FULL PAPER *Toxicology*

Antibiotic Furazolidone Induces CYP1A But Not CYP2E1 Subfamily in Rat Liver

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ABSTRACT. Furazolidone (FZ), one of the nitrofuran fungicides, is used as a veterinary medicine in the Middle and Far Eastern countries. In this study, FZ (125 mg/kg) was administered orally to Wistar rats for 3 days. Results of the Ames test using the S-9 fraction of rats treated with FZ showed a significant increase in the number of revertant colonies. Western blot analysis of hepatic CYP isozymes induced by FZ, revealed a remarkable induction of CYP1A1 apoprotein, but CYP1A2 and CYP2E1 apoproteins were not altered. In addition, the expression of CYP1A1 mRNA level in rats treated with FZ by RT-PCR was significantly enhanced by FZ treatment. We concluded that FZ is apparently mutagenic and induces transcription of the CYP1A1 isozyme, which metabolically activates numerous promutagens, in hepatocytes.

KEY WORDS: CYP1A1, cytochrome P450, furazolidone, mutagenicity, rat.

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Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2 oxazolidinone, FZ) is one of nitrofuran fungicides. There are many studies and reviews of the toxicological effects of FZ [2, 4]. Use of FZ has been banned on food-producing animals in the United States, European Union countries, and Japan owing to its mutagenic and carcinogenic activity. However, it remains in common use in veterinary medicine, especially on poultry, in the Middle and Far Eastern countries [4, 11]. In these countries, FZ is also used on meat-producing and aquatic animals and to treat infectious diseases in humans, especially *Helicobacter pylori* infection [16, 18].

It is known that the injection of furazolidone cause alterations of the phase I drug-metabolizing enzymes in experimental animals. Cytochrome P450 (CYP) participates in the phase I metabolism of numerous drugs, and is reported to be especially affected by furazolidone treatment in rats and chickens [4, 6, 8, 19, 20]. In previous studies, multiple oral doses of FZ (25–200 mg/kg for 4 days) resulted in an increase in hepatic microsomal CYP concentrations in rats [20]. FZ in feed (0.06% for one week) induced hepatic microsomal aniline hydroxylation activity in rats, and 1% FZ in the feed increased the CYP concentration significantly [8]. The authors did not determine the isoform of CYP induced, though the elevation of aniline hydroxylation suggests CYP2E1 induction. In addition, recently, administration of FZ (80 mg/kg by intraperitoneal injection for 3 days) showed induction or inducibility of the hepatic CYP1A subfamily in the Ames test using ethidium bromide (EtBr) as the mutagen [19]. Considering the above reports, it is speculated that repeated administration of FZ may result in the weak induction of hepatic CYP1A and/or CYP2E1 subfamily isoenzymes in rats. However, which CYP isoform is induced by FZ treatment has not been reported. It is necessary to estimate the ability of xenobiotic chemicals to affect drug-metabolizing enzyme activities, particularly in the case of substances that are activated metabolically by the enzymes [9]. Especially, CYP1A1 enzyme has been shown to be responsible for activation of many cyclic aromatic hydrocarbons and related chemical to species binding to DNA and has, therefore, been considered to be involved in initiating steps of carcinogenesis [21]. If a CYP1A or CYP2E1 isoform is induced in FZ-treated food-producing animals, it may enhance the mutagenicity of promutagens. In the current study, we aimed to identify the CYP isoform that is altered by FZ treatment.

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MATERIALS AND METHODS

Materials: FZ was purchased from Ueno Fine Chemicals Industry Co., Ltd. (Osaka, Japan). Acetone was from Kanto Chemical Co. (Tokyo, Japan). Sudan III and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Polyclonal anti-rat CYP1A1 and CYP2E1 goat antibodies were from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). Rabbit horseradish peroxidase-labeled anti-goat IgG was from Sigma Chemical Co. All other chemicals and solvents used were of the highest quality commercially available.

Animals and treatments: Five-week-old female Wistar rats were obtained from Japan SLC Co. (Shizuoka, Japan). They were housed at 24 ± 1°C with a 12 hr light and 12 hr dark cycle, and given laboratory feed (MR Stock, Nosan Co., Yokohama, Japan). They were used for experiments after 1 week of acclimatization. Treatment of all animals

was performed according to the policies of the Institutional Animal Care and Use Committee of Hokkaido University.

Furazolidone was suspended in corn oil and given orally to four female rats at a dose of 125 mg/kg for 3 days. Acetone, a typical CYP2E1 inducer [5, 12], and Sudan III, a potent CYP1A inducer [7], were used as positive controls. Acetone diluted with water was given to four female rats in an oral dose of 5 m*l*/kg for 3 days. Sudan III dissolved in corn oil was given orally at a dose of 40 mg/kg to four female rats for 3 days. Corn oil (5 m*l*/kg) was given orally to three female rats of the control group for 3 days.

Twenty-four hours after the last dose, rats were killed with carbon dioxide, and their livers were removed immediately after sacrifice. The S-9 and microsomal fractions of livers were prepared according to the method of Omura and Sato [17] with modifications. Livers were homogenized with 3 volumes of ice-cold 1.15% KCl. For preparation of the S-9 fraction, liver homogenates were centrifuged at 9,000 g for 20 min. To prepare the microsomal fraction, the S-9 fraction was ultracentrifuged two more times at 105,000 g at 4°C for 70 min. The pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4), frozen in liquid nitrogen, and stored at –80°C until use. The S-9 and microsome protein concentrations were determined by the method of Lowry *et al.* [15].

Ames test: The ethidium bromide mutagenesis assay was performed according to the method of Ames *et al.* [1], with some modifications. An overnight culture of the TA 98 strain of *Salmonella typhimurium* (0.1 m*l*) was pre-incubated for 20 min at 37°C with the S-9 fraction from each treatment group; the S-9 incubation mixture contained a complete NADPH-generating system and ethidium bromide (1.5 μg/plate). After 20 min preincubation, 2 m*l* of soft agar containing 0.5 mM L-histidine and 0.5 mM biotin was added. The mixtures were poured onto a glucose agar plate. The numbers of revertant colonies were counted after 48 hr of incubation at 37°C. Each sample of the three treated and one control groups was assayed in duplicate.

Western blot analysis: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the methods of Laemmli [13] and Towbin *et al.* [22]. Microsomal protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Toyo Roshi Kaisha Ltd., Tokyo, Japan). Anti-rat CYP1A1 and CYP2E1 antibodies were used to detect each CYP isoform. Anti-CYP1A1 antibodies cross-react with CYP1A2. We distinguished CYP1A1 and CYP1A2 from their mobilities after SDS-PAGE. The membrane was immunostained using diaminobenzidine. The results of staining were analyzed by using NIH Image v. 1.63 [14].

RNA extraction: Total RNA was isolated from 50 mg liver using TRIreagent (Sigma). Briefly, liver tissue samples were homogenized in 1 m*l* TRIreagent, then 0.2 m*l* chloroform was added to the sample. The mixture was then shaken, followed by centrifugation at 15,000 g for 20 min. The supernatant layers were transferred to a clean set of tubes, 0.5 m*l* of isopropanol was added to the samples, and they were centrifuged at 15,000 g for 15 min. The RNA pellets were washed with 70% ethanol. RNA was dissolved in RNase-free water. The optical density of all RNA samples was 1.7–1.9 based on the 260/280 ratio. Oligo (dT) primed total RNA was reverse-transcribed to cDNA using reverse transcriptase (Toyobo, Osaka, Japan).

Real-time quantitative PCR: Expression levels of CYP1A1 and GAPDH mRNA were assayed using an ABI PRISM7700 (Applied Biosystems, Foster City, CA, U.S.A.) and the specific primers and TaqMan probe sets (Gene Expression Assay, Applied Biosystems). A 20 μ*l* aliquot of the Universal PCR Master Mix (Toyobo) containing an appropriate amount of the template cDNA, primers, and Taqman probe was prepared, and real-time PCR was performed by 50 cycles of incubation at 95°C for 15 sec, followed by incubation at 60°C for 1 min. Measurement of each CYP enzyme gene was performed in duplicate and repeated three times. The expression of each gene was normalized to the expression of GAPDH and was calculated relative to that of the control.

Statistical analyses: All results are expressed as means ± standard deviation. F-test or Bartlett's test was used to test for homogeneity of variances. Statistical significances were determined by using an unpaired *t*-test or Dunnett's test. P values less than 0.05 were considered statistically significant.

RESULTS

Ames test: The results of the Ames test with the S-9 fraction of rats treated with FZ at a dose of 125 mg/kg for 3 days are shown in Fig. 1. The average number of revertant colonies of the FZ-treated group was approximately double that of the control group. The S-9 fraction of rats treated with acetone also showed enhanced mutagenicity compared with that of control animals. The number of revertant colonies of the acetone-treated S-9 fraction was same as that of the FZ-treated group. The number of *S. typhimurium* colonies of the group given Sudan III, a CYP1A1 inducer, was about 8 times higher than those of the control.

Western blotting: Inductions of CYP1A subfamily members and CYP2E1 were assumed in the previous reports. Therefore, we identified immunologically the hepatic CYP isoenzymes induced by FZ given to rats orally at a dose of 125 mg/kg for 3 days (Fig. 2). We did not detect CYP1A1 expression in liver microsomes of control animals, but FZ treatment produced marked induction of CYP1A1 apoprotein. The expression levels of CYP1A2 and CYP2E1 apoproteins were not altered.

RT-PCR: The CYP1A1 mRNA level in rats treated with FZ was quantitatively measured by RT-PCR in order to determine whether the CYP induction was transcriptional. The CYP1A1 mRNA level was about double that of the control group, which is comparable that of acetone treated group, whereas the CYP1A1 mRNA level in the Sudan III-treated group was over 1000-fold that of the control group.

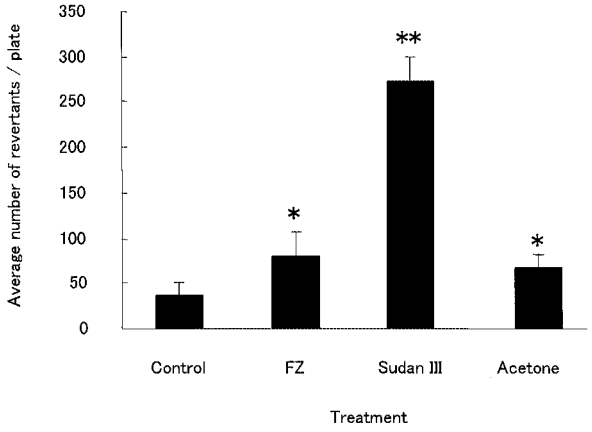

Fig. 1. Revertant colonies in Ames test using FZ-, acetone-, and Sudan III-treated rat livers. The S-9 incubation mixture comprised a complete NADPH-generating system, TA98 strain of *Salmonella typhimurium* and ethidium bromide (1.5 μg/plate). The revertant colonies were counted after 48 hr incubation at 37°C. Each value is expressed as mean ± SD from 3 or 4 rats. Asterisks mean statistical differences compared to data of control animals by Dunnett's test; * $p<0.05$, ** $p<0.01$.

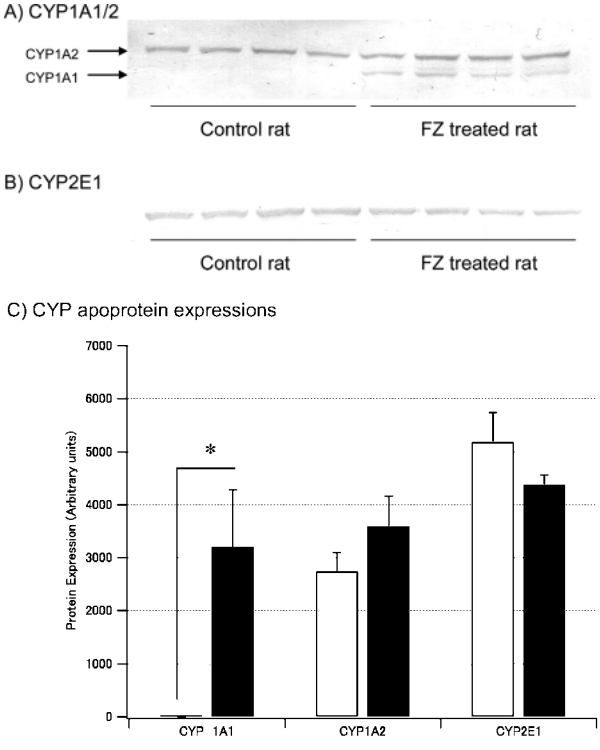

Fig. 2. Expression of CYP1A subfamily and CYP2E1 in liver microsomes of FZ-injected rat liver. Western blotting analyses of liver microsomes from adult female rats were performed using A) anti-CYP1A1 and B) anti-CYP2E1 antibodies. The arrows indicate the position of CYP1A isoforms. C) Results of staining were analyzed by using NIH Image v. 1.63. Open bars indicate the expression of P450 in control rats (n=3), and closed bars show FZ-treated animals (n=4). Each value is expressed as mean ± SD. Significantly different from control; *:$p< 0.01$.

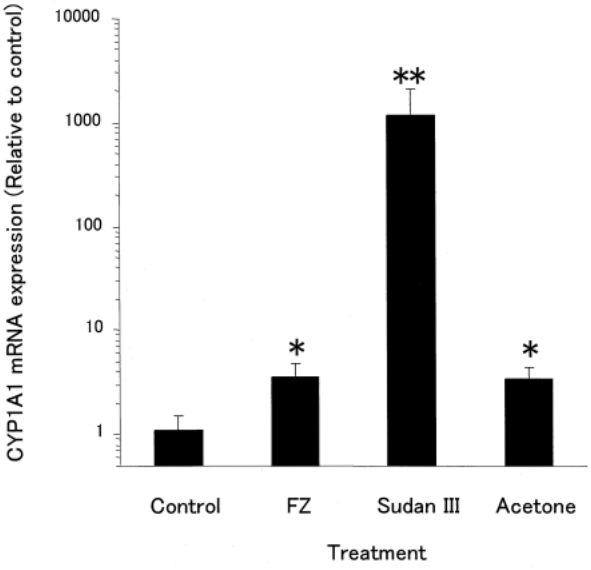

Fig. 3. Expression levels of CYP1A subfamily and CYP2E1 mRNA in FZ-treated rat liver. The relative amount of CYP1A1 mRNA was determined by quantitative RT-PCR. The data represent the relative amounts of CYP1A1 compared to the control. The expression of each gene was normalized to the expression of GAPDH. Each value is expressed as mean ± SD from 3 or 4 rats. Asterisks mean statistical differences compared to control animals by Dunnett's test; * $p<0.05$, ** $p<0.01$.

DISCUSSION

Although numerous studies have indicated high risks of mutagenicity of FZ, several countries, including those in Asia, continue to use FZ on domestic animals, e.g., chickens, fish, and it is clinicaly used for humans. The clinical dose of FZ is 7.5 to 100 mg/kg/day, and previous reports suggested that an appropriate dose for animals is several hundred mg/kg/day [3]. In this study, we injected 125 mg/kg of FZ into rats for 3 days to clarify alterations of CYP isoforms in the liver. We used female rats in the current study because females are more sensitive to the effects of nitrofuran compounds on drug-metabolizing enzymes than male rats [8].

In this study, we carried out the Ames test using the strain *S. typhimurium* TA98 and ethidium bromide as a promutagen that is specifically activated by CYP1A1, and the hepatic S-9 fraction of rats treated with various compounds, including FZ. The FZ-treated S-9 fraction weakly activated ethidium bromide compared to the control S-9. Then, we performed Western blot analysis, and clarified that the increase in reverent colonies in the Ames test using the FZ-treated S-9 fraction was due to the induction of CYP1A1 apoprotein. Our present data on CYP1A1 are consistent with those of Rahden-Staron *et al.* [19], showing that CYP1A was responsible for the enhanced mutagenicity in the Ames test of the S-9 fraction of rats treated intraperitoneally with FZ. We also demonstrated that the CYP1A1 isozyme was regulated transcriptionally, showing induction

of CYP1A1 mRNA in furazolidone-treated rats.

Fukuhara and Takabatake [8] showed the possibility of induction of CYP2E1 by FZ treatment; they reported that 0.06% FZ in feed given to rats for one week resulted in an increase of aniline hydroxylation activity in the hepatic microsomes, and this phenomenon indicated the possibility of induction of CYP2E1 expression. In this study, we found the induction of CYP1A1 by FZ treatment, but there was no alteration in the expression of CYP2E1 apoprotein. In the meanwhile, however, Fukuhara and Takabatake [8] reported that in the case of short-term administration (3 days, as in our current study), nitrofuran compounds caused the suppression of aniline hydroxylase activity in rat liver microsomes. Their result in short-term administration was not inconsistent with the result in our current study. We did not actually detect elevation of the aniline hydroxylase activity in rat liver microsomes after FZ treatment for 3 days (data not shown). Although further study is needed to clear the reason why prolonged-administration of FZ causes the slight induction of aniline hydroxylase activity, we hypothesize that the CYP2E1-induction ability of FZ might be weak. In addition to identification of the isoforms of CYP induced using specific antibodies, we compared the effect of FZ on CYP isoforms with that of acetone, which is a typical inducer of CYP2E1. In the present data, the ability of FZ to enhance mutagenicity in the rat liver S-9 fraction is almost equal to that of acetone, where CYP1A1 is mainly responsible for the mutagenicity in the rat treated with acetone in the Ames test. The elevation of mutagenicity in the Ames test using the acetone-treated S-9 fraction was coincident with the levels of CYP1A1 mRNA induced in liver by acetone treatment. Barnett *et al.* [5] and Iba *et al.* [12] also reported that CYP1A1/2 isoforms, as well as CYP2E1, were induced by acetone given in drinking water or inhaled by rats.

FZ is hydrolyzed to 5-nitrofuran and 3-amino-2-oxazolidone (AOZ), which reveals mutagenicity in the *Salmonella*/microsome test [10]. In addition to the mutagenicity of AOZ, FZ apparently has the ability induce hepatic drug-metabolizing enzymes, especially CYP1A1, which are responsible for increased mutagenicity. Consequently, more attention should be paid to the use of FZ.

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