー・フォストン, ヨコガワ・コイチ, シェイダ・ツトム, ミヤマト・ケニチ |
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Obesity-induced increase of CYP2E1 activity and its effect on disposition kinetics of chlorzoxazone in Zucker rats

Phisit Khemawoot, Koichi Yokogawa, Tsutomu Shimada, Ken-ichi Miyamoto*
Department of Medicinal Informatics, Division of Cardiovascular Medicine, Graduate School of Medical Science, Kanazawa University, Japan

1. Introduction

An increasingly sedentary lifestyle, together with a preference for refined foods containing high levels of meat and saturated fat, has resulted in a drastic increase in the incidence of metabolic abnormalities, obesity, and hypercholesterolemia, which are associated with premature mortality [1,2]. Therefore, animal models, such as genetically obese Zucker (fa/faf) rats, have been developed to study the mechanisms of physiological changes related to obesity. The Zucker (fa/faf) rat does not develop leptin receptors, resulting in impaired regulation of food intake and impaired energy homeostasis.

Abstract

This study was designed to investigate the induction of CYP2E1 in obese Zucker rats and its effect on the disposition kinetics of chlorzoxazone (CZX). CZX 20 mg/kg was administered to three groups of rats: normal Zucker rats fed a normal diet (ND), normal Zucker rats fed a high-fat diet (HF), and genetically obese Zucker rats fed a normal diet (OB). The values of the area under the plasma concentration-time curve from 0 to \( \infty \) (AUC) of CZX were in the order of ND > HF > OB rats. The AUC values of total 6-hydroxychlorzoxazone (6OHCZX-T), which is considered to be a CYP2E1 metabolic marker, were in the opposite order. The values of the AUC ratio (6OHCZX-T/CZX) in ND, HF and OB rats were approximately 0.2, 0.3 and 0.4, respectively. The CZX concentration in fat was much higher than the concentrations in plasma, liver and kidney in all groups. Induction of CYP2E1 protein was greater in both liver and fat of OB rats than in those of HF rats. Microsomal activity of CYP2E1 in liver and fat was also in the order of OB > HF > NM rats. These results suggest that CYP2E1 may be induced in liver and fat of obese patients, thereby potentially altering the disposition kinetics of not only CZX, but also other lipophilic drugs metabolized by CYP2E1.

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[3,4]. The overfed normal rat can also mimic physiological aspects of severe obesity in humans, such as hypercholesterolemia, hyperinsulinemia, etc. [5,6].

Changes in physiological status, such as obesity and hypercholesterolemia, can modulate the metabolic activity of CYP2E1 [7–9]. Interestingly, CYP2E1 is a ubiquitous enzyme that is distributed in various organs of animals, but obesity-related increase of CYP2E1 activity is well documented only in liver.

There are several reports that the hydroxylation of chlorzoxazone (CZX) can be used as an indicator of CYP2E1 activity both in vivo and in vitro [10,11]. CYP2E1 is a muscle relaxant that primarily undergoes hydroxylation, catalyzed mainly by CYP2E1, to 6-hydroxyczoxazone (6OHCZX), which is rapidly glucuronidated and excreted in urine [12,13]. CZX is lipophilic [14], and so may be a suitable probe to examine the activity of CYP2E1 in various tissues of obese animals.

This study was therefore designed to examine the induction of CYP2E1 in genetically obese Zucker rats fed a normal diet (OB) and its effect on the disposition kinetics of CZX and its metabolite 6OHCZX in liver, kidney and fat, compared with those in normal Zucker rats fed a high-fat diet (HF) and normal Zucker rats fed a normal diet (ND).

2. Materials and methods

2.1. Materials

CZX and 6OHCZX were purchased from Sigma–Aldrich Inc. (St. Louis, MO). High-fat diet (LABO H Standard) and normal diet (LABO MR Stock) were purchased from Nusan Corp. (Yokohama, Japan). The high-fat diet contained 8.6% (w/w) fat (total energy 346.7 kcal/100 g), while the normal diet contained 4.1% (w/w) fat (total energy 259.2 kcal/100 g).

2.2. Animal treatment

Male 8-week-old Zucker (+/+ ) rats and genetically obese Zucker (fa/fa) rats were purchased from Japan SLC Inc. (Toyama, Japan). The rats were divided into three groups: normal Zucker (+/+) rats fed with normal diet (ND rats), normal Zucker (+/+) rats fed with high-fat diet (HF rats) and genetically obese Zucker (fa/fa) rats fed with normal diet (OB rats). The animals were housed for 3 months in a climate- and light-controlled environment with free access to water and the designated food. All animal procedures were in accordance with the standards set forth in the guidelines for the care and use of laboratory animals at the Takara-machi Campus of Kanazawa University.

2.3. Disposition kinetic of CZX

CZX (20 mg) was dissolved in 400 μl of 0.5N NaOH and diluted with 600 μl of normal saline solution (20 mg/ml). This solution was administered to rats at a dose of 20 mg/kg by i.v. administration over 2 min via a lateral tail vein. Then, approximately 0.2–0.5 ml of blood was collected from the tail vein on the other side at 0.033, 0.083, 0.25, 0.5, 1, 2, 4, and 8 h after completion of the administration of CZX. The blood was centrifuged at 3000 × g for 10 min, and the plasma was collected. Some rats were killed by decapitation at 4 h after CZX administration, and the liver, kidney and abdominal fat were quickly excised, rinsed well with ice-cold saline, blotted dry, and weighed. Each tissue sample was homogenized with normal saline and stored at −80 °C until analysis.

2.4. Sample preparation

The concentrations of CZX and 6OHCZX in biological samples were analyzed as unchanged and glucuronidated fractions. The assay of glucuronidated CZX and 6OHCZX was performed according to Frye and Stiff [15] with slight modifications. Briefly, a 100 μl sample of plasma or tissue homogenate was added to 300 μl of 0.2 M phosphate buffer (pH 6.5) containing 500 units of β-glucuronidase. The mixture was incubated at 37 °C with shaking for 2 h, and then the reaction was stopped by adding 100 μl of acetonitrile containing phenacetin (1 μg) as an internal standard for HPLC analysis.

2.5. HPLC assay of CZX and 6OHCZX

To a 100 μl of sample solution was added 5 ml of diethyl ether, then the mixture was shaken vigorously for 10 min, and centrifuged at 3000 × g for 10 min. The ether layer was transferred to another tube for evaporation in a vacuum centrifugal concentrator. In the case of adipose tissue, reverse phase extraction from the aqueous phase of homogenated samples was done by adding 0.5N NaOH to the samples and mixing. The aqueous phase was collected and titrated with an equal amount of 0.5N HCl. Next, diethyl ether (5 ml) was added to extract CZX and 6OHCZX from aqueous phase, and the organic solution was further processed as described above.

The residue from evaporation was dissolved in 200 μl of the mobile phase, and a 50 μl aliquot was injected into an HPLC system (LC-9A, Shimadzu Co. Ltd., Kyoto, Japan) equipped with a CAPCELL PAK C18 column, 1.5 mm i.d. × 150 mm (Shiseido Co. Ltd., Tokyo, Japan). The mobile phase consisted of 25% (v/v) acetonitrile in 50 mM KH2PO4 (pH 4.0), pumped at a rate of 0.1 ml/min. The absorbance was detected at wavelengths of 295 and 287 nm for 6OHCZX and CZX, respectively [14,16]. The retention times of 6OHCZX, phenacetin and CZX were approximately 5, 12 and 20 min, respectively. Linear calibration curves (r > 0.999) were obtained for both compounds in plasma over the concentration range from 1 to 150 μg/ml. The limits of detection were estimated to be 0.25 μg/ml for both CZX and 6OHCZX.

2.6. Reverse transcriptase polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from liver, kidney and fat with Isogen (Nippon Gene Co. Ltd., Toyama, Japan). Each RNA sample (1 μg) was reversed-transcribed at 37 °C for 2 h, and the cDNA was amplified with a Peltier Thermal Cycler PTC-100 (Bio-Rad Laboratories Inc., Hercules, CA). The numbers of amplification cycles were 30 for fat cDNA and 25 for cDNAs from other tissues. PCR products were evaluated by electrophoresis on 2% (w/v) agarose gel stained with ethidium bromide, and photographed under UV trans-illumination. The product size was estimated by comparison with a 100 bp DNA ladder.
(Takara Bio Inc., Shiga, Japan). Control reactions to verify the absence of contaminants and genomic DNA were routinely performed. Primers used for rat CYP2E1 were 5'-CTG ATT GGC TGG GCA CCC TGC-3' and 3'-GAA CAC CTC GGC CAA AGT CAC-5', and those for rat β-actin were 5'-TTC TAC AAT GAG CTC CTA GAA-3' and 3'-GAA CAG GTC GGC CAA AGT CAC-5'. PCR was run under the following conditions: initial denaturation at 94 °C for 3 min, repeated denaturation at 94 °C for 45 s, followed by annealing at 66 °C for 45 s for CYP2E1 and 67 °C for 45 s for β-actin, primer extension at 72 °C for 45 s, and final extension at 72 °C for 3 min. The other conditions for RT-PCR were as described previously [17].

2.7. Preparation of microsomes

Tissue microsomes were prepared according to Yokogawa et al. [17] with slight modifications. Liver, kidney or abdominal fat was homogenized with phosphate buffer (50 mM K2HPO4 containing 0.1 mM EDTA, pH 7.4). The homogenate was centrifuged at 10,000 × g for 30 min, and then the supernatant was recentrifuged at 100,000 × g for 60 min. The microsomal pellet was collected, and resuspended in an appropriate volume of 50 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. The protein concentration of the microsomal solution was determined with a protein assay kit from Bio-Rad Laboratories Inc. (Hercules, CA).

2.8. Immunoblotting

Immunoblotting of the microsomes for CYP2E1 and β-actin was carried out essentially as described by Yokogawa et al. [18]. The microsomal protein was resolved by 10% SDS-PAGE, and transferred to a polyvinylidene fluoride membrane or Immobilon-P (Millipore Co., Bedford, MA). The membrane was incubated with blocking buffer for 1 h (5%, w/v, non-fat dried milk in PBS), followed with overnight incubation with a 2000-fold dilution of secondary antibody (rabbit anti-goat IgG from Santa Cruz Biotechnology Inc., Santa Cruz, CA). The membranes were then incubated with a 2000-fold dilution of secondary antibody (rabbit anti-goat IgG from Santa Cruz Biotechnology Inc., Santa Cruz, CA). The immunopositive band was detected with an ECL chemiluminescence detection kit (Amersham Biosciences UK Ltd., Buckinghamshire, UK), and scanned with a Typhoon 9200 scanner (Amersham Biosciences Europe GmbH, Freiburg, Germany).

2.9. Measurement of hydroxylation activity of microsomal CYP2E1

The enzyme activity of microsomal CYP2E1 was determined by the measurement of 6OHCZX formation according to Chittur and Tracy [19], with minor modifications. A mixture of the microsomal solution (equivalent to 0.4 mg protein) and 50 mM phosphate buffer (pH 7.4) containing 50 μM CZX and 1 mM NADPH (final, 200 μl) was incubated at 37 °C for appropriate times. The reaction was stopped by adding 100 μl of acetonitrile containing phenacetin (1 μg) as an internal standard for HPLC assay. The 6OHCZX formed was extracted with diethyl ether and measured by HPLC as described above.

2.10. Measurement of glucuronidation activity of microsomal UGTs

The activity of UDP-glucuronosyltransferase (UGT) microsomes was measured colorimetrically in 0.15 M tris-phosphate buffer pH 7.4 containing 0.8 mM p-nitrophenol (PNP), 14 mM UDP glucuronic acid, 10 mM MgCl2, and 1 mg microsomal protein [20]. The mixture (1.4 ml) was incubated at 37 °C for 30 min, then the reaction was stopped by adding 5 ml of 0.2 M glycine buffer (pH 10.4). The disappearance of PNP was quantified in terms of absorbance at 405 nm with a UV–vis spectrophotometer. The microsomal activities for glucuronidation of CZX and 6OHCVZX were assayed similarly, except that the disappearance of CZX and 6OHCVZX was determined by HPLC.

### Table 1 – Physical and biochemical data in rats with obesity

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ND</th>
<th>HF</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>385 ± 5.0</td>
<td>410 ± 17.3</td>
<td>480 ± 8.3</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>5.94 ± 0.42</td>
<td>6.22 ± 0.64</td>
<td>11.85 ± 0.88</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>2.11 ± 0.08</td>
<td>2.13 ± 0.14</td>
<td>2.23 ± 0.12</td>
</tr>
<tr>
<td>Epididymal fat (g)</td>
<td>3.49 ± 0.64</td>
<td>4.35 ± 1.12</td>
<td>12.74 ± 1.63</td>
</tr>
<tr>
<td><strong>Biochemical data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.26 ± 0.23</td>
<td>4.30 ± 0.10</td>
<td>4.07 ± 0.06</td>
</tr>
<tr>
<td>T-bilirubin (mg/dl)</td>
<td>0.057 ± 0.006</td>
<td>0.057 ± 0.012</td>
<td>0.28 ± 0.030</td>
</tr>
<tr>
<td>T-cholesterol (mg/dl)</td>
<td>75 ± 1.5</td>
<td>81 ± 2.1</td>
<td>88 ± 1.5</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.29 ± 0.02</td>
<td>0.30 ± 0.02</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>81 ± 7.0</td>
<td>79 ± 3.1</td>
<td>83 ± 6.6</td>
</tr>
<tr>
<td>Cholinesterase (IU/l)</td>
<td>≈4</td>
<td>≈4</td>
<td>≈4</td>
</tr>
</tbody>
</table>

Data were presented as mean ± S.D. of four rats. "Significant difference from ND rats at P < 0.05. "Significant difference from ND rats at P < 0.01.

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2.11. Data analysis

The pharmacokinetic parameters were calculated according to model-independent moment analysis as described by Yamaoka et al. [21]. Electrophoregrams after RT-PCR and immunopositive bands were evaluated in arbitrary units by using NIH Image software. Comparisons of numerical data among groups were made by one-way ANOVA, with P < 0.05 as the criterion of a significant difference. For each significant effect, a multiple comparison test was performed with Scheffe's test to verify the difference between groups at P-values of 0.05 and 0.01, using SPSS 10® from SPSS Inc. (Chicago, IL).

3. Results

3.1. Physical and biochemical data

Table 1 summarizes the physical data for ND, HF and OB rats. The body, liver and epididymal fat weights of OB rats were significantly higher than those of ND rats. The fat tissue weight of HF rats, although slightly higher, was not significantly different from that of ND rats. The value of total bilirubin of OB rats was significantly higher than that of ND rats, but liver function (AST) and kidney function (creatinine) were unaffected. Total cholesterol was significantly increased in HF rats and OB rats compared with normal rats.

3.2. Disposition kinetics of CZX

Fig. 1 shows the plasma concentration–time courses of CZX and 6OHCZX-T after an i.v. administration of CZX 20 mg/kg in ND, HF and OB rats. The plasma concentrations of CZX linearly decreased in all cases, but the concentration at 8 h after administration was in the order of ND > HF > OB rats. In this figure, the concentration of 6OHCZX is presented as a total value of 6OHCZX (6OHCZX-T), i.e., the sum of free 6OHCZX and glucuronidated 6OHCZX, after the administration of CZX. The amount of the glucuronide conjugate of CZX (CZX-G) was negligible (data not shown). It was found that the plasma concentration of 6OHCZX-T increased gradually and reached a peak at about 2 h after administration. The peak concentration of 6OHCZX-T in OB rats was significantly higher than that in ND rats. Table 2 shows the pharmacokinetic parameters of CZX and 6OHCZX-T after administration of CZX 20 mg/kg. In OB rats, the AUC∞, T1/2 and Vdss values of CZX were significantly smaller, while the CLtot and Vdss values of 6OHCZX-T in OB rats were significantly higher than those of ND rats. The pharmacokinetic parameters in HF rats were intermediate between those in OB rats and ND rats. Moreover, the AUC∞ values of 6OHCZX-T in HF and OB rats were significantly higher than that of ND rats.

Table 2 - Pharmacokinetic parameters of CZX and 6OHCZX after an i.v. administration of CZX 20 mg/kg over 2 min

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ND</th>
<th>HF</th>
<th>OB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CZX</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC∞ (μg/h/ml)</td>
<td>204 ± 14</td>
<td>181 ± 12</td>
<td>155 ± 21</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>1.67 ± 0.18</td>
<td>1.38 ± 0.19</td>
<td>1.19 ± 0.23</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.40 ± 0.19</td>
<td>2.30 ± 0.28</td>
<td>2.00 ± 0.17</td>
</tr>
<tr>
<td>Cl tot (l/h/kg)</td>
<td>0.104 ± 0.002</td>
<td>0.122 ± 0.010</td>
<td>0.145 ± 0.013</td>
</tr>
<tr>
<td>Vdss (l/h)</td>
<td>0.239 ± 0.014</td>
<td>0.277 ± 0.014</td>
<td>0.285 ± 0.016</td>
</tr>
<tr>
<td><strong>6OHCZX-T</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC∞ (μg/h/ml)</td>
<td>41.2 ± 4.7</td>
<td>60.3 ± 4.2</td>
<td>71.6 ± 5.9</td>
</tr>
</tbody>
</table>

Data were presented as mean ± S.D. of four rats. Significant difference from ND rats at P < 0.05. *Significant difference from ND rats at P < 0.01.

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**Fig. 2** – Tissue and plasma concentrations of CZX and its glucuronide (CZX-G) at 4 h after an i.v. administration of CZX (20 mg/kg) over 2 min in ND (♀), HF (♂) and OB (♀) rats. Each column and bar represents the mean ± S.D. of four rats. *Significant difference from ND rats at P < 0.05. **Significant difference from ND rats at P < 0.01.

The expression levels of CYP2E1 protein in the liver, kidney and fat tissue were examined by immunoblot analysis. The protein levels of CYP2E1 in the liver and fat of HF rats and OB rats were conspicuously increased compared with those of ND rats. Furthermore, the relative expression of CYP2E1/β-actin in liver microsomes and fat microsomes of HF and OB rats was significantly higher than that in ND rats (Fig. 5).

### 3.3. Expression of CYP isoform mRNAs and proteins

The mRNA expression of CYP2E1 in liver of HF rats and OB rats was only slightly higher than that in ND rats, whereas, the relative expression of CYP2E1/β-actin in fat tissue from HF rats and OB rats was apparently higher than that in ND rats (Fig. 4).

**Fig. 4** – Effect of obesity on the expression of CYP2E1 mRNA compared with β-actin in liver, kidney and fat of ND (♀), HF (♂) and OB (♀) rats. Each column and bar represents the mean ± S.D. of four rats. Significant difference from ND rats at P < 0.05. **Significant difference from ND rats at P < 0.01.

### 3.4. In vitro CYP2E1 and UGTs activity

**Fig. 6** shows the hydroxylation activity of microsomal CYP2E1 in terms of 6OHHCXZ formation from CZX, in various tissues from the three groups of rats. The highest hydroxylation rate was found in the liver compared with kidney and fat tissue. The activities in liver and fat from HF rats and OB rats were significantly higher than those in ND rats, whereas, no difference was seen in the kidney.

**Fig. 7** shows the glucuronidation activity of microsomal UGTs in liver and kidney towards PNP, CZX and 6OHHCXZ. The glucuronidation rates in the kidney of HF rats and OB rats were generally significantly higher than those in ND rats. The glucuronidation activity in fat was negligible in all groups. Interestingly, the glucuronidation of CZX was very much lower than that of 6OHHCXZ, although the enzyme activity in the liver was not affected by obesity.

### 4. Discussion

Zucker (fa/fa) rats at 20 weeks of age showed markedly increased body weight, accompanied with an enlarged liver and increased epididymal fat, compared with Zucker (+/+ ) rats. In contrast, Zucker (+/+) rats fed with high-fat diet (twice the level in normal diet) for 12 weeks did not show any significant physical or biochemical changes (Table 1).

CZX is well known to be a specific probe for CYP2E1, being hydroxylated to 6OHHCXZ [10,11], which in turn is rapidly glucuronidated to 6OHHCXZ-G [13]. Therefore, the appropriate
Fig. 5 – Effect of obesity on the content of CYP2E1 protein compared with β-actin in liver, kidney and fat of ND (♂), HF (__) and OB (♀) rats. Each column and bar represents the mean ± S.D. of three rats. *Significant difference from ND rats at P < 0.05. **Significant difference from ND rats at P < 0.01.

Fig. 6 – Effect of obesity on the hydroxylation activity of microsomal CYP2E1 in liver, kidney and fat of ND (♂), HF (__) and OB (♀) rats. Each column and bar represents the mean ± S.D. of four rats. *Significant difference from ND rats at P < 0.05. **Significant difference from ND rats at P < 0.01.

Fig. 7 – Effect of obesity on the glucuronidation activity of microsomal UGTs in liver and kidney of ND (♂), HF (__) and OB (♀) rats. Each column and bar represents the mean ± S.D. of four rats. *Significant difference from ND rats at P < 0.05. **Significant difference from ND rats at P < 0.01.

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did not mention the physical condition of either the lean or obese Zucker rats, so that it is difficult to compare their findings and ours. Interestingly, the CYP2E1 mRNA isofrom was expressed in fat tissue, and its expression level and activity were significantly higher in HF rats and OB rats than in ND rats (Figs. 4–6). Yoshinari et al. [25] and Wan et al. [26] reported that the expression levels of both CYP2E1 mRNA and protein were increased in adipose tissue of fasting rats. However, the amount of adipose tissue in fasting rats was small, and so CYP2E1 in fasting animals may contribute little to the pharmacokinetics of its substrates compared with the situation in obese animals.

The hydroxylated metabolite of CZX (6OHCZX) has been reported to be excreted in bile to only a small extent; rather it undergoes rapid glucuronidation with subsequent excretion of the conjugate in urine [13]. We found that the glucuronidation activity in kidney microsomes was significantly higher in HF rats and OB rats than ND rats (Fig. 7), and the concentration of 6OHCZX glucuronide in the kidney was higher than that in the liver (Fig. 3). Since there is no evidence that a specific UGT subfamily is involved in glucuronide conjugation of CZX and 6OHCZX, we used PNP, a general marker for glucuronidation, to confirm our results. The glucuronidation activity towards PNP in kidney microsomes of HF and OB rats showed the same trend as did the activity towards CZX and 6OHCZX. However, the glucuronidation activity in liver microsomes was not influenced by the high-fat diet or obese status, and was quite different towards different substrates. This suggests the presence of different UGTs isoforms in liver and kidney. Further research will be required to identify the UGTs isoform(s) responsible for 6OHCZX glucuronidation in tissues, and the species that are affected by obese status.

In conclusion, CYP2E1 activity was induced in the liver fat tissues of obese animals, and glucuronidation activity was induced in the kidney. As a result, the disposition kinetics of CZX was markedly changed in obesity, with an increase in hydroxylation of CZX to form 6OHCZX, and an acceleration of 6OHCZX glucuronidation, resulting in rapid excretion in urine. Other drugs that are metabolized by CYP2E1 and UGTs may show similar changes of disposition kinetics in obese patients, resulting in reduced potency and shorter duration of action.

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