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CYP450 enzyme-specific enantioselective species-specific response for metalaxyl in in vitro hepatic cells

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ABSTRACT

Although enantioselective accumulation of chiral pesticide has been reported in organisms, the mechanisms remain unclear. In this study, the effects of chiral pesticide metalaxyl on CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP2E1 and CYP3A were investigated in human hepatoma HepG2, rat hepatic H4IIE, chicken hepatic LMH and grass carp hepatic L8824 cells. Moreover, the residual concentrations and enantiomeric ratios (ERs) of metalaxyl were also detected in the medium. The results showed the responses of these CYP450s to metalaxyl were enzyme-dependent and species-dependent in the four cells. CYP1A1, CYP1A2, and CYP2B1 were induced in HepG2 cells, CYP2A1 and CYP2B1 were induced in H4IIE cells, CYP1A1 and CYP2B1 were induced in LMH cells, and CYP2B1 was induced in L8824 cells. The enantioselective residual of metalaxyl was detected in the medium and found to be species-specific. HepG2, H4IIE and LMH cells were inclined to attenuate *S*-metalaxyl and lead to decrease of ER of metalaxyl, while L8824 cells were inclined to remove *R*-metalaxyl and resulted in an inverse shift of ER. These findings suggest an enantioselective metabolism of metalaxyl in various species which is not only related with CYP450s and CYP450 enzyme-specific, but also species-specific.

1. Introduction

Widespread application of chiral compounds results in their ubiquitous occurrence in environment and accumulation in organisms as well as subsequently toxic effects on organisms (Lewis et al., 1999). In general, the enantiomers of chiral compounds possess enantioselective biological activities and toxicity due to their different structural properties (Sekhon, 2009). However, chiral compounds have been treated as racemic mixtures in their environmental fate and ecotoxicity for a long time. In recent years, the stereoselectivity-related environmental safety of chiral compounds has become a popular focus of attention (Liu et al., 2005; Wang et al., 2014).

The enantiomeric ratio (ER) of chiral compounds accumulated in organisms has been found to be different among species (Borga and Bidleman, 2005; Harner et al., 1999; Warner et al., 2005; Wiberg et al., 2000), indicating enantioselective accumulation of chiral compounds are species-specific. The ER of α-HCH was detected to vary along the polar bear food chain and increase from ≈1.0 in cod to 2.3 in polar bear (Wiberg et al., 2000). In arctic marine invertebrates, depletion of the (+)-α-HCH enantiomer increased from ice fauna to zooplankton, and to benthos (Borga and Bidleman, 2005; Harner et al., 1999). Compared to α-HCH, chlordane and *o,p'*-DDT showed stronger enantioselective bioaccumulation in benthic amphipods than in zooplankton and ice fauna (Borga and Bidleman, 2005). In the similar area, enantioselective species-specific biotransformation of individual PCB stereoisomers has also been reported in marine food web. Greater nonracemic enantiomeric fractions (EFs) of PCBs were observed in several seabird species and ringed seals, but racemic EFs were found in the prey such as zooplankton and fish (Warner et al., 2005). Besides in animals, the accumulation of chiral compounds has also been found to be enantioselective species-specific in plants (Wang et al., 2014; Schneiderheinze et al., 1999). However, the mechanisms of enantioselective species-specific biotransformation of chiral compounds have received little attention and remain unclear so far.

Xenobiotic metabolism primarily occurs in liver, which contains many enzymes to catalyze the transformation of xenobiotic. Usually, cytochrome P-450s (CYP450s) act as the first response for biotransformation of xenobiotic in organisms. CYP450s generally consist of various subfamily enzymes which can catalyze different reactions of xenobiotic (Tang et al., 2006). Previous studies have revealed that the effects of a compound varied among CYP450 enzymes, indicating that the effects of a compound on CYP450s were enzyme-specific. CYP450

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enzyme-specific metabolism has been reported for some pollutants such as PCB, dioxins and other xenobiotics (Guengerich, 2001; Lu et al., 2013; Vijaya Padma et al., 2014). The findings suggest that a compound could be catalyzed by one or several specific CYP450 enzymes during its metabolism by CYP450s (Guengerich, 2008). Therefore, CYP450 enzyme-specific interaction with pollutants provides us to better understand the pathway of metabolism of pollutants.

Metalaxyl is a chiral pesticide with a stereogenic center in the carboxy alkyl moiety, and thus consists of a pair of enantiomers (Fig. S1, Supporting Material). Since first introduced in 1977, metalaxyl has been widely used as a fungicide to control diseases caused by *Oomycetes* on a wide range of plants including crops for more than thirty years (Li et al., 2013; Monkiedje et al., 2003). With the wide application, metalaxyl has been detected in many environmental matrixes, including water, soil, and sediment as well as organisms (Martin et al., 2012; Monkiedje et al., 2003; Sanchez-Gonzalez et al., 2013; Wani et al., 2012; Wightwick et al., 2012). Although metalaxyl was used as a racemic mixture (ER = 1), the enantioselective accumulation of metalaxyl has been reported in organisms and found to vary among the organisms (Wang et al., 2014; Xu et al., 2011; Zhang et al., 2012). However, the mechanisms for species-specific enantioselective biotransformation of metalaxyl have never been investigated.

Although CYP450s consist of various subfamily enzymes, only some of them have been well studies relating to metabolism of xenobiotics such as CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP2E1, and CYP3A (Guengerich, 2001; Lu et al., 2013; Oropeza-Hernández et al., 2003; Vijaya Padma et al., 2014). In this study, therefore, the effects of metalaxyl on the six CYP450 enzymes were investigated in four vertebrate hepatic cell lines, including human hepatic HepG 2 cells, rat hepatic H4IIE cells, chicken hepatic LMH cells and grass carp hepatic L8824 cells. The residual concentrations and ER of metalaxyl were also determined in the medium of the four cells to reveal the enantioselective species-specific responses of CYP450s for metalaxyl.

2. Materials and methods

2.1. Chemicals and reagents

Metalaxyl (99.6%), resveratrol (≥99%), 4-phenylimidazole (97%), ketoconazde (98%), sodium orthovanadate (≥90%) and dicumarol were provided by Sigma-Aldrich (St. Louis, MO, USA). Benzyloxyresorufin, methoxyresorufin, erythromycin, ethoxyresorufin, pentoxyresorufin, fluvoxamine and 4-nitrophenol were purchased from Santa Cruz Biotechnology Ink (Santa Cruz, CA, USA). All of the chemicals were dissolved in dimethyl sulfoxide (DMSO, ≥99.5%, Sigma-Aldrich, St. Louis, MO, USA). Ammonium acetate (AR Grade) was a product of Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Acetylacetone (99%) was purchased from Aladdin Industrial Corporation (Shanghai, China). Dulbecco's minimum essential medium was obtained from Thermo Scientific HyClone (San Jose, CA, USA). Penicillin-streptomycin solution, minimum essential medium, medium 199 basic, trypsin and fetal bovine serum were provided by Biological Industries (Beit-Haemek, Israel). Methanol (LC/MS grade) and acetonitrile (residue analysis grade) were obtained from Fisher Scientific Co. LLC (Fair Lawn, NJ, USA) and J. T. Baker, Inc. (Phillipsburg, NJ, USA), respectively. Other chemicals and solvents used in this study were of cell culture, HPLC, or analytical grade.

2.2. Cell culture

Human hepatoma HepG2 cells, rat hepatic H4IIE cells and chicken hepatic LMH cells were cultured in Dulbecco's minimum essential medium. Grass carp hepatic L8824 cells were cultured in medium 199. The medium was supplemented with 10% fetal calf serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. HepG2, H4IIE and LMH cells were incubated at 37 °C in a humidified 5% CO_2 incubator, while L8824 cells were incubated at 28 °C in a humidified 5% CO_2 incubator. Prior to test, the cells were cultured in plates of 96 wells at 5×10^3 cells/well for 24 h. After attachment for 24 h, the cells were exposed to medium without fetal calf serum but containing the test chemicals described below.

2.3. Cytotoxicity of metalaxyl

Cytotoxicity of metalaxyl on HepG2, H4IIE, LMH and L8824 cells was measured by quantitative colorimetric assay with MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] according to a previous study (Liu et al., 2008). The cells were incubated with medium containing metalaxyl or DMSO as control for 24 h. At the end of exposure, the medium was removed from the wells, and MTT solution was added. After 4 h, the solution was removed and 150 μL DMSO was added in each well. After 10 min of shaking with a micro-mixer, the absorbance was measured at 490 nm with the Bio-Rad 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. CYP450 enzyme activities

CYP1A1 (EROD), CYP1A2 (MROD), CYP2B1 (PROD), CYP2B2 (BROD) were determined by micro-alkoxyresorufin O-dealkylases (AROD) assay. AROD activities were spectrofluorometrically assessed by monitoring the formation of resorufin according to Burke's method with some modifications (Burke et al., 1985). Taking EROD as an example, the cells were exposed to 200 μL medium containing metalaxyl or DMSO as control for 24 h. The concentration of DMSO in medium was 0.1% (v/v) in each exposure. Thereafter, the medium was removed and 100 μL of fresh medium containing 10 μM ethoxyresorufin and 10 μM dicumarol was added for an additional 60 min of incubation for determination of CYP1A1 activity. The medium was then transferred to a new 96-well plate and mixed with 130 μL of absolute ethanol. Resorufin-associated fluorescence was measured in the solution on the Bio-Rad 680 microplate reader. Excitation and emission wavelengths were set at 535 and 590 nm, respectively. For CYP1A2, CYP2B1, and CYP2B2 activity assay, the procedure was the same with the EROD assay except that methoxyresorufin, pentoxyresorufin, and benzyloxyresorufin was used instead of ethoxyresorufin, respectively.

CYP2E1-associated 4-nitrophenol hydroxylase (4-NPH) activity was determined according to the method described by Koop (Koop, 1986). After the cells were exposed to 200 μL medium containing metalaxyl or DMSO as control for 24 h, the medium was replaced with 100 μL of fresh medium containing 0.2 mM 4-nitrophenol. The cells were then incubate at 37 °C for an additional 30 min. Reaction was stopped by addition of 0.5 mL of 0.6 N perchloric acid followed by shaking with a micro-mixer. 4-Nitrocatechol formation was then spectrophotometrically determined in 100 μL of supernatant plus 10 μL of NaOH (10 N) at 510 nm of absorbance.

CYP3A-associated erythromycin N-demethylase (END) activity was spectrophotometrically measured by detecting the production of formaldehyde according to a previous method (Alexidis et al., 1996). The procedure was similar with the EROD assay. At the end of exposure, the medium was removed. The cells were then incubated with 100 μL of fresh medium containing 10 mM erythromycin for an additional 30 min. Reaction was stopped by addition of 50 μL 12.5% trichloroacetic acid followed by blending. 100 μL of supernatant was mixed with 100 μL Nash reagent and heated at 50 °C for 30 min. The absorbance at 412 nm was spectrophotometrically measured in the mixture.

2.5. Metalaxyl enantiomer analysis by UPLC-MS/MS

To explore the enantioselective species-specific biotransformation, the residual concentrations and ERs of metalaxyl were detected in the medium by UPLC-MS/MS at the end of exposure. The medium was