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Bioaccumulation and the expression of hepatic cytochrome P450 genes in marine medaka (*Oryzias melastigma*) exposed to difenoconazole

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ABSTRACT

This study was conducted to assess the effects of difenoconazole (DFZ), a triazole fungicide, on the hepatic biotransformation system and its bioaccumulation in marine medaka (*Oryzias melastigma*). Fish were exposed to DFZ (1, 10, 100, 1000 ng/L) for 180 days. The results showed that: (1) The mRNA levels of hepatic CYP1A1, CYP1B, CYP1C1, CYP27B and CYP3A40 were up-regulated, but those of CYP3A38 and CYP27A1 were down-regulated. (2) The activity of ethoxyresorufin-O-deethylase (EROD) and the content of reduced glutathione (GSH) in the liver were increased in the DFZ-treated groups, and glutathione S-transferase (GST) activity was increased in the 100 and 1000 ng/L groups. (3) DFZ was accumulated in the muscle and the biological concentration factors in the 10, 100, and 1000 ng/L groups were respectively 149, 81 and 25. These results suggested that long-term exposure to DFZ at low concentrations would result in a bioaccumulation of this compound and disturb the biotransformation system.

Introduction

Difenoconazole (DFZ), which belongs to the triazole group of pesticides, is used for the control of fungal diseases on fruits, vegetables, cereals and other field crops (Goetz et al., 2009; Zarn et al., 2002; Haorsfield et al., 2010). Most of these pesticides are not completely degraded after application, and their metabolites as well as some unchanged forms enter the ecosystem. Under environmental conditions and UV exposure, DFZ is a relatively persistent fungicide, degraded by just 15% after 2 months, and may be transported over long distances (Rodriguez-Cabo et al., 2013). DFZ has been detected in rivers from agricultural areas at concentrations from μg to mg per liter of influent (Mu et al., 2015a). Estuaries and embayments are often contaminated by triazole pesticides via agricultural runoff; for example, the concentration of DFZ in the surface water from the Jiulong River Estuary, China was found to range from non-detectable to 125 ng/L (Zheng et al., 2016). However, limited information concerning the effects of triazole pesticides on marine fish is available.

Many previous experiments have shown that triazole pesticides, such as myclobutanil, triadimefon and fluconazole, can induce the metabolism and detoxification process in mice and fish, as well as disturb the expression of some functional genes (Lin et al., 2014; Allen et al., 2006; Sun et al., 2006; Barton et al., 2006; Li et al., 2013). Mu et al. (2013) investigated the effects of DFZ on the three life phases (embryo, larvae and adult fish) of zebrafish, and found that the acute toxicity varied in the order larvae (1.17 mg/L) > adult fish (1.45 mg/L) > embryo (2.34 mg/L); in addition, DFZ induced an extensive suite of symptoms in embryonic development such as hatching inhibition, abnormal

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spontaneous movement, slow heart rate, growth regression and morphological deformities. However, the concentrations of DFZ in coastal environments are as low as ng/L levels (Zheng et al., 2016), and whether which could influence the biotransformation system of marine fish is unclear.

Xenobiotic compounds entering organisms should be metabolized and detoxified. The cytochrome P450 superfamily (CYP) is known as a large group of enzymes involved in catalyzing the oxidation, bioactivation and detoxification of diverse organic substances (Kim et al., 2013). Most P450 genes associated with xenobiotic metabolism have been identified. It has been demonstrated that the CYP1, CYP2 and CYP3 families are responsible for a variety of xenobiotic metabolism functions in the liver. In the present study, we chose marine medaka (*Oryzias melastigma*) as a model organism exposed to DFZ, and investigated the possible bioaccumulation and metabolism of DFZ via analyzing the expression of some CYP genes.

1. Materials and methods

1.1. Chemicals

DFZ was purchased from Sigma-Aldrich (St. Louis, MO, USA), with a purity of greater than 99%. It was dissolved in dimethyl sulfoxide (analytical grade) to produce stock solutions of 1, 10, 100 and 1000 μg/mL. All other chemicals were of analytical grade and were obtained from commercial sources.

Simeconazole and PCB103 were purchased from Witega Laboratories Berlin-Adlershof GmbH (Berlin, Germany) with a purity of greater than 97%. The simeconazole was used as a surrogate and PCB103 was used as an internal standard. Individual stock solutions were prepared in acetone at 100 mg/L, intermediate stock solutions were prepared in acetone at 10 mg/L, and both were stored at –20°C prior to use. Acetonitrile, methyl alcohol, acetone and n-hexane were chromatographically pure grade and were purchased from Tedia, USA. Ultrapure water was prepared with a Milli-Q water system (Millipore, Bedford, MA, USA). Anhydrous sodium sulfate-was heated to 450°C for 4 hr and kept in a desiccator. C18 cartridges (6 mL/1 g) and NH_2-LC cartridges (6 mL/500 mg) were purchased from Supelco (Bellefonte, PA, USA).

1.2. Fish maintenance and exposure

Marine medaka were supplied by the City University of Hong Kong and were acclimatized in 27% ± 1‰ salinity artificial seawater at a water temperature of 28 ± 0.5°C under a constant 14-hr light:10-hr dark photoperiod and fed twice a day with newly hatched brine shrimp. All animal experiments were conducted according to the research protocols approved by the Xiamen University Institutional Animal Care and Use Committee. Fertilized eggs were collected a few hours after oviposition. The fish were exposed to DFZ from 72 hpf larva to adult for 180 days. Medaka were reared in aerated artificial seawater under the same conditions aforementioned. 20–30 fish in each group were reared in 6 L aquaria and each 10 fish occupied 2 L of seawater, which had pH 8.0 ± 0.1, dissolved oxygen at 7.8 ± 0.4 mg/L, and un-ionized ammonia at 0.015–0.025 mg/L. Fish in each group were exposed to nominal concentrations (1, 10, 100 and 1000 ng/L) of DFZ, and the control group received an equal volume of the solvent dimethyl sulfoxide (1 μl/L). Half the water, containing the specified concentrations of DFZ, was changed every day. Each exposure was performed in triplicate. Fish from each group were sampled after exposure for 180 days. The liver and muscular tissue were frozen in liquid N_2 immediately after collection and stored at –80°C until analysis.

1.3. Determination of DFZ in water samples

The analytical procedure for DFZ was based on the method described by Mahara et al. (1998) with slight modification. Briefly, the freshly made exposure dilution was collected randomly three times and extracted using a liquid–liquid extraction method with 20 mL CH_2Cl_2 in a separatory funnel. The organic phase was collected and dried with anhydrous sodium sulfate, and then the extracts were concentrated to dryness under a rotary evaporator. The DFZ concentration was measured using a Gas Chromatography–Triple Quandrupole Mass Spectrometery (GC–MS/MS) system (Thermo Scientific, USA). The recoveries of DFZ were 103% ± 13.5% (n = 3) and the limit of detection of DFZ was 1.3 ng/L. The detected concentration of DFZ in the exposure medium was 0, 0.87 ± 0.44 (lower than the detection limit), 9.56 ± 0.11, 99.67 ± 3.13 and 1025 ± 42.55 ng/L in each group.

1.4. Determination of DFZ in muscle

A Model Trace 1310 gas chromatograph was connected to a Model TSQ 8000 Triple Quadrupole GC–MS/MS and was equipped with a Model AL1310 autosampler (Thermo Scientific, USA). The column used was a DB-5 ms capillary column, 50 m × 0.250 mm × 0.25 μm (Agilent Technologies, Inc.). GC–MS/MS operating conditions: column temperature, 80°C hold 1 min, 30°C /min to 210°C hold 1 min, 5°C/min to 260°C hold 1 min, 40°C/min to 300°C hold 12 min; carrier gas was helium (purity ≥99.999%); collision gas was argon (purity ≥99.999%); Flow rate was 1.2 mL/min; injection port temperature was 290°C; injection volume was 2 μL; injection mode was splitless, and splitless time started after 1 min, splitless flow at 100 mL/min; ion source temperature was 280°C; MS interface temperature was 300°C; selective reaction monitoring mode was selected. Precursor ion, daughter ion and collision energies are listed in Table 1.

The muscle tissue was used for the determination of DFZ. An accurately weighed 1 g portion of muscle tissue from pooled medaka samples was transferred into a 50 mL polypropylene centrifuge tube and spiked with 5 ng of the surrogate standards. The sample was subjected to a two-step extraction process. Briefly, the sample was first extracted with 15 mL acetonitrile by homogenization for 3 min, and centrifuged at 2050 ×g for 5 min (Sun et al., 2005). The muscle tissue residue was then re-extracted with 15 mL acetonitrile by sonication for 10 min, and the two-step supernatant were collected into a 100 mL glass media bottle with 30 mL saturated salt water. Shake for 10 min. Let stand until the solution is clearly separated into layers. Discard the aqueous layer. The acetonitrile supernatants were subjected to clean-up by two different solid phase

Table 1 – Parameters for determination of DFZ (difenoconazole), IS-PCB103 (Internal standard-printed circuit board 103) and surrogate-simeconazole in muscle tissue of medaka by Gas Chromatography–Triple Quandrupole Mass Spectrometery.

Compound	Retention time (min)	Mode	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Simeconazole	10.00	Positive	195.2	75	15
Simeconazole	10.00	Positive	211.1	195.1	10
PCB 103	11.25	Positive	325.8	255.9	8
PCB 103	11.25	Positive	325.8	290.8	8
Difenoconazole1	20.14	Positive	265	202.1	8
Difenoconazole1	20.14	Positive	323	265	8
Difenoconazole2	20.26	Positive	265	202.1	8
Difenoconazole2	20.26	Positive	323	265	8

extraction (SPE) columns. The supernatants were first cleaned-up by C18 (1 g) SPE-columns, which were pre-conditioned sequentially by 6 mL methyl alcohol, 6 mL ultra-pure grade water and 6 mL acetonitrile (Sun et al., 2005). After being concentrated to 1 mL by the vacuum rotary evaporation method, eluents were passed through an 8 mL 1:1 (V/V) acetone-n-hexane pre-conditioned NH2-LC (500 mg) SPE-column, and then washed with 15 mL 1:1 (V/V) acetone-n-hexane. Finally, the analytes were concentrated to approximately 0.1 mL under a gentle stream of nitrogen at 30°C. The residues were reconstituted into 0.2 mL of 1:1 (V/V) acetone-n-hexane followed by addition of 10 μL internal standard solution and mixed thoroughly, and then evaluated by GC–MS/MS. Recoveries of DFZ in different concentrations ranged from 75.3% to 115.0%. The detection limit of DFZ was 1 ng/g.

For method validation, the following validation parameters were considered to evaluate method performance. (1) Calibration range: The calibration curves were obtained by plotting the peak area against the concentration of the corresponding calibration standards at seven concentration levels ranging between 1.0–100.0 ng/mL; (2) sensitivity: The limit of quantification (LOQ) was determined by considering a signal-to-noise ratio of 10. (3) Accuracy-recovery experiments: The recovery experiments were carried out with homogenized muscular tissue (wet, 1 g) at spiking levels of 2 ng/g and 10 ng/g DFZ, with four replicates each (Table 2).

1.5. *Quantitative real-time PCR*

For real-time PCR analysis, the total RNA of liver was extracted using a Trizol kit (TaKaRa, Dalian, China). The mRNA expression was determined based on previously described methods (Sun et al., 2011). The primers were designed using the Primer 5.0 program (PREMIER Biosoft International, Silicon Valley, USA) and are shown in Table 3. Each mRNA level was normalized to GADPH (glyceraldehyde-3-phosphate dehydrogenase). We used the Relative Expression Software Tool (REST-MCS ©-version 2) to calculate the relative expression of target gene mRNA.

1.6. *Biochemical analysis*

Ethoxyresorufin-O-deethylase (EROD) activity was determined according to Peters et al. (1994). The unit of EROD activity is defined as formation of 1 pmol resorufin per min per mg of protein. Glutathione S-transferase (GST) activity was evaluated with 1-chloro-2,4-dinitrobenzene as substrate, following the formation of the conjugate with GSH at 340 nm (ε = 9.6/mM cm) according to Habig et al. (1974). The GSH content was determined using a commercial kit purchased from Nanjing Jiancheng Bioengineering Institute (China) according to the manufacturer's instructions. Protein concentrations in the supernatants were determined by the Bradford (1976) procedure using bovine serum albumin as standard. The fluorometric assays were determined on a Hitachi F-4010 fluorescence spectrophotometer. The absorbance at UV and visible wavelengths was monitored on a UV-2550 UV–Vis spectrophotometer (Shimadzu Corporation, Japan).

1.7. *Statistical analysis*

Results are reported as mean ± standard error. Significant differences between means were analyzed with one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test using SPSS Version 11.0 (SPSS Inc., Chicago, IL, USA). For mRNA expression, the Pair Wise Fixed Reallocation Randomization Test© (Pfaffl et al., 2002) was used. The biological concentration factor (BCF) was calculated as: BCF = (ng DFZ/g muscle)/(ng DFZ/mL water). In all cases, $p < 0.05$ was used to indicate significance.

2. Results

The expression of the selected cytochrome P450 genes is shown in Fig. 1. CYP1A1 transcripts increased in a dose-dependent manner and reached a significant difference in the 1000 ng/L group (3.41-fold) compared to the control group; CYP1B and CYP1C1 showed a dramatic increase by 2.54,

Table 2 – Recoveries of DFZ and surrogate- simeconazole spiked in blank fish muscle tissue.

Compound	Recovery% (RSD, *n* = 4)		Liner range (ng/g)	R^2	LOQ (ng/g)
	2 ng/g	10 ng/g			
Difenoconazole	75.3 ± 15.5	115.0 ± 8.6	1.0–100.0	0.9995	1.0
Surrogate-Simeconazole	83.4 ± 4.7	110.5 ± 3.3	1.0–100.0	0.9998	1.0

Table 3 – Primers used in the real-time quantitative RT-PCR analysis.

Gene	Amplification efficiency	Primer sequences
CYP1A1	89.40%	F:TACAACAAGGGACACATCTCTGA
		R:GGTAACCACTTTCTCTCCTTCAA
CYP1B1	106.50%	F:TTTCAGCCCATGACTTATCTG
		R:GTCTTAAGTTGTCAAAGATCGT
CYP1C1	85.00%	F:AAGTGGCTGCTGCTAACATCA
		R:TAGAGACTGCGGACTGGGTTA
CYP3A38	90.00%	F:GCTCTGGTGTCTATGAAACTGG
		R:TTAGGATGTAGAGCGGGGC
CYP3A40	92.30%	F:AGCGTTCTGTCTCCTTCCTTC
		R:TCTAAGGGTTCATCTTTGTCTGC
CYP27A1	100.00%	F:GTCAGTGCCTCGGAATGGA
		R:CCCCTGAAACACAAGCCTG
CYP27B	99.30%	F:CGCTTTGGACTTGAAGGTAT
		R:GATCTTTAGCAAATTCAAACATG
CYP51A1	98.00%	F:TCTGGAGAAAGCCTACGACC
		R:AGGTCCTCGTTCTTGCTGTT
GAPDH	97.40%	F:CGTTGAGGGCTTGATGAG
		R:GGTCAGTTTGCCGTTCAG

5.26, 7.14 and 2.14, 7.10, 6.02-fold in the 10, 100 and 1000 ng/L groups. CYP3A38 transcripts exhibited a reduction in the 10, 100 ($p < 0.05$) and 1000 ng/L groups. CYP3A40 expression showed a significant increase (1.83-fold) in the 1000 ng/L group. CYP27A1 transcripts significantly decreased by 0.32, 0.49 and 0.28-fold in the 10, 100, and 1000 ng/L groups; The mRNA levels of CYP27B significantly increased by 9.37 and 9.56-fold in the 10 and 100 ng/L groups. Also, we determined the CYP51A1 gene expression, and there was a significant increase (2.52-fold) in the 1 ng/L group, while a significant decrease (0.44 and 0.23-fold) was observed in the 100 and 1000 ng/L groups.

Hepatic EROD activity was significantly elevated by 2.7, 2.76 and 2.19-fold in the 10, 100, and 1000 ng/L groups. The GST activity showed significant elevation by 1.45 and 1.26-fold in the 100 and 1000 ng/L groups, while it significantly decreased by 0.92-fold in the 10 ng/L group compared to the control. Hepatic GSH levels were significantly elevated (1.26, 1.39, 1.89 and 1.79-fold) in the 1, 10, 100 and 1000 ng/L groups (Fig. 2).

No DFZ was detected in the muscle of the control fish. The concentration of DFZ significantly increased in the 10, 100 and 1000 ng/L groups compared to the control (Fig. 3). The biological concentration factor (BCF) of each group was 149.0, 81.4 and 24.7 in the 10, 100 and 1000 ng/L groups.

3. Discussion

Most environmental contaminants cause induction of the biotransformation system of organisms for their detoxification. Previous studies have demonstrated that azole pesticides, such as propiconazole (PCZ), triadimefon, fluconazole and myclobutanil, can induce CYP1A2, CYP2B and CYP3A families in rat liver (Lin et al., 2014; Allen et al., 2006; Sun et al., 2006; Barton et al., 2006). Triadimefon enhances CYP1A activity in medaka (*Oryzias latipes*) (Lin et al., 2014). Exposure to PCZ for 14 days increases hepatic CYP1A activity in sexually immature brown trout (Egaas et al., 1999). Our result showed that the expression of CYP1A1, CYP1B and CYP1C1, accompanied with GST and EROD activity, was increased in the liver of medaka exposed to DFZ, which was consistent with previous studies. GST is an important detoxification enzyme involved in catalyzing the conjugation of a wide variety of electrophilic substrates to reduced glutathione, producing a more water-soluble form, which can be excreted from the body more easily (Van der Oost et al., 2003). In the 50 g trout exposed to 313 mg/L PCZ, the GST activity (cumene hydroperoxide or ethachrynic acid as the substrate) significantly increased (Egaas et al., 1999). Hepatic GST activity is significantly

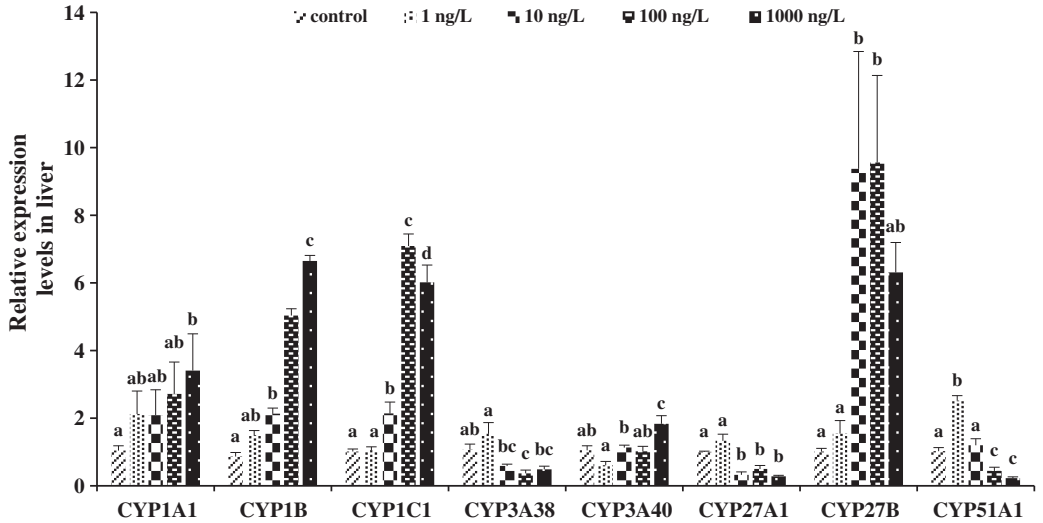

Fig. 1 – The mRNA expression of eight CYP450 genes in liver of marine medaka (*Oryzias melastigma*) exposed to difenoconazole (DFZ) for 180 days. Values were normalized against GAPDH. The data are expressed as mean ± SE ($n = 6$). Means of exposures not sharing a common letter are significantly different at $p < 0.05$ as assessed by one-way ANOVA followed by the Duncan test.

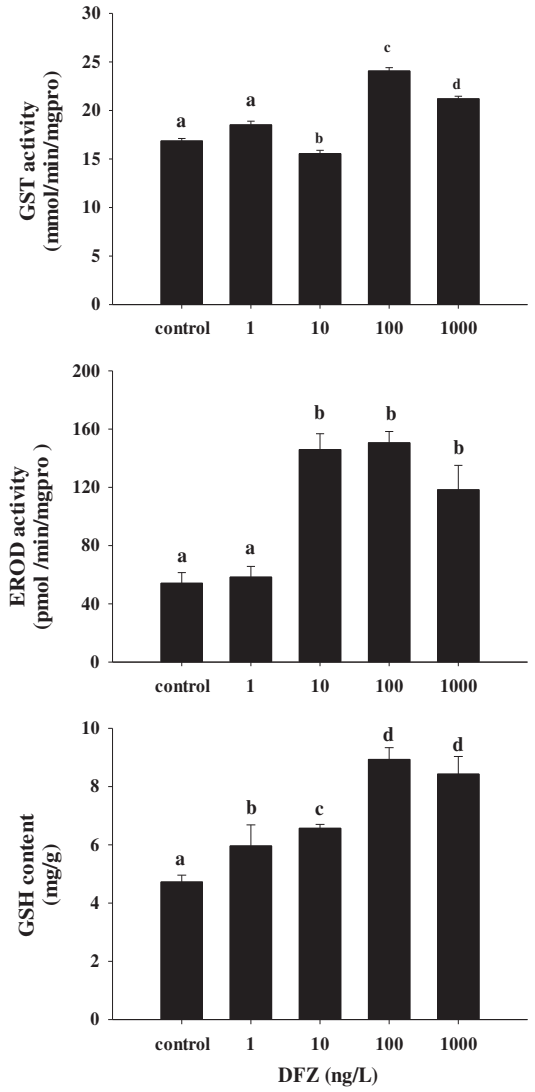

Fig. 2 – EROD (ethoxyresorufin-O-deethylase), GST (glutathione S-transferase) activity and GSH (glutathione) content in the liver of marine medaka (*Oryzias melastigma*) exposed to difenoconazole (DFZ) for 180 days. Data are presented as mean ± SE (n = 6). Means of exposures not sharing a common letter are significantly different at $p < 0.05$ as assessed by one-way ANOVA followed by the Duncan test.

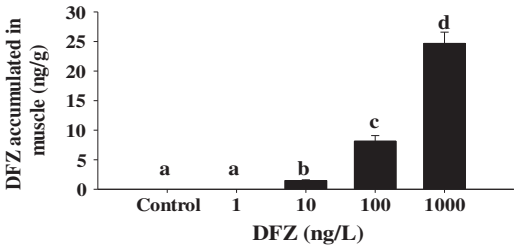

Fig. 3 – Difenoconazole accumulation in the muscles of marine medaka (*Oryzias melastigma*) exposed to difenoconazole (DFZ) for 180 days. The value in the control and 1 ng/L group was lower than the detection limit. Data are presented as means ± SE (n = 3). Means of exposures not sharing a common letter are significantly different at $p < 0.05$ as assessed by one-way ANOVA followed by the Duncan test.

induced in rainbow trout exposed to 50 and 500 μg/L PCZ for 20 days, while GSH levels in the liver are significantly decreased (Li et al., 2010). GSH is a major cytosolic low molecular weight sulfhydryl compound that acts as a cellular reducing and protective reagent against numerous pollutants through the –SH group (Zhang et al., 2004). The results in our study showed that GST activity and GSH levels both were induced by DFZ exposure, which would promote the biotransformation and elimination of DFZ. However, even when the fish were exposed to the lowest concentrations of this compound, the accumulation of this fungicide in muscle was still detected.

As one of the most abundant CYP enzymes, the CYP3A subfamily contributes to the metabolism of >60% of major drugs in human liver (Riley et al., 2000). Triadimefon and myclobutanil induce hepatic CYP3A activity in medaka with 3 to 7-day exposure (2.0 and 3.5 μmol/L), and CYP3A38 and CYP3A40 appear to be the major induced CYP isoforms in triazole metabolism; with 20-day exposure to triadimefon or myclobutanil at 20–2000 nmol/L, the mRNA expression of CYP3A38 and CYP3A40 is slightly enhanced by both triazoles at 20–200 nmol/L but repressed at 2000 nmol/L (Lin et al., 2014). The two triazoles also increase the transcriptional or protein expression of CYP3A1/2 (rats) or CYP3A11 (mice) (Allen et al., 2006; Sun et al., 2006; Barton et al., 2006). However, overexpression of CYP3A may deplete important endogenous substances (*e.g.*, sex hormones or vitamins) by elevating their metabolic rate. The results showed that the CYP3A38 mRNA levels were inhibited after long-term exposure to DFZ, while that of CYP3A40 was increased, which was different from the results for triadimefon and myclobutanil; this might due to the different chemicals or different exposure concentrations used.

CYP27A1 plays an important role in bile acid (BA) synthesis (Lorbek et al., 2012). The present study showed that DFZ was an inhibiter toward CYP27A1 in medaka, which probably disturbed the system of biosynthesis and metabolism of BA. CYP27B plays an important role in the synthesis of vitamin D, metabolism of 25D and secretion of detectable levels of de novo synthesized 1a,25$(OH)_2D_3$ (Schuster et al., 2001). The expression of CYP27B in medaka liver is up-regulated by DFZ, which would disturb the synthesis of vitamin D (Mu et al., 2015b). CYP51 (lanosterol 14α-demethylase) is the most evolutionarily conserved member of the CYP superfamily and is a key enzyme for sterol biosynthesis. Cholesterol provides building blocks for the synthesis of vitamin D, BAs and steroid hormones, all of which play important roles in maintaining the integrity of an organism (Xu et al., 2011; Sun et al., 2007; Parker et al., 2014). Conazole pesticides are based on their inhibition of certain pathways of steroidogenesis by binding to CYP51 (Zarn et al., 2002). The hepatic total cholesterol levels of male zebrafish exposed to 500 μg/L DFZ decrease significantly, and the expression of hepatic cholesterol-genesis genes (including CYP51) declines (Mu et al., 2015b). The results showed that DFZ exposure disturbed

the mRNA levels of CYP51A1, which indicated that the process of vitamin D synthesis would be disturbed.

In the present study, long-term exposure to DFZ at low concentrations led to induction of the hepatic biotransformation and detoxification system, but this compound is still bioaccumulated in the muscle of marine medaka. It was reported that this fungicide was associated with an increase in the incidence of hepatocellular adenomas and carcinomas in a group of male and female mice following long-term dietary exposure (Dong et al., 2013). Since marine fish are an important source of nutrition and protein, DFZ ingestion from marine fish by humans should be of concern.

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