Research Paper

Evaluation of the endocrine disrupting potential of Di-isodecyl phthalate

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ABSTRACT

Low molecular weight *ortho*-phthalates have been implicated in perturbing androgen pathways when administered during the masculinization programming window. Di-isodecyl phthalate (DIDP) is a high molecular weight phthalate and as a high production volume chemical, its ability to disrupt endocrine pathways is important to understand its potential hazard. Both DIDP (and its metabolites) were evaluated to determine the potential to perturb endocrine pathways through a weight of evidence (WoE) assessment in accordance with the European Chemicals Agency (ECHA)/European Food Safety Authority (EFSA) Endocrine Disruptor Guidance (2018). A literature review was performed of toxicological data for DIDP related to estrogen, androgen, thyroid, or steroidogenesis pathways. Literature searches returned 41 relevant articles from which data were extracted and assessed in conjunction with data from 105 high-throughput assays. Because some of the *in vitro* assays lack metabolic capabilities, an *in silico* assessment of estrogen (E), androgen (A), thyroid (T) or steroidogenesis (S) activity was conducted. Based on the available evidence for the T pathway, DIDP did not elicit adverse thyroid outcomes *in vivo*. When considering the T mechanistic data, there was evidence that DIDP induced the liver pregnane X receptor (PXR) and some indication that DIDP increased iodide uptake in the thyroid. As there were no studies evaluating thyroid hormone levels *in vivo*, a data gap was identified because per the ECHA/EFSA guidance, the lack of this information prevents drawing a conclusion on the T pathway. However, the E, A and S pathways were sufficiently assessed to conclude a limited or lack of E, A or S related apical outcomes in *in vivo* studies; there was also a lack of endocrine activity in *in vitro* or *in vivo* mechanistic studies. These results suggest that DIDP does not meet the ECHA/EFSA criteria for an endocrine disruptor, therefore DIDP is unlikely to disrupt the androgen pathway during development.

1. Introduction

An endocrine disruptor (ED) is an exogenous substance or mixture that alters function of the endocrine system and consequently causes adverse health effects in an intact organism, its progeny or subpopulations (WHO/IPCS 2002). In 2023 the European Commission amended the Classification, Labelling and Packaging (CLP) Regulation ((EC) No 1272/2008) to include endocrine disruption as a new hazard class. The CLP regulation requires all manufacturers, importers or downstream users of substances or mixtures to classify, label and

package hazardous chemicals appropriately before placing them on the market. The European Chemicals Agency (ECHA) is currently preparing an update of the Guidance on Application of the CLP criteria to chemicals, including guidance on the new hazard classes. Until then, the European Chemicals Agency (ECHA) has directed reliance on the ECHA/European Food Safety Authority (EFSA) Guidance for biocidal products and plant protection products (ECHA/EFSA, 2018).

In 1998 the Organisation for Economic Co-operation and Development (OECD) began identifying test guidelines for the screening and testing of endocrine disrupting chemicals (EDCs). These test methods are described in the OECD Conceptual Framework for the Screening and Testing of Endocrine Disrupting Chemicals published in 2018 (OECD 2018). This framework is an approach for organizing data and provides guidance on interpreting the strength of the evidence that a chemical may act as an ED. In 2018, EFSA and ECHA relied on the OECD conceptual framework in the development of additional guidance on how to gather, consider, and analyze data to comply with obligations under the Biocidal Products Regulation or the Plant Protection Products regulation to identify EDs (ECHA/EFSA, 2018). Chemicals identified as having ED properties have the potential to disrupt hormone pathways which, in turn, can lead to changes in apical endpoints that could be considered as an adverse event or outcome. Currently the focus of this guidance include estrogen (E), androgen (A), thyroid (T) and steroidogenic (S) pathways. According to the ECHA/EFSA guidance document, the process to evaluate EDCs includes a step-wise approach to data collection, establishing lines of evidence and, if relevant, development of a potential mode of action (MoA) that could establish a biologically plausible link between E, A, T or S activity and adverse events identified in animal models.

Phthalates are a group of synthetic compounds that are dialkyl or aryl/alkyl diesters of phthalic acid. They are widely used in the manufacturing of polymers and are found in a variety of consumer and industrial products. As a chemical class, *ortho*-phthalates have been widely studied as potential male reproductive toxicants. Studies have shown that for some low molecular weight *ortho*-phthalates, such as diethyl hexyl phthalate (DEHP; C6) or dibutyl phthalate (DBP; C4), exposure of rats during the prenatal period, particularly during the window of susceptibility for male development, results in a group of effects referred to as rat phthalate syndrome (e.g., cryptorchidism, hypospadias, impaired spermatogenesis/infertility, focal testicular dysgenesis) (Conley et al., 2018; Fisher et al., 2003; van den Driesche et al., 2017). These effects have not been commonly or consistently found in mice or marmosets (Do et al., 2012; Gaido et al., 2007; McKinnell et al., 2009) and have been linked to phthalates with a C3-C6 carbon backbone (Li et al., 2019).

Di-isodecyl phthalate (DIDP) is a high molecular weight phthalate that is a complex mixture of branched C9-C11 isomers containing mainly C10 isomers and a C7-C9 carbon backbone range, primarily C8. It is a plasticizer used to impart flexibility to polymers primarily in wire, cable, coatings, coated fabrics and sealant applications. In 2019, the EFSA Panel on Food Contact Materials reviewed the toxicological data for DIDP and based on data from a two generation reproductive toxicity (Hushka et al., 2001), two developmental toxicity (Hellwig et al., 1997; Waterman et al., 1999), and *in vitro* mechanistic (Hannas et al., 2012) studies established a NOAEL of 33 mg DIDP/kg bw/day for decreased survival in the F2 off-spring (Hushka et al., 2001). Based on these studies EFSA concluded that DIDP did not exhibit anti-androgenic activity (EFSA, 2019).

Given the inclusion of endocrine disruption as a new hazard class in the CLP regulation, a thorough and comprehensive review of the existing toxicological data for the potential endocrine effects of DIDP exposure occurring through E, A, T or S pathways was undertaken. In alignment with the recommendations by the Agency, the step-wise methodology described in the ECHA/EFSA ED guidance document (2018) was applied and a weight of evidence assessment was performed. Overall, this assessment showed that DIDP does not show ED activity in

the E, A or S pathways based on a lack of evidence for endocrine activity for which a biologically plausible link can be established to EAS-mediated effects *in vivo*. Despite a lack of serum thyroid hormone data, identified, by ECHA/EFSA, as a data gap, DIDP does not show ED activity in the T pathway based on a lack of T-mediated effects *in vivo*.

2. Methods

The ED potential of DIDP (CASRN 68515-49-1 or 26761-40-0) was assessed by applying the ECHA/EFSA guidance (2018) for identification of EATS pathways using the workflow provided in Supplemental Data (A – ED Assessment Strategy).

2.1. Evidence identification

Per ECHA/EFSA guidance (2018), an assessment of ED properties is based on all available relevant scientific data and includes both data generated in accordance with internationally accepted study protocols and other scientific data applying systematic review methodology (ECHA/EFSA, 2018). Structured searches of primary literature were performed for DIDP (February 29, 2024) and DIDP metabolites: mono-isodecyl phthalate (MiDP), mono-oxo-isodecyl phthalate (MOiDP), mono-carboxy-isononyl phthalate (MCiNP), and mono-hydroxy-isodecyl phthalate (MHiDP) (February 6, 2024). Searches were conducted in the PubMed and Embase literature databases for CASRN, chemical name, and synonyms and were not date limited. The full syntax for each literature search is provided in Supplemental Data (B – Literature Search Syntax). In addition, citation mining and hand searching of authoritative assessments of DIDP including ECHA (ECHA, 2013), EFSA (EFSA, 2019) and the US Environmental Protection Agency (EPA) (EPA, 2024) was performed (May 2024) to identify unpublished (industry) toxicology study reports describing DIDP *in vivo* studies. These unpublished reports were included in the assessment only when cited in publicly available sources. Information from high throughput (HT) assays available from the EPA Toxicity Forecaster (ToxCast) program (Kavlock and Dix, 2010) were queried for DIDP, MiDP, and secondary metabolites MOiDP, MCiNP and MHiDP. Assay data including activity calls, cytotoxic concentrations, and assay information, were downloaded from the US EPA's ToxCast downloadable data[1] and reviewed for activity in a battery of assays relevant to each of the EATS pathways.

Relevant studies were identified by title and abstract (TiAb) screening. If study relevance was unable to be determined at the TiAb level, the full text was examined. Inclusion/exclusion criteria were defined by a PECO (Population, Exposure, Comparator and Outcome) as follows: P: Mammalian or non-mammalian species at any life stage; E: DIDP or DIDP metabolites; C: Humans exposed to lower levels of DIDP or experimental animal receiving vehicle-only; O: any EATS pathway endpoints for *in vivo*, *in vitro*, *in silico* or epidemiological studies. Studies considered eligible were moved forward to data extraction.

2.2. Data extraction

Study types included *in vivo* toxicology studies that evaluated effects on the reproductive and thyroid systems, *in vitro* assays in the peer reviewed literature, as well as HT assays[2] that mapped to the E, A, T, or S pathways. All assays/studies were used to identify potential effects and whether these effects occurred through a mechanism that involved perturbation of an endocrine pathway.

For included studies, endocrine-related and general toxicity endpoints were reviewed and extracted into the Excel template provided as

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Appendix E of the ECHA/EFSA guidance document (https://efsa.online library.wiley.com/doi/full/10.2903/j.efsa.2018.5311). Study metadata e.g., study principle, test substance purity and data for each endpoint was captured regardless of the direction or level of the reported change. The approximate relationship between parts per million (ppm) in the diet and dosage in the animals (mg/kg-bw/day) was based on average food consumption in young and old rats with 1 ppm DIDP in the diet approximately equivalent to 0.075 mg/kg-bw/day (Lehman et al., 1954; JECFA, 2000). For each *in vivo* and *in vitro* study, a study reliability assessment was made. Evaluations were performed based on the Klimisch scoring approach. This approach assigns scores of: reliable without restriction (1), reliable with restriction (2), not reliable (3) and not assignable (4) (Klimisch et al., 1997). If studies were scored as either 1 or 2, they were considered reliable and incorporated into the evaluation. If they were not reliable or not assignable, they were not incorporated in the evaluation.

2.3. Extraction and assessment of lines of evidence

In accordance with the ECHA/EFSA guidance document, lines of evidence were assembled using the data extracted from the literature and HT assays (ECHA/EFSA, 2018). Lines of evidence describe groups of parameters/endpoints that together can be used to assess potential endocrine activity and EATS-mediated effects (EFSA Scientific Committee et al., 2017). Endocrine activity was described by two lines of evidence including 1) *in vitro* mechanistic, and 2) *in vivo* mechanistic. Lines of evidence that identify apical and potentially adverse effects were extracted from *in vivo* studies (Supplemental Data C – Lines of Evidence). 'Adversity' and 'adverse' are used throughout the work presented herein as defined by ECHA/EFSA as a "change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences" (ECHA/EFSA, 2018).

EATS-mediated effects were identified in the ECHA/EFSA template as: 1) EATS-mediated, and 2) EATS-sensitive endpoints; generalized effects were identified in the template as systemic toxicity endpoints. Examples of EATS-mediated endpoints include reproductive organ histopathology, and developmental markers such as anogenital distance and vaginal opening (ECHA/EFSA, 2018). EATS-sensitive endpoints are considered as potentially sensitive to, but not diagnostic of EATS pathways e.g., reproductive outcomes such as gestation length, litter size and viability. Systemic toxicity data include endpoints such as body weight changes, as well as liver and kidney weight and histopathology, to support and contextualize the distinction between a chemical effect on the EATS pathways or more generalized toxicity. Epidemiological and *in silico* data were assessed as a line of supportive evidence for signals of effects for EATS-mediated endpoints associated with exposure to DIDP (ECHA/EFSA, 2018).

Each line of evidence was evaluated for each of the EATS pathways by applying a weight of evidence (WoE) assessment. Where data were available, if there were no endpoints mediated by EATS with changes that suggested adversity, the pathway was considered to have no evidence of an endocrine effect. Each line of evidence was categorized based on available data as having 'high', 'medium' or 'low' confidence in the findings. These categories defined as: 1) High – outcomes (positive or negative) observed in one or more study of high reliability (performed according to standardized test guidelines) with no conflicting results; 2) Medium – outcomes (positive or negative) observed in one or more studies or assays of partial reliability (non-guideline), with no conflicting results or one or more studies of high or partial reliability but with conflicting results (e.g., no change versus decreased testosterone levels) that could be explained by differences in study design; 3) Low – outcomes (positive or negative) were observed in one or more studies of high or partial reliability but with conflicting results that could not be

explained by differences in study design. These categories were based on those described by Escriva et al. (Escriva et al., 2021).

Based on ECHA/EFSA guidance, an assessment was performed of whether the available DIDP information was sufficient to support a conclusion on EATS-mediated adversity in humans and mammals (ECHA/EFSA, 2018). To perform this assessment, the DIDP dataset was compared to the available test guidelines for mammals described in Table 14 of the ECHA/EFSA Guidance (ECHA/EFSA, 2018). For assessment of EAS-mediated adversity, the endpoints foreseen to be investigated in an extended one-generation reproductive toxicity study (OECD TG 443) or a two-generation reproductive toxicity study (OECD TG 416) were compared to the endpoints measured in the available DIDP studies. For T-mediated adversity, the thyroid endpoints foreseen to be investigated in the following guidelines studies were compared to the DIDP studies: OECD TGs 407, 408, 416, 443 and 451–3. DIDP guideline and non-guideline studies were used to determine data sufficiency. Following this analysis, and according to the ECHA/EFSA guidance, if 'EATS-mediated' adversity was not sufficiently investigated and no 'EATS-mediated' adversity was observed, EATS-related endocrine activity was assessed. This was performed by determining whether the following information was available for DIDP: ToxCast estrogen receptor (ER) Bioactivity Model output or uterotrophic bioassay (E), Hershberger bioassay (A), H295R steroidogenesis assay and the aromatase assay (human recombinant) (S). Owing to the lack of T *in vitro* mechanistic tests, endocrine activity for the T pathway was considered sufficiently investigated if the T endpoints in the *in vivo* assays described above were investigated. The impact of missing data or a lack of evidence were considered when assessing the confidence in the findings. Missing data can either reduce the level of confidence in the findings or, when the data gap is filled by other lines of evidence, have no effect on the overall level of confidence.

2.4. Quantitative Structure Activity Relationship (QSAR) assessment of DIDP metabolites

DIDP metabolites MiDP, MOiDP, MCiNP and MHiDP were evaluated by means of the full endocrine activity suite within Leadscope Model Applier (v2022.0.2–3). The suite includes the following endpoints, all of which were considered to address potential for endocrine disruption activity:

- Androgen receptor (AR) binding
- AR transactivation antagonist
- Aromatase inhibition
- ER bioactivity
- Sodium iodide symporter (NIS) inhibition
- Thyroid peroxidase (TPO) inhibition
- Thyroid receptor (TR) binding and transactivation

Chemical structures of DIDP metabolites were searched in the EPA CompTox Chemicals Dashboard (v 2.2.1) to identify simplified molecular-input-line-entry system (SMILES) codes associated with each compound of interest. These SMILES codes and those representing the product in commerce were then run through the Leadscope interface for QSAR assessment. Descriptions of models used are provided in Supplemental Data (D – QSAR Model Predictions).

3. Results

3.1. Evidence identification and extraction

The literature searches for DIDP and DIDP metabolites identified 447 articles (after de-duplication) in PubMed and Embase and 6 additional primary peer reviewed articles from review of authoritative sources (Fig. 1). A total of 39 primary peer reviewed publications (containing data for mammalian toxicology and ecotoxicology) met the inclusion
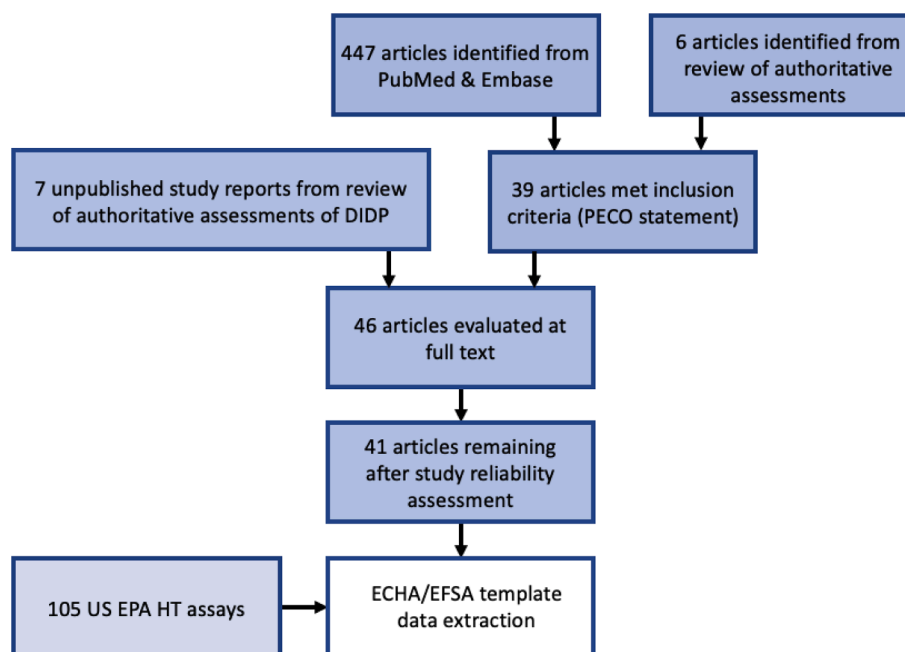
Fig. 1. Total Number of Articles Evaluated for the Comprehensive EATS Assessment of DIDP. Data were extracted into the ECHA/EFSA template from a total of 41 articles (lowest and darker blue box) and 105 HT assays (light blue box) that mapped to EATS-pathways. Key: PECO: Population, Exposure, Compatator and Outcome; EPA: Environmental Protection Agency; ECHA: European Chemicals Agency; EFSA: European Food Safety Authority. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

criteria and were therefore considered relevant and included in the evaluation. Seven industry toxicology study reports containing endocrine endpoints relevant to the EATS assessment were identified for DIDP by review of authoritative assessments of DIDP. In addition, HT assays that mapped to EATS endpoints were identified in the ToxCast database.[3] Data for a total of 105 publicly available ToxCast HT assays relevant to EATS pathways were downloaded and evaluated for activity; no HT data were available for DIDP metabolites.

Relevance and reliability were considered for each study evaluated at full text and used to categorize studies as either reliable (with or without restriction) or not reliable. Overall, five studies were scored as 'not reliable' and were removed from the endocrine weight of evidence assessment (Fig. 1). Studies excluded based on the relevance and reliability of data are identified in Supplemental Data (E – Reasons for Exclusion). Studies were excluded when, for example, no dose levels were provided or when insufficient information was available in the study report for confidence in its reliability.

Thirty-six mammalian toxicology studies were evaluated as part of the assessment and are described in Sections 3.3–3.6. Eleven ecotoxicology studies were evaluated as part of the assessment and are described in Section 3.7. One study contained both mammalian toxicology and ecotoxicology studies. Three of the 36 mammalian toxicology studies and two of the 11 ecotoxicology studies were excluded (Supplemental Data E – Reasons for Exclusion). Data were extracted from the remaining 41 studies (Supplemental Data F – All EATS Data).

Studies were classified according to the OECD Conceptual Framework for assessment of ED substances (OECD, 2018) that lists the OECD test guidelines and standardized test methods relevant to the evaluation of ED potential (Table 1). Only three of the studies available for DIDP were conducted according to standard test guidelines; of these, only two were listed in the OECD Conceptual Framework. Guideline studies

Table 1
OECD Conceptual Framework for Assessment of EDCs Used to Group Studies in the Evaluation of DIDP.

OECD Conceptual Framework [Test Method] Level[1]	Description of Test Methods
1	Non-test information e.g., epidemiology studies, *in silico* tools (read-across, quantitative structure activity relationships (QSAR))
2	*In vitro* assays providing data for endocrine mechanism(s) or pathway(s) e.g., H295R Steroidogenesis Assay (OECD TG 456)
3	*In vivo* assays providing data for endocrine mechanism(s) or pathway(s) e.g., Hershberger (OECD TG 441) or uterotrophic assays (OECD TG 440)
4	*In vivo* assays providing data for adverse effects on endocrine-relevant endpoints e.g., repeated dose 90-day study (OECD TG 408), prenatal developmental toxicity studies, reproduction/developmental toxicity tests (OECD TG 421, 422)
5	*In vivo* assays providing data on adverse effects on endocrine-relevant endpoints over multiple life stages (extended one-generation reproductive toxicity study (EOGRTS; OECD TG 443)), two-generation reproductive toxicity study (OECD TG 416 recent update).

Key: OECD: Organisation for Economic Co-Operation and Development; TG: test guideline; HT: high-throughput; EOGRTS: Extended One-Generation Reproductive Toxicity Study.
[1]OECD (2018), Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, OECD Series on Testing and Assessment, No. 150, OECD Publishing, Paris, https://doi.org/10.1787/9789264304741-en.

included OECD 414 – prenatal developmental toxicity study (Hellwig et al., 1997) and OPPTS 850.1500 – a non-validated, guideline test for fish life cycle toxicity (Patyna et al., 2006). One study was conducted according to OPPTS 870.3800 – reproduction and fertility effects (Hushka et al., 2001) that is not listed in the framework but is a two-

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[3] US EPA ORD, Center for Computational Toxicology (2018): ToxCast Database: invitrodb version 3.5. The United States Environmental Protection Agency's Center for Computational Toxicology and Exposure. Dataset. https://doi.org/10.23645/epacomptox.6062623.v10.

generation reproduction study and is comparable to OECD TG 416. Given the age of the studies and as the guidelines can be updated, it was not assumed that these 3 studies contained sufficient data to meet the current guideline standards. OECD states that the list of test methods provided in conceptual framework is not exhaustive and assays other than those described in the framework can used to assess ED. All studies (those conducted according to standard test guidelines and those that were not) were evaluated, and data extracted into the ECHA/EFSA Excel template provided as an appendix to the endocrine disruption guidance document that describes how to evaluate data to determine whether ED criteria are fulfilled. The DIDP mammalian literature set included three epidemiology studies and eight *in silico* endocrine models at Level 1, 105 HT assays and 12 *in vitro* studies at Level 2, three mechanistic studies at Level 3, 16 *in vivo* studies at Level 4 and one *in vivo* study over extended life stages at Level 5 (Fig. 2). Two studies were conducted at more than one test method level (levels 2 and 4).

Following the assessment of study reliability, as described in the ECHA/EFSA guidance (2018), the *in vivo* and *in vitro* mechanistic study methods (Levels 2 and 3) and the study endpoints measured were grouped to evaluate the ability of DIDP to perturb E, A, T, or S endocrine pathway activity. The available data and lines of evidence for mammals are summarized in Fig. 2.

3.2. Identification of data and integration of lines of evidence

Each line of evidence was organized by E, A, T or S (Supplemental Data F – All EATS Data). Where available, general adversity or systemic toxicity data were considered in conjunction with the EATS-mediated endpoints (OECD, 2018). *In silico* and epidemiological data were considered as supportive evidence only (ECHA/EFSA, 2018).

3.3. Analysis of evidence: Estrogen

In the assessment of potential endocrine activity mediated by E, the evidence for DIDP came from three different sources: *in silico* predictions, HT data and *in vitro* mechanistic assays (Table 2). The ToxCast HT and *in vitro* assays (peer reviewed literature) were not metabolically competent and lacked the capability to activate the DIDP diester to the monoester forms. The evidence available for DIDP metabolites (MiDP, MOiDP, MCiNP and MHiDP) came from *in silico* predictions for ER binding, agonist and antagonist activity and a single *in vitro* study of MiDP, MOiDP or MCiDP that showed overall, no evidence of activity (agonist or antagonist) at 100 μM using human ERα or ERβ reporter gene constructs (Table 2). *In vitro* mechanistic data for the E pathway showed DIDP had no effect on ER and estrogen-related receptor (ERR) binding (ToxCast HT assay data). No DIDP related effects were observed in other *in vitro* assays for ER agonism including an ER transactivation assay and ER-mediated cell proliferation assay (Ghisari and Bonefeld-Jorgensen, 2009; Harris et al., 1997; Lee et al., 2019; Akahori et al., 2005; Akahori et al., 2008; Zacharewski et al., 1998). Assays were conducted in MCF-7, ZR-75, Hela (up to 10^{-5} M DIDP) and MVLN (up to 20 mg/L DIDP) cells as well as yeast (up to 10^{-3} M DIDP). In one study conducted in porcine granulosa cells, an increase in FSH-stimulated progesterone production and decrease in FSH-stimulated estradiol production was reported following exposure to 10^{-4} M DIDP for 72 h although no change in basal progesterone or estradiol production was noted (Mlynarcikova et al., 2007). The CERAPP ER computational models (also trained on HT data) predicted DIDP to be negative for ER-related activity (binding, agonism, and antagonism).

The potential for estrogen-mediated adversity was evaluated in a total of six *in vivo* studies (Table 2). Findings were observed in one guideline study (OPPTS 870.3800, related to OECD TG 416) conducted in rats exposed to DIDP in feed (0, 2000, 4000, 8000 ppm; approximately 150, 300, 600 mg/kg-bw/day) in P1 (ovary and uterus) and F1 (ovary) generations (Hushka et al., 2001). Changes were observed at the highest exposure concentration (8000 ppm, approximately 600 mg/kg/

day) and occurred in the absence of histopathological changes. No changes were observed in vaginal histopathology, age at vaginal opening, anogenital distance, nipple development, or the estrous cycle in this study. Significantly decreased body weight was observed in high dose P1 (~9–10 % lower than controls) and F1 (≥6% lower than controls) animals suggesting the findings occurred due to systemic toxicity. In another guideline prenatal developmental toxicity study (OECD 414), no significant change in gravid uterus weight was observed following oral gavage administration of DIDP (up to 1,000 mg/kg-bw/day) from GD 6 – 15 (Hellwig et al., 1997). No change in gestational (number of implantations and corpora lutea, and pre- or post-implantation loss) or fetal (number viable fetuses or fetal malformations or growth retardation) endpoints were reported (considered sensitive to, but not diagnostic of, EATS and summarized in Supplemental Data (F – All EATS Data)).

Four non-guideline studies (repeat dose and carcinogenicity studies) showed no changes in EATS-mediated endpoints (histopathological evaluations of the uterus, ovary, mammary glands or vagina) following dietary exposure of adult animals up to 10,000 ppm DIDP (approximately 750 mg/kg-bw/day) (Cho et al., 2011; Exxon Biomedical Sciences Inc, 1986; Hazleton Laboratories Inc, 1968a; b). Detailed information for each study is provided in Supplemental Data (F – All EATS Data).

3.4. Analysis of evidence: Androgen

In the assessment of endocrine activity mediated by A, the evidence for DIDP came from three different sources: *in silico* predictions, HT data and *in vitro* mechanistic assays (Table 3). The ToxCast HT and *in vitro* assays (peer reviewed literature) were not metabolically competent and lacked the capability to activate the DIDP diester to the monoester forms. The evidence available for DIDP metabolites (MiDP, MOiDP, MCiNP and MHiDP) came from *in silico* predictions for AR binding, agonist and antagonist activity (Table 3) and a single *in vitro* study of MiDP, MOiDP or MCiDP that showed no evidence of activity (agonist or antagonist) at 100 μM using human AR reporter gene constructs (Table 3). *In vitro* mechanistic data for the A pathway showed DIDP had no effect on AR binding (ToxCast HT assay data). No DIDP related effects were observed in *in vitro* assays for AR agonist or antagonist activity (Takeuchi et al., 2005; Engel et al., 2017; Kruger et al., 2008). Assays were conducted up to concentrations of 100 μM DIDP. Data from a Hershberger assay designed to evaluate the antiandrogenic effects of DIDP (Lee and Koo, 2007) in 4 week old, castrated male rats was extracted. DIDP was administered orally, by gavage, (0, 20, 100, or 500 mg/kg-bw/day) in combination with testosterone propionate (TP) (0.4 mg/kg/day subcutaneously) for 10 days. TP (0.4 mg/kg/d), was used as a positive control. Decreased prostate and seminal vesicle weights were observed in the high dose group (p < 0.05) compared to TP alone, but no change in levator ani/bulbocavernosus muscles (LABC), Cowper's gland or glans penis weights. These changes occurred in conjunction with an increased liver weight (p < 0.05) and a decrease in serum testosterone at the same dose level. No effects were observed on Cowper's glands, glans penis, LABC weights or adrenal weights and an increase in luteinizing hormone (LH) at 100 and 500 mg/kg-bw/day DIDP. No evidence of general toxicity was observed, with no changes in mortality or body weight. According to OECD test guidelines, a statistically significant reduction in any two of the target tissues relative to TP along with some degree of reduced growth in all the target tissues is considered a positive androgen antagonist result (OECD, 2009). Evidence for DIDP related endocrine activity for the A pathway is summarized in Table 3.

Lines of evidence assessing A-mediated adversity that resulted in changes in apical endpoints were evaluated in two groups: gestational exposure and adult exposure (Table 3). Nine studies reported DIDP exposure in adult rats. Dietary exposure concentrations up to 20,000 ppm (approximately 1,500 mg/kg-bw/day) for 14 or 28 days, or three months; or 10,000 ppm (approximately 750 mg/kg-bw/day) for 6, 24,
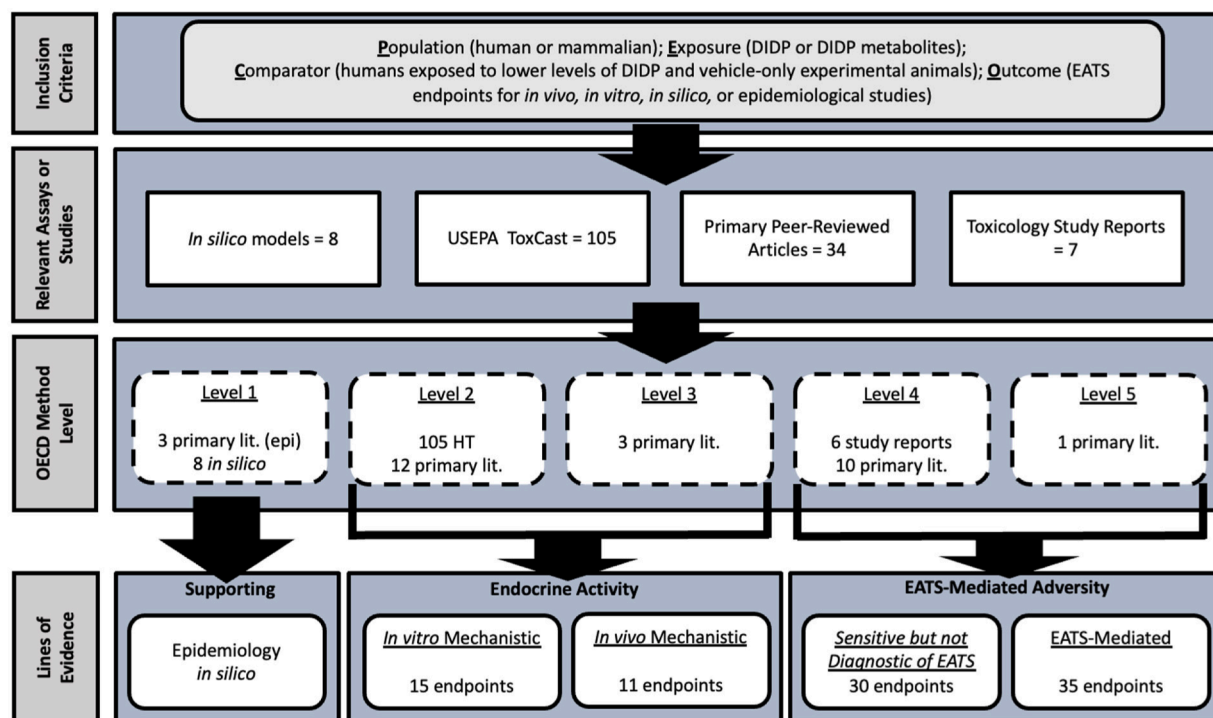
Fig. 2. Available DIDP Studies and Integrated Lines of Evidence for Outcomes and Endocrine Activity for E, A, T or S Pathways in Mammals. The number of studies or HT assays from which data was extracted for each OECD test method level is shown (Relevant Assays or Studies). Five studies excluded from the data assessment are not included in this count. The number of studies or assays at each OECD method level is provided (each study may be counted at more than one method level). The lines of evidence show the number of unique endpoints that provide information on E, A, T, or S endocrine pathway activity or adversity. Key: Lit: Literature.

26 or 104 months reported no dose-related changes in testis weight and histopathology, or prostate, epididymis or seminal vesicle histopathology (Cho et al., 2011; Exxon Biomedical Sciences Inc, 1986; Hazleton Laboratories Inc, 1968a; b; Cho, 2008; Exxon Biomedical Sciences Inc, 1982a; b; Kwack et al., 2010; Kwack et al., 2009). A single study in adult rats administered 500 mg/kg-bw/day DIDP for 14 days by oral gavage reported decreased relative testis weight (no absolute weights provided) without a co-incident decrease in body weight compared to the control group. The single dose level means no dose relationship could be established and no histopathological analysis was performed to assess the relevance of this finding (Kwack et al., 2010). As the results conflict with other studies measuring testis weight, and as no dose response could be established for this finding, the reliability of this finding was considered low. In another study using a single dose level (500 mg/kg-bw/day), rats were administered DIDP for 28 days by oral gavage. An assessment of reproductive endpoints reported decreased sperm motility (p < 0.05) compared to the control group (straight-line velocity, curvilinear velocity, straightness, and linearity) but no change in average path velocity, amplitude of the lateral head displacement, or beat cross frequency) and no change in sperm numbers (Kwack et al., 2009). The study used a small group size (n = 6) and a single dose reducing the confidence in the findings.

Evaluation of the androgen pathway disrupting potential of DIDP was performed in five reproductive or developmental studies (Hushka et al., 2001; Hannas et al., 2012; Zhang et al., 2020). No changes in organ weights (testis, prostate, epididymis or seminal vesicle) or histopathology (testis, prostate, or epididymis) were reported in parental or F1 animals in a two-generation study (OPPTS 870.3800; (Hushka et al., 2001) in which rats were exposed to dietary concentrations of 0, 2000, 4000 or 8000 ppm DIDP (approximately 150, 300, or 600 mg/kg-bw/day). In one study, dose dependent increases in the incidence of multinucleated germ cells and changes in Leydig cell distribution in testis were reported in GD 21 rats following oral gavage of dams with 0, 10,

100, 500 or 1000 mg/kg-bw/day DIDP from GD 14–21. No changes in the number of fetal Leydig or Sertoli cells were reported; however, a non-dose dependent decrease in serum testosterone was detected in GD 21 male pups exposed to the highest dose of DIDP (1000 mg/kg-bw/day) (Zhang et al., 2020). In contrast, two studies in which dams were orally administered 0, 100, 300, 600, 900 mg/kg-bw/day DIDP or 0, 500 mg/kg-bw/day DIDP from GD 14–18, no changes in fetal testicular testosterone were observed in GD 18 rat fetuses (Hannas et al., 2012; Gray et al., 2021). A two-generation study in rats exposed to dietary concentrations of 0, 200, 600, 2000, 4000 ppm DIDP (approximately 0, 16, 48, 160 or 320 mg/kg DIDP) produced no consistent changes in developmental markers, anogenital distance or age at balanopreputial separation in F1 or F2 off-spring. Balanopreputial separation was slightly delayed (1.2 days) following exposure to 4000 ppm DIDP in F2 off-spring; no change was observed in F1 off-spring. In a second two-generation study in rats, exposure to dietary concentrations of 0, 2000, 4000 or 8000 ppm (approximately 150, 300, or 600 mg/kg-bw/day) produced no changes in sperm endpoints, morphology, motility and number in parental animals (Hushka et al., 2001).

Supporting epidemiological evidence was provided by two prospective cohort studies in humans that assessed the association of DIDP urinary metabolites with age of pubertal onset or sperm endpoints (Minguez-Alarcon et al., 2022; Burns et al., 2022). No association was observed between the molar sum of DIDP metabolite concentrations for MHiDP, MOiDP, MCiNP, mono-(3-carboxypropyl) phthalate (MCPP) and delayed pubertal onset (Burns et al., 2022). Similarly, no association was observed between the molar sum of the DIDP metabolite concentration (MHiDP, MOiDP, MCiNP, and MCPP) on sperm motility or count (Minguez-Alarcon et al., 2022).

3.5. Analysis of evidence: Thyroid

In the assessment of endocrine activity mediated by T, the evidence

Table 2

Integration and Assessment of Lines of Evidence for DIDP and DIDP Metabolite Disruption of the E pathway. Key: HT: high throughput; LOAEL: lowest observed adverse effect level; ER: estrogen receptor; E: estrogen; QSAR: quantitative structure activity relationship.

	Line of Evidence	Observed Effects and Confidence in the Line of Evidence[1,2]	Assessment of and Confidence in Integrated Lines of Evidence[3]
Integrated lines of evidence for endocrine activity	ER binding/ transactivation	• No activity in HT assays[4] (Medium) Weak or no activity in *in vitro* assays (Medium) (Ghisari and Bonefeld-Jorgensen, 2009; Harris et al., 1997; Lee et al., 2019; Akahori et al., 2005; Akahori et al., 2008; Zacharewski et al., 1998; Takeuchi et al., 2005) DIDP activated ERα or ERβ (LOAEL 10 μM) in reporter gene assay (Low) (Engel et al., 2017) • MiDP, MOiDP or mono-carboxy-isodecyl phthalate (MCiDP) did not activate ERα or ERβ at 10 μM (Engel et al., 2017) No inhibition of E2 stimulated ERα or ERβ activity by DIDP, MiDP, MOiDP or MCiDP (Medium) (Engel et al., 2017)	**Overall negative for endocrine activity** There were no lines of evidence for endocrine activity.
	ERR binding	• No activity in HT assays[4] (Medium)	
	Uterotrophic (estrogenic / anti-estrogenic)	• No increase in uterine weight or decrease in uterine weight when co-administered with 17β −ethynyl estradiol (High) (Akahori et al., 2008; Zacharewski et al., 1998)	
Integrated lines of evidence for E-mediated adversity (adult exposure)	Organ weight	• No change in ovary weights at up to 6,000 ppm DIDP (Medium) (Exxon Biomedical Sciences Inc, 1986)	**Overall negative for E-mediated adversity (adult exposure to DIDP)** The lines of evidence show a lack of adversity in endocrine mediated endpoints. Findings were consistent in 4 rodent studies.
	Organ histopathology	• No change in ovary, mammary glands, uterus, vagina at up to 10,000 ppm DIDP (Medium) (Cho, 2011; Exxon Biomedical Sciences Inc, 1986; Inc, 1968; Inc, 1968)	
Integrated lines of evidence for E-mediated adversity (*in utero* exposure)	Organ weight	• Decreased ovary and uterus weight at 8,000 ppm DIDP (High) (Hushka, 2001) No change in ovary or uterus weight at up to 1,000 mg/kg-bw/day DIDP (Medium) (Hellwig et al., 1997)	**Overall negative for E-mediated adversity (developmental exposure to DIDP)** The lines of evidence show a lack of adversity in endocrine mediated endpoints. Findings were consistent in rodent studies with effects observed only at dose levels that caused general toxicity.
	Organ histopathology	• No change in ovary, uterus, vagina at up to 8,000 ppm DIDP (Medium) (Hushka, 2001)	
	Estrous cycle	• No change in the estrous cycle at up to 8,000 ppm (High) (Hushka, 2001)	
	Developmental markers	• No change in age at vaginal opening, anogenital distance, or nipple development at up to 8,000 ppm (High) (Hushka, 2001)	
Evidence of general toxicity	Body weight	• Decreased body weight observed at 8,000 ppm DIDP (High) (Hushka, 2001)	
Supportive Evidence	*In silico* predictions	• Negative QSAR prediction for ER agonism and antagonism (MiDP, MCiNP, MHiDP, MOiDP ToxCast DIDP SMILE) [5] Positive QSAR prediction for ER agonism and antagonism (MiDP, MCiNP, MHiDP, MOiDP commercial DIDP SMILE) [5] Negative for CERAPP potency consensus ER model[6]	This evidence provides weak support for a lack of E-mediated activity

[1] Endpoints that are 'sensitive to, but not diagnostic, of EATS' are reported in Appendix E – All EATS data.

[2] High: effects observed in one or more guideline study with no conflicting results; Medium: effects observed in one or more non-guideline studies with no conflicting results; effects observed in one or more guideline or non-guideline studies with conflicting results that could be explained by differences in study design; Low: effects were observed in one or more studies of guideline or non-guideline studies with conflicting results that could not be explained by differences in study design.

[3] Assessment of whether a sufficient dataset was available to support a conclusion on E activity in Section 3.8: Data Sufficiency and Identification of Data Gaps.

[4] https://www.epa.gov/comptox-tools/exploring-toxcast-data#Download (invitroDBv4.1, downloaded May 29, 2024).

[5] Leadscope Model Applier (v2022.0.2–3).

[6] CERAPP (Collaborative Estrogen Receptor Activity Prediction Project) described by (Mansouri, 2016).

for DIDP came from three different sources: *in silico* predictions, HT data and *in vitro* mechanistic assays (Table 4). The ToxCast HT and *in vitro* assays (peer reviewed literature) were not metabolically competent and lacked the capability to activate the DIDP diester to the monoester forms. The evidence available for DIDP metabolites (MiDP, MOiDP, MCiNP and MHiDP) came from *in silico* predictions for NIS and TPO inhibition as well as TR binding and activation (Table 4). *In vitro* mechanistic data for the T pathway showed DIDP had no effect on TR in 24 out of 25 assays (ToxCast HT assay data). There was a single active assay (ATG_trTRa_XSP2) but the reliability of this assay was low as there was only 2 replicates tested at each concentration and there was high

variability between the results for each replicate.[4] In liver nuclear receptor HT assays relevant to thyroid pathways (conjugation and elimination of the thyroid hormone (TH)) (Noyes et al., 2019), DIDP had no effect in constitutive androstane receptor [CAR] binding assays. In three (out of five) assays evaluating PXR, DIDP was active with an AC50 between 1.05 and 45 μM suggesting that DIDP induces drug metabolism enzymes. No activity was observed in thyroid stimulating hormone (TSH) or thyrotropin releasing hormone (TRH) receptor HT assays. *In vitro* mechanistic data for the T pathway showed DIDP increased iodide uptake (NIS stimulation) in rat FTRL-5 thyroid cells and increased *Nis* gene expression in rats PCCL3 thyroid cells. Increased iodide uptake in

[4] https://comptox.epa.gov/dashboard/chemical/invitrodb/DTXSID4025082 (invitroDBv4.1, downloaded May 29, 2024).

FTRL-5 cells occurred at the highest tested concentration only (10^{-3} M DIDP) following a 6-day exposure period) (Wenzel et al., 2005). Increased *Nis* gene expression in PCCL3 cells occurred after 48 h co-exposure to 0.001 M (1 mM) DIDP, and TSH (1.5 mU/ml) (Breous et al., 2005). No dose response was established in this single dose study and no change in expression occurred after 24- and 72-h exposure. In a TH-dependent rat pituitary (GH3) cell growth assay, DIDP induced cell proliferation following six days of exposure at highest tested dose (5×10^{-5} M) which did not appear to be cytotoxic. DIDP exposure at the same does levels had no effect on T3-induced cell proliferation (Ghisari and Bonefeld-Jorgensen, 2009). The Leadscope QSAR model predictions for DIDP metabolites (MiDP, MCiNP, MHiDP and MOiDP) were negative for NIS inhibition, TPO inhibition and TR binding and transactivation. Descriptions of these models are provided in Supplemental Data D – QSAR Model Predictions.

Thyroid-mediated adversity was assessed in a total of eight *in vivo* studies (Table 4). No changes in thyroid gland weight or histopathology were reported. Studies were conducted using dietary DIDP or oral gavage exposure of adult male and female rats, mice or dogs. Subacute, sub-chronic or repeat dose exposures (2, 4 or 13 weeks) were conducted using dietary concentrations up to 20,000 ppm (approximately 1500 mg/kg-bw/day) (Hazleton Laboratories Inc, 1968a; b; Exxon Biomedical Sciences Inc, 1982; Kwack et al., 2010; Kwack et al., 2009). Chronic exposures (12 and 24 months) were conducted using dietary concentrations up to 10,000 ppm (approximately 750 mg/kg-bw/day) in rats or mice (Cho et al., 2011; Exxon Biomedical Sciences Inc, 1986; Cho et al., 2008). There was no evidence of an apical effects that resulted from an increase in iodide uptake (an effect that was only observed in one *in vitro* assay).

In a supporting epidemiology study examining the association of the molar sum of DIDP urinary metabolites (MHiDP and MCiNP) with serum TH levels in pregnant women, higher levels of the sum of these metabolites were associated with lower total thyroxine (T4) levels but was not associated with changes to free triiodothyronine (T3), total T3, or TSH levels (Derakhshan et al., 2021). These results do not align with increased iodide uptake which would result in increased levels of TH.

3.6. Analysis of evidence: Steroidogenesis

In the assessment of endocrine activity mediated by S, the evidence for DIDP came from four different sources: *in silico* predictions, ToxCast HT assay data, *in vitro* and *in vivo* mechanistic assays (Table 5). The HT assay data and *in vitro* assays (peer reviewed literature) were not metabolically competent and lacked the capability to activate the DIDP diester to the monoester forms. The evidence available for DIDP metabolites (MiDP, MOiDP, MCiNP and MHiDP) came from *in silico* predictions for aromatase inhibition activity (Table 5). No *in vitro* studies of DIDP metabolites were available. *In vitro* mechanistic data for the S pathway showed DIDP had no effect in an HT aromatase assay (Table 5). No DIDP related changes were observed in other *in vitro* assays for S, including a H295R steroidogenesis assay (Lee et al., 2019). The assays were conducted in human adrenocortical carcinoma cells (H295R) exposed to 0, 0.8, 4 or 20 mg/L DIDP for 48 h. DIDP had no effect on testosterone levels and while a statistically significant increase in the level of estradiol was detected at 20 mg/L, the difference compared to control was small (less than 2-fold) and the authors concluded there was no estrogenic effect based on no change in the estradiol (E2) to testosterone ratio. Analysis of steroidogenic gene expression also showed no change in steroidogenic enzymes, steroid regulatory, and transport protein genes in GD 18 or 21 rat pups following *in utero* exposure to up to 1,500 mg/kg-bw/day DIDP (GD 14 – 18 or 21). In a mechanistic study with a comparable design, decreased expression of *Cyp11a1*, *Hsd17b3*, and *StAR* genes and proteins was reported in GD 21 rats following *in utero* exposure to 1,000 mg/kg-bw/day DIDP from GD 14–21. The Leadscope Aromatase Inhibition QSAR model prediction showed DIDP metabolites to be negative. This model used a training set of 4953

structures intended to predict the outcome of the Tox21 Aromatase Inhibition assay (Supplemental Data D – QSAR Model Predictions).

The effects of DIDP on hormone levels in different *in vivo* or *in vitro* test systems showed an increase in progesterone production and decrease in estradiol production in porcine granulosa cells following FSH-stimulation and exposure to 10^{-4} M DIDP for 72 h (Mlynarcikova et al., 2007). However, no changes were observed in fetal testicular testosterone in GD 18 or 21 rat pups following *in utero* exposure to up to 1,500 mg/kg-bw/day DIDP (GD 14 – 18 or 21) (Hannas et al., 2012; Gray et al., 2021). Decreased serum testosterone was observed in GD 21 pups at the high dose only following *in utero* exposure to 10, 100, 500 or 1,000 mg/kg-bw/day DIDP (GD 14–21) (Zhang et al., 2020).

3.7. Analysis of evidence: Non-mammalian species

Data were extracted from eight ecotoxicology studies (Supplemental Data F – All EATS Data). Data were available for fish (*Danio rerio* and *Oryzias melastigma*) and amphibian (*Xenopus laevis*) species as well as *Daphnia magna* and *Chironomus riparius*. One Level 5 assay was conducted according to OECD test guidelines (OECD TG 240[5]) to assess endpoints relevant to E, A, or S pathways (OECD, 2018; Patyna et al., 2006). The study was an extended one generation assessment of dietary exposure of Medaka fish to 19.3 mg DIDP /kg feed. There were no significant effects on survival, development, growth, and reproduction after 284 days of exposure (Supplemental Data G – Summary Non-mammalian Data). The authors conclude that DIDP had no estrogenic/antiestrogenic or androgenic/antiandrogenic effects based on the lack of changes. Data for all studies are provided in Supplemental Data (F – All EATS Data).

3.8. Data sufficiency and identification of data gaps

According to the ECHA/EFSA guidance, an assessment of the sufficiency of the dataset is required to support a conclusion on the absence of ED potential. Firstly, a determination of whether a dataset is sufficient to support a conclusion on the absence of EATS-mediated adversity is made. According to the assessment strategy, if 'EATS-mediated' adversity was not sufficiently investigated (i.e., the dataset was insufficient) and no 'EATS-mediated' adversity was observed, then EATS-related endocrine activity should be considered to support a conclusion on the absence of ED potential (ECHA/EFSA, 2018). To perform the data sufficiency assessment, endpoints measured in all DIDP studies (guideline and non-guideline) were compared to the endpoints included in regulatory test guidelines based on the ECHA/EFSA recommendation to consider all available information (Supplemental Data H – Sufficiency Assessment).

To assess the sufficiency of the DIDP dataset to conclude on EATS-mediated adversity, the endpoints measured in the DIDP guideline two-generation reproductive toxicity study (OPPTS 870.3800, related to OECD TG 416) (Hushka et al., 2001) were compared to the ECHA/EFSA recommended extended one- or two-generation reproduction toxicity studies (OECD TG 416 or 443, OECD Level 5 test methods). Overall 30 out of the 46 recommended E, A, T, or S endpoints in OECD TG 416 (Table 14; (OECD, 2018) were measured by Hushka et al. (Supplemental Data H – Sufficiency Assessment). The two-generation reproductive toxicity study (OECD TG 416) does not require assessment of thyroid hormones. Guideline and non-guideline repeated dose or reproductive toxicity studies for DIDP (OECD Level 4 test methods) were next considered to determine whether the remaining recommended endpoints had been investigated for DIDP. This assessment provided information for an additional 7 endpoints including thyroid weight and

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[5] OECD (2023), *Test No. 240: Medaka Extended One Generation Reproduction Test (MEOGRT)*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264242258-en.

Table 3

Integration and Assessment of Lines of Evidence for DIDP and DIDP Metabolite Disruption of the A Pathway. Key: HT: high throughput; AR: androgen receptor; DHT: dihydrotestosterone; LABC: levator ani/bulbocavernosus muscles; LH: luteinizing hormone; GD: gestational day; QSAR: quanitative structure activity relationship.

	Line of Evidence	Observed Effects and Confidence in the Line of Evidence[1,2]	Assessment of and Confidence in Integrated Lines of Evidence[3]
Integrated lines of evidence for endocrine activity	AR binding/ transactivation	• No activity in AR binding or transactivation in HT assays[4] (Medium) No AR agonist or antagonist activity in *in vitro* assays (Medium) (Takeuchi, 2005; Engel, 2017; Kruger et al., 2008) MiDP, MOiDP or MCiDP did not activate AR at 10 µM (Medium) (Engel, 2017) No inhibition of DHT stimulated AR activity by DIDP, MiDP, MOiDP or MCiDP (Medium) (Engel, 2017)	**Overall negative for endocrine activity** The lines of evidence show a lack of endocrine activity. Increased liver weight and reduction in serum testosterone levels in Hershberger assay suggests induction of liver enzymes and increased metabolism and clearance of TP leading to decreased tissue weight.
	Hershberger (antiandrogen effects in castrated immature rats)	• Decreased prostate and seminal vesicle weight, increased liver weight. No effect on Cowper's glands, glans penis, LABC weights or adrenal weight. Decrease in serum LH and exogenously administered testosterone levels (High) (Lee and Koo, 2007)	
Integrated lines of evidence for A-mediated adversity (adult exposure)	Organ weight	• No change in testis or epididymis weight at dose levels up to 20,000 ppm (Medium) (Cho, 2011; Exxon Biomedical Sciences Inc, 1986; Inc, 1968; Inc, 1968; Cho, 2008; Exxon Biomedical Sciences Inc, 1982a; b; Kwack, 2010; Kwack, 2009) Decreased relative testis weight at 500 mg/kg-bw/day DIDP. No change in body weight. Single dose study with no dose response established (Low) (Kwack, 2010)	**Overall negative for A-mediated adversity (adult exposure to DIDP)** The lines of evidence show a lack of adversity associated with endocrine perturbation in adult-exposed rodents. A single dose study reported decreased motility in adult males exposed to 500 mg/kg-bw/day DIDP for 28 days. These findings were mitigated by the lack of changes in reproductive endpoints in a two-generation study.
	Histopathology	• No change in testis, prostate, or epididymis histopathology at dose concentrations up to 10,000 ppm (Medium) (Cho, 2011; Inc, 1986; Inc, 1968; Inc, 1968; Cho, 2008; Exxon Biomedical Sciences Inc (as cited by EPA), Thirteen Week Pre-chronic Oral Feeding Study in Fischer 344 Rats., 1982)	
	Reproductive parameters	• Decreased sperm straight-line and curvilinear velocity, straightness, and linearity at 500 mg/kg-bw/day. No change in average path velocity and amplitude and no change in sperm numbers. Single dose study with no dose response established (Low) (Kwack, 2009)	
	Organ weight	• No change in testis, epididymis, prostate, or seminal vesicle weights in parental and F1 generation animals (High) (Hushka, 2001)	**Overall negative for A-mediated adversity (developmental studies of DIDP)** The lines of evidence show a lack of adversity associated with endocrine perturbation resulting from *in utero* exposure during the male rat programming window. Inconsistent evidence of changes in fetal (GD 18) and serum testosterone at GD 21.
Integrated lines of evidence for A-mediated adversity (*in utero* exposure)	Histopathology	• No change in number of fetal Leydig or Sertoli cells; increased incidence of multinucleated gonocytes and dose-dependent change in Leydig cell distribution (Medium) (Zhang, 2020) No change in testis, prostate, or epididymis in parental or F1 generation animals (High) (Hushka, 2001)	
	Hormone levels	• Decreased serum testosterone at GD 21; dose dependency not established (Medium) (Zhang, 2020) • No change in fetal testicular testosterone production at GD 18 (Medium) (Hannas, 2012; Gray, 2021)	
	Developmental markers	• No change in age at balanopreputial separations, or anogenital distance in F1 or F2 off-spring (High) (Hushka et al., 2001)	
	Reproductive parameters	• No change in sperm morphology, sperm motility or sperm numbers in parental animals (High) (Hushka et al., 2001)	
Evidence of general toxicity	Body weight	• Decreased body weight observed at up to 10,000 ppm DIDP in adult, parental and F1 generation animals (High) (Hushka et al., 2001; Hellwig et al., 1997; Cho et al., 2011; Kwack et al., 2010; Kwack et al., 2009) • No change in body weight (Medium) (Hannas et al., 2012; Kwack et al., 2010; Zhang et al., 2020)	
Supporting evidence	Epidemiology	• No association between the molar sum of DIDP metabolite concentrations and delayed pubertal onset (Burns et al., 2022). • No association was observed between the molar sum of DIDP metabolite concentration and sperm motility or count (Minguez-Alarcon et al., 2022)	Epidemiological data provides lines of evidence for a lack of A-mediated adversity. The QSAR evidence provides weak support for a lack of AR binding for DIDP metabolites.
	In silico prediction	• Positive QSAR predictions for AR transactivation antagonism (MiDP and MHiDP ToxCast and commercial DIDP SMILES) [5] • Negative QSAR predictions for AR transactivation antagonism (MCiNP and MOiDP MHiDP ToxCast and commercial DIDP SMILES) [5] • Negative QSAR predictions for AR binding (MiDP, MCiNP, MHiDP and MOiDP ToxCast and commercial DIDP SMILES) [5]	

histopathology, seminal vesicle histopathology (Exxon Biomedical Sciences Inc, 1982; Kwack et al., 2010; Kwack et al., 2009), number of implantations/corpora lutea, pre- and post-implantation loss and the presence of anomalies (external, visceral, skeletal) (Hellwig et al., 1997; Waterman et al., 1999). Genital abnormalities and dystocia were not specifically identified as measured endpoints in any of the studies describing the effects of DIDP exposure; however, no gross external abnormalities or histopathological lesions of the reproductive tract were observed in adult animals (P, F1 and F2) or F1/F2 neonates and no maternal lethality was reported (Hushka et al., 2001; Hellwig et al., 1997). Overall, the following EATS-mediated endpoints were not evaluated for DIDP: TH level (T3, T4 or TSH), cervix, coagulating gland, oviduct and vagina histopathology, and pituitary weights, thyroid follicular cell height, and time to mating. This analysis performed according to the sufficiency criteria outlined in ECHA/EFSA guidance (2018) showed that the dataset for E, A, and S pathways were minimally insufficient and for the T pathway was insufficient.

As the E, A, T and S datasets all showed at least minimal data gaps, an assessment of whether 'EATS-mediated' adversity was observed was performed next. Regarding the A, and S pathways, one study showed evidence of DIDP-mediated adversity (Zhang et al., 2020) with a decrease in expression of genes and proteins associated with the steroidogenesis pathway and a concomitant decrease in serum testosterone in GD 21 off-spring at 1;000 mg/kg-bw/day DIDP (Zhang et al., 2020). These results conflicted with other studies showing no change in steroidogenic gene expression in fetal rats (up to 1;500 mg/kg-bw/day) and no change in fetal testicular testosterone production (Hannas et al., 2012; Gray et al., 2021). Two other (low confidence) studies showed potential adversity resulting from DIDP exposure including a decrease in testis weight following a 14-day oral gavage exposure in adult rats (500 mg/kg-bw/day DIDP) and a decrease in some sperm parameters (straight-line velocity, curvilinear velocity, straightness, and linearity) following a 28-day exposure to 500 mg/kg-bw/day DIDP (Kwack et al., 2010; Kwack et al., 2009). Both studies used a small sample size (n = 6) and were single dose studies which precluded an assessment of a potential dose response effect and reduced confidence in the findings. No apical effects were observed for the E pathway and for the T pathway, one study showed a 'minimal increase in thyroid activity' in rats exposed to 10,000 ppm (approximately 750 mg/kg-bw/day) DIDP (Hazleton Laboratories Inc, 1968a). Overall, and despite some weak signals of effects, the WoE suggests no EATS-mediated adversity.

According to the ECHA/EFSA criteria, when the E, A, T and S dataset do not meet the sufficiency criteria and the available data reflect a lack of endocrine-mediated adversity, an assessment of endocrine activity is required. This can be assessed using *in vitro* and *in vivo* mechanistic endpoints. Sufficient investigation of EAS-related endocrine activity includes either information from the ToxCast ER Bioactivity Model or uterotrophic bioassay in rodents (OECD TG 440; E pathway), Hershberger bioassay in rats (OECD TG 44; A pathway), and the H295R steroidogenesis and aromatase *in vitro* assays (OECD TG 456 and OPPTS 890.1200 respectively; S pathway) (ECHA/EFSA, 2018). The ED potential of DIDP was investigated using each of these assay types. Therefore, the dataset to support E, A, and S endocrine activity was sufficient to support a conclusion on endocrine activity.

As summarized in Section 3.3, DIDP had no estrogenic activity in two uterotrophic assays (Akahori et al., 2008; Zacharewski et al., 1998). In a Hershberger assay, DIDP administration decreased two androgen-dependent tissue weights (seminal vesicle and prostate) in castrated rats (Lee and Koo, 2007). These changes occurred in conjunction with an increased liver weight and a decrease in exogenously administered testosterone. These results imply induction of liver enzymes, as well as increased metabolism and clearance of testosterone, which results in decreased androgen-sensitive tissue weights. The Hershberger assay, therefore, was determined to be negative for anti-androgenic activity.

Overall DIDP was negative for steroidogenic activity based on one Tox21 aromatase inhibition assay and negative findings in the H295R steroidogenesis assay (Lee et al., 2019). Overall, the sufficiency of the dataset based on the ECHA/EFSA sufficiency guidance (2018) and lack of findings demonstrated DIDP had no endocrine activity.

In vitro mechanistic test guidelines and *in vivo* mechanistic tests are not available for the T pathway in mammals. Therefore, to consider T-related endocrine activity to be sufficiently investigated, information for *in vivo* mechanistic endpoints from Repeated Dose studies (OECD TG 407, 408, 409, 416, and 451–3) (OECD, 2018) are required. No measurements of thyroid hormones (T3, T4, and TSH), thyroid follicular cell height, or colloid area were performed following exposure to DIDP. Overall, there was insufficient data to conclude on T-related endocrine activity.

As DIDP is rapidly and completely metabolized after oral administration in mammals, the potential endocrine activity of DIDP metabolites should be considered when assessing data sufficiency. Because metabolism is integral to the *in vivo* studies conducted to assess 'EATS-mediated' adversity endpoints (e.g., OECD TG 416), the data sufficiency assessment conducted for DIDP includes the metabolites. As the WoE suggests no EATS-mediated adversity; however, the presence of data gaps (TH level [T3, T4 or TSH], cervix, coagulating gland, oviduct and vagina histopathology, and pituitary weights, thyroid follicular cell height, and time to mating) means an assessment of EATS-related endocrine activity for DIDP metabolites is required. *In vivo* mechanistic evidence for DIDP is inclusive of DIDP metabolites, therefore, uterotrophic bioassays in rodents (E pathway) and a Hershberger bioassay in rats (A pathway) provide sufficient evidence to support a conclusion on E- and A-related endocrine activity. For the S pathway, the *in vitro* mechanistic assays do not include metabolism. As well, DIDP metabolites were not evaluated in the H295R steroidogenesis or aromatase assays, therefore, the S pathway was not considered sufficiently assessed to conclude on endocrine activity for the DIDP metabolites. For the T-related endocrine activity, a data gap was noted for DIDP (parent) and for the DIDP metabolites, and therefore, according to ECHA/EFSA (2018) sufficiency criteria, there was insufficient data to conclude on T-related endocrine activity or adversity.

3.9. Mode of action (MoA) assessment

A MoA assessment is performed when *in vivo* or *in vitro* mechanistic data align with adversity outcomes. For DIDP, there was very limited evidence of endocrine activity in the E, A or S pathways that was consistent with apical and potentially adverse outcomes (e.g., reproductive abnormalities). Some evidence showed changes in steroidogenic gene expression, testosterone levels in fetal males, and testis weight and sperm endpoints in adult animals but taking into consideration the confidence in the studies describing changes and the weight of evidence, the likelihood that DIDP induced apical and potentially adverse outcomes via E, A or S pathways was low. As such, an endocrine MoA was not supported, with no biologically plausible connection between endocrine activity and changes in apical endpoints.

Table 4

Integration and Assessment of Lines of Evidence for DIDP and DIDP Metabolite Disruption of the T Pathway.Key: TR: thyroid hormone receptor; TSH: thyroid stimulating hormone; HT: high throughput; NIS: sodium iodide symporter; TPO: thyroid peroxidase; QSAR: quantitative structure activity relationship; LH: luteinizing hormone; CAR: constitutive androstane receptor; TRH: thyrotropin releasing hormone; T4: thyroxine; T3: triiodothyronine; PXR: pregnane X receptor.

	Line of Evidence	Observed Effects and Confidence in the Line of Evidence[1,2]	Assessment of and Confidence in Integrated Lines of Evidence[3]
Integrated lines of evidence for endocrine activity	Receptor binding	• No activity in TR binding in 24/25 HT assays[4] (Medium) • No activity in TSH receptor binding in HT assay[3] (Medium) • No activity in TRH receptor binding in HT assay[3] (Medium)	**Evidence suggests DIDP induces Phase I metabolism enzymes and potentially stimulates iodide uptake in thyroid.** The lines of evidence show a lack of activity across KEs in the thyroid pathway (TR, TSH receptor and CAR binding). Two non-guideline studies show potential changes in NIS expression or iodide uptake.
	Iodide uptake	• Increased human NIS promoter activity and endogenous rat *Nis* mRNA levels in PCCL3 thyroid cells (Medium) (Breous et al., 2005) • Increased uptake of iodide in NIS uptake assay (Medium) (Wenzel, 2005)	
	Cell proliferation	• No effect on thyroid hormone mediated pituitary cell proliferation (Medium) (Ghisari and Bonefeld-Jorgensen, 2009)	
	Nuclear receptor induction	• No activity in hepatic CAR binding HT assays[3] (Medium) Activity detected in 3 of 4 assays of PXR binding HT assays[3] (Medium)	
Integrated lines of evidence for T-mediated adversity	Organ weight	• No change in thyroid weight at dietary concentrations up to 10,000 ppm (approx. 750 mg/kg-bw/day) (Medium) (Exxon Biomedical Sciences Inc, 1986; Hazleton Laboratories Inc, 1968a; b; Kwack, 2010; Kwack, 2009)	**Overall negative for T-mediated adversity.** The lines of evidence support the lack of adversity mediated by perturbation in the thyroid pathway. A consistent lack of effects was observed even at doses that produced general toxicity.
	Histopathology	• No changes were observed in thyroid histopathology in animals exposed to dietary concentrations up to 20,000 ppm (approx. 1500 mg/kg-bw/day) (Medium) (Cho et al., 2011; Exxon Biomedical Sciences Inc, 1986; Hazleton Laboratories Inc, 1968a; b; Cho, 2008; Exxon Biomedical Sciences Inc, 1982)	
Evidence of general toxicity	Body weight	• Decreased body weight at in high dose groups (Medium) (Hazleton Laboratories Inc, 1986a; b; Cho, 2008; Exxon Biomedical Sciences Inc, 1982)	
Supporting evidence	Epidemiology	• DIDP urinary metabolites negatively associated with total T4 levels in pregnant women (Derakhshan et al., 2021) DIDP urinary metabolites not associated with changes to free T3, total T3, or TSH levels (Derakhshan et al., 2021)	Epidemiological provides weak evidence for a lack of T-mediated adversity. The QSAR evidence provides weak support for a lack of NIS and TPO inhibition as well as TR binding and activation for DIDP metabolites.
	In silico prediction[1]	• Negative QSAR prediction in TPO inhibition and TR binding and activation model (MiDP, MCiNP, MHiDP and MOiDP commercial DIDP SMILE)[5] • Negative QSAR prediction in NIS inhibition (MCiNP, MHiDP and MOiDP) and indeterminant for MiDP (commercial DIDP SMILES)[5] • Negative QSAR predictions for TPO and NIS inhibition and TR binding and activation (MiDP, MCiNP, MHiDP and MOiDP ToxCast SMILES)[5]	

[1] Endpoints that are 'sensitive to, but not diagnostic of, EATS' are reported in Appendix E – All EATS data.

[2] High: effects observed in one or more guideline study with no conflicting results; Medium: effects observed in one or more non-guideline studies with no conflicting results; effects observed in one or more guideline or non-guideline studies with conflicting results that could be explained by differences in study design; Low: effects were observed in one or more studies of guideline or non-guideline studies with conflicting results that could not be explained by differences in study design.

[3] Assessment of whether a sufficient dataset was available to support a conclusion on T activity in Section 3.8: Data Sufficiency and Identification of Data Gaps.

[4] https://comptox.epa.gov/dashboard/chemical/invitrodb/DTXSID4025082 (invitroDBv4.1, downloaded May 29, 2024).

[5] Leadscope Model Applier (v2022.0.2–3).

4. Discussion

Due to their widespread industrial use and the well documented effects of low molecular weight phthalates on male reproductive tract development, as a chemical group, phthalates have been extensively reviewed by multiple regulatory authorities. With the upcoming addition of endocrine disruption to the list of information requirements considered under EU REACH regulations, this comprehensive review of the literature was conducted both to inform the likelihood that the high molecular weight phthalate, DIDP, could potentially act as an ED and to identify any limitations or data gaps in the existing evidence. Data limitations that were identified included the lack of guideline studies conforming to the most recent updates to OECD test guidelines. Many of the DIDP studies were conducted before the development and/or revision of test method guidelines. While these studies provided valuable and consistent information, the confidence in these methods cannot be classified as 'high' given the ECHA/EFSA sufficiency criteria requires data across all endpoints to be aligned with the latest test guidelines.

"Rat phthalate syndrome" describes a group of effects observed in male rats from exposure to low molecular weight phthalates (C3-C6 backbone) during a critical developmental window of male sexual differentiation. These effects include reproductive abnormalities characterized by malformations of the epididymis, vas deferens, seminal vesicles, prostate, and external genitalia (hypospadias or cryptorchidism) together with retention of nipples/areolae and demasculinization of the growth of the perineum resulting in a reduced anogenital distance (AGD) (Foster, 2006). Rat phthalate syndrome can be experimentally induced in rats by exposure to chemicals that block androgen synthesis or action during the embryonic period, GD 15.5–18.5 in rat (van den Driesche et al., 2017; Welsh et al., 2008). Phthalates shown to experimentally induce the syndrome include DEHP, DBP, and butyl benzyl phthalate (Fisher et al., 2003; Benson, 2009; Borch et al., 2006; Howdeshell et al., 2008; Kallsten et al., 2022) although in the case of DEHP (at least) this may not occur through the EATS pathways. DIDP is a high molecular weight phthalate that is a complex mixture of branched C9-C11 isomers containing mainly C10 isomers. A structure–activity

relationship has been described for phthalates with C3-C6 carbon chain length having a more potent effect on testis developmental and function than phthalates with higher chain length (Li et al., 2019).

Overall, there was sufficient data to support a conclusion on the potential for EATS-mediated adversity for DIDP in E, A, and S pathways. For the T pathway, a data gap was identified in the form of a lack of *in vivo* mechanistic data (TH levels), and the lack of morphometric measures of thyroid, including follicular cell height and colloid area. For DIDP metabolites, there was sufficient evidence to support a conclusion on EATS-mediated adversity for E and A pathways, but a lack of *in vitro* mechanistic data meant there was insufficient evidence to support a conclusion on endocrine activity the S and T pathways. ECHA/EFSA requires an assessment of the absence of EATS-related endocrine activity when both of the following are true 1) EATS-mediated adversity has not been sufficiently investigated as outlined in the guidance sufficiency criteria and 2) Within the available dataset, no EATS-mediated adversity was observed. In the case of DIDP and its metabolites, an assessment of EATS-related endocrine activity was triggered because there were slight inconsistencies in the adversity outcomes within the dataset and minimal data gaps identified when considering the stringent data sufficiency requirements in the ECHA/EFSA guidance. When evaluating the absence of EATS-mediated activity, if activity has not been sufficiently investigated the guidance reflects a need to generate additional information. However, while generating *in vitro* mechanistic data for these compounds may fill the data gap, the biological/molecular events that the new studies would measure are ultimately driving apical outcomes that were not consistently observed in *in vivo* studies. The analysis herein relies on the ECHA/EFSA guidance for the identification of EDs for biocidal and plant protection products as this is serving as an interim guidance until the Agency releases the guidance on application of CLP criteria for EDs. It remains to be understood if the sufficiency criteria and new data generation requirements will be harmonized across regulations. As an approach, the ECHA/EFSA guidance provides a structured approach to classifying large amounts of heterogenous data types into a defined numbers of endpoint categories albeit with some limitations such as the endpoint categories for non-mammalian data.

In the present analysis, four studies reported androgen pathway related outcomes resulting from exposure during the male programming window in rats (GD 15.5 – 18.5) (Hushka et al., 2001; Hannas et al., 2012; Zhang et al., 2020; Gray et al., 2021); however, effects were observed in only one of these studies (Zhang et al., 2020). In this study, animals (exposed from GD 14–21) examined at GD 21 showed altered testicular morphology at 1,000 mg/kg-bw/day DIDP and a reduction in the INSL3 protein hormone produced by the Leydig cells (Zhang et al., 2020). INSL3 plays a key role in testicular descent which begins around GD 15.5 in rats (Sharpe, 2020). In the study described by Zhang et al. (2020) evaluation of reproductive toxicity was restricted to histopathology of the testis at GD 21 and did not include evaluation at other timepoints or of other endpoints (e.g., AGD, testis descent or reproductive function), reducing the ability to evaluate if these changes culminated in a functional consequence. However, Zhang et al. (2020) did show decreased serum testosterone at GD 21 when DIDP was administered at 1,000 mg/kg-bw/day but not at lower dose levels. The changes were coincident with decreased expression for *Cyp11a1*, *Hsd17b3*, and *StAR* in male rat pups on GD 21. These genes encode enzymes that convert cholesterol to pregnenolone (CYP11A1 and STAR) and dehydroepiandrosterone (DHEA) to androstenediol (HSD17B3) (Miller & Auchus, 2011). In a 2-generation reproduction study, evaluation of AGD, nipple development, age at vaginal opening or blanopreputial separation and reproductive function (e.g., fertility) showed no consistent effects (Hushka et al., 2001). A slight delay in balanopreputial separation observed following exposure to 4000 ppm DIDP (approximately 320 mg/kg-bw/day) occurred in F2 off-spring, yet body weights on day of acquisition were similar to those of the concurrent control. This effect was not considered adverse as it was coincident with a significant, but transient, treatment related reduction in body weight

(6–9 % loss) of F2 males around the time of weaning (PNDs 14 and 21). As growth retardation prior to weaning has been associated with a delay in attainment of pubertal landmarks (Engelbregt et al., 2000), the change was small and in the range of unknown biological relevance (i.e., ~1 day) (EPA, 1996), and the effect was not observed in F1 off-spring this effect was not considered endocrine-related. In two other studies using a comparable study design and dose level (oral exposure between GD 14 – 18), there was no evidence of changes in expression of steroidogenic genes including *Cyp11a1* and *StAR* as well as *Cyp11b1*, *Scarb1*, *Cyp17a1*, *Insl3*, and *Hsd3b*, or changes in fetal testicular testosterone on GD 18 at 900 mg/kg-bw/day DIDP (Hannas et al., 2012) or 1;500 mg/kg-bw/day (Gray et al., 2021). Given the similarity in study design, it is difficult to reconcile the difference in findings of these studies. Both used small group sizes (n ≤ 12), and Zhang (2020) used a slightly longer exposure period, and measured serum as opposed to testicular testosterone which may account for the difference in findings. Phthalates that affect testosterone production do so by down-regulation of genes *StAR*, Hmg-CoA synthase, and *Srb1* (cholesterol uptake/transport), and those in the steroidogenic cascade: *Cyp11a*, 3 β −*Hsd*, and *Cyp17* (Hannas et al., 2012). Effects have been shown to be dose dependent occurring at doses above 100–250 mg/kg/day for DEHP and DBP (Gray et al., 2021). Other phthalates including DIDP and diethyl phthalate, dimethyl phthalate, dioctyl phthalate are not thought to affect fetal rat testicular testosterone production or AGD (Dean and Sharpe, 2013; McIntyre et al., 2001). Indeed, while the highest dose (1,000 mg/kg-bw/day) of DIDP (GD 14–21) decreased serum testosterone, at 500–900 mg/kg-bw/day DIDP (GD 14 – 18), it failed to produce changes in fetal testicular testosterone. One study showed no change in AGD in F1 and F2 off-spring exposed to 4,000 ppm (approximately 300 mg/kg-bw/day) DIDP throughout gestation. AGD can be considered a reliable indicator or biomarker of DHT levels present during the entire male programming window (Dean and Sharpe, 2013; McIntyre et al., 2001). Together, the weight of evidence supports no change in testosterone and likely no change in steroidogenic gene expression following exposure to DIDP at dose levels lower than 1,000 mg/kg-bw/day. To support this evaluation, data from a Japanese medaka (*Oryzias latipes*) extended one generation guideline study (OPPTS 850.1500) was considered (Patyna et al., 2006). DIDP exposure (19.3 mg/kg diet) did not result in a change in testosterone hydroxylase activity in males indicating no change in testosterone metabolism. In addition, no reproductive toxicity was observed in F2 generation fish including egg production, body weight, post hatch survival, gonad weight and histopathology. Overall, the weight of evidence suggests DIDP was unlikely to elicit endocrine adversity with no evidence of changes in AGD, no evidence of apical and potentially adverse reproductive outcomes, and limited and inconsistent evidence for reduced fetal testosterone and steroidogenic gene expression.

When considering endocrine activity for the androgen pathway, no DIDP-related effects were observed in ToxCast HT androgen receptor binding, or transactivation assays. *In vivo* mechanistic studies showed the potential for androgen antagonism with decreased weights for two androgen-dependent tissues (seminal vesicle and prostate) in the Hershberger assay when castrated rats were administered 500 mg/kg-bw/day DIDP in conjunction with TP (Lee and Koo, 2007). These changes occurred in conjunction with an increased liver weight but in the absence of effects on testosterone level. Compounds that significantly decrease weights of two or more androgen-sensitive tissues in the presence of TP as well as displaying some degree of reduced growth in all other target tissues are considered positive for anti-androgenicity (OECD, 2009; Marty and O'Connor, 2014). However, it has also been shown that an apparently positive Hershberger result (when being performed to assess antiandrogens) could be achieved when the test compound induces liver drug metabolizing enzymes (Freyberger et al., 2007; Freyberger and Schladt, 2009). Exposure of castrated rats to DIDP increased liver weights suggesting induction of liver enzymes that could increase the metabolism and clearance of serum testosterone. DIDP

Table 5

Integration and Assessment of Lines of Evidence for DIDP and DIDP Metabolite Disruption of the S Pathway. Key: HT: high throughput; GD: gestational day; h: hours; QSAR: quantitative structure activity relationship.

	Line of Evidence	Observed Effects and Confidence in the Line of Evidence[1]	Assessment of and Confidence in Integrated Lines of Evidence[2]
Integrated lines of evidence for endocrine activity	Aromatase activity	• No change in aromatase inhibition HT assay[3] (Medium)	**Potentially positive for endocrine activity based on changes in gene expression.** The lines of evidence show a lack of change in testosterone synthesis and a small change in estradiol synthesis in the H295R steroidogenesis assay. Supporting transcriptomic assays provide conflicting evidence of changes to steroidogenic gene/protein expression.
	H295R assay	• Small (~<1.5 fold compared to control) but significant increase in estradiol biosynthesis at 20 mg/L DIDP but no change in E2 to testosterone ratio (Medium) (Lee et al., 2019)	
		• No change in testosterone biosynthesis (Medium) (Lee, 2019)	
	Gene/protein changes	• No change in expression of steroidogenic enzymes, steroid regulatory, or transport protein genes in GD 18 rat pups (Low) (Hannas et al., 2012; Gray et al., 2021)	
		• Decreased gene and protein expression for *Cyp11a1*; *Hsd17b3*, and *StAR* in male rat pups (GD 21) at 1000 mg/kg-bw/day (Low) (Zhang, 2020)	
Integrated lines of evidence for S-mediated adversity	Hormone levels	• Decreased estradiol and increased progesterone synthesis in FSH-stimulated porcine granulosa cells at 10^{-4} M DIDP for 72 h (Low) (Mlynarcikova et al., 2007).	**Overall negative for S-mediated adversity.** The lines of evidence show inconsistent changes in fetal serum and testicular testosterone levels.
		• No change in fetal testicular testosterone at GD 18 or 21 (Low) (Hannas et al., 2012; Gray et al., 2021)	
		• Decreased serum testosterone at GD 21 at highest dose tested (1,000 mg/kg-bw/day DIDP) (Low) (Zhang et al., 2020)	
Evidence of general toxicity	Body weight	• No change in maternal body weight gain (Medium) (Hannas et al., 2012; Zhang et al., 2020)	
Supporting Evidence	*In silico* prediction[1]	• Negative QSAR predictions for aromatase inhibition models (MiDP, MCiNP, MHiDP and MOiDP ToxCast DIDP SMILES)[4]	The QSAR evidence provides weak support for a lack of aromatase inhibition for DIDP metabolites.
		• Negative QSAR predictions for aromatase inhibition models (MCiNP, MHiDP and MOiDP) and indeterminant prediction for MiDP (commercial DIDP SMILES)[4]	

[1] High: effects observed in one or more guideline study with no conflicting results; Medium: effects observed in one or more non-guideline studies with no conflicting results; effects observed in one or more guideline or non-guideline studies with conflicting results that could be explained by differences in study design; Low: effects were observed in one or more studies of guideline or non-guideline studies with conflicting results that could not be explained by differences in study design.

[2] Assessment of whether a sufficient dataset was available to support a conclusion on S activity in Section 3.8: Data Sufficiency and Identification of Data Gaps.

[3] https://comptox.epa.gov/dashboard/chemical/invitrodb/DTXSID4025082 (invitroDBv4.1, downloaded May 29, 2024).

[4] Leadscope Model Applier (v2022.0.2–3).

induction of PXR (a xenobiotic nuclear receptor that up-regulates expression of phase I and II metabolic enzymes and phase III uptake and efflux transporters which may accelerate hormone metabolism and clearance) in HT assays also provides evidence that the activity of the enzymes that metabolize testosterone are induced (Supplemental Data F – All EATS Data) as well as by reports of increased liver weights in the literature (Cho et al., 2008). The reduction in testosterone observed in this study means less is available to interact with the AR to maintain androgen-sensitive tissue weight. As the rats used in this assay were castrated, there is no endogenous testosterone that could compensate for the increased metabolism. When considering endocrine activity for the S pathway, DIDP had no effect on steroidogenesis based on the Tox21 aromatase inhibition assay and the H295R steroidogenesis assay. Overall, considering the data for both endocrine activity and adversity, a plausible biological link cannot be established.

For the E-mediated adversity, the only apical changes observed were coincident with decreased body weight indicative of generalized toxicity and likely not related to DIDP exposure (Hushka et al., 2001). In mechanistic assays of E-mediated endocrine activity, DIDP did not show estrogenic or anti-estrogenic responses in the uterotrophic assay, and the ER HT results were negative. When assessing the estrogenic activity of DIDP using the H295R steroidogenesis assay, there was a slight, but significant increase in the level of estradiol following exposure to 20 mg/L DIDP for 48 h (Lee et al., 2019), however the authors of this (non-guideline) study concluded that the ER binding potential for DIDP was very weak and as the estradiol to testosterone ratio was unchanged, that DIDP did not produce an estrogenic effect. These results contrasted with another study that showed decreased estradiol production in FSH-stimulated porcine granulosa cells following exposure to 10^{-4} M DIDP for 72 h (Mlynarcikova et al., 2007). While the design of these studies is

clearly different, the divergent outcomes reduce confidence that DIDP affects the biosynthesis of estradiol. Overall, there are no adverse effects mediated by E or S pathways and therefore, according to ECHA/EFSA criteria, a plausible biological link between endocrine activity and adverse effects cannot be established.

For the T pathway, none of the *in vivo* studies showed evidence of histopathological or weight changes in the thyroid gland which would represent an apical outcome resulting from changes in the thyroid pathway. There were no direct measurements of serum THs in the DIDP *in vivo* studies; however, a supporting epidemiological study of pregnant women did not show an association between urinary MHiDP and MCiNP concentration and serum T3 and TSH levels with a negative association with T4 (Derakhshan et al., 2021). Decreased T4 could be the result of an increase in the conjugation of T4, but as this failed to produce a change in TSH or the biologically active TH, T3, an observation of a change in T4 alone does not support an overall perturbation in the thyroid pathway that could lead to adverse thyroid outcomes. The lack of TH data in experimental models represents a potential data gap for DIDP and possibly precludes drawing a definitive conclusion on T-mediated adversity. However, while measuring changes in serum TH levels is considered a useful marker of T pathway perturbation it is perhaps an over-simplification; serum TH levels are not always aligned with apical events as regulation of THs occurs at the tissue level by deiodinase (DIO) enzymes, cell membrane transporters, and thyroid receptor expression (Noyes et al., 2019). For DIDP, the available data for the T pathway were consistently negative yet, according to the ECHA/EFSA criteria, there was insufficient data to support a conclusion on T-mediated adversity.

5. Conclusions

In conclusion, according to the ECHA/EFSA criteria to evaluate a substance as an ED, as defined by WHO/IPCS (International Programme on Chemical Safety), based on the available evidence base DIDP does not show ED activity in the E, A, or S pathways. This conclusion is based on a lack of evidence for endocrine activity across these pathways (E, A, and S) for which a biologically plausible link can be established to EAS-mediated relevant adverse effects noted *in vivo*. DIDP does not show T-mediated adverse effects in thyroid gland, but insufficient evidence was available to evaluate endocrine activity (as defined by ECHA/EFSA).

CRediT authorship contribution statement

I.A. Lea: Data curation, Writing – original draft. **D. Feifarek:** Data curation. **A. Mihalchik:** Investigation. **M. Heintz:** Conceptualization, Project administration. **L. Haws:** Funding acquisition, Supervision. **H. Nyambego:** Writing – review & editing. **K. Goyak:** Conceptualization, Resources, Writing – review & editing. **C. Palermo:** Conceptualization, Resources, Writing – review & editing. **S.J. Borghoff:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: ToxStrategies LLC reports financial support and article publishing charges were provided by ExxonMobil Biomedical Sciences Inc. H. Nyambego reports a relationship with ExxonMobil Biomedical Sciences Inc that includes: employment. K. Goyak reports a relationship with ExxonMobil Biomedical Sciences Inc that includes: employment. C. Palermo reports a relationship with ExxonMobil Biomedical Sciences Inc that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2025.100221.

Data availability

The data used in the assessment are made available in the Supplementary Data file

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