Lack of human relevance for procymidone’s developmental toxicity attributable to species difference in its kinetics and metabolism

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The agricultural fungicide procymidone can cause external genitalia abnormalities in rats but not monkeys or rabbits. To investigate the relevance of developmental findings in rats to humans, we conducted in vitro plasma protein binding studies, in vitro metabolism (biotransformation) studies using liver S9 fractions and hepatocytes, and in vivo metabolism and excretion studies using chimeric mice with humanized hepatocytes. On the basis of these results, we concluded that the metabolic and excretion profiles of procymidone in humans are similar to those in monkeys and rabbits but differ from those in rats. From the findings of this and previous studies, we judge the developmental toxicity potential of procymidone to be very low in humans. © Pesticide Science Society of Japan

Keywords: species differences, developmental toxicity, human relevance, biotransformation, chimeric mouse, procymidone.

Introduction

Procymidone (Sumilex®) is a fungicide with both protective and curative properties. It is used to control plant diseases, such as fruit rot; gray mold on fruits, vines, and vegetables; and Sclerotinia rot of kidney beans and vegetable crops.1–3) The metabolic pathways of procymidone in mammals are shown in Fig. 1.4–6) The imide (cyclic) compounds (procymidone, PCM-CH2OH, and PCM-COOH) and their amide metabolites (PCM-NH-COOH, PA-CH2OH, and PA-COOH, respectively) are in equilibrium,7) and the sum of both components (amounts and/or concentrations) is considered to be an appropriate index for comparing the amounts (concentrations) of metabolites. Therefore, in this article, the following abbreviations are used for procymidone and its metabolites: PCM (representing the sum of procymidone and PCM-NH-COOH), Hydroxylated-PCM (sum of PCM-CH2OH and PA-CH2OH), Carboxylated-PCM (sum of PCM-COOH and PA-COOH), and Hydroxylated-PCM-glucuronide (sum of PCM-CH2OH-glucuronide and PA-CH2OH-glucuronide).

Procymidone is hydroxylated at the methyl group of the imide ring. Hydroxylated-PCM is then metabolized in one of two ways: one is oxidation at the hydroxymethyl group to form Carboxylated-PCM, and the other is conjugation with glucuronic acid to form Hydroxylated-PCM-glucuronide. Carboxylated-PCM and Hydroxylated-PCM-glucuronide are highly hydrophilic and thus are excreted more readily than Hydroxylated-PCM.4,5) Many toxicity studies have been conducted to evaluate the safety of procymidone. Toxicity studies revealed the development of external anomalies such as a short anogenital distance in male rats but not in rabbits or monkeys.

To investigate species differences in developmental toxicity, we conducted a series of mechanistic studies. In one mechanistic study, we measured the in vitro anti-androgenic activity of procymidone and its metabolites. The anti-androgen assay found no differences between rats and humans, and it was clear that PCM and Hydroxylated-PCM have only very weak anti-androgenic activity, while other metabolites (Carboxylated-PCM and Hydroxylated-PCM-glucuronide) have no anti-androgenic activity at all.8,9) Our pharmacokinetic, metabolism, and excretion studies10) showed significant differences in the plasma concentration of Hydroxylated-PCM in rats, rabbits, and monkeys, with the Hydroxylated-PCM concentration being several times higher than the procymidone concentration in rat plasma, and both being much higher in rat plasma than in rabbit or monkey plasma. It was concluded that Hydroxylated-PCM could be a key factor in developmental toxicity in rats. In rabbits and monkeys, procymidone is hydroxylated to Hydroxylated-PCM and transformed in the liver to Hydroxylated-PCM-glucuronide. The glucuronide is then passed into the bloodstream and rapidly excreted in urine as one of the main metabolites (Fig. 2). Hydroxylated-PCM-glucuronide was a minor metabolite in rat urine but a significant one in rat bile. It was deconjugated in the...
gastrointestinal tract to a form reabsorbed by the liver, resulting in its recycling through enterohepatic circulation (Fig. 2). This recycling is considered to be important because it sustains high plasma concentrations of Hydroxylated-PCM. Therefore, it was concluded that an important species difference related to the developmental toxicity of procymidone is in the plasma Hydroxylated-PCM concentration, which is attributable to the biliary excretion rate of Hydroxylated-PCM-glucuronide. Placental transfer studies of procymidone were also conducted to investigate species differences. In these studies, much higher
amounts of PCM and Hydroxylated-PCM were transferred to rat fetuses than to rabbit or monkey fetuses. Furthermore, rat fetuses tended to accumulate Hydroxylated-PCM upon repeated oral administration of procymidone. Taken together, these results suggest that Hydroxylated-PCM plays an important role in developmental effects in vivo in rats.

To investigate the relevance to humans of procymidone’s developmental effects in animals, we conducted in vitro metabolism (biotransformation) studies using liver S9 fractions and hepatocytes, in vitro protein binding studies, and in vivo metabolism and excretion studies using chimeric mice with humanized hepatocytes. The present article deals with this issue.

Materials and Methods

1. Chemicals

14C-procymidone (specific radioactivity: 4.48 GBq/mmol; radiochemical purity: >99%; chemical purity: >98%) and 14C-PCM-CH2OH (specific radioactivity: 0.98 GBq/mmol; radiochemical purity: >99%; chemical purity: >98%) were synthesized by Sumitomo Chemical Co., Ltd. (Japan) for 10 min at 20°C and 1,000×g. After centrifugation, an aliquot (100 µL) of the ultrafiltrate was analyzed to confirm the concentration of 14C-procymidone or 14C-PCM-CH2OH (at final concentrations of 1, 3, 10, and 30 µg/mL) was placed in a Centrifree YM-30® ultrafiltration device for approximately 5 min. An aliquot (100 µL) of the sample was analyzed to confirm the concentration of 14C-procymidone or 14C-PCM-CH2OH [concentration in the case of (A), ConcA; concentration in the case of (B), ConcB]. The Centrifree YM-30® device was centrifuged in a Hitachi 20B3 centrifuge (Hitachi, Japan) for 10 min at 20°C and 1,000×g. After centrifugation, an aliquot (100 µL) of the ultrafiltrate was analyzed to confirm the concentration of 14C-procymidone or 14C-PCM-CH2OH [concentration in the case of (A), ConcA; concentration in the case of (B), ConcB]. The radioactivity in samples was measured with a Tri-Carb® 2500TR Liquid Scintillation Analyzer (PerkinElmer, Inc., USA) after mixing the samples with 10 mL of Hionic-Fluor scintillator fluid (PerkinElmer, Inc.). All samples were analyzed in triplicate. The binding rate of procymidone or PCM-CH2OH to plasma protein was calculated using the following equation.

\[
\text{Binding} \% = \left\{1 - \frac{\text{ConcA}}{\text{ConcB}}\right\} \times 100
\]

2. Analysis of metabolites

In the manner described previously,10 samples obtained (as described below) were subjected to TLC analysis using silica-gel 60F254 plates (thickness: 0.25 mm; E. Merck, Germany) and an image analyzer (BAS-1800 II, Fuji Photo Film Co., Ltd., Japan). The solvent system was as follows: (1) toluene/ethyl formate/formic acid (5 : 7 : 1, by vol.) and/or (2) butanol/acetic acid/water (6 : 1 : 1, by vol.) for urine and feces samples. The metabolites were quantitated via image analysis of the visible TLC plate spots resolved by using solvent system (1). TLC co-chromatography with procymidone and its metabolites was conducted to identify metabolites. To identify glucuronide, enzymatic hydrolysis was carried out with representative urine samples. Urine samples (1 mL) were added to 1 mL of 0.5 M acetate buffer (pH 5.0) and incubated with β-glucuronidase (approximately 5,000 units, containing sulfatase from Helix pomatia, Roche Diagnostics, Japan) at 37°C for approximately 4 hr for hydrolysis.

TLC co-chromatography (with solvent systems (1) and (2)) was used to identify aglycones in the resulting mixture. In this manner, Hydroxylated-PCM-glucuronide was detected.

3. In vitro study

3.1. Plasma protein binding of procymidone and PCM-CH2OH

Samples from female humans (Caucasian), rats (CD(SD)IGS), monkeys ( cynomolagus), and rabbits (New Zealand white) were purchased from Biopredic International (France). The plasma was stored at −80°C until use. The plasma protein binding of 14C-procymidone and 14C-PCM-CH2OH was determined at concentrations of 1, 3, 10, and 30 µg/mL using Centrifree YM-30® micropartition devices (Millipore, USA). These plasma concentrations were determined from in vivo pharmacokinetic studies in animals.10 One milliliter of (A) 0.1 M phosphate buffer (pH 7.0)/acetonitrile = 9/1 (v/v) or (B) plasma fortified with 10 µL of an acetonitrile solution of 14C-procymidone or 14C-PCM-CH2OH (at final concentrations of 1, 3, 10, and 30 µg/mL) was placed in a Centrifree YM-30® ultrafiltration device for approximately 5 min. An aliquot (100 µL) of the sample was analyzed to confirm the concentration of 14C-procymidone or 14C-PCM-CH2OH [concentration in the case of (A), ConcA; concentration in the case of (B), ConcB]. The Centrifree YM-30® device was centrifuged in a Hitachi 20B3 centrifuge (Hitachi, Japan) for 10 min at 20°C and 1,000×g. After centrifugation, an aliquot (100 µL) of the ultrafiltrate was analyzed to confirm the concentration of 14C-procymidone or 14C-PCM-CH2OH [concentration in the case of (A), ConcA; concentration in the case of (B), ConcB]. The radioactivity in samples was measured with a Tri-Carb® 2500TR Liquid Scintillation Analyzer (PerkinElmer, Inc., USA) after mixing the samples with 10 mL of Hionic-Fluor scintillator fluid (PerkinElmer, Inc.). All samples were analyzed in duplicate. The binding rate of procymidone or PCM-CH2OH to plasma protein was calculated using the following equation.

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\text{Binding} \% = \left\{1 - \frac{\text{ConcA}}{\text{ConcB}}\right\} \times 100
\]

3.2. Assessment of the enzyme activities responsible for procymidone and PCM-CH2OH biotransformation in rat, monkey, rabbit, and human liver S9 fractions

The liver S9 fractions from female humans (pooled from two Caucasian and one African American individual), rats (pooled from 95 CD(SD)IGS rats), monkeys (pooled from six cynomolgus monkeys), and rabbits (pooled from two New Zealand white rabbits) were purchased from Xenotech LLC (USA) and stored at −80°C until use. The reaction mixtures consisted of 100 mM phosphate buffer (pH 7.4), 1.5 mg of liver S9 protein (final concentration was 2 mg/mL), 3 mM NADPH (Oriental Yeast Co., Ltd., Japan), and 7.5 µL of acetonitrile solution containing 14C-procymidone or 14C-PCM-CH2OH (final concentration was 50 µM) in a volume of 0.75 mL. All samples were analyzed in duplicate. The reaction mixtures were incubated at 37°C for 15, 30, and 60 min. The reaction was terminated by the addition of 0.75 mL of ice-cold acetonitrile and centrifuged at 20,400×g for
5 min at 4°C. The supernatant was removed and the metabolites in an aliquot (100 µL) of each supernatant were analyzed via TLC using solvent systems (1) and (2). The extents of hydroxylation from PCM ([14C-procymidone]) to Hydroxylated-PCM and oxidation from Hydroxylated-PCM ([14C-PCM-CH2OH]) to Carboxylated-PCM were calculated using the following equations. The extent of hydroxylation from PCM to Hydroxylated-PCM (nmol/mg protein) = sum of Hydroxylated-PCM and Carboxylated-PCM/sum of procymidone and all metabolites × 25
The extent of oxidation from Hydroxylated-PCM to Carboxylated-PCM (nmol/mg protein) = sum of Carboxylated-PCM/sum of PCM-CH2OH and all metabolites × 25

3.3. Assessment of the enzyme activities responsible for procymidone biotransformations in human hepatocytes
The following four lots of hepatocytes were used in this study: two (Lot Nos. AEX and KCT) purchased from In Vitro Technologies (USA), one (Lot No. 426) from Xenotech, LLC (USA) and one (Lot No. 045396380002) from Tissue Transformation Technologies (USA). Samples of the cryopreserved human hepatocytes were thawed in a 37°C water bath for 1.5 min and then isolated using the Hepatocyte Isolation Kit (XenoTech, LLC) as follows. The thawed human hepatocyte suspension was poured into 13 mL of isotonic Percoll solution. After centrifugation (100 × g, 20°C, 2 min), the supernatant was aspirated. The cell pellet was gently suspended in 10 mL of DMEM by inverting the tube. After another centrifugation (100 × g, 20°C, 2 min) and supernatant aspiration, the cell pellet was gently resuspended in 2 mL of DMEM by inverting the tube. One hundred and fifty microliters of 14C-procymidone solution (74 µM acetonitrile solution) was added to 30 mL of culture medium, and 7 mL of the mixture was added to 2 mL of the cell suspension in DMEM. Two milliliters of this mixture was transferred to a plate (7.11 × 10^5 cells/plate) and incubated at 37°C for 46 hr. The reaction was terminated by the addition of 3 mL of acetonitrile, then the reaction mixture was collected in a tube, the plate was rinsed with 3 mL of acetonitrile, and the rinsate was combined with the reaction mixture. The supernatant resulting from centrifugation at 16,000 × g (4°C, 10 min) was concentrated to dryness under a stream of nitrogen gas. The residue was redissolved in 200 µL of 50% acetonitrile solution, and aliquots of the sample were analyzed via TLC using solvent systems (1) and (2).

3.4. Assessment of the enzyme activities responsible for PCM-CH2OH biotransformations in rat and human hepatocytes
The following four lots of cryopreserved hepatocytes were used in this study: two were from female humans (Lot No. 548, Caucasian, and Lot No. 88, Caucasian, purchased from Xenotech, LLC and In Vitro Technologies, Inc., respectively) and two from female rats (Lot No. 143, Sprague Dawley, and Lot No. 106, Sprague Dawley, purchased from Xenotech, LLC, and In Vitro Technologies, Inc., respectively). Samples of the cryopreserved hepatocytes were each thawed in a 37°C water bath and then isolated using the Hepatocyte Isolation Kit as follows. Each thawed hepatocyte suspension was poured into 13 mL of isotonic Percoll solution and centrifuged (100 × g, 20°C, 2 min), and the supernatant was aspirated. The cell pellet was suspended in 10 mL of DMEM. After centrifugation (100 × g, 20°C, 2 min), the supernatant was aspirated. The resulting cell pellet was resuspended in 2 mL of hepatocyte incubation media and a 30 µL aliquot of the suspension was added to 270 µL of trypsin blue solution. Dead and viable cells were counted in a hemocytometer. The concentration of viable cells was adjusted by dilution with Hepatocyte Incubation Media to 10^5–10^6 cells/mL. Each reaction mixture consisting of 39 µL of [14C-PCM-CH2OH solution (510 µM acetonitrile solution), 20 µL of distilled water, and 2 mL of cells suspended in culture medium was pipetted onto a plate and incubated at 37°C. At 0.5, 1, 2, 4, and 8 hr after the start of incubation, an aliquot (200 µL) of each reaction mixture was analyzed. A sample of each reaction mixture was added to 1 mL of acetonitrile, mixed well by vortexing, and centrifuged (16,000 × g, 4°C, 10 min). The supernatant was concentrated to dryness under a stream of nitrogen gas. The residue was redissolved in 200 µL of 50% acetonitrile solution, and an aliquot was analyzed via TLC using solvent systems (1) and (2). All samples were analyzed in duplicate. The extent of Hydroxylated-PCM ([14C-PCM-CH2OH] oxidation and glucuronidation to Carboxylated-PCM and Hydroxylated-PCM-glucuronide, respectively, was calculated using the following equations.

Extent of oxidation from Hydroxylated-PCM to Carboxylated-PCM (nmol/10^6 cells) = sum of Carboxylated-PCM/sum of PCM-CH2OH and all metabolites × cell numbers/10^6
Extent of glucuronidation from Hydroxylated-PCM to Hydroxylated-PCM-glucuronide (nmol/10^6 cells) = sum of Hydroxylated-PCM-glucuronide/sum of PCM-CH2OH and all metabolites × cell numbers/10^6

4. Metabolism and excretion studies in chimeric mice
4.1. Animal husbandry
Chimeric mice with human hepatocytes (humanized liver) were generated by injecting human hepatocytes into urokinase-type plasminogen activator transgenic SCID mice (uPA/SCID mice). The transplanted human hepatocytes can grow in the livers of uPA/SCID mice, which are immunodeficient and would otherwise undergo liver failure. Four uPA/SCID mice (control) and four chimeric mice with a humanized liver were supplied by PhoenixBio Co., Ltd. (Japan). Animals were maintained in an air-conditioned room at 20–26°C with an alternating 12 hr light and 12 hr dark cycle for at least 4 days prior to use in the study. For control mice, water and pellet diet (CRF-1, Oriental Yeast Co., Ltd., Japan) were provided ad libitum. For chimeric mice, water sterilized in an autoclave containing 120 µL of sodium hypochlorite per 1000 mL of water and CRF-1 containing Vitamin C sterilized by X-ray irradiation were provided ad libitum. Dur-
ing the period from quarantine to $^{14}$C-procymidone administration, the general condition of the mice was observed once per day.

4.2. Animal treatment
After quarantine (at least 4 days), animals received $^{14}$C-procymidone orally at 37.5 mg/kg/5 mL corn oil. The dose level (37.5 mg/kg) was selected as the lowest toxicologically effective dose. The dose of radioactivity was set at about 55.2 MBq/kg. After dose administration, animals were housed individually in metabolic cages to allow separate collection of urine and feces 24, 48, and 72 hr after administration. After each of these times, the cages were washed with approximately 50 mL of water (cage washing). Following the cage washing at 72 hr, an additional cage washing with approximately 50 mL of methanol and the occasional removal of discarded food and other debris from the cages were carried out. The study protocol was approved by the committee concerned with animal protection at the laboratory and the study was performed in accordance with established ethical standards.

4.3. Sample processing and radioanalysis
Urine collected at each sampling time was weighed and assayed (0.05 g of urine per assay). Feces collected at each sampling time was diluted to 10 mL with acetonitrile, homogenized using a Polytron homogenizer and solubilized (0.1 mL of the fecal homogenate in 2 mL of the tissue solubilizer Soluene-350 (PerkinElmer, Inc.)). Cage washing collected at each sampling time was weighed and assayed (0.01 g of the cage washing per assay). Radioactivity in the sample was included in the urinary $^{14}$C excretion. Debris recovered occasionally was diluted to 10 to 40 mL with water, homogenized using a Polytron homogenizer, and solubilized (0.1 mL of the debris homogenate in 2 mL of the tissue solubilizer Soluene-350 (PerkinElmer, Inc.)). The radioactivity of each sample after the addition of 10 mL of Hionic-Fluor scintillator fluid (PerkinElmer, Inc.) was measured with a Tri-Carb® 2500TR Liquid Scintillation Analyzer (PerkinElmer, Inc.). The fecal acetonitrile homogenate (0.5 g) was mixed with 0.5 mL of Milli-Q water, shaken after the addition of 1 mL of ethanol, and centrifuged (1,800×g, 4°C, 10 min) to remove the supernatant. Another 1 mL of ethanol was added to the residue, and the same extraction procedure was repeated twice. All of the obtained supernatants were combined. The residue was solubilized in tissue solubilizer Soluene-350 and diluted to a designated volume, and an aliquot of this solution was mixed with 10 mL of Hionic-Fluor to measure the radioactivity. The combined supernatants were evaporated to dryness, and the resulting solid was reconstituted in 90% ethanol and subjected to TLC analysis. Urine was subjected to TLC analysis directly.

Results

1. In vitro study

1.1. Plasma protein binding of procymidone and PCM-CH$_2$OH
The rates of procymidone and PCM-CH$_2$OH binding to plasma protein are shown in Tables 1 and 2. The binding rate of procymidone was approximately 96%, 97%, 94%, and 97% for humans, rats, monkeys, and rabbits, respectively. It was constant over the concentration range of 1 to 30 µg/mL. The rate of PCM-CH$_2$OH binding to human plasma protein was approximately 91% and greater than that to plasma protein from other species. In other species, the protein binding became weaker at higher concentrations of PCM-CH$_2$OH. In other words, the level of free (unbound) PCM-CH$_2$OH was increased at a higher concentration in plasma from non-human species.

1.2. Enzyme activity level in liver S9 responsible for the hydroxylation of procymidone to Hydroxylated-PCM and the oxidation of PCM-CH$_2$OH to Carboxylated-PCM
The extent of the biotransformations from procymidone to Hydroxylated-PCM and from PCM-CH$_2$OH to Carboxylated-PCM catalyzed by enzymes in the liver S9 of rats, monkeys, rabbits, and humans is shown in Table 3. These hydroxylation and oxidation activities were constant over 60 min for every species (Figs. 3 and 4). Regarding hydroxylation (procymidone to Hydroxylated-PCM) in all species, rabbits had the highest enzyme activity. The enzyme activity in humans, monkeys, and rats was 60%, 50%, and 16% of that in rabbits, respectively. For the oxidation of Hionic-Fluor to measure the radioactivity. The combined supernatants were evaporated to dryness, and the resulting solid was reconstituted in 90% ethanol and subjected to TLC analysis. Urine was subjected to TLC analysis directly.

<p>| Table 1. Binding rate of procymidone to female human, rat, rabbit, and monkey plasma |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
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<th>Concentration [µg/mL]</th>
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<th>Rabbit</th>
<th>Monkey</th>
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<td>a) Data represent the mean value (n=2).</td>
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</table>

<p>| Table 2. Binding rate of PCM-CH$_2$OH to female human, rat, rabbit, and monkey plasma |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
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<th>Rabbit</th>
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<td>a) Data represent the mean value (n=2).</td>
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</tbody>
</table>

| Table 3. Biotransformation activities of procymidone and PCM-CH$_2$OH in liver S9 of rats, rabbits, monkeys, and human |
|---------------------------------|-----------------|-----------------|-----------------|
| Species | Activity (pmol/min/mg protein)$^a$ | Hydroxylated-PCM to Carboxylated-PCM $^b$ |
|----------------------|-----------------|-----------------|-----------------|
| Rat                   | 9.8 (1.0)       | 1.0 (1.0)       |
| Rabbit                | 60.7 (6.2)      | 26.4 (26.4)     |
| Monkey                | 30.4 (3.1)      | 32.6 (32.6)     |
| Human                 | 36.4 (3.7)      | 12.9 (12.9)     |
| a) Data represent the mean value (n=2). b) Rat data were defined as 1.0.
1.3. Biotransformation of procymidone in human hepatocytes

Glucuronides have been detected as metabolites in in vivo procymidone metabolism studies in rats, rabbits, and monkeys. To clarify the fate of glucuronides as procymidone metabolites in humans, we conducted an in vitro metabolism study using human hepatocytes and detected Hydroxylated-PCM-glucuronide in human hepatocytes as a main metabolite (Fig. 5).

1.4. Enzyme activity level responsible for the oxidation of PCM-CH₂OH to Carboxylated-PCM and the glucuronidation of PCM-CH₂OH to Hydroxylated-PCM-glucuronide in rat and human hepatocytes

Each of the rat and human hepatocytes was incubated with PCM-CH₂OH as a substrate, and its oxidation product (Carboxylated-PCM) and glucuronidation product (Hydroxylated-PCM-glucuronide) were quantified. The levels of enzyme activity responsible for these biotransformations in rat and human hepatocytes are shown in Table 4. These activities were constant over 8 hr in human hepatocytes, but it appeared that the activities were maintained only for up to 4 hr in rat hepatocytes (Figs. 6 and 7). Human hepatocytes generated Carboxylated-PCM and Hydroxylated-PCM-glucuronide at a faster rate than rat hepatocytes.

![Fig. 3. Time–course change in the amount of Hydroxylated-PCM produced when procymidone is incubated with liver S9 at 37°C. Data represent the mean value (n=2).](image1)

![Fig. 4. Time–course change in the amount of Carboxylated-PCM produced when PCM-CH₂OH is incubated with liver S9 at 37°C. Data represent the mean value (n=2).](image2)

![Fig. 5. Typical autoradiogram of TLC of procymidone metabolites in reaction mixtures obtained from human hepatocytes. A: Solvent system (1); B: solvent system (2).](image3)
cytes. The rate of conversion from PCM-CH$_2$OH to Carboxylated-PCM was 0.34 and 0.16 nmol/hr/10$^6$ cells in human and rat hepatocytes, respectively. The rate of glucuronidation was 0.43 and 0.11 nmol/hr/10$^6$ cells in human and rat hepatocytes, respectively. Both oxidation and glucuronidation rates were clearly higher in human hepatocytes than in rat hepatocytes.

2. In vivo study metabolism and excretion studies in chimeric mice

To investigate the metabolism of procymidone in humans, metabolite profiles in urine and feces were compared between control mice (uPA/SCID mice) and chimeric mice after a single oral administration of 14C-procymidone at 37.5 mg/kg. The cumulative 14C-excretions in urine and feces are shown in Table 5. The excretions in urine and feces within 72 hr after administration were 75.7% and 19.5% of the dose, respectively, in control mice and 73.3% and 24.2% of the dose, respectively, in chimeric mice. The metabolite amounts in urine and feces 0–72 hr after administration are shown in Table 6. Typical autoradiograms of the TLC maps of metabolites in urine after a single oral administration of 14C-procymidone to control and chimeric mice are shown in Fig. 8. In the urine samples obtained from control and chimeric mice up to 72 hr after administration, unchanged procymidone accounted for 1.4% and 0.1% of the dose, respectively. Carboxylated-PCM and Hydroxylated-PCM accounted for 59.4% and 2.5% of the dose, respectively, in control mice and 33.7% and 1.6% of the dose, respectively, in chimeric mice. Hydroxylated-PCM-glucuronide was a major metabolite in urine, accounting for 35.3% of the dose in chimeric mice, and a minor urinary metabolite accounting for 7.1% of the dose in control mice. In the fecal samples obtained up to 72 hr after administration, unchanged procymidone accounted for 0.8% and 1.8% of the dose, respectively, in control and chimeric mice. Carboxylated-PCM and Hydroxylated-PCM accounted for 9.5% and 0.6% of the dose, respectively, in control mice and 7.2% and 0.6% of the dose, respectively, in chimeric mice. Though Hydroxylated-PCM-glucuronide accounted for 2.1% of the dose in control mice and 7.5% of the dose in chimeric mice, this fecal glucuronide was considered to be a contaminant from urine resulting from insufficient separation of urine and feces in the metabolic cage. In general, glucuronides are not detected in feces because they are deconjugated by bacteria in the intestinal tract.

Discussion

To investigate the relevance to humans of the developmental effects caused by procymidone in rats, we conducted an in vitro plasma protein binding study, an in vitro metabolism study with human hepatocytes, and in vivo metabolism and excretion studies in chimeric mice with humanized liver. The extent of plasma protein binding of procymidone was almost the same in humans, rats, monkeys, and rabbits. The amount of plasma protein binding of Hydroxylated-PCM was greater in humans than in other species, especially in the higher concentration range (10–30 µg/mL) where toxicity may be produced in rats. In other

<table>
<thead>
<tr>
<th>Species</th>
<th>Hydroxylated-PCM to Carboxylated-PCM</th>
<th>Hydroxylated-PCM to Glucuronide</th>
</tr>
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<tbody>
<tr>
<td>Rat</td>
<td>0.16 (1.0)</td>
<td>0.11 (1.0)</td>
</tr>
<tr>
<td>Human</td>
<td>0.34 (2.1)</td>
<td>0.43 (3.9)</td>
</tr>
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</table>

$^{(a)}$ Data represent mean value ($n=2$). $^{(b)}$ Rat data were defined as 1.0.
words, the concentration of free (unbound) Hydroxylated-PCM is lowest in humans as compared with the other species in the present study. Therefore, the proportions of PCM and Hydroxylated-PCM transferred from mother to fetus were considered to be the lowest in humans relative to the other species, because only free compounds (not bound to plasma proteins) are expected to cross the placenta. Likewise, the placental transfer ratios of procymidone and Hydroxylated-PCM are expected to be lower in humans than in the other species. To compare the ratios of procymidone and Hydroxylated-PCM are expected to cross the placenta. Likewise, the placental transfer ratios of procymidone and Hydroxylated-PCM are expected to be lower in humans than in the other species. To compare the rate of biotransformation from procymidone (PCM) to Hydroxylated-PCM and from PCM-CH$_2$OH (Hydroxylated-PCM) to Carboxylated-PCM in humans, rats, monkeys, and rabbits, \textit{in vitro} studies were conducted. The rate in human S9 was much higher than that in rat S9 but was similar to that in monkey and rabbit S9. Although it occurred in human, monkey, and rabbit S9, the oxidation of PCM-CH$_2$OH (Hydroxylated-PCM) to Carboxylated-PCM hardly occurred in rat S9. These results suggested that metabolism of procymidone (PCM) and PCM-CH$_2$OH (Hydroxylated-PCM) occurs faster in humans, monkeys, and rabbits than in rats. Therefore, it was considered that the metabolic detoxification of PCM-CH$_2$OH (Hydroxylated-PCM) occurs more readily in humans, monkeys, and rabbits than in rats.

\textit{In vivo} studies of procymidone metabolism have detected Hydroxylated-PCM-glucuronide as a metabolite in rats, rabbits, and monkeys. In an \textit{in vitro} metabolism study, we confirmed that human hepatocytes also produce this glucuronide (as a metabolite of procymidone). The enzyme activity responsible for converting PCM-CH$_2$OH (Hydroxylated-PCM) to Carboxylated-PCM, and PCM-CH$_2$OH (Hydroxylated-PCM) to Hydroxylated-PCM-glucuronide, was quantified by \textit{in vitro} study using rat and human hepatocytes and PCM-CH$_2$OH as a substrate. The oxidation activity and glucuronidation activity were 2.1 and 3.9 times greater in human hepatocytes than in rat hepatocytes, respectively. It is reported that the number of cells per liver weight is almost the same in rats and humans,$^{12)}$ and the liver weight per body weight (bw) in rats is 0.024 (male)

### Table 5. Cumulative $^{14}$C-excretions in urine and feces after single oral administration of $^{14}$C-procymidone at 37.5 mg/kg to control mice and chimeric mice

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control mice</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Chimeric mice</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>0–24</td>
<td>65.1±4.8</td>
<td>17.4±4.1</td>
<td>82.5±5.0</td>
<td>59.0±2.1</td>
<td>21.5±6.2</td>
<td>80.5±4.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–48</td>
<td>72.2±4.9</td>
<td>19.0±4.1</td>
<td>91.2±3.3</td>
<td>69.5±5.5</td>
<td>23.7±5.2</td>
<td>93.2±0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–72</td>
<td>75.7±4.4</td>
<td>19.5±4.2</td>
<td>95.1±2.4</td>
<td>73.3±7.6</td>
<td>24.2±4.8</td>
<td>97.6±2.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Debris</td>
<td>—</td>
<td>—</td>
<td>0.6±0.7</td>
<td>—</td>
<td>—</td>
<td>1.7±1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{14}$C-Recovery 95.7±2.1 99.3±2.6

$^{a)$ Data represent the mean value±S.D. of four mice.

### Table 6. Amounts of metabolites in urine and feces within 72 hr after single oral administration of $^{14}$C-procymidone at 37.5 mg/kg to control mice and chimeric mice

| Metabolite | Control mice | | | | | | Chimeric mice | | | |
|------------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|
|            | Urine        | Feces    | Total    | Urine    | Feces    | Total    | Urine    | Feces    | Total    |
| Procymidone| 1.4±0.5      | 0.8±0.7  | 2.2±0.8  | 0.1±0.0  | 1.8±1.2  | 1.9±1.2  |
| PCM-COOH   | 33.8±4.6     | 6.0±2.3  | 39.8±2.3 | 21.4±1.8 | 4.6±2.6  | 26.0±2.3 |
| PA-CH$_2$OH| 2.5±0.4      | 0.6±0.2  | 3.1±0.3  | 1.6±0.5  | 0.6±0.2  | 2.2±0.4  |
| PA-COOH    | 25.6±1.4     | 3.4±1.2  | 29.1±2.5 | 12.3±1.7 | 2.6±0.9  | 14.9±2.1 |
| Glucuronide| 7.1±1.0      | 2.1±0.2  | 9.2±0.9  | 35.3±5.7 | 7.5±2.2  | 42.8±4.2 |
| Others     | 5.3±0.5      | 3.6±0.5  | 8.8±0.8  | 2.7±1.0  | 3.8±0.4  | 6.5±1.3  |
| Unextractable | —$^b)$   | 2.9±0.4  | 2.9±0.4  | —$^b)$   | 3.3±0.5  | 3.3±0.5  |
| Total      | 75.7±4.4     | 19.5±4.2 | 95.1±2.4 | 73.3±7.6 | 24.2±4.8 | 97.6±2.8 |

$^{a)$ Data represent the mean value±S.D. of four mice. $^{b)$ Not applicable. $^{c)$ Data were produced by the following equation: PCM=procymidone, Hydroxylated-PCM=PA-CH$_2$OH, Carboxylated-PCM=PCM-COOH+PA-COOH, Hydroxylated-PCM-glucuronide=glucuronide

and 0.026 (female) g liver/g bw\textsuperscript{13} and is 0.020 (male) and 0.019 (female) g liver/g bw in humans.\textsuperscript{14,15} By using the above data, the number of cells per bw was derived (Table 7). From those results, the activities per g bw were calculated and are shown in Table 8. The oxidation and glucuronidation rates per bw are 1.9 times and 3.5 times greater, respectively, in humans than in rats. When exposed to the same amount of chemical per bw, both reactions were found to proceed faster in humans.

Therefore, it was concluded that the detoxification of PCM-CH\textsubscript{2}OH (Hydroxylated-PCM) to Carboxylated-PCM and Hydroxylated-PCM-glucuronide occurs more readily in humans than in rats.

The chimeric mice express human metabolic enzymes and transporters and are, therefore, known to be a useful model for investigating the metabolism and excretion of chemicals in humans.\textsuperscript{16–21} Similar to rabbits and monkeys, the chimeric mice in the present study produced Hydroxylated-PCM-glucuronide and excreted it in urine.\textsuperscript{10} It can therefore be concluded that the metabolic and excretion profiles of procyromide in humans will be similar to those in monkeys and rabbits and dissimilar to those in rats. In humans, Hydroxylated-PCM, a key developmental effector in rats and an active developmental toxicant, will most likely be excreted via the urine and not undergo enterohepatic circulation, suggesting that Hydroxylated-PCM might not be retained in the body and fetus. On the basis of these results, we conclude that the developmental toxicity observed in procyromide treated rats at 37.5 mg/kg/day or higher would never occur in humans.

Chimeric mice with rat hepatocytes can be prepared similarly and these are expected to be a useful model for investigating the metabolism and excretion of chemicals in rats. If the metabolic and excretion patterns in chimeric mice with rat hepatocytes are similar to those in rats, this will provide further evidence that metabolic and excretion patterns in chimeric mice with human hepatocytes reflect actual human metabolism and excretion. Therefore, we have conducted metabolism and biliary excretion studies using chimeric mice with rat hepatocytes. Those results will be described elsewhere.

### Table 7. Amounts of hepatocytes per body weight in human and rat

<table>
<thead>
<tr>
<th>Species</th>
<th>10\textsuperscript{6} cells/g liver\textsuperscript{a}</th>
<th>g liver/g body weight</th>
<th>10\textsuperscript{6} cells/g body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Rat</td>
<td>114.2</td>
<td>0.024\textsuperscript{b}</td>
<td>0.026\textsuperscript{b}</td>
</tr>
<tr>
<td>Human</td>
<td>136.8</td>
<td>0.020\textsuperscript{b}</td>
<td>0.019\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Reproduced from a previous article 12. \textsuperscript{b} Reproduced from a previous article 13. \textsuperscript{c} Reproduced from a previous article 14. \textsuperscript{d} Reproduced from a previous article 15.

### Table 8. Biotransformation activities of PCM-CH\textsubscript{2}OH in rat and human female hepatocytes

<table>
<thead>
<tr>
<th>Species</th>
<th>Activity (nmol/hour/g body weight)\textsuperscript{a} (relative activity)\textsuperscript{b}</th>
<th>Hydroxylated-PCM to Carboxylated-PCM</th>
<th>Hydroxylated-PCM to Glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>0.47 (1.0)</td>
<td>0.32 (1.0)</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>0.88 (1.9)</td>
<td>1.12 (3.5)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data represent mean value (n=2). \textsuperscript{b} Rat data were defined as 1.0.

### References