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Flumioxazin metabolism in pregnant animals and cell-based protoporphyrinogen IX oxidase (PPO) inhibition assay of fetal metabolites in various animal species to elucidate the mechanism of the rat-specific developmental toxicity

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ABSTRACT

Flumioxazin, an *N*-phenylimide herbicide, inhibits protoporphyrinogen oxidase (PPO), a key enzyme in heme biosynthesis in mammals, and causes rat-specific developmental toxicity. The mechanism has mainly been clarified, but no research has yet focused on the contribution of its metabolites. We therefore conducted *in vivo* metabolism studies in pregnant rats and rabbits, and found 6 major known metabolites in excreta. There was no major rat-specific metabolite. The most abundant component in rat fetuses was APF, followed by flumioxazin and 5 identified metabolites. The concentrations of flumioxazin and these metabolites in fetuses were lower in rabbits than in rats. *In vitro* PPO inhibition assays with rat and human liver mitochondria showed that flumioxazin is a more potent PPO inhibitor than the metabolites. There were no species differences in relative intensity of PPO inhibition among flumioxazin and these metabolites. Based on the results of these *in vivo* and *in vitro* experiments, we concluded that flumioxazin is the causal substance of the rat-specific developmental toxicity. As a more reliable test system for research on *in vitro* PPO inhibition, cell-based assays with rat, rabbit, monkey, and human hepatocytes were performed. The results were consistent with those of the mitochondrial assays, and rats were more sensitive to PPO inhibition by flumioxazin than humans, while rabbits and monkeys were almost insensitive. From these results, the species difference in the developmental toxicity was concluded to be due to the difference in sensitivity of PPO to flumioxazin, and rats were confirmed to be the most sensitive of these species.

1. Introduction

Flumioxazin ([7-fluoro-6-(3,4,5,6-tetrahydrophthalimido)-4-(2-propynyl)-2*H*-1,4-benzoxazin-3(4*H*)-one], Sumisoya®) is an *N*-phenylimide herbicide that is widely used for controlling annual broadleaf weeds in soybeans (Yoshida et al. 1991). The herbicidal activity of flumioxazin was elucidated to be derived from its inhibitory activity on protoporphyrinogen oxidase (PPO), one of the key enzymes in porphyrin biosynthesis. The PPO inhibition in plants causes accumulation of its substrate protoporphyrinogen IX and yields its further auto-oxidized compound of protoporphyrin IX (PPIX), which liberates peroxide on irradiation with light (Matringe and Scalla, 1988) to produce herbicidal activity.

It is well identified that PPO exists also in mammals, and works as a key enzyme in heme biosynthesis (Dailey, 1990). Flumioxazin has developmental toxicity in rats, causing embryolethality, teratogenicity (mainly ventricular septal defects, VSD, and wavy ribs), and growth retardation (Kawamura et al. 1995, 1996b). The mechanism of this developmental toxicity in rats has already been elucidated. Flumioxazin inhibits PPO in rat embryos, thereby suppressing normal heme biosynthesis and producing erythroblastic degeneration, resulting in embryonic anemia. As a compensatory reaction to anemia, stroke volume is increased, which leads to the enlargement of heart, and then VSD is produced by mechanical distortion of the heart or abnormal cardiac hemodynamics (Kawamura et al. 2014). Even though all mammals have PPO for heme biosynthesis, which is essential for vital activity, a

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remarkable species difference in the toxicity caused *via* the PPO inhibition by flumioxazin has been reported. It was demonstrated that the oral administration of flumioxazin at 3000 mg/kg, which was two orders of magnitude larger than the teratogenic dose of 30 mg/kg in rats, showed no developmental toxicity in rabbits (Kawamura et al. 1995). Another study demonstrated that remarkable PPIX accumulation, which is an indicator of PPO inhibition, was observed in rat embryos, but not in rabbit embryos following the oral administration of 1000 mg/kg of flumioxazin (Kawamura et al. 1996a). These phenomena observed in *in vivo* studies are consistent with *in vitro* experiments in which rat, rabbit, and human liver mitochondrial fractions were used to compare the sensitivity of PPO to flumioxazin among species (Kawamura et al. 2016). The results showed that rabbit mitochondria are less sensitive to flumioxazin than those of rat, which corresponded to the species difference in the developmental toxicity observed in *in vivo*. Considering all of these observations, it was concluded that the developmental toxicity is caused by the PPO inhibitory activity of flumioxazin, and the difference in the sensitivity to flumioxazin on PPO is related to the species difference in developmental toxicity observed in *in vivo*. In the meantime, although these mechanism studies have been conducted with the parent flumioxazin, the contribution of metabolites of flumioxazin has not been studied. In addition, the species difference in PPO inhibition was confirmed only in mitochondrial assays, but further cell-based assays have been expected, because a cell-based assay can reflect the accessibility of flumioxazin to PPO in the cells, while the accessibility may not be fully evaluated in a mitochondrial assay. Previous reports on metabolic profiles of flumioxazin in rats revealed that flumioxazin is easily metabolized and dominantly forms the metabolites of 3-OH flumioxazin, 4-OH flumioxazin, APF, AcAPFA, 3-OH flumioxazin-SA, and 4-OH flumioxazin-SA (Tomigahara et al. 1999a, 1999b, structures are shown in Fig. 1). Some of these metabolites are estimated to have a potential of PPO inhibitory activity, since they are structurally and physicochemically related to the parent, flumioxazin. However, metabolic profiles of flumioxazin in pregnant rats and rabbits, distribution of possible causal substances in fetuses, and the inhibitory activity of the metabolites against PPO have not been determined.

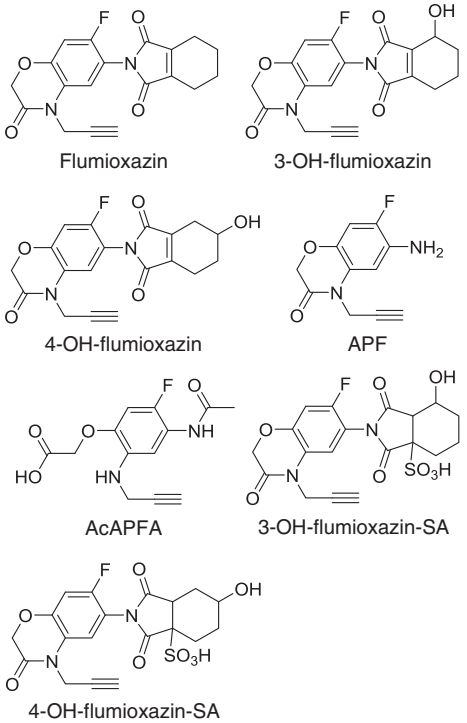

Fig. 1. Chemical structures of flumioxazin and its metabolites.

The objectives of the present study were to identify the active form of the developmental toxicity in rat fetuses and to compare its intensity among species. First, we conducted single- and repeated-dose *in vivo* metabolism studies in pregnant rats and rabbits to elucidate the abundant components and their distribution in the animals, and then *in vitro* enzyme inhibition assays with rat and human mitochondria were conducted to compare the PPO inhibitory activity of the possible causal substances. Because PPO exists in complex and highly organized structures in mitochondria, another PPO inhibition assay was conducted under cell-based conditions with hepatocytes of 4 mammalian species to further elaborate the species differences in the developmental toxicity.

2. Materials and methods

2.1. Materials and reagents

[phenyl-U-^{14}C]Flumioxazin (4.80 GBq/mmol), non-labeled flumioxazin, and the authentic standards of 3-OH flumioxazin, 4-OH flumioxazin, and APF were supplied by Sumitomo Chemical Co., Ltd. AcAPFA, 3-OH flumioxazin-SA, and 4-OH flumioxazin-SA were prepared in accordance with previous methods (Tomigahara et al. 1999a, 1999b). The R_f values in thin layer chromatography (TLC) analysis are shown in Supplemental Table 1. Rat liver mitochondrial fraction was prepared as described later. Human liver mitochondrial fraction was purchased from XenoTech, LLC (KS, USA). Cryopreserved female hepatocytes of SD rats and Cynomolgus monkeys from Biopredic International (Saint-Gregoire, France), NZW rabbits from Cellz Direct (NC, USA), and Caucasian humans (3 individuals) from Celsis IVT (MD, USA) were used. Other chemicals were of reagent grade.

2.2. In vivo rat and rabbit metabolism study

2.2.1. Animal treatment

Procedures involving animals and their care conformed to the institutional guidelines, which are in compliance with the Japanese laws and were approved by the IACUC in the laboratories. Animals had free access to pelleted diet and water throughout the study.

2.2.2. Single oral dose study

Pregnant SD rats (10–12 weeks old) from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and JW/NBIS rabbits (6 months old) from Nisseiken Co., Ltd. (Tokyo, Japan) were given a single oral dose of [phenyl-^{14}C]flumioxazin at 30 mg/kg/5 mL (2.94 MBq/mg for rats and 0.294 MBq/mg for rabbits) on gestational day (GD) 12 at 4 or 2 animals per group. The animals were euthanized at 1, 2, 4, or 24 h after administration and the blood was collected from abdominal artery with a heparinized syringe. The blood was then separated by centrifugation to obtain plasma. Liver, kidney, ovary, uterus, placenta, amniotic fluid, and fetus were dissected from the residual carcass for the radioanalysis. Each tissue (except plasma and amniotic fluid) was weighed and radioactivity in whole or a part was analyzed. The animals of the 24-h group were housed in metabolic cages to collect urine and feces prior to their euthanasia.

2.2.3. Repeated oral dose study

Pregnant (GD 6) Wistar Hannover rats (10–11 weeks old) from Clea Japan, Inc. (Tokyo, Japan) and NZW rabbits (6–7 months old) from Kitayama Labes Co., Ltd. (Nagano, Japan) were given repeated oral doses of [phenyl-^{14}C]flumioxazin at 30 mg/11.3 MBq/5 mL/kg/day for rats and 30 mg/1.12 MBq/5 mL/kg/day for rabbits, for 7 consecutive days. Approximately 400 μL of blood was collected from 3 rats and 3 rabbits at the defined sampling intervals with a heparinized capillary, and was centrifuged to obtain plasma. Separately, 3 rats and 3 rabbits per time point were euthanized and the blood, plasma, tissues and excreta were collected in a similar manner to those in the single dose