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The pyrethroid metabolites 3-phenoxybenzoic acid and 3-phenoxybenzyl alcohol do not exhibit estrogenic activity in the MCF-7 human breast carcinoma cell line or Sprague–Dawley rats

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ABSTRACT

Synthetic pyrethroids are one of the most frequently and widely used class of insecticides, primarily because they have a higher insect to mammalian toxicity ratio than organochlorines or organophosphates. The basic structure of pyrethroids can be characterized as an acid joined to an alcohol by an ester bond. Pyrethroid degradation occurs through either oxidation at one or more sites located in the alcohol or acid moieties or hydrolysis at the central ester bond, the latter reaction being important for mammalian metabolism of most pyrethroids. The primary alcohol liberated from the ester cleavage is hydroxylated to 3-phenoxybenzyl alcohol, which for most pyrethroids is then oxidized to 3-phenoxybenzoic acid. These products may then be conjugated with amino acids, sulfates, sugars, or sugar acids. *In vitro* studies have suggested that some of the pyrethroids may have estrogenic activity. Interestingly, the chemical structure of specific pyrethroid metabolites indicates that they may be more likely to interact with the estrogen receptor than the parent compounds. Two of the pyrethroid metabolites, 3-phenoxybenzoic acid (3PBA) and 3-phenoxybenzyl alcohol (3PBalc) have been reported to have endocrine activity using a yeast based assay. 3PBAlc exhibited estrogenic activity with reported EC_{50}s of 6.67×10^{-6} and 2×10^{-5} while 3PBAcid exhibited anti-estrogenic activity with a calculated IC_{50} of 6.5×10^{-5}. To determine if the metabolites were able to cause the same effects in a mammalian system, the estrogen-dependent cell line, MCF-7, was utilized. Cells were treated with 1.0, 10.0 or 100.0 μM concentrations of each metabolite and cytotoxicity was assessed. The two lowest concentrations of both metabolites did not induce cell death and even appeared to increase proliferation over that of the control cells. However, when cellular proliferation was measured using a Coulter counter neither metabolite stimulated proliferation (1.0 nM, 10.0 nM, or 10.0 μM) or induced an estrogen receptor α/ERE-controlled luciferase reporter in the MCF-7 cells. Following the *in vitro* screenings, the metabolites were then evaluated for estrogenic activity *in vivo* using the uterotrophic assay in Sprague–Dawley rats. Animals were orally gavaged (10.0, 5.0, and 1.0 mg/kg) once daily for 3 days. Neither metabolite had any effect on uterine wet weight, body weight, or organ weight. Lastly, in order to determine if either metabolite was able to alter the onset of puberty, immature female rats were orally gavaged (10.0, 5.0, and 1.0 mg/kg) once a day with the metabolites beginning 1 day post-weaning until the onset of puberty as evidenced by vaginal opening (VO). Again, neither metabolite had any effect on the onset of VO.

1. Introduction

Synthetic pyrethroids are used world wide to control insects in both agriculture and the home (Roberts and Hutson, 1999). These pesticides are synthetic analogs of the naturally occurring toxin, pyrethrin, derived from the flowers of *Chrysanthemum cinerariaefolium*. They have been further classified as type I or type II based on symptoms due to toxic exposure and the presence or absence of a cyano group at the carboxyl alpha position. Because of their high insect to mammalian toxicity ratio, pyrethroids have generally been regarded as safe for humans; and their use over the years has expanded. The neurotoxic effects, in both insects and mammals, are due to prolongation of the open state of the voltage-dependent sodium channels resulting in repetitive firing of the neurons (Soderlund et al., 2002; Vijverberg and van den Bercken, 1990).

While pyrethroids are less acutely toxic to mammals, there is concern that these synthetic pesticides may be endocrine disruptors; and they have been listed as such by the Environmental Protection Agency (Colborn et al., 1993; US EPA,

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1997). The endocrine disruptor designation is based primarily on *in vitro* studies indicating that specific pyrethroids may possess estrogenic activity. Deltamethrin, fenvalerate, cypermethrin, lambda-cyhalothrin, and permethrin have been shown to induce MCF-7 cell proliferation using the E-screen assay (Chen et al., 2002; Zhao et al., 2008). Fenvalerate, lambda-cyhalothrin, and sumithrin increased the expression of pS2, an estradiol (E_2) inducible gene in MCF-7 cells (Go et al., 1999; Chen et al., 2002; Zhao et al., 2008). Fenvalerate at 10.0 μM concentrations also induced alkaline phosphatase at the same level as 10.0 nM 17α-ethynylestradiol in Ishikawa Var-I cells (Garey and Wolff, 1998) as well as the proto-oncogene Wnt10B expression in MCF-7 cells (Kasat et al., 2002). Fenvalerate and deltamethrin were also shown to competitively bind to the estrogen receptor (ER) (Chen et al., 2002). However not all of the studies demonstrate estrogenic activity. Kim et al. (2004) found that neither fenvalerate nor deltamethrin had any effect on pS2 mRNA expression, ER binding, or ERα expression. Permethrin, fenvalerate, cypermethrin, and d-trans-allerthrin all failed to elicit any response in three ERα mediated assays (luciferase, yeast two-hybrid, competitive ligand binding) (Saito et al., 2000). Furthermore, when the pyrethroids fenvalerate, esfenvalerate, and permethrin were evaluated *in vivo* via the uterotrophic and Hershberger assays, the results were negative for both estrogenic and androgenic activity (Kunimatsu et al., 2002). Interestingly, our laboratory recently demonstrated that immature female rats exposed once per day by oral gavage to low doses (1.0 mg/kg) of the pyrethroid esfenvalerate beginning on postnatal day 22 and continuing until vaginal opening occurred, exhibited delayed pubertal onset and decreased serum estradiol, indicating this particular pyrethroid was able to perturb the neuroendocrine system (Pine et al., 2008).

Current dogma states that the neurotoxic effects are due to the parent compounds and that the metabolites are inactive. Both type I and type II pyrethroids undergo ester hydrolysis which results in a cyclopropyl acid and either 3-phenoxybenzyl alcohol (type I) or a cyanohydrin (type II) (Casida, 1983; Crawford et al., 1981). The aromatic alcohol is further oxidized to 3-phenoxybenzoic acid (3PBAcid) while the cyanohydrin is able to spontaneously rearrange to the phenoxybenzaldehyde in aqueous solutions and then undergo hydroxylation to 3PBAcid.

Interestingly, the *metabolites* are more likely to be xenoestrogen candidates than the parent compounds based on structure–activity relationships (Fang et al., 2001). In two separate recombinant yeast assays expressing the human estrogen receptor (hER), 3PBAlc exhibited estrogenic activity with a reported EC_{50} of 6.67×10^{-6} (McCarthy et al., 2006) and 2×10^{-5} (Tyler et al., 2000), while 3PBAcid exhibited anti-estrogenic activity with a calculated IC_{50} of 6.5×10^{-5} (Tyler et al., 2000). 3PBAcid is a nonspecific urinary metabolite, meaning that it is a breakdown product common to most of the pyrethroids with the exception of cyfluthrin. It is also the most frequently detected urinary metabolite in humans. 3PBAcid has been detected in urine from the general population in the United States (0.32 μg/l; CDC, 2005), Germany (2.0 μg/l reference value; Schulz et al., 2009), Italy (0.88 μg/l urban and 0.71 μg/l rural; Saieva et al., 2004) and Japan (0.29 μg/l; Ueyama et al., 2009). Levels in occupationally exposed populations such as pest control workers tend to be higher (6.8 μg/g creatinine; Hardt and Angerer, 2003).

While the recombinant hER assay is a useful screening tool, it is only able to detect interaction of a chemical with the estrogen receptor. To date the potential endocrine-disrupting capabilities of pyrethroid metabolites have not been examined *in vivo,* and what impact if any this should have on risk and safety evaluations of pyrethroids has not been established. This study was undertaken to measure the effects of two of the major metabolites, 3PBAlc and 3PBAcid, for possible endocrine-disruptive activity using both *in vitro* and *in vivo* mammalian assays, and thereby determine if these metabolites, should be addressed separately in risk assessments and safety evaluations.

2. Materials and methods

2.1. Cell culture

MCF-7 breast cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained as recommended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1.0% penicillin streptomycin (Gibco, Grand Island, NY), 0.01 mg/ml bovine insulin (Sigma Chemical Co., St. Louis, MO) and 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) in a humidified incubator kept at 5% CO^2 and 37 °C.

2.2. Pyrethroid pesticide metabolites

3PBAcid and 3PBAlc (Sigma Chemical Co., St. Louis, MO) were dissolved in corn oil (Sigma Chemical Co., St. Louis, MO) for the dosing studies. Dosing solution was made every other day and stored at room temperature protected from light.

2.3. Cytotoxicity assays

MCF-7 cells were challenged with 3PBAcid and 3PBAlc (0, 1.0, 10.0, or 100.0 μM) dissolved in DMSO. Briefly, cells were seeded onto a 96-well plate at 5×10^3 cells per well and allowed to attach over night. The next day growth medium was removed, cells were washed with phosphate buffered saline and test medium (DMEM w/out phenol-red, 2.5% charcoal-stripped FBS, and metabolite) was added. Cytotoxicity was assessed on days 3 and 5 using the CellTiter 96® Aqueous One Solution according to manufacturer's protocol (Promega, Madison, WI). $N = 6–8$ wells per treatment and each experiment was performed in duplicate.

2.4. Proliferation assays

MCF-7 cells were plated at a concentration of 2.5×10^4 cells per well in 12-well culture dishes and grown in phenol-red free DMEM, 2% charcoal-stripped FBS, and antibiotics. For the first experiment, cells were grown in either 10.0 nM estrogen; 10.0 μM, 10.0 nM, or 1.0 nM 3PBAcid; 10.0 μM, 10.0 nM, or 1.0 nM 3PBAlc; or DMSO vehicle. Concentrations of metabolite were chosen based on the results from the cytotoxicity assay. For the second experiment, cells were treated as previously described and in addition each treatment group received the anti-estrogen, ICI 182,780 also known as fulvestrant, at 100.0 nM concentration. Cells were harvested via trypsinization and counted using a Coulter Z1 particle counter (Beckman Coulter, Fullerton, CA) on days 3 and 6 post-treatment. $N = 3$ wells per treatment group and each experiment was performed in triplicate.

2.5. Luciferase reporter assays

MCF-7 cells were transfected with 200 ng of ERE3-luciferase reporter plasmid (a kind gift from Dr. Stephen Safe) and 100 ng of the internal control pβ-galactosidase using GeneJuice transfection reagent (EMD Biosciences, San Diego, CA). Briefly, MCF-7 cells were seeded at a concentration of 6×10^5 cells per well in 12-well plates in phenol-red-free DMEM containing 2% charcoal-stripped serum 24 h prior to transfection. Cells were then transfected with the reporter construct and the internal control, according to the manufacturer's protocol. The following day, medium was changed to phenol-red-free DMEM containing 2% charcoal-stripped FBS and either 10.0 nM estrogen; 10.0 μM, 1.0 μM, 10.0 nM, or 1.0 nM 3PBAcid, 10.0 μM, 1.0 μM, 10.0 nM, or 1.0 nM 3PBAlc; or DMSO vehicle, with and without anti-estrogen (100.0 nM ICI). After another 24 h cells were harvested and lysed, and the luciferase and β-galactosidase activities of the cell lysates were determined by dual luciferase assay using Luciferin (Promega, Madison, WI) and Galacto-Light (Tropix/Applied Biosystems, Foster City, CA). Luciferase activities were normalized to the internal control values and are represented as the mean ± S.E.M. $N = 4$ wells per treatment and each experiment was performed in triplicate.

2.6. Animals

Female Sprague–Dawley rats raised in our colony at the Texas A&M University Department of Comparative Medicine were used for these experiments. The animals were housed under controlled conditions of photoperiod (lights on, 06:00 h, lights off, 18:00 h) and temperature (23 °C), with *ad libitum* access to food and water. All procedures were approved by the University Animal Care and Use Committee and in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Adult female rats were bred and allowed to deliver pups normally. On postnatal day 2 (PND), pups were culled, if necessary, to equalize the litter size among dams. Litter sizes were maintained at 10–12 pups total with at least 5–6 females per litter. For all experiments littermates were randomly assigned to treatment groups.