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ToAD Improved Tools to Assess whether biocides and plant protection products have endocrine Disrupting properties

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Preface

This report describes the ToAD project, which aimed to enhance the ability to test active substances in biocides and plant protection products (BP/PPP) for their potential endocrine disrupting (ED) activities. A key objective was to incorporate new knowledge and methods, particularly for assessing disruptions to the retinoid signalling pathway, which is currently not included in any standardized test guidelines under the OECD program.

In this project, we focused on the retinoid signalling pathway as a relevant non-EATS modality. Initially, we aimed to develop an in vitro assay for CYP26 enzyme activity, critical in maintaining retinoid homeostasis in tissues and potentially targeted by many CYP enzyme-inhibiting chemicals such as azole fungicides. Additional efforts were directed towards assessing the overlap, or lack thereof, of pesticides known to inhibit CYP19 (aromatase) activity, central to the S modality in EDC assessments. Unfortunately, establishing a CYP26 activity assay proved unfeasible within the project's remit, so we focused on another molecule in the retinoic acid biosynthesis pathway, namely RALDH.

We would like to acknowledge the Danish Environmental Protection Agency for funding the ToAD project (j. no. 2020-68044), as well as all our colleagues in the Research Group for Molecular and Reproductive Toxicology at the National Food Institute, Technical University of Denmark.

Dansk resume

Nuværende EU-forordninger foreskriver, at alle aktivstoffer i biocider og plantebeskyttelsesmidler (BP/PPP) skal vurderes for potentielle hormonforstyrrende (ED) egenskaber (EU, 2017/2100; EU, 2018/605). Dette er en kompliceret opgave, da de dækker alle potentielle ED-effekter og ikke kun dem, der er medieret af østrogene, androgene, thyroid- eller steroid virkningsmåder (EATS-modaliteterne). Det kompliceres yderligere af det faktum, at mange ED-modaliteter stadig diskuteres med hensyn til relevansen af visse endepunkters påvirkning. Det er også et faktum at mange ED-modaliteter stadig ikke undersøges i standardiserede 'OECD test guidelines' og vejledninger i ECHA/EFSA og OECD fokuserer kun på EATS. Som en konsekvens af dette, kan standardinformationskravene til ED-vurderinger af biocidaktivstoffer/pesticidaktivstoffer vise sig at være utilstrækkelige, hvilket gør de videnskabelige tolkninger af virkningerne til genstand for diskussion (Escrivá et al., 2021).

Denne rapport beskriver ToAD-projektet, som havde til formål at forbedre evnen til at teste biocid- og pesticidaktivstoffer for deres potentielle hormonforstyrrende aktiviteter. Et hovedformål var at inkludere ny viden og metoder til at vurdere forstyrrelser i retinoid signaleringsvejen.

I dette projekt har vi udviklet og implementeret flere modeller som har relevans for retinoid signaleringen, herunder et aldehyde dehydrogenase (ALDH) hæmnings assay og retinoid receptor aktivering og hæmning. Receptor hæmning blev både undersøgt i en cellemodel, samt ved at udvikle en QSAR-model for denne effekt. Desuden blev der foretaget yderligere udvikling af Adverse Outcome Pathways (AOP'er), der beskriver de mekanistiske årsagssammenhænge mellem forstyrret retinoidsignalering og reproduktionsskadelige effekter hos gnavere og mennesker. Hensigten med disse AOP'er er at bidrage til mekanistisk forståelse og dermed til forbedret vurdering af om kemiske stoffer er skadelige for mennesker. Vi testede 21 kemikalier, der inkluderer aktivstoffer fra BP og PPP'er, samt nogle lægemidler. Disse blev undersøgt i alle de tilgængelige in silico og in vitro modeller og resultaterne blev sammenholdt med allerede rapporterede in vivo effekter, som kunne være medieret igennem en retinoid ("R")-modalitet. Generelt var der en god overensstemmelse mellem resultaterne fra de forskellige modeller, samt eksisterende data for de 21 test kemikalier på det molekylære niveau. Billedet blev mere uklart når vi sammenholdt data fra vores alternative metoder med rapporterede effekter i dyremodeller. Overvejelser omkring inklusion af ADME, hvorvidt de molekylære assays er dækkende for retinoid systemet, og om effekterne i dyremodellerne er konserverede for forstyrrelser af retinoid signalering bliver diskuteret. Resultater fra dette projekt kan fungere som et første skridt hen imod bedre håndtering af en af mange ikke-EATS modaliteter i en regulatorisk sammenhæng.

I fremtiden vil det være en udfordring at integrere alternative modeller der undersøger retinoid signalering i internationale testmetoder, især da alternativer til dyreforsøg forventes at spille en mere markant rolle i ED-identifikation og regulering. I de nuværende gnaver toksicitetsstudier bliver effekter på retinoid signalvejen betragtet som skadelige effekter i intakte organismer; men i en potentiel fremtid, hvor dyreforsøg bliver udfaset, skal vi sikre, at vi inkluderer de nødvendige alternative modeller til at forudsige skadelige virkninger i hele organismer.

Contents

List of Abbreviations

ALDH	- Aldehyde dehydrogenase
AO	- Adverse Outcome
AOP	- Adverse Outcome Pathway
AOP-KB	- Adverse Outcome Pathway Knowledge Base
atRA	- all-trans Retinoic Acid
BP/PPP	- Biocides and Plant Protection Products
DRP	- Detailed Review Paper
EATS	- Estrogen, Androgen, Thyroid, and Steroidogenesis
ECHA	- European Chemicals Agency
ED	- Endocrine Disruptors
EFSA	- European Food Safety Authority
ESCA	- Advisory Group on Emerging Science in Chemicals Assessment
EU	- European Union
GD	- Guidance Document
IATA	- Integrated Approaches to Testing and Assessment
KE	- Key Event
KER	- Key Event Relationship
LOEC	- Lowest Observed Effect Concentration
LPDM	- Leadscope Predictive Data Miner
MIE	- Molecular Initiating Event
MoA	- Mode of Action
NIH	- National Institute of Health
OECD	- Organisation for Economic Co-operation and Development
QSAR	- Quantitative Structure-Activity Relationship
RAR	- Retinoic acid receptor
RXR	- Retinoid X receptor
SD	- Standard deviation
SPSF	- Standard Project Submission Form
US EPA	- United States Environmental Protection Agency
WNT	- Working Party of the National Coordinators of the Test Guidelines Programme

1. Project aims

The overall aim of the ToAD project was to enhance safety assessment strategies for active substances in biocidal and plant protection products (BP/PPPs) within the EU regulatory framework by developing and enhancing relevant *in vitro* assays and QSAR models for identifying endocrine disruptors (EDs). Specifically, this included establishing an *in vitro* assay for RALDH1 activity, developing QSAR models for retinoic acid receptor (RAR) inhibition, applying existing QSAR models for CYP19A1 (aromatase) inhibition, then integrating new developments with existing assays and knowledge on relevant endocrine disrupting modalities to assess chemicals. This toolbox of assays, organized in a conceptual testing strategy, was aimed to improve the capacity to prioritize compounds for *in vivo* testing and ultimately reduce the reliance on animals for chemical toxicity testing. A final improved assessment framework emphasizing *in vitro* and *in silico* methods in ED identification would be achieved by integrating new assays and knowledge on mechanism-of-action into OECD's adverse outcome pathway (AOP) framework.

2. Background

In 2018, EFSA/ECHA guidance for identifying ED properties in BP/PPP was introduced (ECHA/EFSA et al., 2018). The ED identification process in this advanced framework relies on linking mechanistic data with in vivo adverse effect data to establish a biologically plausible link between chemical exposure and adverse outcomes, typically centered around the EATS modalities (estrogen, androgen, thyroid, and steroidogenesis). However, assessing biological plausibility presents a significant challenge, often necessitating deep biological understanding from studies on model substances or animal knockout models. To address these challenges, constructing a shared knowledge base of causal pathways, such as the Adverse Outcome Pathway Knowledge Base (AOP-KB) managed by the OECD, can provide a solid foundation for decision-making by collecting mechanistic knowledge from various sources.

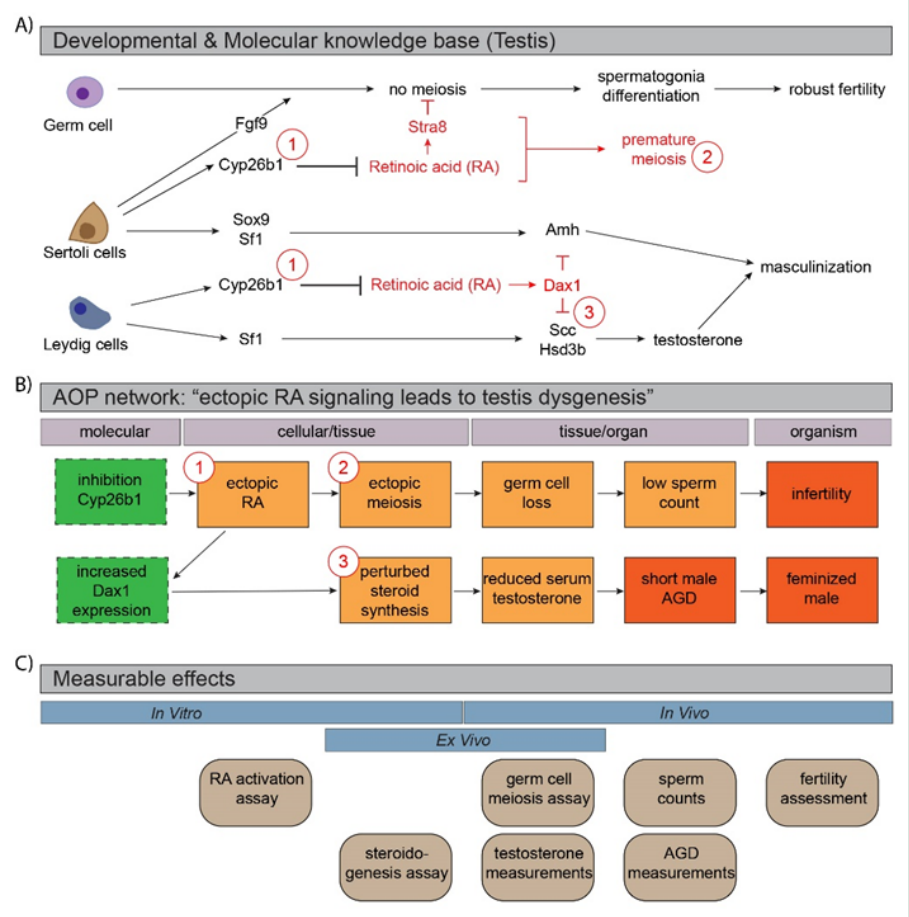

FIGURE 1. Example of how A) scientific knowledge can be extracted to B) develop pragmatic adverse outcome pathways (AOPs) that only make use of those key events (KEs) that are essential to driving the causal pathway towards an adverse outcome (AO). Importantly, to be useful in a regulatory context, C) individual KEs must be measurable, which typically involves in silico and in vitro methods for low-tier KEs (green/orange) and in vivo studies for high-tier KEs (or-ange/red). By having established the plausible links between the KEs, one can potentially predict an AO based on data from upstream KEs. Figure from (Draskau et al., 2020).

The concept of Adverse Outcome Pathways (AOPs) was developed within regulatory toxicology to enhance the use of alternative test methods in risk assessments of chemical substances (Ankley and Edwards, 2018). AOPs provide mechanistic insights into cause-effect relationships, facilitating the integration of non-animal data sources such as in vitro assays and in silico models (Villeneuve et al., 2014; Vinken et al., 2017).These pathways are constructed based on comprehensive reviews of experimental evidence and expert knowledge, offering versatile panels of test methods to address specific toxicological questions across various disciplines. Importantly, AOPs are not specific to individual chemicals and can be utilized to infer biological plausibility for any substance that disrupts the pathways, including biocides and plant protection products (BP/PPPs). Figure 1 illustrates how mechanistic knowledge of toxicity pathways can aid in the construction of AOPs that are underpinned by relevant test methods used to obtain empirical data necessary for risk assessment.

There are currently 300-400 BP/PPPs (active substances) approved for use in the EU[1]. The process of identifying which active substances could have ED properties is continually ongoing, with ED assessments being performed both when new active substances seek approval and when existing ones seek renewal. In these assessments most emphasis is put on evaluating the classical EATS modalities (as described in GD 150) (OECD, 2018). Established AOPs and AOP networks for these modalities can help the ED identification process by clearly describing the plausible link between the mode of action and the adverse effects. However, AOPs or MoA knowledge for non-EATS modalities causing ED effects are still largely missing.

The OECD has acknowledged the relevance of non-EATS modalities in causing ED effects, as outlined in a detailed Review Paper (OECD, 2012). Despite this recognition, these additional modes of action are seldom considered in existing guidelines, particularly concerning in silico or in vitro data. Retinoid signalling (R) has emerged as a particularly relevant non-EATS modality for developmental and reproductive toxicity (Grignard et al., 2020), with certain BP/PPPs, such as azole fungicides, being identified as disruptors of retinoid signalling. However, there is currently a lack of assays for retinoid signal disruption incorporated in OECD test guidelines and a scarcity of developed AOPs addressing retinoic acid signal disruption and its effects outcomes within the OECD's AOP framework. This knowledge gap has been highlighted in various regulatory reports and reports from workshops (European Commission, 2018; Nilsson, 2020; Knudsen et al., 2021), indicating a significant challenge in the current testing and assessment regimens for BP/PPPs in terms of their potential to cause developmental and reproductive effects. Therefore, addressing this issue through comprehensive research endeavours would lead to a more robust and less resource-intensive testing regimen for assessing the ED-related effects of BP/PPPs.

Based on this background knowledge, we have developed an overall research objective to enhance the ED assessment strategy for BP/PPPs through three main approaches: i) developing novel alternative test methods (WP1 and WP2), ii) evaluating a selection of BP/PPPs using these newly developed methods alongside established ones (WP1 and WP2), and iii) advancing current understanding of the modes-of-action for key components of ED assessment requirements, specifically focusing on the S and R modalities (WP3). This includes updating OECD’s AOP Knowledge Base (AOP-KB) framework. Figure 2 illustrates this project plan visually.

[1] https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances

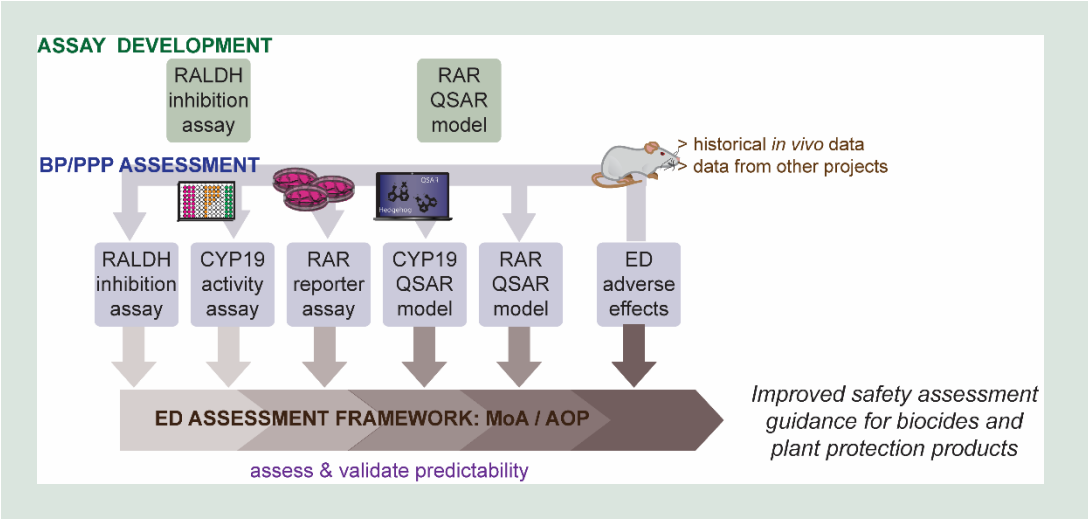

FIGURE 2. Overview of the research plan. Two new non-animal methods for assessing substances for ED activities will be developed or implemented: a RALDH enzyme activity assay and retinoic acid receptor (RAR) quantitative structure-activity relationship (QSAR) models (green boxes). These models will complement existing tools, including a CYP19A1 enzyme activity assay and CYP19A1 QSAR models developed under the EU Horizon 2020 project FREIA (van Duursen et al., 2020). The developed methods will be evaluated against in silico, in vitro, and in vivo data from various sources (blue boxes). These enhancements are envisioned to improve the current testing regimen and ED identification, offering improved modes-of-action knowledge and elaborated AOPs for chemical risk assessment (brown).

3. Materials and Methods

3.1 Test chemicals

Twenty-one test substances were included in this project. The selection of test substances was based on the Detailed Review Paper (DRP) on retinoid signalling and endocrine disruption (OECD, 2012) and a list of endocrine disruptors(Hass et al., 2018) prepared under the Danish Center for Endocrine Disruptor (CeHoS). We focussed on selecting substances with reported effects that could potentially be caused by disruption of the retinoid signalling pathway, as well as including substances that led to reproductive toxicity that was not expected to be caused by the R-modality. As many pesticides were included as possible, but some pharmaceuticals were also included. For a full list of test substances see Table 1.

TABLE 1. Test substances, CAS numbers, use, and in vivo reported effects that could be related to a disruption of the retinoid signalling pathway. Approval status is located here for the biocides: https://echa.europa.eu/da/information-on-chemicals/biocidal-active-substances) and here for the pesticides: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances. "-" if pharmaceutical

Test substance	CAS	Use	In vivo effects	Approval/ Assessment status (BPR/PPPR)	Ref.
Cyproconazole	94361-06-5	Pesticide, biocide	Gene expression changes related to retinoid-related signalling in rat and zebrafish embryo studies	Expired, not approved	(Hermsen et al., 2012; Robinson et al., 2012)
Deltamethrin	52918-63-5	Pesticide, biocide	In utero, exposed adult male rats display lower sperm count, motility, and viability, and a higher percentage of abnormal sperm (and histological changes). Several studies were sperm parameters affected by exposure in adult rats.	Approved – Renewal in progress	(Ben Halima et al., 2014; Ben Slima et al., 2017)
Dimethomorph	110488-70-5	Pesticide	R-related effects?	Not approved	-
Endrin	72-20-8	Pesticide	Cleft palate and malformations in mice	Not approved	(ATSDR Agency for Toxic Substances and Disease Registry, 2021)
Fenitrothion	122-14-5	Pesticide	Effects on sperm parameters in adult rats	Not approved	(Ito et al., 2014; Saber et al., 2016)
Flusilazole	85509-19-9	Pesticide	Gene expression changes related to retinoid-related signalling in rat and zebrafish embryo studies.	Not approved	(Hermsen et al., 2012; Robinson et al., 2012)

Test substance	CAS	Use	In vivo effects	Approval/ Assessment status (BPR/PPPR)	Ref.
Imazalil	35554-44-0	Pesticide, biocide	R-related effects?	No longer supported (BPR) Approved(PPPR)	-
Isotretinoin	4759-48-2	Pharmaceutical	Cause histological changes in all stages of spermatogenesis and affect the retinoid system	-	(Al-Shahed et al., 2022)
Ketoconazole	65277-42-1	Pharmaceutical	Meiosis induction in mouse fetal testis, premature meiotic entry in male germ cells in male rats. Ectopic RAR signalling, data indicates strong inhibition of CYP26B1	-	(Bowles et al., 2006)
Molinate	2212-67-1	Pesticide	Testicular toxicant in rats, an inhibitor of RALDH; inhibits the conversion of retinal to RA, decreases testicular RA levels in exposed rats	Not approved	(Zuno-Floriano et al., 2012)
Permethrin	52645-53-1	Pharmaceutical, Pesticide, biocide	Craniofacial abnormalities in zebrafish	Approved (BBR) Not approved (PPPR)	(DeMicco et al., 2010)
Prochloraz	67747-09-5	Pesticide	R-related effects?	Not approved	-
Procymidone	32809-16-8	Pesticide	R-related effects?	Not approved	-
Salicylic acid	69-72-7	Pharmaceutical, biocide	Impaired spermatogenesis. Failure of closure of the neural tube resulting in cranium bifidum and/or spina bifida, rib abnormalities, cranioraschischisis* and umbilical hernia, eye defects	Initial application for approval in progress (biocide)	(BEALL, 1977; Didolkar et al., 1980; Overman and White, 1983)
Talarozole	870093-23-5	Pharmaceutical	Potent inhibitor of CYP26A1 and CYP26B1	-	(Stevison et al., 2017)
Tebuconazole	107534-96-3	Pesticide, biocide	Skeletal malformations in rats, mice, and rabbits.	Approved, ongoing classification process (RAC)	(EFSA, 2014)

Test substance	CAS	Use	In vivo effects	Approval/ Assessment status (BPR/PPPR)	Ref.
Tributyltin chloride	1461-22-9	Pesticide	Craniofacial skeletal deformities in rockfish embryos	No info	(Zhang et al., 2012)
Triphenyltin chloride	639-58-7	Pesticide	R-related effects?	No info	-
Triticonazole	131983-72-7	Pesticide	R-related effects?	Approved	-
Vinclozolin	50471-44-8	Pesticide	R-related effects?	Not approved	-
WIN 18,446	1477-57-2	RALDH2 inhibitor, experimental drug	Neonatal exposure causes meiotic defects in spermatocytes. Progression from A spermatogonia to A1 spermatogonia blocked	-	(Griswold and Hogarth, 2018)

*Craniorachischisis is the most severe form of neural tube defect in which both the brain and spinal cord remain open to varying degrees. It is a very rare congenital malformation of the central nervous system

3.2 RAR activity

The establishment of this protocol at DTU was done as part of the ToAD project. Stably transfected HEK293 cells containing the human retinoic acid receptor alpha (RARα) and a reporter construct were purchased from BPS Bioscience. Cells were seeded at a concentration of 32,000 cells/well into 96-well plates and incubated for 4 h after which they were exposed to chemicals for approximately 24 h. The assay was performed in agonist and antagonist mode with test substance concentrations ranging from 0.4-100 µM. A vehicle control was also included and the vehicle concentration was constant across all treatments. In antagonist mode cells were co-exposed to 1 nM all-trans retinoic acid. After 24 h exposure, the culture medium was removed from wells and a lysis buffer was added to incubate for 20 min and then a luciferin solution was added. Luciferase activity was measured on a LUMIstar Galaxy microplate reader. Positive control substances were tested to ensure assay performance in both agonist, *all-trans* retinoic acid, and antagonist mode, Ro 41-5253. Several assays for cell viability were tested, but visual evaluation proved the most sensitive method and thus used for all the presented experiments.

3.3 RALDH inhibition

The cells used for the RALDH inhibition assay was kindly gifted by Jisun Paik from Washington State University. They represent a stably transfected cell line that was generated as described previously (Amory et al., 2011). In short, ALDH1A1 and ALDH1A2 cDNA cloning was done into lentiviral plasmids, which were virally transduced into H1299 cells, a lung cancer cell line with low baseline expression of ALDH. Stably transfected cells were seeded into 96-well plates and plates were left to incubate for 24 h at 5% CO_2 and 37°C in a humidified atmosphere. Cells were then exposed to test chemicals at concentrations ranging from 0-5 µM in concert with 1 µM all-trans retinal for approximately 24 h. The culture medium was harvested and analyzed for retinoic acid by high-performance liquid chromatography (KANE et al., 2005; PAIK et al., 2005). A positive control substance, WIN 18,446, was tested to ensure assay performance.

In addition to the measurements of all trans-RA (atRA) by analytical tools, we also did initial trials to measure the production of atRA from the stably transfected ALDH cells by using the RARα reporter gene assay. We seeded ALDH1A2 cells in 96 well plates and left them to incubate for approximately 24 h. Then we co-exposed cells to 1 µM all-trans retinal and increasing concentrations of the positive control WIN 18,446. After 24 h, the medium was transferred to RARα (HEK293) cells that were seeded 24 h previously. After exposure in RARα cells to ALDH medium for 24 h, luciferase activity was measured. The RARα activity was hence a measure of the production of atRA in ALDH cells. If ALDH was inhibited, this would lead to less activity of the receptor in the reporter gene assay, as less at-RA was produced from retinal. A schematic overview of the two-cell set-up is shown in Figure 3.

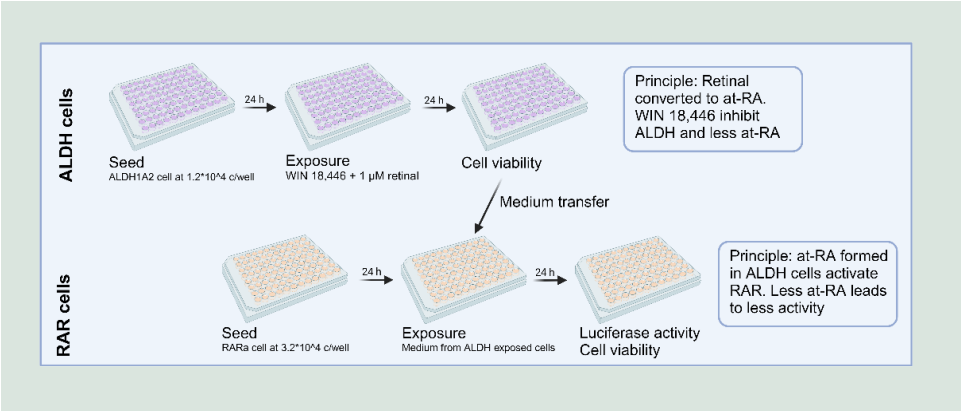

FIGURE 3. Workflow for two cell system for detection of ALDH inhibition. Created using BioRender.com.

3.4 CYP19 inhibition

The protocol was established at DTU under the ToAD project. The CYP19 inhibition assay was purchased from Abcam and performed according to the manufacturer's protocol. The assay is cell-free and relies on the conversion of a fluorogenic substrate by recombinant human aromatase in concert with a NADPH regenerating system. The substrate is converted to a highly fluorescent metabolite that can be measured in the visual range. Test compounds were added in concentrations ranging from 0.001-1000 µM together with the fluorogenic substrate and fluorescence was measured. A positive control for CYP19 inhibition was included to ensure assay performance.

3.5 QSAR modelling

Training and validation set for the development and validation of QSAR models for RAR inhibition were based on experimental in vitro data for around 10,000 substances from the US Tox21 programme (NTP Tox21)[2]. The bioassay record (tox21-rar-antagonist-p2, Pubchem AID 1159555) is denoted as a qHTS assay to identify small molecule antagonists of the retinoic acid receptor (RAR) signaling pathway. The nuclear receptor assay is performed in the C3H10T1/2 murine embryo fibroblast cell line. The detailed assay results including activities at all 16 tested concentrations, Hill curve fitting parameters, *etc* were downloaded from the US National Institute of Health (NIH) Dashboard (Tox21 Gateway 2024)[3].

Two separate, binary QSAR models were developed based on Tox21 data, using the same definition for the negative class but two different definitions for the positive class:

1. High potency positives, only, defined as absolute IC_{25} occurring at a maximum concentration of 10 µM
2. High and low potency positives, defined as absolute IC_{25} occurring at any concentration, denoted NUL – No Upper Limit

Thorough and transparent data and structure curation are crucial elements to achieve highly performing QSAR models with clear interpretable endpoint definitions. For this purpose, a previously developed comprehensive procedure to analyze the Tox21 raw data and Hill curves fittings was applied(Nikolov et al., 2023). To extract only the most robust experimental results for actives and inactives, we applied the following criteria:

- Selecting actives for the two separate QSAR models, results with good Hill curve fitting (having inflection, p-value < 0.05 and efficacy > 3 SD of control) and exhibiting 'absolute' activity, here chosen to apply IC_{25}, at maximum 10 µM or with no concentration threshold, respectively, and requiring non-cytotoxicity at effect concentration defined as min. 80% of cells being alive
- Extracting only the most robust inactives, i.e. tested up to high concentration, here chosen to be 50 µM, without cytotoxicity
- Filtering out substances tested in low purity, i.e. all others than Tox21 purity class A corresponding to at least 90% purity, to have higher certainty that correlations are made to the correct chemical structures
- Filtering out results giving assay interference according to a strict DTU-interpretation of artifacts by use of Tox21 counter screens *etc.* – for Tox21 RAR inhibition luciferase inhibitors may lead to FN results

[2] NTP Tox21, Toxicology in the 21st Century (Tox21).
Available: https://ntp.niehs.nih.gov/results/tox21/index.html, (Accessed 4 July 2024)

[3] Tox21 Gateway, Public Data, https://tripod.nih.gov/pubdata/ (accessed 4 July 2024)

Structure curation was performed, including a procedure to represent tautomeric groups in a consistent manner. Additionally, the traditional structure curation procedure used to remove duplicate structures (removing only one if results agree, requiring at least two carbon atoms, de-salting and neutralizing allowing only non-toxic counterions, etc.) was applied. All training sets used for modeling and validation underwent these data and structure generic curation procedures, and no experimental results were excluded due to high leverage (outliers) etc. (as this may lead to over-optimistic validation results). Results from individual steps in the data and structure curation procedure for all data sets were transparently reported.

The modelling was performed in a binary fashion with both a positive and negative class. Two models were made, with the same definition of the negative class and with the above-mentioned two different definitions of minimum potencies for the positive class, i.e. requiring IC_{25} at a maximum of 10 µM and IC_{25} with no concentration threshold, i.e. with no-upper-limit (NUL), respectively.

QSAR modeling was performed using state-of-the-art QSAR technology in the Leadscope Predictive Data Miner (LPDM)[4], a component of Leadscope Enterprise Server 3.5.3–5 (Instem plc, Stone, Staffordshire, UK), with the application of, among other things, a huge pre-defined library of around 27,000 hierarchically organized chemical fragments as descriptors. For binary response variables, LPDM uses partial logistic regression (PLR). For skewed data sets such as for RAR inhibition an averaging modelling method can be applied, where the smaller class (here actives) is used repeatedly in a series of sub-models, containing disjoint (as much as possible) and random portions of the bigger class (here inactives). A maximum of 10 sub-models can be developed in this procedure, leading us to use a maximum of 10 times as many inactive as active.

Initial models were developed with 80% of actives and inactives (maximum 10 times as many inactives as actives were included in the models for technical reasons). The 20% which were left out by random before modelling were used for independent external validation of the initial models (i.e., the initial models were not chosen by use of the external validation set). After that, the external validation sets were integrated into the models' training sets, and the final extended models were developed. The developed models underwent robust validation according to the OECD QSAR validation principles. All models, initial and extended, were cross-validated with DTU robust 10*5-fold cross-validation to measure robustness and external predictivity. If there were remaining extra inactives not used for the extended models due to technical reasons (maximum 10 times as many inactives as actives), they were used for external validation of the specificity of the extended models. Cross-validations and external validations were not used to choose models or domains. The overall workflow steps of the procedure are depicted in Figure 4.

[4] Leadscope commercial in silico software by Instem, accessed 21 June 2024 at: https://www.instem.com/solutions/insilico/computational-toxicology.php

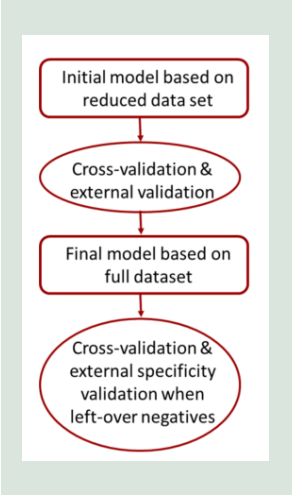

FIGURE 4. Workflow for the development of QSAR models from the initial model to robust validation.

The models were included in the Danish (Q)SAR Models free website for real-time predictions of user-defined structures and download of detailed prediction reports in the (Q)SAR Prediction Reporting Format (QPRF). Predictions for 650,000 substances, including 448 EU pesticide active organic mono-constituent substances and 232 EU biocide organic mono-constituent substances were included in the freely available Danish (Q)SAR Database. Advanced search functionalities, as well as structure similarity sorting to find the closest structure analogues, are available in the database user interface.

In addition to the developed RAR inhibition models, already developed QSAR models for aromatase (cyp19) inhibition were used to make predictions for the 21 test chemicals. The aromatase models were developed as part of the above-mentioned EU H2020 FREIA research project and details on performance are currently prepared for a scientific publication. Like the RAR inhibition models, the aromatase models are based on Tox21 data. The procedures for data and structure curation, modelling, and validation were as described above. Two models were developed, based on a negative class defined as in the RAR inhibition training set, and with the positive classes containing substances with IC50 as maximum 10 µM and 50 µM, respectively. No model with a positive class with substances having activity at any concentration (NUL) was developed. The resulting final models had high predictive performance with specificities well above 90% and sensitivities well above 80%.

3.6 AOP development

The OECD's Handbook for Development of AOPs was followed during the development of knowledge units within the AOP framework, as well as alignment with pragmatic AOP development approaches as described by (Svingen et al., 2021). In brief, KEs were developed by narrative approaches to render description as general as possible to allow for including in additional AOPs, especially upstream KEs that will also cover more animal taxa. KERs, on the other hand, were developed using a systematic weight-of-evidence approach to collect, evaluate, extract, and integrate evidence from in vivo (animal) and epidemiological (human) studies. Scientific support for causal relationships between linked KEs in the AOPs was obtained by the overall weight of evidence assessments using modified Bradford-Hill criteria. The domain of applicability for AOPs was defined as the most narrowly restricted of its KE/KERs.

The AOPwiki was screened for all relevant KE/KERs and AOPs. A total of 9 AOPs were identified as related to retinoid signalling, but only two contained information beyond simple titles: AOP-398 (as developed herein) and AOP-436 (“Inhibition of RALDH2 causes reduced all-trans retinoic acid levels, leading to the transposition of the great arteries”). This latter AOP has been added after 398 and used two of the KEs of AOP-398, namely KE1880 and 1881.

4. Results

4.1 Retinoic acid receptor-α agonism and antagonism

Several of the test substances led to increased activity of the RARα (Figure 5), whereas none led to decreased activity that was not attributed to compromised cell viability (SI Figure 1). The positive control for agonism, atRA (Figure 5), and antagonism, Ro 41-5253 (SI Figure 1), led to expected responses that were reproducible between independent experiments.

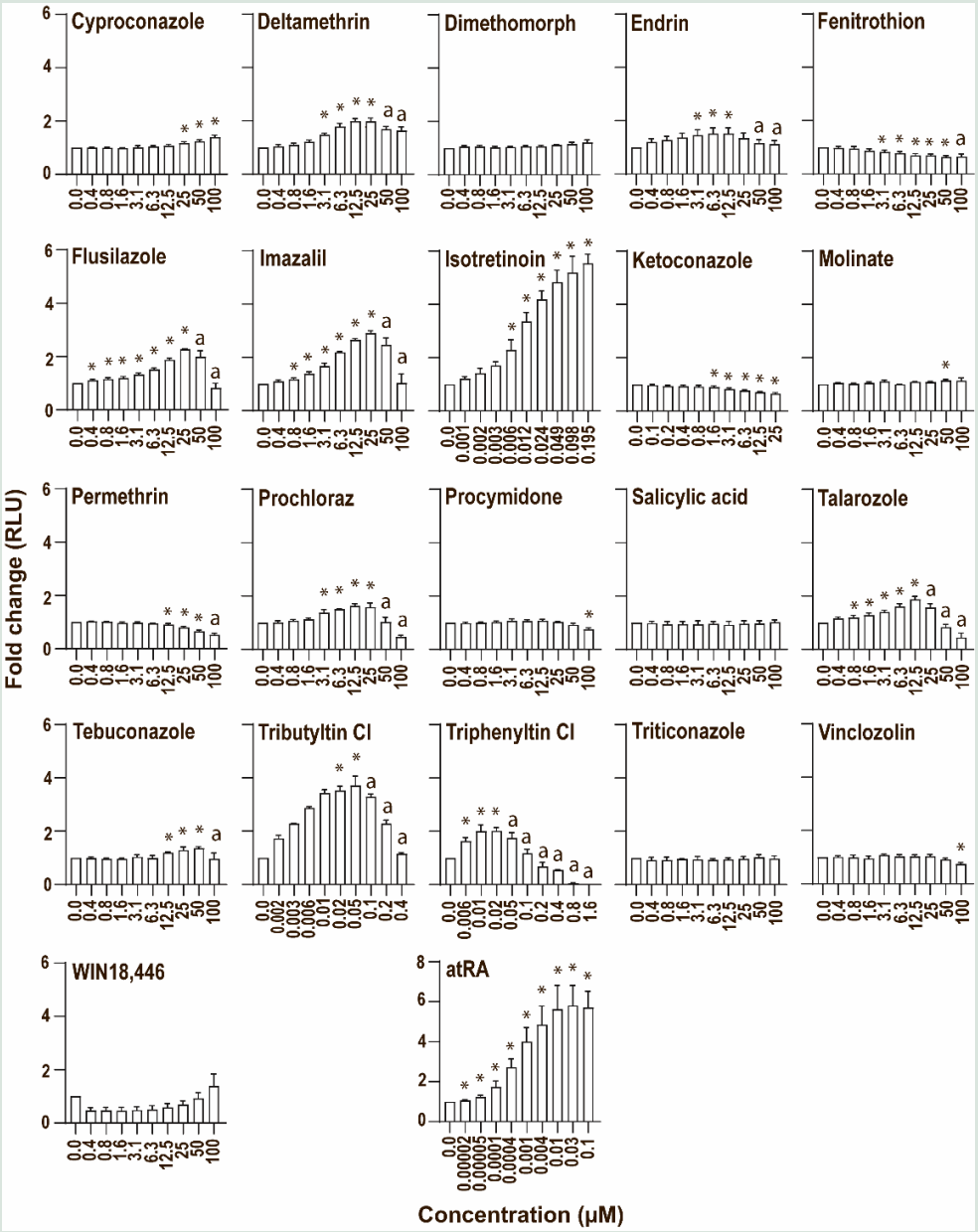

FIGURE 5. RARα agonism. Activity with exposure to test substances. Graphs represent pooled means from three independent experiments (n=3, mean ± SD). Asterisk for p ≤ 0.005, a: concentrations considered to compromise cell viability evaluated visually (not included in statistical analysis).

The most prominent effects were seen for deltamethrin, flusilazole, imazalil, isotretinoin, talarozole, tributyltin chloride, and triphenyltin chloride, which all increased the activity of the receptor by more than 100% compared to the vehicle control at relatively low concentrations.

4.2 Retinal dehydrogenase inhibition

The retinal dehydrogenase activity was affected by several of the test compounds (Figure 6). WIN 18,446 was used as a positive control and showed responses comparable to historical data (data not shown). Dimethomorph and fenitrothion both led to decreased ALDH1A1 enzyme activity, whereas only fenitrothion affected ALDH1A2. Both tributyltin chloride and triphenyltin chloride led to decreased activity, which was only significant for ALDH1A2.

However, these two substances exhibited unusual concentration-response, as an increased activity was observed at higher test concentrations. Prochloraz, procymidone, and salicylic acid led to decreased response; however, these did not appear to be concentration dependent. None of the remaining test substances affected the activity of ALDHs (SI Figure 2).

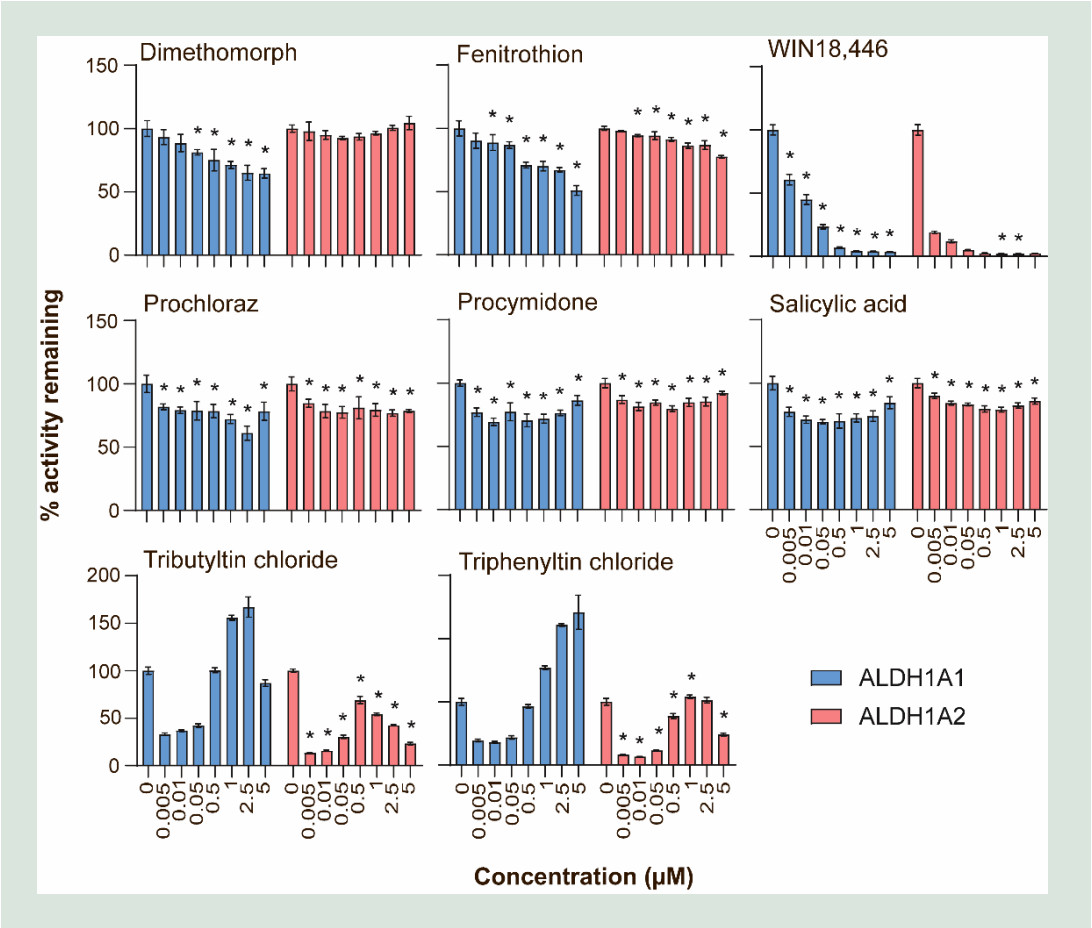

FIGURE 6. ALDH1A1 (blue) and ALDH1A2 (red) enzyme activity after exposure to test substances. All groups were normalised to a vehicle control group set to 100%. Graphs represent data from one experiment in technical triplicates.

The two-cell system for measuring ALDH inhibition gave promising results (Figure 7). We tested WIN 18,446, the positive control substance for ALDH inhibition, in the ALDH1A2 cells and transferred the medium from these cells to RARa cells in three independent experiments. WIN 18,446 led to decreased responses in the RARa reporter gene assay with increasing concentrations suggesting a decreased synthesis of at-RA in ALDH cells. The maximum efficacy is approximately 70% compared to control.

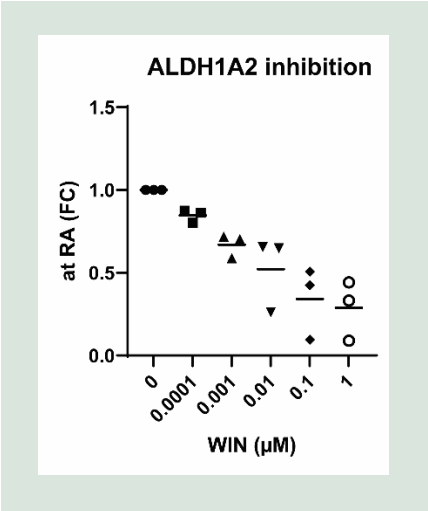

FIGURE 7. Two cell system for ALDH inhibition. A decreased response was observed in the RARa report-er gene assay after exposure to medium from ALDH cells for 24 hours. ALDH medium con-tained produced atRA and increasing concentrations of WIN 18,446. atRA concentrations were derived from a standard curve for at-RA tested in the RAR reporter gene assay and data was normalised to control. Data is derived from three independent experiments (n=3).

4.3 Aromatase (CYP19) inhibition

Among the test substances, the azoles were the most potent and efficacious, though not all azoles led to significant effects. Cyproconazole, flusilazole, imazalil, ketoconazole, prochloraz, and tebuconazole led to significant effects with LOECs at or below 12.5 µM and a fold change compared to controls of around 50 %, whereas talarozole, triticonazole, and vinclozolin did not lead to significant effect. Fenitrothion, isotretinoin, molinate, and tributyltin chloride also led to significant effects with LOECs from 100 µM or above. The remaining test substances did not lead to significant effects.

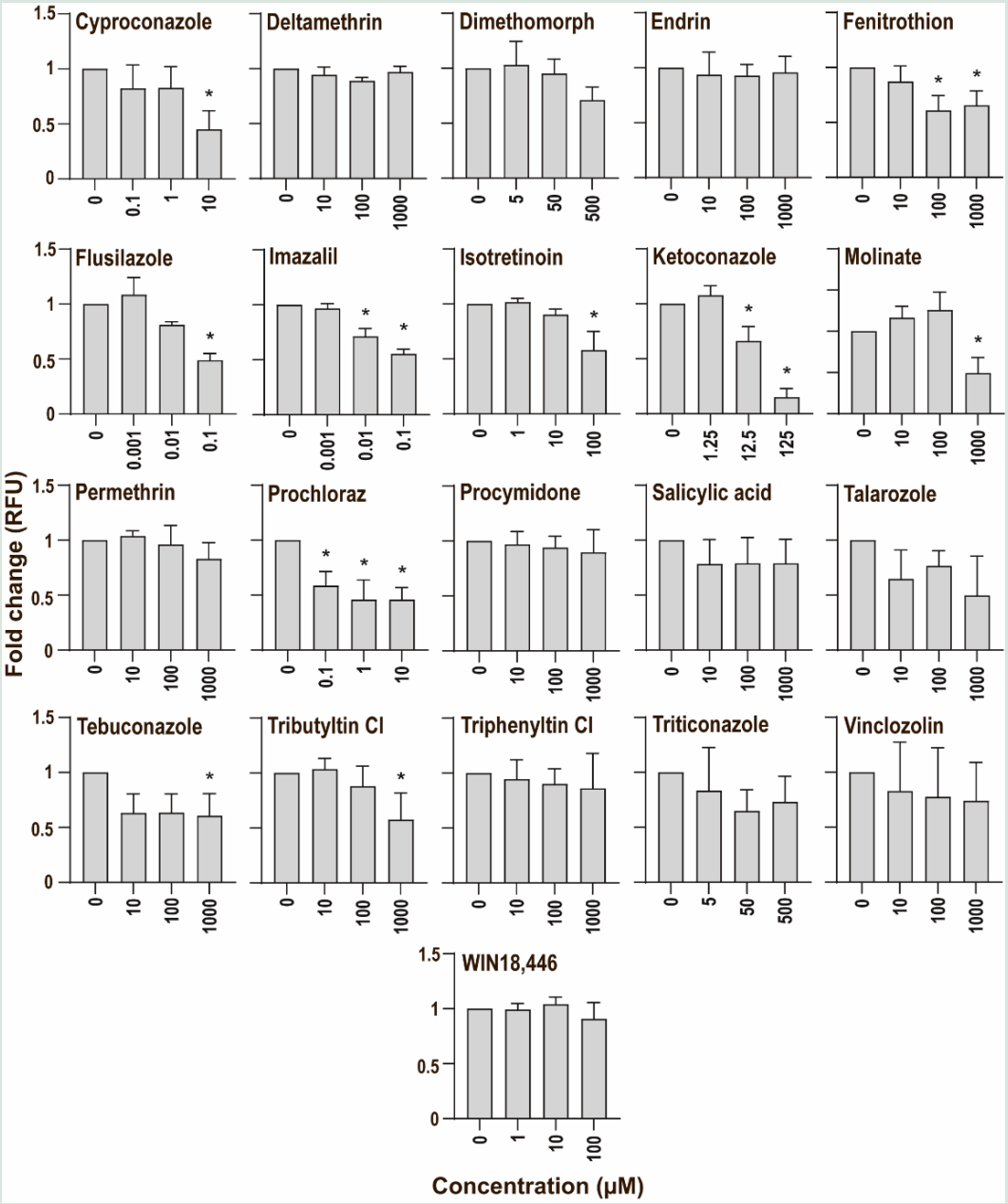

FIGURE 8. CYP19 inhibition assay. Graphs represent pooled normalised means from three independent experiments (n=3, mean ± SD). Asterisks for $p \leq 0.005$.

4.4 QSAR models

Table 2 lists results from the individual data and structure curation steps, as well as numbers for the training and validation sets. Furthermore, the external predictivity of the models, as determined by the cross-validations and external validations are given in the Table 3.

The new QSAR models for RAR inhibition, as well as an existing model for aromatase (Cyp19) inhibition, were used to generate predictions for the 21 chemicals tested in vitro to allow for comparison of results. These predictions are shown in Table 4

TABLE 2. Data for QSAR models on RAR inhibition. Results of individual data and structure curation steps, and resulting training and validation set sizes.

RAR inhibition	Active		Inactive	
	10μM	NUL	10μM	NUL
Initial substances (SIDs)		9,667		9,667
SIDs with purity at least 90%		6,457		6,457
SIDs identified by data curation procedure as active or inactive	130	627		3,291
SIDs after cell viability filter	122	432		3,277
SIDs after excluding auto-fluorescent substances	122	430		3,126
CAS'es after resolving SIDs to CAS	104	369		1,940
CAS'es with (Q)SAR-acceptable structures (organic mono-constituent etc.)	76	320		1,883
Structures after duplicates removal at structure level (i.e. between CAS'es)	73	303		1,798
Structures for initial model and external validation, randomly split into 80% (reduced training set) and 20% (external validation set)*	58 : 15	242 : 61	580 : 1,218	1,438 : 360
Structures for final models (integration of reduced training set + 20% external validation set)*	73	303	730 : 1,068	1,798

* Maximum ration between active and inactive substances in the applied modeling system is 1:10, and therefore there were left-over inactive substances for external validation of specificity of the model based on only most potent actives (activity at maximum 10μM).

TABLE 3. External predictivity results for the developed RAR inhibition QSAR models, based on cross-validation and external validation.

Model	Sens, %* (SD)	Spec, % (SD)	BA, % (SD)	TP	TN	FP	FN	Coverage, % (SD)
10 uM								
Initial model, cross-validation	60.8 (±20.0)	95.7 (±2.6)	78.3 (±10.4)	167	3,567	157	105	62.6 (±4.8)
Initial model, external validation	80.0	93.9	87.0	8	756	49	2	66.2
Final model, cross-validation	70.2 (±17.0)	95.6 (±2.0)	82.9 (±8.5)	290	4,659	212	119	65.8 (±3.6)
Final model, external validation	-	94.1	-	-	642	40	-	63.9
NUL								
Initial model, cross-validation	78.8 (±7.6)	93.8 (±1.5)	86.3 (±3.7)	1,172	9,470	626	321	69.0s (±2.4)
Initial model, external validation	75.0	90.6	82.8	27	261	27	9	77.0

Final model, cross-validation	77.6 (±6.7)	93.9 (±1.5)	85.8 (±3.3)	1,498	12,063	786	432	70.3 (±2.7)

TABLE 4. QSAR predictions from the RAR inhibition and aromatase inhibition models and experimental results displayed when they were part of the QSAR training sets.

	RAR inhibition						Aromatase inhibition					
	High-potency positives model ($IC_{25} \leq 10$ µM)			All positives model (IC_{25} at any concentration, NUL)			High-potency positives model ($IC_{25} \leq 10$ µM)			Low-potency positives model ($IC_{25} \leq 50$ µM)		
Structure Name	**EXP**	**PREDICT**	**PROB**	**EXP**	**PREDICT**	**PROB**	**EXP**	**PREDICT**	**PROB**	**EXP**	**PREDICT**	**PROB**
Cyproconazole		NEG	0.05		POS	0.80		POS	0.99		POS	0.99
Deltamethrin		NEG	0.02		NEG	0.26		OUT	0.74		POS	0.97
Dimethomorph		POS	0.73		OUT	0.63		POS	0.89		POS	0.84
Endrin		OUT	0.45		OUT	0.54		OUT	0.46		POS	0.73
Fenitrothion		OUT	0.46		OUT	0.42		NEG	0.14		OUT	0.42
Flusilazole		NEG	0.25		OUT	0.35	POS	POS	0.74	POS	POS	0.79
Imazalil		OUT	0.44		POS	0.96	POS	POS	0.97	POS	POS	0.94
Isotretinoin		OUT	0.53		OUT	0.43		POS	0.91		POS	0.91
Ketoconazole		POS	0.85		POS	0.92		POS	1.00	POS	POS	1.00
Molinate		NEG	0.14	NEG	OUT	0.34	NEG	OUT	0.05	NEG	OUT	0.07
Permethrin		NEG	0.12		OUT	0.36		POS	0.85		POS	0.96
Prochloraz		OUT	0.38	NEG	OUT	0.44	POS	POS	0.94	POS	POS	0.99
Procymidone		OUT	0.76	NEG	NEG	0.20		NEG	0.22	NEG	OUT	0.52
Salicylic acid	NEG	NEG	0.12	NEG	NEG	0.03	NEG	NEG	0.02	NEG	NEG	0.01
Talarozole		OUT	0.86		OUT	0.74		OUT	0.99		OUT	1.00
Tebuconazole		NEG	0.08	NEG	OUT	0.50	POS	POS	0.99	POS	POS	1.00
Tributyltin Chloride		OUT	0.42		OUT	0.41		OUT	0.24		OUT	0.41
Triphenyltin Chloride		OUT	0.31		OUT	0.39		OUT	0.24		OUT	0.45
Triticonazole		OUT	0.55		POS	0.76		POS	0.98		POS	0.95
Vinclozolin		NEG	0.21	NEG	OUT	0.38	NEG	OUT	0.61	NEG	POS	0.76
WIN 18,446		POS	0.94		POS	0.89		POS	0.72		OUT	0.70

POS: Positive predictions in the applicability domain or positive experimental values, NEG: Negative prediction in the applicability domain of the model or positive experimental values, OUT: out of domain, EXP: experimental values, PREDICT: Predictions, PROB: probability.

4.5 AOP development

This project builds on two previously developed putative AOPs proposing a causal link between disrupted retinoid signalling and reproductive toxicity in mammals. These were originally developed under a separate project from Directorate-General for Environment (DGEM, Belgium), with AOP 398 *('Inhibition of ALDH1A (RALDH) leading to impaired fertility via disrupted meiotic initiation of fetal oogonia of the ovary'*) being the most mature and AOP 400 ('*Increased (ectopic) concentration of atRA in fetal testis leading to reduced sperm count, males'*) being more naïve regarding content. AOP 398 (Figure 9) has been developed under different projects, whereas some further developments on AOP 400 have been performed here in adherence with the OECD's "AOP Developer's Handbook".

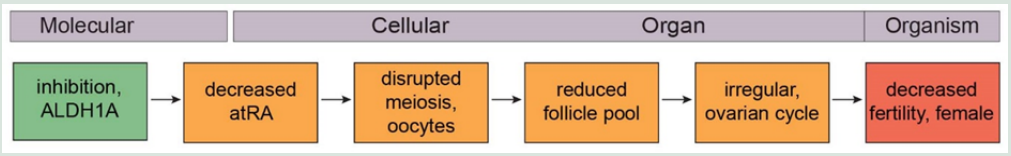

FIGURE 9. AOP for inhibition of RALDH leading to decreased fertility in females. The development of this AOP was initiated under a separate project from DGEM-Belgium and then further developed under the EU Horizon project FREIA (Johansson et al., 2020).

AOP 398 links inhibition of ALDH1A (MIE) to decreased fertility in females (AO) via four intermediate key events (a full AOP description can be found on AOP-wiki). The first KE, decreased atRA levels, is a direct consequence of enzyme inhibition and can be assessed by enzyme activity assays. The next KE disrupted oocyte meiosis, builds on the knowledge that atRA initiates meiotic entry in fetal (mouse) ovaries. The following two KEs, reduced follicle pool and irregular ovarian cycle, are a consequence of disrupted meiotic regulation. This causal pathway builds on the knowledge that oocytes need to enter meiosis prophase I during fetal development, an event involving atRA initiation of Stra8 expression, to ensure a healthy follicle pool for adult fertility. Meiotic initiation is essential for subsequent oocyte health and establishment of the finite follicle pool; oocytes failing to enter meiosis will be cleared by apoptosis. In consequence, a reduced ovarian pool will lead to irregular ovarian cycling in adulthood and may cause infertility by a diminished egg reserve, but also through disrupted hormone regulation.

AOP 400 links ectopic RA expression in fetal testis to reduced sperm count in adult males via two intermediate KEs (Figure 10). KE1889 follows from the untimely expression of RA in fetal testis, pushing gonocytes into meiotic cell division at a developmental stage when they should go into mitotic arrest. This leads to KE 1890, decreased gonocyte number, as meiotic cells may be cleared by apoptosis during fetal life. It follows that a reduced gonocyte pool may compromise adult spermatogenesis, although the quantitative relationship here is not well characterized.

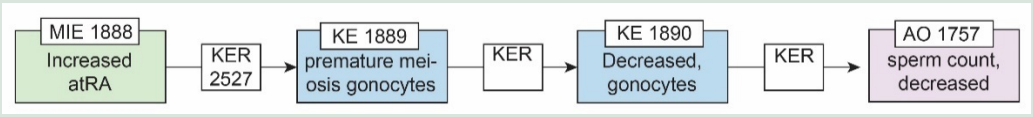

FIGURE 10. AOP 400: 'Ectopic all-trans retinoic acid (atRA) in fetal testis leading to reduced sperm count, males' (https://aopwiki.org/aops/400). This AOP is not yet fully developed, but all KEs have been described, and KER 2527 was developed by systematic approaches as per OECD's AOP Developers Handbook.

KER 2527 links ‘increased atRA concentration’ (KE 1888) to ‘premature meiosis, gonocytes’ (KE 1889) see Table 5 (below). This KER was developed by querying the open literature with the review question “Does ectopic/increased retinoic acid concentration during fetal development lead to ectopic meiosis in male mammals?” Search terms ("retinoic acid" OR "ATRA" OR "Vitamin A"[MeSH Terms[5]] OR "Tretinoin"[MeSH Terms] OR "Retinoids"[MeSH Terms] OR "retinoid*") AND ("meiosis*" OR "Meiosis"[MeSH Terms] OR "meiotic initiation") AND (“male*” OR “Male"[MeSH Terms] OR “testis” OR “Testis"[MeSH Terms] OR “gonocyte*” OR “germ cell*” OR "Germ Cells"[MeSH Terms]) were used to source PubMed in ‘All Fields’. Exclusion criteria for retrieved literature were: i) reviews (non-primary literature), ii) publications written in languages other than English; iii) studies in non-mammalian species; iv) studies with effects induced during other life stages than fetal development; v) studies with females only. The final literature search was conducted on March 7th, 2023.

Germ cells undergo meiotic cell division to produce haploid sperm or eggs from diploid gonocytes or oocytes, respectively. Meiotic entry occurs during fetal life in germ cells of the ovaries but postnatally in germ cells of the testes (Spiller et al., 2017). During late fetal life, germ cells in the testes remain in a state of mitotic quiescence and initiate meiotic entry just before puberty. RA is suggested to induce meiosis in both males and females at the appropriate life stages. In developing ovaries, atRA prompts germ cells to initiate the first round of meiosis during fetal life, while in developing testes, male germ cells are prevented from entering meiosis until puberty due to the breakdown of atRA by CYP26 enzymes. In the absence of RA, fetal male germ cells enter cell cycle quiescence (Spiller et al., 2017). However, if RA is ectopically expressed in the fetal testis, it induces premature meiotic initiation in fetal male germ cells, ultimately disrupting gonocyte development. This is the rationale for KER 2527. Most of the evidence derives from mouse studies, with gonad explants predominating experimental evidence from humans.

Biological plausibility rests on the fact that, in mammalian germ cells, the expression of the pre-meiotic marker *Stra8* is the critical factor for meiotic onset and progression (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006). In male mice lacking *Stra8*, germ cells fail to initiate meiosis at puberty (Anderson et al., 2008), and this failure is also observed in fetal females (Baltus et al., 2006), highlighting that Stra8 is required for correct meiotic initiation in both sexes. Although the mechanistic details of the spatiotemporal regulation of *Stra8* are still unclear, robust evidence suggests that RA is a main regulator of *Stra8* expression (Griswold et al., 2012). Germ cells from embryos of pregnant vitamin A-deficient rats fail to upregulate Stra8 and enter meiosis (Li and Clagett-Dame, 2009), and several functional RA response elements have been identified within the *Stra8* promoter (Feng et al., 2021).

In the fetal male, somatic expression of CYP26B1 ensures RA degradation, thus avoiding the initiation of meiosis (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007; Li and Clagett-Dame, 2009). However, if RA is not effectively cleared, male germ cells will initiate expression of *Stra8* and aberrantly enter meiosis (Bowles et al., 2006; Koubova et al., 2006). Exogenous RA can also stimulate male germ cells to induce Stra8 expression and meiosis in mouse testis explants (Bowles et al., 2006; Koubova et al., 2006; Trautmann et al., 2008). This effect seems to be conserved in humans, though limited studies are available. Following the above-mentioned rodent studies, single-cell suspension cultures of human fetal testes showed increased STRA8 expression in response to exogenous RA; however, no effect was observed on the gene expression of meiosis markers SYCP3 and DMC1 (Childs et al., 2011).

The quantitative understanding of this KER is very limited beyond the observation that germ cells, whether from the testis or ovary, rapidly induce Stra8 expression when cultured in the presence of atRA at concentrations as low as 10 nM (Spiller and Bowles, 2019).

[5] MeSH-Medical Subject Headings, The Medical Subject Headings (MeSH) thesaurus is a controlled and hierarchically-organized vocabulary produced by the National Library of Medicine.

TABLE 5. Overview of empirical evidence supporting KER 2527.

Study type	Species	Compound	Effect Dose	Duration	Results
Embryonic stem cells	Mouse	atRA	100 nM	4-10 d	Activates meiosis-related gene network
Fetal testes in culture	Mouse	atRA	1 µM	48 h or 72 h	Ectopic expression of meiotic markers (*Stra8, Sycp3, Dmc1*) and decrease in pluripotency marker *Oct4*
Fetal testes in culture	Mouse	Ketoconazole (Cyp26 inhibitor)	40 µM	48 h or 72 h	Ectopic expression of meiotic markers (*Stra8, Sycp3, Dmc1*) and decrease in pluripotency marker *Oct4*
Fetal testes in culture	Mouse	BMS-194753 (RAR-α agonist)	0.5 µM	2 d	Ectopic *Stra8* expression
Fetal testes in culture	Mouse	BMS-213309 (RAR-β agonist)	0.5 µM	2 d	Ectopic *Stra8* expression
Fetal testes in culture	Mouse	BMS-270394 (RAR-γ agonist)	0.5 µM	2 d	Ectopic *Stra8* expression
Fetal testes in culture	Mouse	atRA	0.7 µM	2 d	Ectopic *Stra8* expression
Fetal testes in culture	Mouse	Ketoconazole (Cyp26 inhibitor)	0.7 µM	2 d	Ectopic *Stra8* expression
Fetal testes in culture	Mouse	R115866 (Cyp26 inhibitor)	0.7 µM	2 d	Ectopic *Stra8* expression
Fetal testes in culture	Rats	atRA	1 µM and 0.031 µM	2 or 3 d	Increased apoptosis of germ cells, reduction in total number of germ cells
Culture of fetal testis single-cell suspension	Human	atRA	1 µM	24 h	Increased *STRA8* expression, but had no effect on *DMC1 or SYCP3* expression

5. Discussion

The current regulatory framework for BP/PPPs relies heavily on animal studies to assess hazards and safety. However, there is an ongoing shift towards developing alternative methods that reduce, replace, and refine the use of animals for toxicity testing. For instance, ECHA published in 2023 guidance on using non-animal methods for risk assessment (ECHA, 2023), but notably, these guidelines focus solely on EATS-modalities for ED identification; retinoid signaling is not mentioned. The persistent paucity of including RA-relevant testing methods for regulatory purposes is of concern since there is substantial evidence suggesting disrupted RA signalling as a prominent modality also for endocrine disruption. A detailed review paper (DRP) on retinoid signalling pathways commissioned by the OECD provided strong evidence for the involvement of RA-disrupting chemicals in causing RA-mediated reproductive, skeletal, and neurodevelopmental effects in mammals (OECD, 2012). Although this project did not aim to develop assays to a level of OECD validation or endorsement, it has provided additional support for the necessity of doing so alongside new QSAR models for retinoid receptor inhibitions that can be used for screening and prioritising purposes. In the 2024 report "Key Areas of Regulatory Challenge" by ECHA, the DRP on retinoids from OECD is specifically highlighted in the section addressing developmental and reproductive toxicity. The report underscores the need for further research into these areas to better understand the potential impacts of retinoids on human health and to inform regulatory measures. This focus on retinoids is part of a broader effort to enhance the regulation of chemicals that may pose risks to development and reproduction (European Chemicals Agency, 2023).

5.1 Readiness evaluation of in vitro tests

There are ongoing activities under the OECD test guideline program for an RAR reporter gene assay, including the PEPPER[6] Project 4.167: New TG on a stably transfected human retinoic acid receptors hRARs transcriptional activation assay for detection of agonistic and antagonist activity of chemicals towards hRARs. However, this activity was postponed for one year at the 36th Meeting of the Working Party of the National Coordinators of the Test Guidelines Program, which took place in Paris in April 2024 (personal communication Sofie Christiansen, DK National Coordinator).

To our knowledge, there are no current activities related to validation under the OECD test guideline programme for either CYP19 or RALHD inhibition assays, as no such project are on the current workplan of OECD test guideline programme[7]. However, under the US EPA's endocrine disruptors screening program (EDSP), an aromatase assay is used as a Tier 1 assay to test for the ability of chemicals to inhibit the human aromatase enzyme activity (OPPTS 890.1200). This method has not undergone vigorous validation under OECD oversight but is mentioned in the EFSA/ECHA guidance (2018) as a possible CF level 2 test that can be used for investigation of the S-modality, and it may prove eligible for inclusion in the test guideline program if submitted as a Standard Project Submission Form (SPSF)[8].

[6] PEPPER: Public-private platform for the validation of endocrine disruptors characterization methods, https://ed-pepper.eu/en/

[7] https://www.oecd.org/chemicalsafety/testing/work-plan-test-guidelines.pdf

[8] SPSF: Standard Project Submission Form - a project proposal under the OECD Test Guideline programme https://www.oecd.org/chemicalsafety/testing/standard-project-submission-form.pdf

5.2 Retinoid signalling disruptions and aromatase inhibition in vitro

Overall, there is good comparability between the in vitro results obtained here and activity calls in the ToxCast and Tox21 models from the US EPA when evaluating across the different assays applied[9]. While there were a few positive 'activity calls' for antagonism in ToxCast and Tox21, none of the test compounds inhibited RAR activity in our tests. These discrepancies could at large be attributed to compromised cell viability, inconsistencies across experimental runs, or borderline antagonism at the highest tested concentration in the ToxCast dataset. Agonist data also generally aligned, with a few exceptions.

Comparing in vitro results with activity calls from the Tox21 assay for aromatase inhibition, they are largely consistent, albeit with a few exceptions[10]. Fenitrothion and molinate showed significant effects in our CYP19 kit but did not show a positive activity call in Tox21 data. For fenitrothion, Tox21 data indicates aromatase inhibition at the maximum tested concentration of 100 µM, whereas no effect was observed for molinate at this concentration. The discrepancies may be due to differences in the maximum concentrations tested (1000 versus 100 µM). Likewise, vinclozolin did not show significant effects here but was deemed active in the Tox21 model. Vinclozolin did show a decreasing tendency (Figure 8), which was not significant, due to relatively large variations between independent experiments for this test substance.

To our knowledge, this is the first report of the ability of fenitrothion, dimethomorph, tributyltin chloride and triphenyltin chloride to inhibit ALDH enzyme activity, whereas the inhibitory effect of WIN 18,446 is well described (Amory et al., 2011; Paik et al., 2014). The test substances that did not affect ALDH activity had, to our knowledge, not been tested previously, except molinate, which has some inhibitory potential for hALDH2 (Allen et al., 2010).

5.3 QSAR models for RAR inhibition

A rigorous data and structure curation procedure was implemented to eliminate, as far as possible, positive and negative results which may be due to cytotoxicity or other experimental artifacts, forming a robust foundation for QSAR model development with clearly defined endpoints and minimized 'artifact-based' predictions (detailed results are reported in Table 2). The QSAR models for RAR inhibition were developed using a binary (pos/neg) classification with two active class definitions: requiring an absolute activity of 25% at maximum of 10 µM, and with no-upper-limit for the concentration (NUL). This dual approach allows the models to predict both more or less potent substances, which may be relevant for different purposes.

The models were validation using both robust cross-validation and external validation, as reported in Table 3. The final models demonstrated decent external predictivity for sensitivity (70-78%) and high external predictivity for specificity (around 94%), indicating they miss about 20-30% of actives but generate few false-positives (around 6%). Comparing initial models based on a reduced training set to final models with larger training sets showed improved sensitivity and reduced standard deviation, indicating more robust models. External validation results were close to cross-validation results but showed higher uncertainty regarding sensitivity due to few active substances in the external validation sets. Predictions for RAR inhibition for the 21 substances analysed in this project are shown in Table 4. Six substances, included as inactives in one or both training sets, were predicted negative or out-of-domain. Of the 21 substances, five were predicted positive by one or both models.

Predictions for aromatase inhibition were also generated using models developed in the FREIA project (van Duursen et al., 2020) for the 21 substances analysed here, as shown in Table 4. Nine of these substances were in the training sets, with five actives and four inactives; all were predicted correctly, except for Vinclozolin, which was included as inactive

[9] https://comptox.epa.gov/dashboard/

[10] https://comptox.epa.gov/dashboard/assay-endpoints/TOX21_Aromatase_Inhibition

but predicted as low-potency-active. For example, Ketoconazole, filtered out from the RAR inhibition QSAR models due to cytotoxicity, showed inhibition at the highest concentrations in the Tox21 set. Despite cytotoxicity concerns, the RAR inhibition QSAR models predicted it as positive, indicating its structural properties correlate with RAR inhibition. Comparing QSAR predictions for both RAR and aromatase inhibition with in vitro results showed general alignment but also some discrepancies. These discrepancies have multiple possible explanations, without a single common cause identified for all chemicals.

5.4 From retinoid signalling disruption in alternative models to associated effects in vivo

The first AOP for disrupted retinoid signalling has been fully developed. This AOP links reduced RA levels in the fetal ovary with decreased fertility in adult females. With detailed scrutiny of available literature and an overall AOP assessment for biological plausibility and KE essentialities, this AOP (ID# 398) is currently under peer review (as of Medio 2024); it is envisaged that this AOP will be endorsed by the Advisory Group on Emerging Science in Chemicals Assessment (ESCA) and the Working Party for the Test Guideline Program (WNT) in OECD, after which it will provide a descriptive causal pathway of reproductive toxicity mediated by the retinoid modality.

With RA acting as a ligand for the nuclear retinoic acid receptors (RAR and RXR) regulating key genes such as *Stra8*, both QSAR models and in vitro reporter assays are valuable screening tests for potential RA disrupters. For instance, RA mediates meiotic initiation in germ cells by directly regulating *Stra8* expression through RA response elements (RAREs) in the upstream promoter region, elements that are targeted by RAR/RXR (Feng et al., 2021). Based on this, RAR and RXR interference should be incorporated as MIE is the emerging AOP network for retinoid signal disruption. Since these MIE also have a broad applicability domain extending beyond mammalian taxa, the utility is testing regimens is potentially substantial and can cover a wide pallet of adverse outcomes in both environmental and human health.

RA plays a role in the meiotic division of germ cells, which can affect spermatogenesis in adults During development, atRA also serves as a regulator of the vertebrate body plan. Disruptions of the retinoid signalling pathway can lead to adverse effects such as congenital skeletal-, eye-, urogenital tract-, defects, and cleft palate (Piersma et al., 2017). Focusing on the above effects in vivo – affected meiotic division markers, sperm parameters, craniofacial and skeletal defects, and cleft palate – we aimed to determine if the applied alternative methods could be used to explain in vivo effect profiles. For this purpose, we defined three cases (Table 6). These cases were based on the in vitro results, as well as the in vivo effects gathered from the initial analysis of data to select the test chemicals used in this project (Table 1).

TABLE 6. Three cases for evaluation of correspondence between results from in vitro methods and in vivo effects. Cases are based on in vivo data forming the basis for test chemical selection initially in this project, and hence, some substances may move to another group after a thorough literature review.

CASE 1: No effect in vitro, effect in vivo		
	In vitro	**In vivo**
Ketoconazole	No effects	Specific meiotic effects
Molinate	No effects	Decrease testicular RA levels
Salicylic acid	No effects	Sperm parameters affected

CASE 2: Effect in vitro, no effect in vivo		
	In vitro	**In vivo**

Dimethomorph	Inhibition ALDH	Effects in vivo?
Imazalil	RAR agonism	Effects in vivo?

CASE 3: Effect in vitro, effect in vivo		
	In vitro	**In vivo**
Fenitrothion	Inhibition ALDH	Sperm parameters affected
Isotretinoin	RAR agonism	Sperm parameters affected
WIN 18,445	Inhibition ALDH	Specific meiotic effects

The cases clearly demonstrate inconsistent relationships between the in vitro results and reported in vivo effects. For instance, RA-related effects were observed in vivo, but no in vitro activity was observed for ketoconazole, molinate, and salicylic acid (CASE 1). Likewise, dimethomorph inhibited ALDH, but there were no reports of any RA related effects in vivo (CASE 2). There were, however, examples where in vitro and in vivo effects were observed for the same substances (CASE 3). Imazalil, originally deemed a CASE 2 chemical, may belong to CASE 3 chemicals after a more thorough literature review, as there are, for instance, indications of impaired spermatogenesis in frogs after exposure to imazalil (Svanholm et al., 2024).

The lack of consistency is not surprising, with the most obvious shortcoming being the lack of ADME considerations in vitro. Besides this very common limitation with in vitro to in vivo extrapolations, the two models – ALDH inhibition and RAR activity – do not fully cover all MIEs and KEs within the retinoid signalling pathway that could lead to these types of effects in vivo. For instance, the synthesis of atRA is a two-step process where Vitamin A is first converted to retinal by the enzyme retinol dehydrogenase, a reversible reaction, and subsequently retinal is converted to atRA by ALDH, an irreversible reaction. Furthermore, atRA is metabolized by CYP26 and thereby inactivated, also an irreversible reaction. These factors collectively control the local level of atRA and hence indirectly the ability of atRA to bind to the RAR and activate it (Piersma et al., 2017). In fact, ketoconazole, which showed no effects on RAR activity or ALDH inhibition in vitro, did seemingly induce inhibition of Cyp26 in vivo (Bowles 2006), which could be an explanation for the observed discrepancies between in vitro and in vivo results. The panel of in vitro assays should, therefore, be expanded to also include more mechanisms of disruption of retinoid signalling in the future, but for now, can serve as a good first step alongside also providing additional data sources or knowledge foundation for ED assessing BP/PPPs with potential RA-related effects. In addition to limitations in coverage for the early molecular events, disruption of the retinoid signalling pathway is not the only factor that may lead to affected sperm parameters, meiosis effects, or craniofacial and skeletal defects. Therefore, mapping the upstream network of the in vivo effects is needed, and in the future, it should be a continuous endeavour within the sphere of chemical risk assessment.

5.5 Improving the framework for assessing and evaluating hazard and safety of BP/PPPs

By developing and enhancing relevant *in vitro* assays and QSAR models, the current testing framework for assessing BP/PPPs for endocrine activity, focusing mainly on non-EATS modalities, may be enhanced. These non-EATS modalities are often not included in ED-assessments due to a lack of test methods as well as limited knowledge of how such results are best included. More specifically, we have established an in vitro assay for RALDH1 activity, developed two new QSAR models for retinoic acid receptor (RAR) inhibition, and applied an existing QSAR model for CYP19A1 (aromatase) inhibition for the overall assessment of multiple modalities. These developments were integrated with existing assays and knowledge of relevant endocrine-disrupting modalities, aiming to improve the overall predictivity related to non-EATS modalities

The ToAD project has advanced the current MoA framework for disruption of the retinoid signalling system by further developing AOPs, including incorporating test methods into

relevant KEs of the causal pathways. We integrated data from *in vitro* assays and the QSAR prediction models to enhance the knowledge base for assessing BP/PPPs for potential endocrine activities, including disrupted retinoid signalling. This integrated framework builds on the AOP work within ToAD, proposing causal links between disrupted retinoid signalling and adverse reproductive effects in mammals. These adverse outcomes include impaired fertility in females due to disrupted meiotic initiation of fetal oogonia of the ovary and reduced sperm count in males caused by increased (ectopic) concentration of all-trans retinoic acid in fetal testis. This emerging framework should serve as a solid foundation for which to build additional AOPs and incorporate additional test methods for overall ED identification using complex AOP networks in the future that make use of a broad panel of alternative test methods for predicting in vivo outcomes.

The Integrated Approaches to Testing and Assessment (IATA) concept uses multiple data sources, including all existing information, but also data from in vitro assays, in vivo studies, and in silico models (such as QSARs), to evaluate the hazard and risk of chemicals more efficiently and comprehensively than more restrictive test strategies. An overall aim of IATA is to reduce the reliance on animal testing by simultaneously improving the robustness of chemical assessments, ultimately supporting regulatory decision-making. IATAs commonly leverage the Adverse Outcome Pathway (AOP) framework, as depicted in Figure 11 as they describe sequences of biological events leading from initial molecular perturbation by chemicals to an adverse health outcome in intact organisms. In other words, AOPs can provide mechanistic knowledge in support of predictive models used for chemical risk assessment. The AOPs developed in ToAD could be applied to inform and structure such an IATA in a decision context. Moreover, the QSARs, as well as the new in vitro assays generated in ToAD, could be integrated into the framework and used for regulatory decisions based on a weight of evidence (see Figure 7).

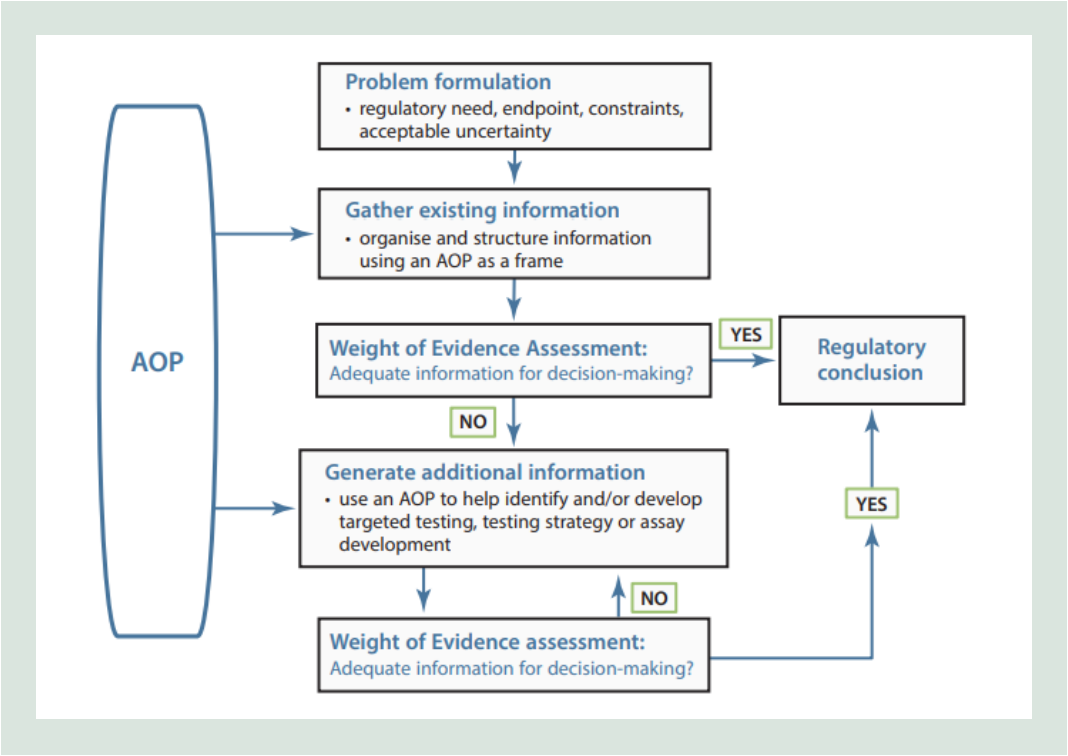

FIGURE 11. Framework for how an AOP can be applied to inform and structure IATA in a decision context from Figure 4 in OECD GD 260 OECD (2017), Guidance Document for the Use of Adverse Outcome Pathways in Developing Integrat-ed Approaches to Testing and Assessment (IATA), OECD Series on Testing and Assessment, No. 260, OECD Publish-ing, Paris, https://doi.org/10.1787/44bb06c1-en and OECD (2020), Overview of Concepts and Available Guidance related to Integrated Approaches to Testing and Assessment (IATA), OECD Series on Testing and Assessment, No. 329, Environment, Health and Safety, Environment Directorate, OECD. https://www.oecd.org/chemicalsafety/risk-assessment/concepts-and-available-guidance-related-to-integrated-approaches-to-testing-and-assessment.pdf

In the ToAD project, we aimed to create a simplified flow chart to help predict the effects of substances acting via endocrine-active substances or the retinoid signalling pathway. This tool would help assessors evaluate the biological plausibility of the link between MoA, including relying on fully developed and endorsed AOPs. However, the development of a CYP26B1 enzyme activity assay, initially critical to this objective, was not feasible. Instead, we focused on a RALDH assay, with future efforts aimed at developing QSAR models for RAR/RXR agonism and antagonism. These models, alongside RAR/RXR reporter gene assays and an expanded AOP network for R-modalities, will enhance in vitro assessment methods and overall ED evaluation.

6. Conclusion

The ToAD project has improved the methodology used for assessing the ED activity of BP/PPPs by integrating retinoid signal disruption (R modality) into the AOP-KB. This can enhance hazard and safety assessments, particularly for substances with multiple effect modalities. While the 2018 EFSA/ECHA guidance is comprehensive for evaluating ED activities of BP/PPPs, it primarily focuses on EATS modalities and recognizes the challenge of accounting for all potential endocrine-disrupting modalities. The project's contributions, including QSAR models and AOPs, support OECD initiatives to include R-relevant assays in ED-screening. Notably, the project developed two QSAR models for RAR inhibition with good predictivity, enhanced in vitro methods for RAR agonism and ALDH inhibition, and created an AOP for disrupted retinoic acid signalling leading to impaired fertility.

We tested 21 BP/PPP active substances and a few pharmaceuticals, across various models and assays. Several test chemicals led to increased RARα activity, but none decreased the receptor's activity. In the aromatase inhibition assay, several chemicals were inhibitors, with azoles being the most potent. In vitro, results were largely consistent with Tox21 data. Dimethomorph and fenitrothion inhibited one or both ALDH isoforms, while triphenyltin and tributyltin chloride showed non-monotonic concentration-response relationships. The RARα reporter gene assay effectively measured at-RA production in ALDH cells, showing a clear decrease in receptor activity when inhibited by WIN 18,446.

In sum, while in silico and in vitro data alone may not suffice to determine if a BP/PPP is an ED, these low-tier screening methods can significantly aid in identifying additional concerns related to non-EATS modalities relevant to reproductive toxicity for chemicals more broadly. As for current guidelines for BP/PPP testing, the methods can be regarded as supplementary. Regardless, these alternative (or supplementary) methods can provide essential mechanistic insights for overall ED assessment under ECHA/EFSA guidance, which requires demonstrating an ED-related adverse effect caused by a plausible ED mechanism. To optimize the testing process, future work should focus on developing methods that capture multiple mechanisms of action in one assay or a battery of assays for a comprehensive and efficient evaluation of potential endocrine disruptors.

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Appendix 1. In vitro results

Appendix 1.1 RARα antagonism

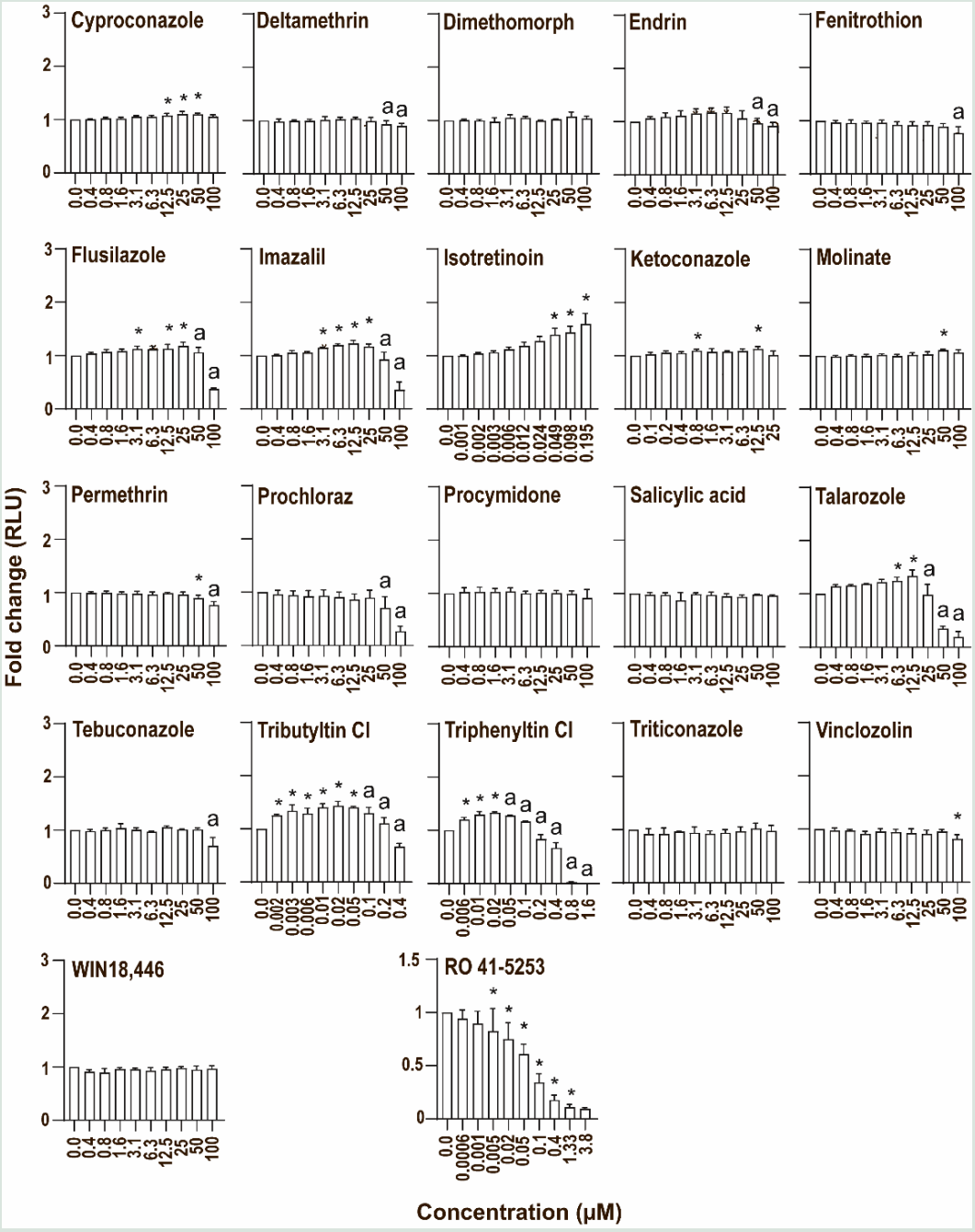

FIGURE SI 1. Retinoic acid receptor alpha antagonism. Activity with exposure to test substances in concert with at-RA. Graphs represent pooled means from three independent experiments (n=3, mean ± SD). Asterisks for p ≤ 0.005, a: concentrations considered to compromise cell viability (not included in statistical analysis).

Appendix 1.2 ALDH inhibition

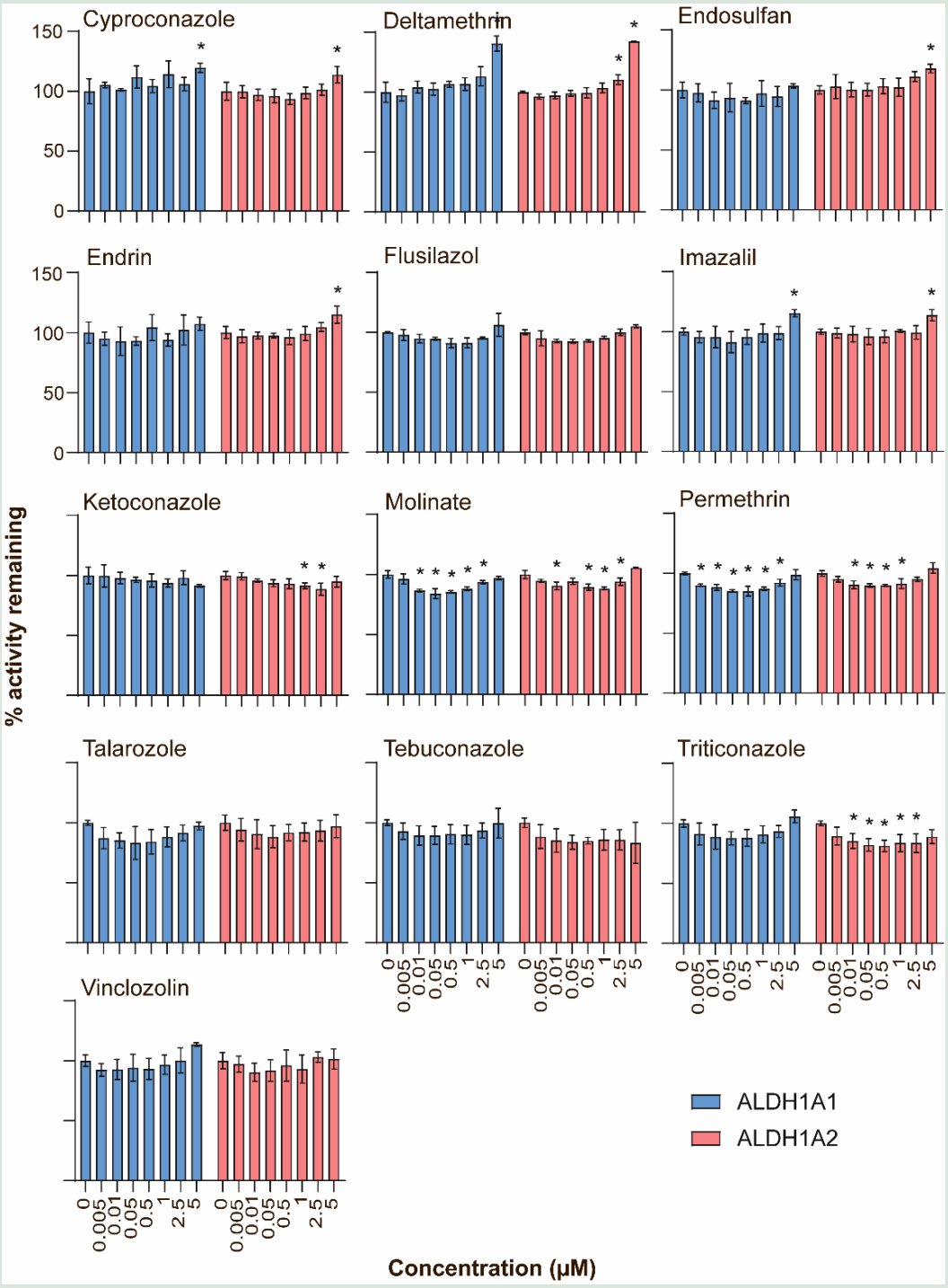

FIGURE SI 2. ALDH1A1 (blue) and ALDH1A2 (red) enzyme activity after exposure to test substances. All groups are normalised to a vehicle control group set to 100%. Graphs represent data from one experiment in technical triplicates (n=3).

ToAD

The 2018 EFSA/ECHA guidance for identifying endocrine-disrupting (ED) properties in biocidal and plant protection products (BP/PPPs) requires linking mechanistic data with in vivo adverse effects, focusing on EATS modalities (estrogen, androgen, thyroid, and steroidogenesis). Non-EATS modalities, such as retinoid signaling, also play critical roles in ED effects. This project explored chemical effects on retinoid signaling by targeting a key enzyme in retinoic acid biosynthesis (RALDH) and the retinoic acid receptor (RAR) as well as inhibition of CYP19 (aromatase) as part of the steroidogenesis pathway.

We tested 21 BP/PPP active substances and selected pharmaceuticals using RAR activity, RALDH inhibition, and CYP19 inhibition assays. Several chemicals increased RARα activity, whereas none antagonized the activity of the receptor. Ten test chemicals inhibited aromatase activity with azoles causing most marked effects. RALDH inhibition was observed for dimethomorph and fenitrothion, while triphenyltin and tributyltin chloride displayed non-monotonic concentration-response patterns. In vitro results were largely consistent with US Tox21 data, supporting model robustness. QSAR models for RAR inhibition were developed and validated using available experimental data from ~10,000 compounds. Finally, Adverse Outcome Pathway (AOP) relevant for perturbed retinoid signaling were developed, focusing on reproductive effects.

These methods, if combined, can enhance the ability to screen for non-EATS-mediated ED effects and provide mechanistic insights into reproductive toxicity, supplementing EFSA/ECHA guidelines. While in vitro and in silico methods alone are insufficient to confirm ED properties, they improve chemical prioritization, reduce reliance on animal testing, and advance the development of integrated testing strategies emphasizing multiple mechanisms of action.

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