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Evaluation of potential endocrine activity of 2,4-dichlorophenoxyacetic acid using *in vitro* assays

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ABSTRACT

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was evaluated in five *in vitro* screening assays to assess the potential for interaction with the androgen, estrogen and steroidogenesis pathways in the endocrine system. The assays were conducted to meet the requirements of the *in vitro* component of Tier 1 of the United States Environmental Protection Agency's Endocrine Disruptor Screening Program (EDSP), and included assays for estrogen receptor (ER) binding (rat uterine cytosol ER binding assay), ER-mediated transcriptional activation (HeLa-9903-ERα transactivation assay), androgen receptor (AR) binding (rat prostate cytosol AR binding assay), aromatase enzymatic activity inhibition (recombinant human CYP19 aromatase inhibition assay), and interference with steroidogenesis (H295R steroidogenesis assay). Results from these five assays demonstrated that 2,4-D does not have the potential to interact *in vitro* with the estrogen, androgen, or steroidogenesis pathways. These *in vitro* data are consistent with a corresponding lack of endocrine effects observed in apical *in vivo* animal studies, and thus provide important supporting data valuable in a comprehensive weight of evidence evaluation indicating a low potential of 2,4-D to interact with the endocrine system.

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D; CAS No. 94-75-7) is an herbicide active ingredient that has been registered and in use since 1946. The herbicide mode of action of 2,4-D is via increased cell-wall plasticity and abnormal increases in biosynthesis of proteins and ethylene resulting in uncontrolled cell division and damage to the vascular tissue of plants (USEPA, 2005). 2,4-D and its derivatives are currently registered for use on field, fruit, and vegetable crops and for use on pasture, turf, lawns, rights-of way, as well as aquatic and forestry applications and can be applied preplant, preemergence, post-emergence, prior to harvest, or during the dormant season (USEPA, 2005).

In 2009, 2,4-D was included in the first list of chemicals for Tier 1 screening in the United States Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP). Chemicals on this list were selected for evaluation based on exposure potential only, and not on any established suspicion of endocrine-like activity (Federal Register 74: 71 (April 15, 2009) p. 17579). Tier 1 EDSP screening evaluates a test substance for potential endocrine pathway interactions in the estrogen, androgen, and thyroid hormone systems using a weight of evidence approach that considers the results from the battery of eleven separate Tier 1 screening assays as well as other scientifically relevant information (USEPA, 2011). Five of the assays in the Tier 1 battery are *in vitro* assays; the remaining six assays in the battery are performed *in vivo* unless waived by EPA based on the availability of other scientifically relevant information.

The five *in vitro* assays in the Tier 1 battery include the estrogen receptor (ER) binding assay, the estrogen receptor transactivation assay (ERTA), the androgen receptor (AR) binding assay, the recombinant aromatase assay, and the steroidogenesis assay. The ER binding assay is an *in vitro* method for measuring the receptor-binding affinity of chemicals by their ability to displace the bound reference estrogen, radiolabeled [^{3}H]-17β-estradiol (E2), from the estrogen receptor in rat uterine cytosol (homogenate), which is interpreted as due to binding of the test material to ER (USEPA, 2009a). The ERTA uses the hERα-HeLa-9903 cell line stably transfected with the human estrogen receptor-alpha (hERα)-expression

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construct and a firefly luciferase reporter construct linked to ER-responsive promoter elements (USEPA, 2009b). The assay system measures the ability of a test chemical to induce hERα-mediated transactivation as indicated by expression of luciferase chemiluminescence (Takeyoshi et al., 2002). The AR binding assay is an *in vitro* method for measuring the AR-binding affinity of chemicals by their ability to displace the bound reference ligand, radiolabeled R1881 ([^{3}H]-methyltrienolone), from the AR present in rat prostate cytosol (homogenate), which is interpreted as due to binding of the test material to the AR (USEPA, 2009c). The recombinant aromatase assay is an *in vitro* method for detecting inhibition of aromatase enzymatic activity (USEPA, 2009d). Aromatase, also known as CYP19, is a member of the P450 superfamily of monooxygenase enzymes, and it plays an important role in catalyzing the conversion of androgens to estrogens during steroidogenesis. The steroidogenesis assay is an *in vitro* method for detecting test chemicals that may affect the steroidogenic pathway. The assay evaluates changes in the production of testosterone (T) and E2 in the human adrenocortical carcinoma cell line (H295R) (USEPA, 2009e; Hecker et al., 2006).

The results of the five *in vitro* EDSP tests reported herein provide important confirmatory data complementing a series of fish, amphibian, and apical rat *in vivo* endocrine evaluations which did not identify endocrine activity associated with 2,4-D treatment (Coady et al., 2013; Marty et al., 2013).

2. Materials and methods

2.1. Test materials

2,4-D acid (CAS No 94-75-7) from Nufarm Americas, Inc., (lot# 2006 2433 8006-USA; purity: 98.5%) was used in all five *in vitro* assays (Fig. 1). Positive and negative control chemicals for the assays, including 17β-estradiol (E2), 19-norethindrone, octyltriethoxysilane, 17α-estradiol, 17α-methyltestosterone, corticosterone, dexamethasone, forskolin, prochloraz, and 4-hydroxyandrostenedione, were all obtained from Sigma (St Louis, MO, USA). Methyltrienolone (radioinert), ^{3}H-methyltrieneolone, ^{3}H-estradiol, and ^{3}H-androstenedione were obtained from Perkin Elmer (Boston, MA, USA). Radioinert androstenedione was obtained from Steraloids (Newport, RI, USA). The vehicle controls, ethanol and dimethylsulfoxide (DMSO) were ordered from Sigma (St. Louis, MO, USA).

The highest concentration of 2,4-D in each of the assays was limited to 10^{-4} M based on *in vivo* toxicokinetic analyses; higher concentrations were not considered relevant for testing in this assay as they are substantially above the inflection point for linear toxicokinetics (Saghir et al., 2012, 2013) and far exceed actual exposure concentrations established through biomonitoring studies (Aylward and Hays, 2008; Aylward et al., 2010; Hays et al., 2012). The selected concentrations of 2,4-D in stock dosing solutions were verified by high performance liquid chromatography (HPLC) with ultraviolet (UV) detection in the recombinant aromatase assay and the ERTA and by high performance liquid chromatography with negative ion electrospray ionization and multiple reaction ion monitoring detection (HPLC/-ESI-MRM) in the ER and AR binding assays and the steroidogenesis assay.

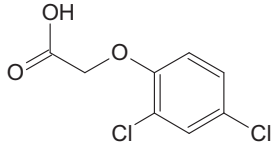

Fig. 1. Chemical structure of 2,4-D (acid).

2.2. ER binding assay

The ER binding assay was conducted according to the US EPA OPPTS 890.1250 test guideline (USEPA, 2009a), except that the highest concentration of 2,4-D was adjusted as discussed above. A total of eight log concentrations of 2,4-D were evaluated in the ER binding assay at concentrations ranging from 1×10^{-11} to 1×10^{-4} M. On the days of treatment, 2,4-D dosing solutions were prepared such that the final concentration of the solvent (ethanol) in the binding assay was less than 3%.

To conduct the ER binding assay, ^{3}H-E2, uterine cytosol, and either radioinert E2, 19-norethindrone, octyltriethoxysilozane, ethanol solvent, or 2,4-D were added to each sample tube as described in the OPPTS 890.1250 guideline (USEPA, 2009a). All samples were run in triplicate within each assay run, and incubation, wash and extraction steps were carried out as described in the guideline (USEPA, 2009a). The radiolabel in the final eluent was determined by liquid scintillation counting to measure the amount of ^{3}H-E2 retained in each sample as described in the guideline (USEPA, 2009a). Compounds that interact with the ER displace bound ^{3}H-E2, which was lost during the washing steps and results in lower radioactivity counts.

The binding of 2,4-D (*i.e.*, displacement of radiolabeled E2) was analyzed by a standard curve and 4-parameter (Hill) non-linear regression analysis (GraphPad Prism, version 5.0), and relative binding affinity of the test material compared to that of E2 was calculated. The ER binding assay with 2,4-D was replicated three times (*i.e.,* three independent runs). The criteria for classification of a response for a given run was based on the guideline where the curve-fit for the test material's lowest point was determined to be "interacting" if it displaced at least 50% of the ligand, "non-interacting" if it displaced less than 25%, and "equivocal" if it displaced between 25% and 50%. The average of the three independent runs was utilized for the ultimate characterization of a test chemical's response in the ER binding assay.

2.3. Estrogen receptor transactivation assay (ERTA)

The ERTA assay was performed with a stably transfected hERα-HeLa-9903 cell line that had recovered from freezing by culturing for at least two passages (USEPA, 2009b). A total of seven log serial concentrations of 2,4-D were evaluated in the assay at concentrations ranging from 1×10^{-10} to 1×10^{-4} M. As noted above, the high concentration specified in the test guideline was modified based on *in vivo* TK and biomonitoring data. DMSO was used to dissolve 2,4-D and was therefore included as the vehicle control treatment at a final concentration of 0.1% (v/v).

Following a 3-h attachment period with cell culture media the cells were dosed in triplicate and exposed for approximately 24 h at 37 °C and 5% CO_2 to either the vehicle control (DMSO), multiple concentrations of 2,4-D, or concurrent reference controls as described in the guideline (USEPA, 2009b). Following incubation and wash steps, firefly luciferase activity was quantified using a standard assay kit (Promega, Madison, WI, USA), per manufacturer's instructions. Chemiluminescence was measured immediately using a Packard TopCount NXT luminescence counter (Packard Instrument Company, Meriden, CT, USA). Cell viability/cytotoxicity testing was conducted on a separate plate in parallel to the 2,4-D transactivation exposure plate using CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Madison, WI, USA).

The luminescence response curves of the reference chemicals and 2,4-D were fitted using a non-linear regression program (Graph Pad Prism version 5.0). Results were expressed as relative transcriptional activity for each well compared to the response of the positive control, E2. The assay results were considered negative