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Early life exposure to a rodent carcinogen propiconazole fungicide induces oxidative stress and hepatocarcinogenesis in medaka fish

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ABSTRACT

Conazole pollution is an emerging concern to human health and environmental safety because of the broad use of conazole fungicides in agriculture and medicine and their frequent occurrence in aquifers. The agricultural pesticide propiconazole has received much regulatory interest because it is a known rodent carcinogen with evidence of multiple adverse effects in mammals and non-targeted organisms. However, the carcinogenic effect and associated mechanism of propiconazole in fish under microgram-per-liter levels of environmental-relevant exposure remains unclear. To explore whether early life of propiconzaole exposure would induce oxidative stress and latent carcinogenic effects in fish, we continuously exposed larvae of wild type or $p53^{-/-}$ mutant of medaka fish (*Oryzias latipes*) to propiconazole (2.5–250 μg/L) for 3, 7, 14 or 28 days and assessed liver histopathology and/or the oxidative stress response and gene expression during exposure and throughout adulthood. Propiconazole dose-dependently induced reactive oxygen species (ROS) level, altered homeostasis of antioxidant superoxide dismutase, catalase and glutathione *S*-transferase and caused lipid and protein peroxidation during early life exposure in wild type medaka. Such exposure also significantly upregulated gene expression of the cytochrome P450 *CYP1A*, but marginally suppressed that of tumor suppressor *p53* in adults. Furthermore, histopathology revealed that $p53^{-/-}$ mutant medaka with early life exposure to propiconazole showed increased incidence of hepatocarcionogensis, as compared to the $p53^{-/-}$ control group and wild type strain. We demonstrated that propiconazole can initiate ROS-mediated oxidative stress and induce hepatic tumorigenesis associated with *CYP1A*- and/or *p53* -mediated pathways with the use of wild type and $p53^{-/-}$ mutant of medaka fish. The toxic response of medaka to propiconazole is compatible with that observed in rodents.

1. Introduction

As technology of analytical chemistry rapidly develops, various anthropogenic organic contaminants such as pesticides, pharmaceuticals and personal care products (PPCPs) are frequently detected at nanogram- to microgram-per-liter levels in aquifers worldwide. PPCPs include a large and diverse group of medicinal or agricultural compounds used for the diagnosis, cure, mitigation, treatment, or prevention of diseases in humans and animals (Corcoran et al., 2010; Jones et al., 2001; Petrovic, 2004). Conazoles are a class of imidazole- or triazole-containing antifungal agents commonly used in agricultural and pharmaceutical products. Medically, conazoles are used to treat or prevent local and systemic fungal infections in humans and animals and as anti-estrogens for treating human cancers. In agriculture, they are applied as fungicides to trees, grasses, fruit, vegetables, and cereal crops and for lawn care and wood preservation (Kahle et al., 2008; Zarn et al., 2003).

The antifungal activity of conazoles is based on their inhibition of certain pathways of steroidogenesis by binding the enzymes 14-alpha-demethylase (CYP51) from demethylating a precursor to ergosterol, an important steroid of fungal cell membranes. The therapeutic function of conazoles in human is based on inhibition of cytochrome P450 aromatase (CYP19), which is responsible for the conversion of androgens into estrogen (Zarn et al., 2003). However, the specificity of the enzyme inhibition of several conazoles is poor, with evidence of adverse health effects in humans and experimental animals (Allen et al., 2006; Taxvig, 2008; Ward et al., 2006). For instances, propiconazole (1-(2-(2,4-dichlorophenyl)-

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4-propyl-1,3-dioxolan-2-ylmethyl)-1*H*-1,2,4-triazole) is a heavily used agricultural agent carcinogen of emerging environmental concern. It induced liver tumors in mice in a 2-year feeding study (Edwards, 2006; INCHEM, 1987).

With the broad use of conazole fungicides in agriculture and medicine, environmental pollution by conazole fungicides is becoming a major threat to aquatic ecosystems. The main routes of conazoles entering the environment are via manufacturing, hospital or domestic effluent, whereby these pharmaceuticals (as the parent compound or as metabolites) from treated patients is directly released into the wastewater system or via agricultural runoff and irrigation return waters (as the agricultural pesticides) (Kahle et al., 2008). Indeed, many conazole compounds occur ubiquitously in the aquatic environment such as wastewater treatment work effluent, surface and ground water and sediment, the public and regulatory agencies have raised concerns about environmental safety (Battaglin et al., 2011; Corcoran et al., 2010; Haith and Rossi, 2003; Kahle et al., 2008). Environmental exposure to propiconazole tends to be low, with detected concentrations of <100 μg/L in surface waters, 0.17–0.24 μg/L in groundwater and 0.01–0.22 mg/kg in winemaking residue (Battaglin et al., 2011; Haith and Rossi, 2003; Van De Steene and Lambert, 2008).

The bioaccumulation of several chiral triazoles was reported in fish (Konwick et al., 2006). Several conazoles, including propiconazole, inhibit steriodogenic CYPs in mature fish and depress circulating sex steroid concentrations, thereby decreasing hepatic production and ovarian deposition of egg yolk protein (e.g., vitellogenin), thus reducing egg production (Ankley et al., 2002; Liao et al., 2014; Skolness et al., 2013). Propiconazole also alters multiple physiological hematologic indices and brain Na^+-K^+-ATPase activity in rainbow trout (Dong et al., 2010; Li et al., 2010a). Subchronic to chronic exposures of propiconazole (0.2–500 μg/L) modulate the glutathione-related antioxidant defense system and also induce reactive oxygen species (ROS), oxidative stress and lipid peroxidation in the intestine, liver and muscle of trout (Dong et al., 2010; Egaas et al., 1999; Li et al., 2010b). Propiconazole is a nongenotoxic rodent liver carcinogen, which acts as a liver tumor promoter in rats (Hasegawa and Ito, 1992; INCHEM, 1987). Genomic and biochemical studies in rodents suggest that carcinogenic effects of propiconazole act through complex pathways involving oxidative stress, hepatomegaly, CYP activity (e.g., CYP1A, 2B and 3A etc) and cell proliferation (Nesnow et al., 2011a; Sun et al., 2005; Ward et al., 2006). However, few scientific publications have reported on carcinogenic potency and associated mechanisms of propiconazole in fish, especially at an early life stage, the vulnerable stage of development.

Various non-genotoxic carcinogens exert their effects by generating ROS during their metabolism by CYP-enzymes and induce oxidative stress through modification of cellular antioxidant defense mechanisms (Klaunig et al., 1998; Morita et al., 2013; Waris and Ahsan, 2006). Excess ROS accumulation can cause oxidative DNA and protein damage, damage to tumor suppressor genes (e.g., p53) and/or enhanced expression of oncogenes and then lead to chronic inflammatory pathways and a series of carcinogenesis events (Reuter et al., 2010). Mutations in p53 gene are the most frequently observed genetic lesions in human cancers (Donehower et al., 1992). Mice lacking p53 show increased ROS levels and the ability to induce oxidative stress with increased susceptibility to cancer (Donehower et al., 1992; Sablina et al., 2005). In analogy to the mammalian situation, fish p53 was expected to play a similar crucial role in tumorigenesis (Chen et al., 2001). Whether propiconazole-induced oxidative stress and liver toxicity are associated with CYP- or p53-mediated carcinogenesis in fish needs further investigation. Knowledge of its effects on lower vertebrate species may also provide valuable information on the toxic action of carcinogenic chemicals, thus unraveling or adding evidence for effects that are suspected in humans.

Medaka (*Oryzias latipes*) is a well characterized small fish model widely used for carcinogenicity testing because of their extended similarity in mutagenesis and carcinogenesis processes with rodent models and possibly humans (Hawkins et al., 2003; Ishikawa, 2000). As well, an unique mutant strain of medaka carries a p53 gene deficiency (a homozygote $p53^{-/-}$ mutation in the tumor suppressor gene *p53*, (Taniguchi et al., 2006)), is a superior model organism for loss-of-function experiments that help to elucidate the process of tumorigenesis and accelerate the identification of carcinogens (Taniguchi, 2011). Here, we treated medaka fish of the wild type and $p53^{-/-}$ mutant at an early life stage with environmentally relevant or sublethal concentrations of propiconazole for 3–28 days. We aim to assess the oxidative stress responses, gene expression of p53 and CYP1A and carcinogenic potency to understand their correlation with propiconazole-induced hepatic tumorigenesis in the juvenile stage throughout adulthood in medaka fish. We discuss these results in terms of environmentally relevant conditions or rodent findings.

2. Materials and methods

2.1. Propiconazole preparation and concentration analysis

The stock of propiconazole (Orbit; 94.2% active ingredient; Syngenta Crop Protection, NC, USA) was prepared in dimethyl sulfoxide (DMSO) and serially diluted with embryo-rearing medium (ERM; containing NaCl, $CaCl_2$, KCl, $MgSO_4$, pH 7.2) (Iwamatsu, 2004) for fish exposure. Test concentrations of 2.5, 25 and 250 μg/L propiconazole were based on an initial range-finding test from a short term exposure. The actual concentrations of propiconazole in dosing solutions were determined before and after water renewal at appropriate intervals during the exposure period, described as follows. Collected water samples (for 25 and 250 μg/L propiconazole) were directly analyzed in an aliquot (20 μL) of sample directly injected onto a C18 column (Purospher STAR, RP-18e, 5 μm, 4 × 250 mm, Merck) of a high-performance liquid chromatography (HPLC) system (Hitachi Model D-2000 Elite station, Hitachi Technologies, Japan) equipped with a diode array detector. The column was eluted isocratically at a flow rate of 1.0 ml/min with a mobile phase composed of acetonitrile (70%) and water (30%) at 40 °C. UV detection of propiconazole was set at 204 nm. For 2.5 μg/L of propiconazole solution, a water sample (50 ml) was loaded on a cartridge (Oasis HLB-columns, Waters, CT, USA) for solid-phase extraction and then eluted with 10% MeOH in diethyl ether for chemical extraction based on the manufacturer's instructions. The eluent was dried, and the residue was reconstituted in 25% MeOH. The chromatography and UV absorbance of propiconazole standards and related samples is provided in supporting information (Figs. A1 and A2, Appendices). Aqueous concentrations of propiconazole were calculated with reference to an external standard curve. All chemicals were above analytical grade (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Fish culture and care

The medaka fish were bred at the Department of Agricultural Chemistry, National Taiwan University, according to the animal research protocol approved by the Institutional Animal Care and Use Committee. The *p53* null mutant strain ($p53^{-/-}$), $p53^{E241X}$ (Strain ID: MT894) generated by (Taniguchi et al., 2006), was provided by the National Institute for Basic Biology, Japan, supported by the National BioResource Project Medaka (Sasado et al., 2010).