Comparative hepatotoxicity of a herbicide, epyrifenacil, in humans and rodents by comparing the dynamics and kinetics of its causal metabolite

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A new herbicide, epyrifenacil (S-3100), inhibits protoporphyrinogen oxidase (PPO) in plants. Repeated administration of epyrifenacil in laboratory animals led to some toxicological changes related to PPO inhibition, e.g., hepatotoxicity caused by porphyrin accumulation and anemia caused by the inhibition of heme biosynthesis. In vitro studies revealed that an ester-cleaved metabolite, S-3100-CA, is predominant in mammals, exhibits PPO-inhibitory activity, and thus is the cause of epyrifenacil-induced toxicity. To assess the human risk, the effects of species differences on the dynamics (PPO inhibition) and kinetics (liver uptake) of epyrifenacil were evaluated separately. The results of in vitro assays revealed an approximately tenfold weaker inhibition of PPO by S-3100-CA in humans than in rodents and six- to thirteen-fold less hepatic uptake of S-3100-CA in humans than in mice. Finally, it was suggested that humans are less sensitive to the toxicity of epyrifenacil than are rodents, although further mechanistic research is highly anticipated.

Keywords: S-3100, epyrifenacil, subchronic toxicity, PPO, human risk, hepatotoxicity.

Introduction

Epyrifenacil (ethyl[(3-[2-chloro-4-fluoro-5-[3-methyl-2,6-dioxo-4-(trifluoromethyl)-3,6-dihydropyrimidin-1(2H)-yl]phenoxy]pyridin-2-yl)oxy]acetate, S-3100) is a new herbicide that acts via the inhibition of protoporphyrinogen oxidase (PPO) in plants. PPO is an essential enzyme involved in the synthesis of chlorophyll in plants. Inhibition of the enzyme in plant cells causes the accumulation of intermediate tetrapyroles, including protoporphyrin IX (PPIX), and subsequent photoreactions that produce oxygen free radicals to destroy the cell membranes.1,2) Regarding the toxicity of PPO inhibitors in mammals, similar key events of PPO inhibition and further accumulation of PPIX leading to cell damage may also occur. Thus, it is believed that PPO inhibitors can cause toxicity especially in PPO-generating organs such as the liver and occasionally induce hepatocellular tumors as a consequence of hepatotoxicity.3,4) Additionally, PPO catalyzes the oxidation of protoporphyrinogen IX to PPIX during heme biosynthesis in both plants and mammals. In mammals, the inhibition of PPO blocks the production of hemoglobin and erythrocytes, resulting in anemia.5–7) Since the molecular mechanism of heme biosynthesis is common to all mammals, the possibility that PPO inhibitors are hepatotoxic and/or cause anemia in humans must be considered, especially when such toxicity is observed in rodents and other mammals. However, as is generally recognized, it is impossible to administer a pesticide to humans for toxicological evaluation. On the other hand, it is generally known that species differences in toxicity occur when significant variations in dynamics and kinetics arise among species.8–11) Thus, it was considered very important to evaluate the species differences in the dynamics and kinetics of PPO inhibition separately to precisely assess the risk to humans.

For the identification of toxicological profiles including the adverse outcomes related to PPO inhibition, subchronic toxicity studies were conducted for epyrifenacil in some animal spe-
cies. Since epyrifenacil has an ester bond which is expected to be easily cleavable in mammals, it was considered that epyrifenacil is very rapidly metabolized and the metabolites may work as a causal substance of the toxicity in body. In order to determine the elimination rate of epyrifenacil and to identify the causal metabolites in the body, we conducted an in vitro metabolism study to find a major metabolite and then conducted a PPO-inhibition assay to determine whether the identified metabolite had PPO-inhibitory activity. Lastly, in order to evaluate the risks of epyrifenacil in humans, we focused on species differences in the dynamics (i.e., PPO-inhibitory activity) and kinetics (i.e., exposure, especially hepatic uptake) of the major metabolite.

Materials and methods

1. Chemicals

Epyrifenacil (S-3100) and S-3100-CA ((3-[2-chloro-4-fluoro-5-[1,2,3,6-tetrahydro-3-methyl-2,6-dioxo-4-(trifluoromethyl)-pyrimidin-1-yl]phenoxy]pyridin-2-yl)oxy)acetic acid) were synthesized in our laboratory. The chemical purity of these compounds was determined to be 99.8% and 99.7%, respectively, for the in vitro assays. For the subchronic toxicity study, technical-grade epyrifenacil (chemical purity: 96.8%) was used. Pyridyl-4(6)-14C-labeled epyrifenacil was synthesized in our laboratory and had a specific activity of 2.78 GBq/mmol with radiochemical purity of 98.1%. Phenyl-14C-labeled S-3100-CA was also synthesized in our laboratory and had a specific activity of 4.26 GBq/mmol with radiochemical purity of 97.8%. The structures of these compounds are shown in Fig. 1. Krebs–Henseleit buffer (KHB), silicone oil, and mineral oil were purchased from Merck KGaA (Darmstadt, Germany). The hepatocyte isolation kit was supplied by Sekisui XenoTech, LLC (Kansas City, KS, USA). PPIX and sodium amalgam were supplied by Sigma-Aldrich (St. Louis, MI, USA). Other chemicals were of reagent grade.

2. Biochemicals

Liver microsomes from male Sprague Dawley (SD) rats (pool of 711), male CD-1 mouse (pool of 1200), and humans (pool of 100 males and 100 females), and liver mitochondrial fractions from SD rats (pool of 15) and humans (pool of 5) were purchased from Sekisui XenoTech, LLC. Liver mitochondrial fractions from mice, rabbits, and dogs were prepared in-house as described later. Cryopreserved primary hepatocytes of male SD rats (pool of 8), female SD rats (pool of 12), male CD-1 mice (pool of 16), and female CD-1 mice (pool of 23) were from Sekisui XenoTech, LLC. Cryopreserved primary hepatocytes of humans (pool of 10 males and 10 females) were purchased from Biopredic International (Saint Grégoire, France).

3. Subchronic toxicity studies with epyrifenacil

Subchronic (90-day) studies with epyrifenacil in Cr:CD(SD) rats, Cr:CD1(ICR) mice, and beagles were conducted. The design of these studies was based on the relevant test guidelines, and the studies were conducted in compliance with the principles of Good Laboratory Practice (GLP). All experiments in these studies complied with all applicable sections of the Final Rules of the Animal Welfare Act (Code of Federal Regulations, Title 9), the Public Health Service Policy on Humane Care and Use of Laboratory Animals from the Office of Laboratory Animal Welfare, and the Guide for the Care and Use of Laboratory Animals from the National Research Council. Detailed study conditions, including animal husbandry, observation and examination items, and toxicokinetics, are presented in the Supplemental Information.

Briefly, rats and mice were dosed with epyrifenacil via dietary administration, and the concentrations of epyrifenacil in the diet were 0 (basal diet only), 50, 300, and 800 ppm in rats and 0, 5, 25, and 100 ppm in mice. Rats and mice were allowed free access to the diet containing epyrifenacil for 90 consecutive days. Dogs were dosed with epyrifenacil in gelatin capsules for 90 consecutive days at dose levels of 0, 100, 300, and 1000 mg/kg/day. The initial day of administration was counted as “Day 0.” Animals were randomly distributed into groups consisting of 10 male and 10 female rats and mice, and 4 male and 4 female dogs. Additionally, for toxicokinetic (TK) analyses, 4 male and 4 female rats and 24 male and 24 female mice were allocated to TK satellite groups.

4. In vitro metabolism study of epyrifenacil

An in vitro metabolism study of epyrifenacil was conducted in male rat, male mouse, and human (both genders) liver microsomes. [Pyridyl-4(6)-14C]epyrifenacil (10 μM) was incubated in 100 mM sodium phosphate buffer (pH 7.4, containing 1% v/v methanol) with 3 mM NADPH, 3 mM MgCl₂, and 1 mg of microsomal protein/mL at 37°C (final volume: 200 μL). The reaction was terminated after 0, 1, 5, 10, and 30 min by adding 200 μL of acetonitrile. Then the samples were vortexed and centrifuged at 20,400 × g for 10 min. The supernatant was collected and subjected to HPLC analysis. Radio-HPLC analysis was performed using a Shimadzu LC-20A system (Shimadzu Biopredic International, Saint Grégoire, France).

![Fig. 1. Structures of epyrifenacil and its major metabolite, S-3100-CA.](image)
7.5 mL of 10 mM KOH containing 60 mM ascorbic acid. Next, in the dark. Then 2.5 mL of the stock solution was mixed with 10 mM KOH containing 20% ethanol and mixed for 20 min and easily oxidized to PPIX. It is not commercially available.

6. Preparation of liver mitochondrial fractions
For mice, rabbits, and dogs, mitochondrial fractions were prepared in-house using a mitochondrial isolation kit (Cosmo Bio Co., Ltd., Tokyo, Japan). The conditions were as follows: mobile phase, 0.1% formic acid in water (A) and acetonitrile (B); flow rate, 1.0 mL/min; gradient conditions, 20% B at 0 min, 80% B at 20 min, 100% B at 20–23 min, and 20% B at 23–30 min; injection volume, 60 µL; and column oven temperature, 40°C. In an UltimaFlo AP liquid scintillation cocktail (PerkinElmer, Inc., Waltham, MA, USA), the radioactivity of the peaks was measured with an online radio detector, β-RAM (LabLogic Systems, Ltd., Sheffield, UK).

5. Preparation of liver mitochondrial fractions
For mice, rabbits, and dogs, mitochondrial fractions were prepared in-house using a mitochondrial isolation kit (Cosmo Bio Co., Ltd., Tokyo, Japan) just after the livers were dissected from the animals. Crl:CD1 mice (five 12-week-old males and five 10-week-old females) were purchased from Charles River Laboratories Japan (Kanagawa, Japan). NZW rabbits (five 36-week-old males and six 27- to 32-week-old females) and TOYO beagles (two 7-month-old males and two 7-month-old females) were purchased from Oriental Yeast (Tokyo, Japan). Procedures involving animals and their care conformed to institutional guidelines, which are in compliance with Japanese laws and were approved by the IACUC at our institution.

6. Preparation of protoporphyrinogen IX
In the PPO-inhibition assay, the enzyme activity was evaluated by measuring the amount of PPIX converted from the substrate protoporphyrinogen IX. Since the substrate is unstable and easily oxidized to PPIX, it is not commercially available. Thus, protoporphyrinogen IX was prepared in-house according to the previously described method with slight modifications. Briefly, approximately 5 mg of PPIX was added to 15 mL of 10 mM KOH containing 20% ethanol and mixed for 20 min in the dark. Then 2.5 mL of the stock solution was mixed with 7.5 mL of 10 mM KOH containing 60 mM ascorbic acid. Next, approximately 5 g of sodium amalgam was gradually added to the reaction mixture under a nitrogen stream. After becoming colorless, the solution was purified using a 0.45 µm cellulose acetate filter to remove the unreacted PPIX. MOPS was added to adjust the pH to ca. 8, and then the solution was stored in liquid nitrogen. Separately, rat mitochondria were used to assay an aliquot of the prepared solution. At plateau fluorescence intensity, the PPIX concentration in the stock was determined to be 35.4 µM.

7. PPO-inhibition assay
The inhibitory activity of the test substances against PPO was measured according to the previously described method. Assay solutions that contained the test substances and mitochondria at a final concentration of 0.2 mg protein/mL were prepared with 100 mM Tris-HCl buffer (pH 7.5) containing 0.1% Triton-X, 1 mM EDTA, and 40 mM ascorbic acid and added to a 96-well plate at a volume of 50 µL per well. For the background measurement, which was used to correct for the rate of auto-oxidation of protoporphyrinogen IX, heat-denatured mitochondria (denatured at 80°C for 15 min) were prepared and assayed. After 10 min of preincubation at 37°C in a dry incubator, 50 µL of protoporphyrinogen IX solution was added to each well to achieve a final protoporphyrinogen IX concentration of 0.5 µM. The 96-well plate was loaded into a plate reader (FlexStation3, Molecular Devices, San Jose, CA, USA) prewarmed to 37°C, and the changes in fluorescence (excitation wavelength: 410 nm; emission wavelength: 630 nm) resulting from the conversion to PPIX were monitored for 60 min at 1 min intervals. Separately, 0.25, 0.5, 1, and 2 µM PPIX solutions were prepared to obtain a standard regression line.

8. Calculation of PPO activity
Fluorescence intensity was plotted versus time, and a 10 min linear range was selected for each sample. The amount of produced PPIX was calculated, and the PPO activity was calculated as ng PPIX/min. Vehicle control samples were regarded as full activity controls, and the PPIX production rate obtained from heat-killed mitochondria samples was subtracted as a background. The concentration-response curves were plotted as relative PPO activity (Y, %) versus the logarithm of the test-substance concentration (X, logM). The inhibition curves were fitted to the 4-parameter sigmoidal inhibition model using GraphPad Prism Ver. 5 (GraphPad Software, Inc., San Diego, CA, USA) with weights equal to 1/Y to obtain the IC50 values.

9. Thawing of cryopreserved primary hepatocytes
Cryopreserved primary hepatocytes were thawed according to the hepatocyte isolation kit instructions. Briefly, the hepatocytes were thawed in a water bath at 37°C and immediately transferred to a tube containing 25 mL of thawing medium warmed to 37°C. Cells were centrifuged at 100×g for 5 min at r.t. to remove the supernatant and then resuspended in 2 mL of KHB (containing 118 mM NaCl, 23.8 mM NaHCO3, 4.8 mM KCl, 1.0 mM KH2PO4, 1.2 mM MgSO4, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM CaCl2) adjusted to pH 7.4) at 4°C. To remove the thawing medium completely, cells were centrifuged at 100×g for 5 min at r.t., the supernatant was removed again, and the cells were resuspended in 2 mL of KHB at 4°C. Cell concentration was measured and adjusted to 2.0×106 cells/mL.

10. Hepatocyte uptake assay
A hepatocyte uptake assay was conducted using the previously described method with modifications. In short, the cells were incubated in a medium containing radiolabeled S-3100-CA and finally separated on the basis of difference in specific gravity using an oil layer of intermediate density. An aliquot (100 µL) of each suspension of cryopreserved primary hepatocytes (2.0×106 cells/mL) was pre-incubated for 5 min at 37°C or 4°C (on ice). The uptake assay was initiated by adding an equal volume of [phenyl-14C]S-3100-CA solution (final concentration: 5 µM) in
KHB. After incubation at 37°C or 4°C, 100 µL of each culture solution was transferred into a microcentrifuge tube containing 100 µL of 2 N NaOH under 100 µL of the oil layer (silicone oil/mineral oil = 81.7/18.3, v/v; density: 1.016 g/mL). Next, the uptake was terminated at 0.25, 0.5, 1.0, and 2.0 min after the start of incubation by centrifuging the tube at 20,400 × g for 1.0 min at 4°C to transfer the cells from the upper culture solution to the bottom NaOH layer. After the centrifugation, each tube was stored overnight at 50°C to allow dissolution of the cells in the NaOH layer. Each tube was frozen at −80°C and cut at the oil layer using a tube cutter. Aliquots of the NaOH layer were transferred into a scintillation vial, and 10 mL of scintillation cocktail, Hionic-Flour™ (PerkinElmer, Inc., Waltham, MA, USA), was added. The radioactivity in the cell lysate was measured by liquid scintillation counting (LSC, Tri-Carb® 3110TR, PerkinElmer, Inc.).

11. Calculation of hepatic uptake volume
The total radioactivity in the NaOH layer [dpm/10⁵ cells] was divided by the radioactivity concentration in the culture solution [dpm/mL] to obtain the volume of substrate uptake in the hepatocytes [µL/10⁶ cells]. The active uptake volume was defined as the difference in uptake volume between 37°C and 4°C, and then the time-dependent increase in the active uptake volume was transformed into the rate of S-3100-CA active uptake by hepatocytes (active uptake rate).

Results

1. Subchronic study
Detailed results of the 90-day subchronic studies conducted in rats, mice, and dogs are presented in the Supplemental Information, and the summary results are presented in Table 1. Briefly, 90-day subchronic toxicity studies in mice, rats, and dogs showed similar profiles of toxicity, which were limited to anemia and hepatotoxicity. Similar anemic changes were noted in all species tested. Regarding hepatotoxicity, hepatocyte injury, which was mainly characterized by hepatocellular degeneration/necrosis and/or single-cell necrosis/apoptosis, was observed in both rats and mice, but dogs did not show the alterations indicative of evident hepatocyte injury. Among the three species tested, the mice showed the lowest NOAEL based on anemia and/or hepatotoxicity, followed by the rats and then the dogs. In all species, males tended to be more susceptible than females.

| Species     | NOAEL (mg/kg bw/day) | Cmax (µg/mL) of S-3100-CA at the relevant systemic dose after 13w dose period | AUC₀–₂₄ (µg·hr/mL) of S-3100-CA at the relevant systemic dose after 13w dose period |
|-------------|----------------------|---------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Mouse       | Male: 0.7            | Male: 0.0128                                                                      | Male: NC                                                                        |
|             | Female: 4.1          | Female: 0.135                                                                     | Female: 2.24                                                                     |
| Rat         | Male: 3 (4.3)        | Male: 0.0207 (1.6)                                                                | Male: 0.467                                                                      |
|             | Female: 19 (4.6)     | Female: 0.266 (2.0)                                                               | Female: 5.02 (2.2)                                                              |
| Dog         | Male: 100 (143)      | Male: 0.947 (74)                                                                  | Male: 10.6                                                                       |
|             | Female: 300 (73)     | Female: 5.65 (42)                                                                 | Female: 51.2 (23)                                                                |

Values in parenthesis represent the fold change vs. corresponding mouse value. NC: not calculated

Table 1. Summary of subchronic toxicity study results.

| Species     | Dosage | AUC₀–₂₄ [µg·hr/mL] | Cmax [µg/mL] |
|-------------|--------|--------------------|--------------|
|             | Male   | Female             | Male         | Female       |
| Rat         | 50 ppm | 0.467              | 0.879        | 0.0207       | 0.0465       |
|             | 300 ppm| 4.01               | 5.02         | 0.248        | 0.266        |
|             | 800 ppm| N.A.               | 13.2         | N.A.         | 0.756        |
| Mouse       | 5 ppm  | N.C.               | 0.403        | 0.0128       | 0.0227       |
|             | 25 ppm | 4.58               | 2.24         | 0.0649       | 0.135        |
|             | 100 ppm| 10.1               | 14.8         | 0.55         | 0.86         |
| Dog         | 100 mg/kg bw/day | 10.6               | 17.5         | 0.947        | 1.92         |
|             | 300 mg/kg bw/day | 25.9               | 51.2         | 2.86         | 5.65         |
|             | 1000 mg/kg bw/day| 85.8               | 70.6         | 6.76         | 7.05         |

Values represent the mean of calculated parameters. N.A.: Not applicable. N.C.: Not calculated because 2 over 3 of the time points were calculated as below the limit of quantification thus it was considered unlikely to obtain sufficient value. For rats and mice, Tₘₚₙ are presented in terms of 6, 8, or 14 hr for sampling intervals 6:00 to 7:00 a.m., 8:00 to 9:00 a.m., or 2:00 to 3:00 p.m., respectively.
2. Toxicokinetics
The toxicokinetics data obtained in the subchronic study is presented in Table S4 and summarized in Table 2. The exposure to the metabolite (S-3100-CA) increased as the dose increased, in terms of AUC₀–₂₄ and Cₘₐₓ, in a nearly dose-proportional manner in all species tested, whereas no parent epyrifenacil was detected. There were no notable differences in exposure to S-3100-CA between males and females. Furthermore, there was no accumulation of S-3100-CA following 90 days of oral administration of epyrifenacil to all species tested. Only a limited amount of the parent epyrifenacil was detected in dog plasma (not more than 0.04 µg/mL), whereas no measurable amount was detected in rats or mice (data not shown), suggesting that a majority of epyrifenacil was converted to S-3100-CA in all species tested.

3. In vitro metabolism study
In order to determine the elimination rate of epyrifenacil and to identify the major metabolites in rodents and humans, we conducted an *in vitro* metabolism study. The result is shown in Fig. 2. When incubated with microsomal fractions, epyrifenacil was rapidly metabolized and almost disappeared within 5 min in all species tested. As a major metabolite, S-3100-CA was detected with no less than 80% of the applied radioactivity. No quantitative or qualitative difference in the production of the metabolite was observed among rats, mice, or humans in this experiment. To evaluate the contribution of the non-enzymatic reaction, epyrifenacil was assayed without microsomal fractions, which did not present any degradants after 30 min of incubation.

4. PPO-inhibition assay
To investigate the inhibitory activity of epyrifenacil and its major metabolite S-3100-CA against PPO, an *in vitro* enzyme-inhibition assay of test substances in mitochondrial fractions from mouse, rat, rabbit, dog, and human livers was conducted. The results are shown in Fig. 3, and the calculated IC₅₀ values are presented in Table 3. When the inhibitory activities of epyrifenacil and S-3100-CA were compared, the IC₅₀ values were approximately the same for each species, but especially in mice, S-3100-CA was slightly stronger than the parent compound. This result suggests that hydrolysis of the ethyl ester did not reduce the PPO-inhibitory potential. When compared among the species, the IC₅₀ values were comparable in mice and rats. However, the PPO-inhibitory activity in dogs, rabbits, and humans was 3- to 5-fold smaller for epyrifenacil and 7- to 14-fold smaller for S-3100-CA against mitochondrial PPO fractions in each species.

| Animal | Epyrifenacil (IC₅₀ values, [nM]) | S-3100-CA (IC₅₀ values, [nM]) |
|--------|---------------------------------|-------------------------------|
| Mouse  | 2.2 ± 0.03                      | 0.9 ± 0.30                    |
| Rat    | 2.6 ± 0.05                      | 1.5 ± 0.36                    |
| Rabbit | 12.1 ± 1.69                     | 12.1 ± 2.12                   |
| Dog    | 7.6 ± 0.18                      | 11.1 ± 3.12                   |
| Human  | 10.2 ± 0.66                     | 12.5 ± 2.08                   |

Data represent the mean ± standard deviation of 3 runs.
S-3100-CA than that in mice and rats, suggesting the existence of a significant difference in the dynamics of PPO inhibition between rodents and other species, including humans.

5. Hepatocyte uptake assay
The active hepatic uptake of S-3100-CA was evaluated using hepatocytes of rodents and humans. The results are summarized in Fig. 4. In all species tested, the uptake volume of S-3100-CA was larger at 37°C, which includes the volume of active uptake via hepatic transporters, than at 4°C, which includes the volume of uptake by passive diffusion and cell surface adsorption. Therefore, it was suggested that S-3100-CA is taken up into hepatocytes by the hepatic transporters of all tested species. The active uptake volume (active uptake clearance, hereafter abbreviated as CL) was increased in a time-dependent manner. Comparing CL values among the species, the largest was in male mice, followed in order by those in female mice, male rats, female rats, and humans (both genders). From the CL values obtained, the rate of active S-3100-CA uptake in each species was determined during a time interval when the CL increased linearly with good repeatability (i.e., male rat, female rat, and human: 0.5 to 2.0 min; male mouse: 0.25 to 2.0 min; female mouse: 0.25 to 1.0 min). The calculated uptake rates are presented in Fig. 5. The uptake rate in the hepatocytes of humans (3.83 µL/10⁶ cells/min) was significantly lower than that in the hepatocytes of male mice (49.51 µL/10⁶ cells/min), female mice (21.58 µL/10⁶ cells/min), and male rats (8.10 µL/10⁶ cells/min) and tended to be lower than that in female rats (6.66 µL/10⁶ cells/min), although the difference was not statistically significant. When the uptake was compared between males and females of the same species, uptake was significantly higher in male mice, while in male rats, it was higher but not statistically significant.

Discussion
As mentioned above, PPO inhibitors can cause hepatotoxicity stemming from the accumulation of PPIX in hepatocytes and anemic changes due to the inhibition of heme biosynthesis. Hepatotoxicity caused by epyrifenacil in rodents was characterized by hepatocellular degeneration/necrosis and/or single-cell necrosis/apoptosis, which were associated with bile duct hyperplasia in rats or brown pigment deposition in Kupffer cells in mice, as summarized in Table S2. It has been reported that the administration of porphyrinogenic compounds to rodents induces the accumulation of a porphyrin-containing pigment in combination with hepatocyte injury, which is observed as dark brown to red-brown pigment deposition in hepatocytes, Kupffer cells, and portal macrophages, and also in bile ducts, leading to bile duct proliferation. These pathologic natures were clearly in line with the histopathological findings observed in the subchronic studies with epyrifenacil and other PPO inhibitors. In contrast to the above results in rodents, the hepatotoxicity observed in dogs was limited to increased glycogen and an associated increase in ALP, which was not suggestive of porphyrin accumulation. Furthermore, subchronic studies identified the hematopoietic system as the target of epyrifenacil in all species tested, as characterized by decreases in RBC, HGB, HCT, MCH, MCV, and/or MCHC, and associated findings indicative of increased erythropoiesis. These alterations were clearly in line with the reports about other PPO inhibitors and thus were attributed to PPO inhibition and the subsequent disruption of heme biosynthesis. From these results, the adverse outcomes caused by epyrifenacil in rats and mice were determined to be limited to hepatotoxicity and anemia, both derived from PPO inhibition. As presented in Table 1, males tended to be more susceptible to these toxicological effects than females across species. The reason has not been identified, but an in vitro hepatocyte assay revealed the higher uptake of S-3100-CA in the male liver especially in mice, suggesting that sex differences in the exposure of S-3100-CA to the main target organ (i.e., the liver) might cause the higher toxicity in males.

As presented in Fig. 1, epyrifenacil has an ester bond in its structure, which is expected to cleave easily and thereby produce S-3100-CA. In order to identify the major metabolites in the body and to estimate the species differences in exposure to

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**Fig. 4.** Active uptake of S-3100-CA into male mouse (▲), female mouse (○), male rat (○), female rat (□), and human (×) hepatocytes. The active uptake volume was defined as the difference in uptake volume between 37°C and 4°C. Data represent the mean ± standard deviation of 3 runs.

**Fig. 5.** Active uptake rate of S-3100-CA into hepatocytes. The active uptake volume was defined as the difference in uptake volume between 37°C and 4°C. Data represent the mean ± standard deviation of 3 runs. Statistical analysis was performed via the Student t-test: *p* < 0.01 vs. humans and *p* < 0.01 vs. corresponding male group in the same species.
metabolites, we conducted an in vitro metabolism study with liver microsomal fractions. The results showed that the ester cleavage in epyrifenacil was very rapid in all species tested, suggesting that epyrifenacil is mostly metabolized by the first-pass effect when dosed orally. In addition, the ester-cleaved metabolite, S-3100-CA, was predominant and commonly detected. A metabolism study using 14C-labeled epyrifenacil was conducted separately28,29; S-3100-CA was detected as a major metabolite in the plasma of rats and mice, whereas no conjugate of S-3100-CA was detected in urine or feces of rats. In addition, approximately half the amount of dosed 14C was excreted as S-3100-CA into urine and feces, suggesting that the renal and biliary excretion takes a significant part of the rate-limiting process for the elimination of S-3100-CA; conversely, the predominant circulat-
ing metabolite in the body is S-3100-CA.25 Combining these observations with the in vitro data above, it was expected that S-3100-CA would be the major component in mice and rats, and probably also in humans. In order to conclude that it was the main cause of epyrifenacil-induced hepatotoxicity in rodents, we also conducted an in vitro PPO-inhibition assay. The results showed that S-3100-CA inhibited PPO in rodents, and the extent of inhibition was similar to that by the parent epyrifenacil. From these results, we concluded that the major causal substance of epyrifenacil-induced hepatotoxicity in rodents is S-3100-CA.

Since the molecular mechanism of heme biosynthesis is common to all mammals, the possibility of PPO-inhibitor-induced hepatotoxicity and/or anemia in humans should be taken into consideration for the human-health-risk assessment of epyrifenacil. For precise risk assessment, we planned to look for evidence of species differences in the dynamics (i.e., PPO-inhibitory activity) and kinetics (i.e., uptake by the liver) of PPO inhibition separately. Analysis of the PPO-inhibition assay results showed a significant species difference. When focused on S-3100-CA, the IC50 in humans was 8.3 times greater than that in rats and 13.9 times greater than that in mice, demonstrating that the sensitivity to PPO inhibition by S-3100-CA is lower in humans than in rodents. The IC50 values in dogs and rabbits were almost equal to those in humans. When comparing the NOAELs in the tested species based on hepatotoxicity and anemia, the mouse was the most sensitive species, followed by the rat and then the dog, which is consistent with the difference in sensitivity to PPO inhibition. These PPO-inhibition results suggested that dogs were the most relevant species to humans, but the reference values of epyrifenacil for risk assessment (e.g., acceptable daily intake or acceptable operator-exposure level) are most likely to be derived from the mouse studies, ensuring that these reference values are protective of human health. The details underlying species differences have not been revealed. Regarding the molecular dynamics toward PPO, a docking simulation study of the PPO inhibitor flumioxazin in several species clearly demonstrated that the binding affinity of the substrate to PPO varies among species.30 There are no direct data or literatures available, but it is estimated that a similar variation of the binding affinity among species is also occurred for S-3100-CA.

Regarding the kinetics, the in vitro metabolism data showed no quantitative or qualitative difference in the production or metabolic degradation of S-3100-CA among the species. As shown in Fig. 1, S-3100-CA contains a carboxylic acid moiety, which is highly likely to be ionized and to exist as a COO− anion under physiological conditions. It is known that such anions are sometimes taken up into organs via organic anion transporters (OATs) or organic anion transporting peptides (OATPs) and cause significant exposure to the chemicals.31,32 Furthermore, it has been reported that there are species differences in the active transport of chemicals and thereby species differences in exposure and toxicity.33–35 When considering the target organs of epyrifenacil, the active transport is mainly contributed to the liver rather than the hemopoietic system, and thus we focused on evaluating species differences in the hepatic uptake of S-3100-CA. The results of in vitro assays using hepatocytes from rodents and humans demonstrated that the uptake of S-3100-CA is 6 to 13 times greater in mice and 2 times greater in rats than in humans. This result clearly indicates that exposure to S-3100-CA in the liver is lower in humans than in rodents, especially mice. Therefore, it was concluded that the risk of hepatotoxicity by S-3100-CA in humans is lower than that in rodents based on species differences in both dynamics and kinetics. Another proposal that involves the contribution of OATPs is the notable sex-related difference in the uptake of S-3100-CA. It has already been reported that the expression of some OATPs is significantly higher in the liver of male mice and male rats,36–39 whereas no sex-related difference in OATPs has been noted in humans.40,41 These previous studies are likely to explain the sex-related difference in S-3100-CA uptake observed in rodents. Our research provides only phenomenological results, and thus further mechanistic research to explain the significant difference between species or sexes is highly anticipated.

From these results, it was suggested that humans are significantly less sensitive to the hepatotoxicity of epyrifenacil than are rodents, and this difference stems from species differences in the dynamics and/or kinetics of the causal metabolite, S-3100-CA. The findings of this research were considered to enable an interpretation of the species differences between rodents and humans and provide a robust basis for the human-health-risk assessment of epyrifenacil, though further mechanistic research of species differences is highly expected.

Electronic supplementary materials
The online version of this article contains supplementary materials (Supplemental Information), which are available at https://www.jstage.jst.go.jp/browse/pestics/.

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