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Research Article

# Identifying Candidate Reference Chemicals for *In Vitro* Testing of the Retinoid Pathway for Predictive Developmental Toxicity

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**Abstract**
Evaluating chemicals for potential *in vivo* toxicity based on their *in vitro* bioactivity profile is an important step toward animal-free testing. A compendium of reference chemicals and data describing their bioactivity on specific molecular targets, cellular pathways, and biological processes is needed to bolster confidence in the predictive value of *in vitro* hazard detection. Endogenous signaling by all-trans retinoic acid (ATRA) is an important pathway in developmental processes and toxicities. Employing data extraction methods and advanced literature extraction tools, we assembled a set of candidate reference chemicals with demonstrated activity on ten protein family targets in the retinoid system. The compendium was culled from Protein Data Bank, ChEMBL, ToxCast/Tox21, and the biomedical literature in PubMed. Finally, we performed a case study on one chemical in our collection, citral, an inhibitor of endogenous ATRA production, to determine whether the literature would support an adverse outcome pathway explaining the compound's developmental toxicity initiated by disruption of the retinoid pathway. We also deliver an updated Abstract Sifter tool populated with these reference compounds and complex search terms designed to query the literature for the downstream consequences to support concordance with targeted retinoid pathway disruption.

## 1 Introduction

Opportunities exist for refining and supplanting current developmental and reproductive toxicity (DART) testing protocols with *in vitro* data and *in silico* models that advance alternatives to animal testing (Scialli et al., 2018). The term 'new approach methods' (NAMs) has been recently adopted in reference to any non-animal technology, methodology, approach, or combination thereof that can be used to provide information on chemical hazard and risk assessment that reduces the use of intact animals (USEPA, 2018).

Regulatory interest in the retinoid system is growing because of the increasing recognition that the retinoid pathway plays a critical role in many biological functions, especially during embryofetal development, and because of the growing number of environmental chemicals suspected to disrupt the pathway (Grignard et al., 2020; Mark et al., 2009; Duester, 2008). In 2012 the OECD Test Guidelines Programme in a Detailed Review Paper (DRP 178) identified a need for harmonized regulations on the

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retinoid system for toxicity screening and evaluation. The Endocrine Disruption Testing and Assessment Advisory Group (EDTA AG) initiated work on a DRP to review knowledge on the retinoid signaling pathway in multiple organ systems, which was subsequently narrowed to four areas: Overview, Reproductive System (Annex A), Skeletal Patterning (Annex B), and CNS Development (Annex C) (OECD, 2021, 2014). Currently, there are no validated assays in OECD guidelines probing the retinoid system despite its critical role in development. The ongoing work has resulted in a number of publications (Knudsen et al., 2021; Grignard et al., 2020; Damdimopoulou et al., 2019; Chen et al., 2020). Researchers who develop or employ NAMs for DART testing of the retinoid pathway will need a comprehensive set of reference chemicals for testing and to vet and establish confidence in their assays (Judson et al., 2019). Here, our primary research aim was to compile information from the open literature and public databases to build a collection of chemical compounds with demonstrated activity against key targets in the retinoid pathway. The compilation will be delivered as a list of compounds and assays linked to the publications in which they were identified. The results will serve as a resource for researchers developing new approach assays for the retinoid system.

While much of the retinoid toxicity research focuses on functional disruption of signaling by all-trans retinoic acid (ATRA), this work will survey ten significant targets in the retinoid pathway (Figure 1 and Table 1). The proteins and enzymes that mediate retinoid transport, metabolism, and transcription can influence levels of ATRA activity, thereby regulating - or dysregulating - other key pathways in development (Metzler and Sandell, 2016).

In the blood, dietary retinol (vitamin A) circulates bound to a complex containing retinol binding protein (RBP, sometimes referred to as plasma or serum retinol binding protein) and transthyretin (TTR - *trans*porter of *thyr*oxin and *retin*ol) (Mujawar et al., 2014). This complex breaks apart when retinol leaves the complex at the cell surface to bind to the cell membrane receptor stimulated by retinoic acid 6 (STRA6) for transport into the cell. STRA6 plays dual physiological roles as a transporter and a cell surface receptor that upon binding sets off a signaling cascade (Berry et al., 2012). Once in the cell, retinol is bound by cellular retinol binding proteins (CRBPs) (Kelly and von Lintig, 2015; Noy, 2016; Napoli, 2016). CRBPs regulate retinoid biology through dual intracellular functions, both as a transport mechanism and as a sink or storage for retinol (Napoli, 2017).

Before it can become biologically active, retinol must be transformed first to retinal and then from retinal to ATRA. (Figure 1.) The first step is performed by enzymes in the alcohol dehydrogenase (ADH) family, specifically retinol dehydrogenase (RDH) members. The second oxidation step in the retinoid pathway converts retinal (retinaldehyde) to ATRA, the active signaling molecule. The metabolism of aldehydes – endogenous and exogenous – is the role of enzymes in the aldehyde dehydrogenase (ALDH) superfamily and specifically the retinal / retinaldehyde (RALDH) forms.

In the cytoplasm cellular retinoic acid binding proteins 1 and 2 (CRABP1 and CRABP2) bind ATRA with high affinity and deliver this molecule to the nucleus. It is thought that CRABP1 shuttles ATRA to metabolic enzymes (e.g., cytochrome p450s) to buffer against ATRA excess, and CRABP2, in contrast, transports ATRA into the nucleus and delivers it to the RAR/RXR receptor complex to regulate gene expression (Napoli, 2017; Wei, 2016).

Retinoic acid 4-hydroxylases comprise a subfamily of cytochrome P450 enzymes that break down ATRA. The three known isoforms, CYP26A1, CYP26B1, and CYP26C1, all metabolize ATRA efficiently but differ in their tissue localization. In the developing embryo, they exhibit differential cell-specific developmental regulation (Helvig et al., 2011), an action that in part accounts for regional ATRA gradients.

The three retinoic acid receptor forms are alpha (RARa), beta (RARb) and gamma (RARg). All isoforms heterodimerize with the retinoid X receptors (RXR) to regulate transcription at the retinoic acid response element (RARE) binding sites in ATRA-responsive genes. There is evidence for at least 27 genes under direct control of the RAR/RXR complex and many more under indirect control (Balmer and Blomhoff, 2002; Grignard et al., 2020). Compounds that bind one of the RAR receptors often bind one or more of the other isoforms.

The RARs form dimers with the RXRs, and while this activity is critical to downstream gene expression in the retinoid pathway, RXRs also form dimers with other receptors, e.g., peroxisome proliferator-activated receptors, liver X receptors, and farnesoid X receptors (Brtko and Dvorak, 2020; Knudsen et al., 2021). Because of the toxicological and metabolic complexity of RXR biology, RXRs were not considered in-scope for this work in order to focus on the RAR-mediated pathway alone. Other molecules that play roles in the retinoid pathway and could arguably be included in Figure 1 and this study. Metabolizing enzymes such as the cytochrome P450 isoforms CYP1A1 and CYP1B1 and glucuronosyltransferases have been linked to ATRA metabolism (Rowbotham et al., 2010; Maguire et al., 2020; Li et al., 2017). These enzymes are less studied in the context of the retinoid pathway and, except for including CYP1A1 ToxCast results, they have been omitted from this work.

While reference chemicals are key to building confidence in *in vitro* assays (Judson et al. 2019), establishing confidence in the reference chemicals is also important. For example, evidence that a compound known to disrupt a protein target in the retinoid pathway also causes the adverse effects associated with altering that target activity will increase the confidence in that chemical as a potential reference compound in retinoid assays. In other words, demonstrating that a chemical not only shows activity in an *in*

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**Abbreviations:** ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; AOP, adverse outcome pathway; ATRA, all-trans-retinoic acid (tretinoin for pharmaceutical use); CRABP, cellular retinoic acid binding protein; CYP26, cytochrome P450 family 26; DART, developmental and reproductive toxicology; DHAND, heart and neural crest derivatives protein; DR5, direct repeats of 5 nucleotides for RAR/RXR transactivation; DRP, detailed review paper; FGF, fibroblast growth factor; Hoxa1, homeobox A1; NAM, new approach methods; OECD, Organization of Economic Cooperation and Development; RALDH, retinal dehydrogenase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RBP, retinol binding protein (plasma or serum); RDH, retinol dehydrogenase; RXR, retinoid X receptor; SDR, short-chain dehydrogenase/reductase; STRA6, stimulated by retinoic acid 6; ZPA, zone of polarizing activity

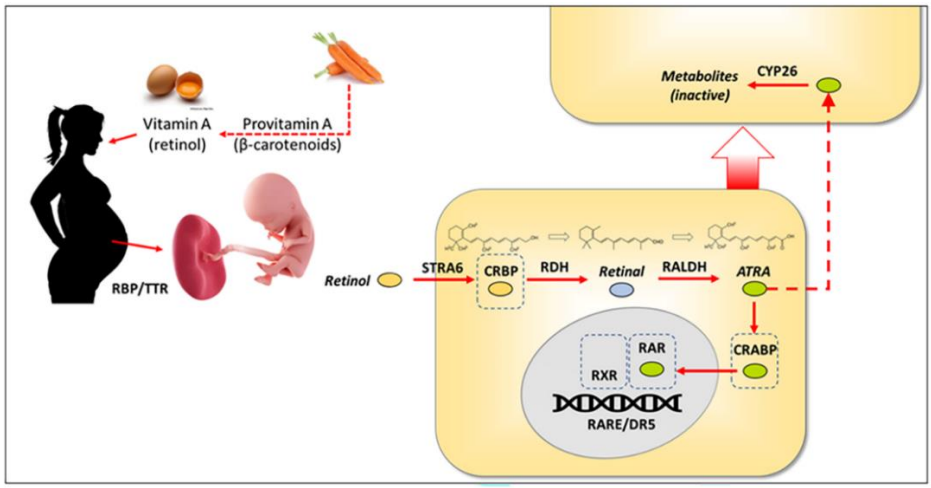



**Fig. 1: Diagram of retinoid pathway in human pregnancy**
In development, dietary Vitamin A (retinol) is transferred to the fetus through the placenta into the bloodstream. Dehydrogenase enzymes (RDH and RALDH) transform retinol to retinal and then to the active molecule ATRA. Transport proteins such as STRA6, CRBP, CRABP move retinol and its forms (retinal, ATRA) from the bloodstream into the nucleus where ATRA binds the retinoic acid receptor / retinoid X receptor complex to initiate gene transcription.

**Tab. 1: Overview of retinoid pathway targets reviewed**

| Section | Retinoid target | Long name | Basic function | Data source and entries | Table |
|---|---|---|---|---|---|
| 3.1 | RBP | Retinol binding protein (serum / plasma) | Transports dietary derived and in situ synthesized retinol in serum | PDB entries | S1[1] |
| | | | | ChEMBL assays | S2[1] |
| 3.2 | STRA6 | stimulated by retinoic acid gene 6 protein | Transports retinol across membrane into cell from bloodstream; receptor for retinol uptake; signaling | PDB entries | S3[1] |
| 3.3 | CRBP (CRBP1, CRBP2, CRBP3) | Cellular retinol binding protein | Binds retinol inside the cell | PDB entries | S4[1] |
| 3.4 | CRABP (CRABP1, CRABP2) | Cellular retinoic acid binding protein | Binds ATRA; Facilitates transfer of ATRA from cytosol to nucleus | PDB entries | S5[1] |
| | | | | ChEMBL assays | S6[1] |
| | | | | Fogh et al., 1993 | 2 |
| | | | | Chaudhuri et al., 1999 | 2 |
| 3.5 | CYP26 | Retinoic Acid 4-Hydroxylase OR Cytochrome P450 Family 26 | Degradation of ATRA | ChEMBL assays | S7[1] |
| | | | | Thatcher et al., 2011; Buttrick, 2013; Foti et al., 2016a,b | 3 |
| 3.6 | RDH | Retinol dehydrogenase | Transforms retinol to retinal through dehydrogenation | ChEMBL assays | S8[1] |
| 3.7 | ALDH / RALDH | Aldehyde dehydrogenase / retinal dehydrogenase | Transforms retinal to ATRA through dehydrogenation | PDB entries | S9[1] |
| | | | | ChEMBL assays | S10[1] |
| | | | | Koppaka et al., 2012 | 6 |
| 3.8 | RARa | Retinoic acid receptor alpha | ATRA receptor | PDB entries | S11[1] |
| | | | | ChEMBL assays | S12[1] |
| | | | | ToxCast assay results | S17[1] |
| 3.9 | RARb | Retinoic acid receptor beta | ATRA receptor | PDB entries | S13[1] |
| | | | | ChEMBL assays | S14[1] |
| | | | | ToxCast assay results | S17[1] |
| 3.10 | RARg | Retinoic acid receptor gamma | ATRA receptor | PDB entries | S15[1] |
| | | | | ChEMBL assays | S16[1] |
| | | | | ToxCast assay results | S17[1] |
| 3.11 | Retinoid pathway | Retinoid pathway | | Chen and Reese, 2013; Chen et al., 2016b | 7 |
| | | | | ToxCast / Tox21 assay results | S17[1] |
| | | | | | S17[1] |

*vitro* assay on a target in the retinoid system but that the chemical has evidence linking it to the other key events and outcomes in an adverse outcome pathway (AOP) will bolster confidence in the chemical as a retinoid disruptor (Villeneuve et al., 2014).

Limb development is one of the developmental processes in which the retinoid pathway is crucial and disruption of that pathway causes defects. ATRA gradients direct the morphology of the developing limb where a precisely orchestrated interplay of ATRA and fibroblast growth factor (FGF) gradients control the normal development of the stylopod, zeugopod, and autopod regions (Knudsen et al., 2021). Exogenous ATRA disrupts this gradient balance and causes limb defects, including phocomelia and digital defects in mice, rats, and chicks (Kochhar, 1973; Wiley, 1983; Yu et al., 2003). Excess ATRA caused by absence or down-regulation of the ATRA metabolizing enzyme CYP26 also has limb effects. Cyp26b1-/- mutant mice lack the CYP26 enzyme that eliminates ATRA, causing excess levels of the morphogen. These mice exhibit multiple forelimb, hindlimb, and digit deformities (Yashiro et al., 2004). Too little ATRA in locations where it is required is also teratogenic (Lee et al., 2004). Reducing synthesis of ATRA through imbibition of RALDH2 has been associated with limb defects. Mouse knockouts of RALDH2 result in several abnormal limb phenotypes including small or absent forelimb and hindlimb buds, abnormal digits, and syndactyly (Niederreither et al., 2002; Vermot et al., 2005). We previously assembled a provisional AOP describing a pathway starting with RALDH2 down-regulation and leading to limb defects as the adverse outcome (Figure 2).

A chemical with a substantial history of use or environmental exposure may indeed have other published supporting evidence of its molecular activity, including effects on cells, tissue, organs, and adverse outcomes in an intact organism or population. Our second aim was to gather and assess the literature support for our chemical set causing adverse outcomes associated with retinoid disruption. We draw connections between the chemicals and outcomes first broadly by querying the biomedical literature for areas of developmental toxicity co-occurring with the candidate reference chemicals, and then we focus on one chemical – citral, an inhibitor of endogenous ATRA production, to perform an in-depth literature review looking for evidence that the chemical participates in an AOP linking RALDH inhibition to developmental limb defects through the steps illustrated in the AOP in Figure 2.

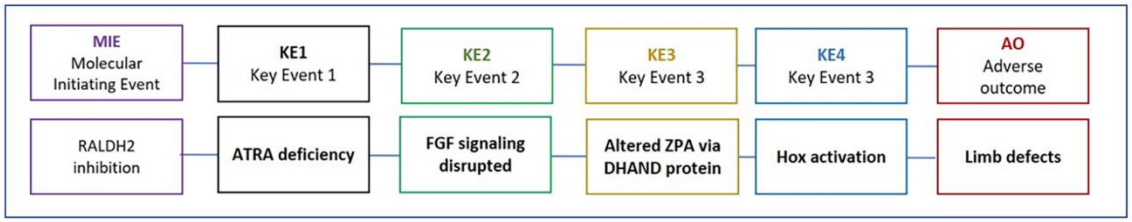



**Fig. 2: Provisional AOP for limb defects caused by RALDH2 inhibition**
The molecular initiating event (MIE) is inhibition of RALDH2, which in turn decreases the synthesis of ATRA (Key Event or KE), followed by disruption of the balance in the fine-tuned gradients of ATRA and its signaling antagonist FGF8 in the limb bud (Knudsen et al., 2021). The imbalance affects the zone of polarizing activity (ZPA) and expression of DHAND (heart and neural crest derivatives expressed) protein and is followed by alterations in RARa/RARg regulated expression of Homeobox genes (Hox) (Niederreither et al., 2002). The imbalance disrupts conditions for normal limb growth, particularly digit formation.

## 2 Methods

The molecular targets in the retinoid pathway that are the focus of this work are listed and briefly described in Table 1. While the primary interest is human data, information on model organisms from different vertebrate species is considered.

### 2.1 Data bases mined for chemical retinoid system disruptors/modulators

The information on the retinoid system is abundant – a search using the term "retinoid' in PubMed returns over 64,000 entries – and each of the proteins and enzymes in the retinoid pathway has substantial literature on its own. To make the project tractable, we started with structured data in publicly available databases that describe *in vitro* assay results of a chemical's activity against one of the pathway targets. These databases included Protein Data Bank (PDB) (Berman et al., 2003), ChEMBL (Gaulton et al., 2017), and ToxCast/Tox21 (Richard et al., 2016; Judson et al., 2010). PDB is a repository of protein structures, often bound with small molecule ligands. Some ligands are present to stabilize the protein for crystal formation, but sometimes the researchers test potential drug candidates for binding specificity against a target. ChEMBL is a source of assays and chemical data curated from publications, and ToxCast/Tox21 is a repository of high-throughput assay data. We elected not to use PubChem at this point because its data are deposited by contributors and not curated (Kim et al., 2019). The other source of information was literature occurrences of chemical-target interactions in PubMed's MeSH terms. Database retrieval was performed originally in 2020 and reviewed for additional records in May, 2022.

For each target, we queried PDB (Berman et al., 2003) using the protein name or official identifier as a query term. Results from PDB were downloaded and are presented in a supplemental table[1]. To condense these results, some information was summarized or excluded (e.g., the chain information for each entry). Hyperlinks for the PDB Identifier (PDB ID) and the PubMed

[1] doi:10.14573/altex.2202231s1

Identifier (PMID) literature record were added to the table. If multiple ligands were used for crystallization, the chemical names are separated by a backslash "/". The column headed "Yr dep" is the year of the deposition to PDB.

ChEMBL was also queried for each target (Gaulton et al., 2017). Resulting assay records were downloaded using web services and formatted into a table. The table contains the assay description and hyperlinks to the assay at ChEMBL and to PubMed when a PMID is provided.

When available for a target, the ToxCast and Tox21 assays were downloaded and included (Richard et al., 2016; Judson et al., 2010). The ToxCast/Tox21 portfolio includes >600 high-throughput screening (HTS) *in vitro* assays for a wide variety of biochemical and cellular responses, including three retinoid pathway receptor targets and the retinoid pathway (described in Table S18[1]). The ToxCast/Tox21 chemical universe comprises environmental toxicants such as pesticides as well as chemicals in consumer products, cosmetics, and pharmaceuticals (Richard et al., 2016). These data were downloaded from the EPA CompTox Chemicals dashboard[2] (downloaded December, 2019) (Williams et al., 2017). In this database, bioactivity is recorded as a 'hit' based on AC50 (micromolar concentration resulting in 50% change in maximum activity observed) or related measurements, as well as automated concentration curve responses generated in the ToxCast data pipeline (Filer et al., 2017). For this work, we limited hits to those chemical-assay pairs where the AC50 < 2.0 micromolar to allow focus on the most potent chemicals. Next, each record was reviewed and curated to remove equivocal hits. We inspected the cytotoxicity values and caution flags associated with each automated hit call and included only hits that were associated with fewer than three caution flags (Filer et al., 2017; Judson et al., 2010). On the EPA CompTox Chemicals Dashboard, these flags are found below the curve charts for each chemical – assay pair and the cytotoxicity cutoff line is colored red. The chemicals remaining after the curation step were inserted into tables with the AC50 values and chemical identifiers used by the EPA CompTox Chemicals Dashboard (DSSToxIDs) hyperlinked to the dashboard. The ToxCast / Tox21 assays that test targets associated with the retinoid pathway and are discussed here are described in Table S18[1]. For the retinoid pathway assays in Tox21, in addition to the filtering steps applied to all ToxCast/Tox21 results, additional data quality filters were applied to remove activity hit calls flagged for questionable quality. Results retained were for compounds active at concentrations below the cytotoxicity cutoff, had fewer than three caution flags and did not have either of the caution flags: "Less than 50% efficacy" or "AC50 less than lowest concentration tested".

Next, the EPA's literature database of Medical Subject Heading (MeSH) terms extracted from PubMed (Judson et al., 2019; Baker and Hemminger, 2010) was queried for chemical annotations associated with the MeSH annotations of the protein targets. These target – chemical annotation pairs were identified and exported to an Excel workbook[3] and subsequently reviewed and curated for chemicals of interest.

### 2.2 Connecting the chemicals to the biological literature

The chemical-target sets from each of these retrieval steps were inserted into the PubMed Abstract Sifter literature mining tool (Baker et al., 2017) CuratedChemicals sheet. Using the batch search capability of the EPA CompTox Chemicals Dashboard, the DSSTox identifier for each chemical was retrieved and added as a hyperlink. A query string consisting of the chemical name, CAS-RN, and MeSH synonym (if found) were added to the sheet. The functionality of the Abstract Sifter is described in the user guide in detail and will not be reproduced here. Both the Abstract Sifter and user guide are available[4].

A previous study compiled potential AOPs that cause skeletal defects from retinoid pathway disruption during embryonic development (Knudsen et al. 2021). We took each of these AOP key events and built PubMed queries designed to retrieve the citations describing that key event's biological activity. For example, the complex query designed to find citations describing limb defects reads "(Limb deformities, Congenital[tw] OR limb defects OR polydactyly OR Brachydactyly OR digits OR Fingers/abnormality OR toes/abnormality OR limb bud/drug effects OR Ectromelia OR amelia OR hemimelia OR phocomelia OR and sirenomelia)) AND (embryo OR fetus OR fetal OR embryonic)". The queries for each step in the skeletal disruption AOPs were stored on the Pathway_Queries sheet of the Abstract Sifter.

The connections between the chemical corpus and the literature describing biological activity were explored using the Landscape sheet of Abstract Sifter. Chemical entities were copied from the CuratedChemicals sheet to the Landscape sheet column C and pathway queries were copied to the Landscape sheet into Row 3. The article counts resulting from queries composed of the chemicals and the biological queries were retrieved and results sorted.

### 2.3 Citral case study and AOP for developmental limb defects

We selected one chemical – citral – and performed a case study to determine whether there was literature evidence to support a complete putative AOP starting with RALDH inhibition and leading to developmental limb defects. Citral was chosen because it has been used for several decades in experiments to disrupt the retinoid pathway through blocking of RALDH (Connor and Smit, 1987; Kikonyogo et al., 1999). Composed of two isomers, nerol and geraniol, citral is a naturally occurring substance in the oils of several plants such as lemon verbena. The first article about citral in PubMed was in 1947 but the first in English was in 1949 describing the application of citral in glaucoma (Kaminskaia, 1949). Since then, there have been articles describing its potential use in cancer, glaucoma, parasitic diseases, atherosclerosis, fungal diseases and as an insect repellant. It has been tested for safety because it is commonly used in perfumes and cosmetics for its lemon aroma. Because of its many uses, citral has accumulated a

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

[3] doi:10.14573/altex.2202231s2

[4] https://comptox.epa.gov/dashboard/downloads

substantial literature presence. The Abstract Sifter literature mining tool (Baker et al. 2017) was used to search PubMed for literature evidence connecting citral to each of the key events in the putative developmental AOP in Figure 2.

## 3 Results

The candidate reference chemicals identified in the following work can be found in the accompanying Abstract Sifter tool[5] organized by target on the RefChemSet sheet, in a simple list on the AllChems sheet, and on the Landscape sheet, shown with sample toxicity-related queries and resulting article counts.

### 3.1 Collection of data on the specific targets in the retinoid pathway

#### *3.1.1 Retinol binding protein – serum/plasma (RBP)*

The structure of serum RBP has been studied extensively, resulting in many PDB entries, which are summarized in Table S1[1]. Some of these crystal structures include transthyretin and retinol; retinoids are the most commonly used ligands. ChEMBL contains 22 assays for serum RBP curated from seven publications. Descriptions of these assays are in Table S2[1]. Most of the assays are binding assays against recombinant human serum RBP. Researchers interested in RBP as a potential therapeutic target have identified retinoid and non-retinoid (without retinoid structure) compounds that bind RBP and inhibit its activity (Cioffi et al., 2014; Wang et al., 2014). Retinol is the physiological ligand and is the *de facto* reference chemical in many assays. Other chemicals of interest are described below.

Fenretinide is a synthetic retinoid used as a positive control in some ChEMBL studies. This drug disrupts the RBP-TTR complex by competing with retinol in binding to RBP. Because the drug is associated with a number of side effects, other non-retinoids that bind RBP have been sought (Racz et al., 2018). Compound A1120 was one such non-retinoid found to bind RBP in a way that disrupted the association and interaction of RBP and TTR. It was originally developed as a treatment for diabetes, but more recently compound A1120 has been studied as a potential treatment for macular degeneration (Cioffi et al., 2014; Hussain et al., 2018; Wang et al., 2014). With A1120 as a starting point, a set of compounds was rationally designed and tested by Racz and colleagues for RBP activity, leading to the identification of BPN-14136, a non-retinoid that effectively binds RBP, disrupts the RBP-TTR complex, and thus blocks the *in vivo* delivery of retinol to cells. (Racz et al., 2018). Additionally, in studies in women with gestational diabetes, the drug sitagliptin has been found to downregulate RBP protein levels (Sun et al., 2017).

#### *3.1.2 Stimulated by retinoic acid 6 (STRA6)*

STRA6 structure is well-described and its function has been characterized in assays testing for the uptake of retinol into cells (Breen et al., 2015; Chen et al., 2016a; Kawaguchi and Sun, 2010). The PDB entries investigate STRA6 (Table S3[1]) bound to endogenous substances, primarily the protein calmodulin. The binding of STRA6 and calmodulin is thought to be an important regulation step of the retinoid pathway and a potentially important target for pharmaceuticals (Zhong et al., 2020).

Mutations in *Stra6* are associated with severe developmental defects (Pasutto et al., 2007) and diseases such as diabetes (Chen et al., 2019); however, there are no records in ChEMBL or PubChem for STRA6 nor literature connecting it with drug therapies or chemical toxicity.

#### *3.1.3 Cellular retinol binding proteins (CRBP)*

CRBP has two main forms with the official gene symbol of *Rbp1* (commonly reported as CRBP1 or CRBP-I) and *Rbp2* (commonly reported as CRBP2 or CRBP-II). Their structure has been explored through crystal structures and other methods. PDB has over 50 records for CRBPs (Table S4[1]); however, there is less information on CRBP1 and CRBP2 binding data or functional assays in ChEMBL or ToxCast datasets.

In the PDB entries, retinol and retinal are the most commonly seen ligands. In more recent years, interest has grown in CRBP as a pharmacological target, particularly in ocular disorders and diseases. While retinoid derivatives are often tested (e.g., retinylamine), the search for analogues or antagonists has focused on non-retinoid compounds that will not be as likely to produce adverse effects associated with retinoids. High-throughput screening (HTS) methodology was used to measure displacement of retinol from CRBP1 to test a library of over 900 compounds (Silvaroli et al., 2019). A cannabidiol derivative called abnormal cannabidiol (abn-CBD) was identified as a potent ligand and inhibitor of CRBP1 (6E5L). With this compound as a starting point, researchers found that derivatives such as cannabidioricin (6E6M) and 2-AG (2-arachidonoylglycerol) were also strong ligands. In recent work, a series of bioactive lipids were found to bind to CRBP2 with an affinity comparable to retinol, including the endocannabinoids 2-arachidonoylglycerol, 2-arachidonoylglycerol, 2-oleoylglycerol, and 2-lineoylglycerol (Lee et al., 2020; Silvaroli et al., 2021).

#### *3.1.4 Cellular retinoic acid binding protein (CRABPI and CRABP2)*

While the structures of CRABP1 and CRABP2 are similar, their localization and functions appear to be distinct (Donovan et al., 1995; Ruberte et al., 1992; Wei, 2016). This distinction holds in the differing associations between the proteins and diseases,

[5] doi:10.14573/altex.2202231s3

particularly cancer (Favorskaya et al., 2014). Whereas elevated CRABP1 is associated with poor outcomes in breast cancer (Liu et al., 2015), elevated CRABP2 has the opposite correlation. In some cancers such as Wilms tumors, the pattern is reversed, and a poor outcome is associated with high CRABP2 (Takahashi et al., 2002).

The associations between each CRABP form and disease have motivated the search for compounds that specifically bind each protein. PDB has many entries for CRABP2 and some for CRABP1 (Table S5[1]) and ChEMBL has assays for both proteins (Table S6[1]). The compounds tested have mainly been new synthetic retinoids. With the identification of these compounds, researchers hope to find drugs that have some of the activity of ATRA, but without the adverse outcomes, particularly teratogenesis. (Fogh et al., 1993) measured the binding affinity of a series of retinoid compounds against CRABP1 and CRAPB2. The active compounds results are listed in Table 2, and the complete results can be found in the publication. Binding affinity of a series of retinoids to CRABP1 and CRAPB2 (Chaudhuri et al., 1999) is summarized in Table 2. The chemical set includes AM80 (tamibarotene), a known RAR alpha agonist (Kagechika et al., 1988) that is available commercially and tested for chemotherapeutic efficacy (Anonymous, 2004). The set also includes TTNPB, a synthetic retinoid that is more active than ATRA but often used in in vitro studies such as the mouse limb bud assay (Pignatello et al., 1997). There are ChEMBL data for isotretinoin and alitretinoin in addition to newly synthesized compounds with undefined names. Finally, 4-amino-2-trifluoromethyl-phenyl retinate is a ATRA derivative that has been used in cell assays since 2013 (Wang et al., 2013) and in 2018 was found to regulate CRABP2 (Ju et al., 2018).

**Tab. 2: Chemicals active in binding assays for CRABP1 and CRABP2 in selected publications**

| Source | Chemical name |
|---|---|
| Fogh et al., 1993 | CD 367 |
| | All-trans retinoic acid (ATRA) |
| | TTNPB |
| | 4-oxoretinoic acid |
| Chaudhuri, Kleywegt et al. 1999 | All-trans retinoic acid (ATRA) |
| | Ro13-6307 |
| | Ro12-7310 |
| | Am80 (tamibarotene) |
| | TTNPB |

### *3.1.5 Retinoic acid 4-hydroxylase (CYP26 subfamily)*

Retinoic acid 4-hydroxylases comprise a subfamily of cytochrome P450 enzymes that break down ATRA. The three known subforms, CYP26A1, CYP26B1, and CYP26C1, all metabolize ATRA efficiently but differ in their tissue localization. In the developing embryo, they exhibit differential cell-specific developmental regulation (Helvig et al., 2011), an action that in part accounts for regional ATRA gradients.

Recent interest in the CYP26 subfamily has focused on pharmacological manipulation of endogenous retinoids in the treatment of a number of diseases, particularly cancer (Bruno and Njar, 2007; Thatcher et al., 2011). PDB has no structural entries for any of the CYP26 forms, although primary genomic structure has been investigated (Foti et al., 2016a). ChEMBL has 29 assay entries for CYP26A1 and two entries for CYP26B1, summarized in Table S7[1]. The most common active chemicals were liarazole, talarozole, ketoconazole, fenretinide, and bexarotene. The azole ring system is a common feature among compounds that disrupt ATRA signaling at CYP26 but lack a typical retinoid chemical structure.

A survey of the literature reveals other interesting assays. Helvig et al. developed a binding assay for the binding pocket of CYP26A1, CYP26B1, and CYP26C1 that allows for comparison of affinity measures (Helvig et al., 2011). For reference, they tested ATRA, 9-cis-retinoic acid, 13-cis-retinoic acid, and ketoconazole. A set of 42 compounds was evaluated for activity against CYP26A1 in a screening phase at 10 µM and then a second assay at 1 µM to identify the inhibitory potency (Thatcher et al., 2011). The 10 most potent inhibitors are listed in Table 3. Three of them are azoles, including talarozole (R115866), liarozole, and ketoconazole. Surprisingly, two of the compounds were PPAR agonists – pioglitazone and rosiglitazone. Three other azoles tested: itraconazole, fluconazole, and voriconazole passed the initial screening at the 10 µM level, but not the 1 µM threshold. In 2016, Foti and colleagues used a homology model to predict how CYP26 would metabolize the drug tazarotenic acid (Agn 190299) and were able to support their prediction by measurement, leading to the inference that CYP26 metabolized this xenobiotic compound (Foti et al., 2016a). Based on the observation that the binding region of CYP26 is similar to that of CYP2C8, they tested a set of known CYP2C8 inhibitors against CYP26A1 and CYP26B1. The initial set of 29 compounds assembled from literature reports was tested first in a 10 uM concentration. The 17 compounds that showed greater than 50% inhibition in the screen were tested with multiple concentrations and the IC50 was calculated. Clotrimazole was the most potent inhibitor tested. For the tested compounds, as a whole, there was a positive and statistically significant correlation between CYP26A1 and CYP2C8 IC50 values and only a weak correlation between CYP26B1 and CYP2C8 (Foti et al., 2016b). Chemicals used as reference compounds in the Foti studies and those that passed the 50% inhibition screen are found in Table 3.

**Tab. 3: Chemicals tested for CYP26A1 and / or CYP26B1 inhibition in selected publications**
For (Foti et al., 2016b) the 17 chemicals that passed the single concentration inhibition screen are listed in addition to results for known CYP26 inhibitors. For all chemicals, consult the publications for measured values, experimental conditions and cut-offs used.

| | Thatcher et al., 2011 | Foti et al., 2016a | | Foti et al., 2016b | | Buttrick, 2013 | |
|---|---|---|---|---|---|---|---|
| **Chemical** | **CYP26A1** | **CYP26A1** | **CYP26B1** | **CYP26A1** | **CYP26B1** | **CYP26A1** | **CYP26B1** |
| 17-alpha-ethinyl estradiol | | | | X | X | | |
| AM580 | | X | X | | | X | X |
| AM80(tamibarotene) | | X | X | X | X | X | X |
| Benzbromarone | | | | X | X | | |
| Bexarotene | | | | X | X | X | X |
| BMS753 | | X | X | | | X | X |
| BMS961 | | X | X | | | X | X |
| Candesartan | | | | X | X | | |
| Candesartan cilexetil | | | | X | X | | |
| CD1530 | X | | | | | | |
| CD437 | | | | X | X | | |
| Clotrimazole | | | | X | X | | |
| CS5 | X | | | | | | |
| EC23 | | | | X | X | X | X |
| Fluconazole | | | | X | X | | |
| Itraconazole | | | | X | X | | |
| Ketoconazole | X | | | X | X | X | X |
| L-165,041 | X | | | | | | |
| Liarozole | X | | | X | X | X | X |
| MM11253 | | | | X | X | X | X |
| Mometasone | | | | X | X | | |
| Montelukast | | | | X | X | | |
| Pioglitazone | X | | | X | X | | |
| Quercetin | | | | X | X | | |
| R115866 (talarozole) | X | | | X | X | X | X |
| R116010 | X | | | | | X | X |
| Raloxifene | | | | X | X | | |
| Repaglinide | | | | X | X | | |
| Ritonavir | | | | X | X | | |
| Rosiglitazone | X | | | X | X | | |
| SR11237 | | | | X | X | X | X |
| Tamoxifen | | | | X | X | | |
| Tazarotene | | | | | | X | X |
| Tazarotenic Acid (Agn 190299) | | X | X | | | X | X |
| TTNPB | X | X | X | | | X | X |
| Zafirlukast | | | | X | X | | |

The Foti work is interesting because, although ToxCast does not contain an assay for CYP26, it does contain an assay for CYP2C8. CYP2C8 is a potential surrogate for CYP26A1 and CYP26B1 bioactivity. The enzyme CYP1A1 has also been shown to metabolize ATRA (Lampen et al., 2000). Results from the ToxCast assays for CYP2C8 and CYP1A1 are included on Table S17[1].

### *3.1.6 Retinol dehydrogenase (RDH)*

RDHs are alcohol dehydrogenases (ADH) and therefore members of the short-chain dehydrogenase/reductase family of enzymes (SDR). The SDR family is large, with over 46,000 members in all species; in humans, 70 genes in the superfamily have been identified (Persson et al., 2009). The historical nomenclature is bewildering: names have changed over the years and are still not firm (Napoli, 2020). Furthermore, SDR enzymes often share substrates: members of the ADH subfamily can play roles in the oxidation of more than one alcohol-containing chemicals, though with varying affinity (Kumar et al., 2012; Persson et al., 2009; Wang et al., 2011). It is thought that while *in vitro* retinol is a substrate for many ADH forms, *in vivo* the retinol dehydrogenases

**Tab. 4: Major RDH genes from (Napoli, 2012).**

| Mouse | Rat | Human | Note |
|---|---|---|---|
| Rdh1 | Rdh7 and Rdh2 (originally RodhI and RodhII) | Rdh16 (originally Rodh4, RDH-E) | Sometimes referred to as ADH Class IV |
| Rdh10 | Rdh10 | Rdh10 | |
| Dhrs9 | Dhrs9 (originally eRolDH2) | Dhrs9 (originally retSdr8, RDHL, Rdh-TBE, RoDH-$E_2$, 3α-HSD) | |

are the only form that can act on retinol bound to RBP (Napoli, 2020). A table from (Napoli, 2012) of the enzymes in mouse, rat, and human that contribute significantly to the retinol dehydrogenase step in ATRA biosynthesis is reproduced in Table 4.

PDB and ChEMBL nomenclature reflects the fact that retinol dehydrogenase is often referred to as alcohol dehydrogenase Class IV in the literature. In PDB, a search using this term resulted in two entries, both from a publication describing the binding of 4-methylpyrazole to the class IV (retinol dehydrogenase) isoform of human alcohol dehydrogenase (Xie and Hurley, 1999). ChEMBL assays found by searching on "retinol dehydrogenase" surfaced over 10 results from the SDR family that act on alcohols nonspecifically and can dehydrogenate retinol to some extent., and then focusing on alcohol dehydrogenase class IV brought up one publication that studied a set of formamide derivatives (Schindler et al., 1998) and highlighted N-heptylformamide for its potency and compared it to all-trans-retinol.

Alcohols, and specifically ethanol, have received attention for their metabolism by the dehydrogenase/reductase family. A review of the ChEMBL publications indicates that inhibitors of these enzymes are sought to counteract the toxic effects of alcohol and therefore the assays focus on enzymes in the liver (Chen et al., 1981; Schindler et al., 1998; Venkataramaiah and Plapp, 2003). Because of the similarity between the phenotype associated with fetal alcohol syndrome and the phenotype linked to vitamin A deficiency (VAD), ethanol has been studied for its effect on retinol oxidation. Mammalian alcohol dehydrogenases from multiple families accept ethanol and retinol as substrates and studies show that ethanol can competitively inhibit alcohol dehydrogenases leading to a decrease in retinal and ultimately ATRA production (Duester, 1991; Molotkov and Duester, 2002). Ethanol will continue to be a topic in the next section of this work. The search for drugs to treat alcohol abuse powers much of the in vitro research on ALDH. In addition to ethanol, other compounds studied for effects on retinol or alcohol dehydrogenases are summarized below.

Cimetidine was found to be a competitive inhibitor of human class IV ADH when tested against ethanol (Allali-Hassani et al., 1998), the fungicide ziram was found to weakly inhibit rat RDH2 (Su et al., 2018), resveratrol inhibited rat RDH2 when tested against steroid substrates (Wang et al., 2017), and the insecticide methoxychlor and its metabolite hydroxychlor (HPTE) both inhibited rat RDH2 with HPTE being the more potent compound (Mao et al., 2018). RDH2 has been shown to be inhibited by gossypol, a compound that disrupts male reproduction (Cao et al., 2019; Lim et al., 2019). The compounds carbenoxolone and phenylarsine oxide have been used *in vitro* to inhibit RDH (Napoli, 2020; Boerman and Napoli, 1995).

In a study published in 1969, pyrazole and pyrazole derivatives were tested against an unnamed form of ADH and the oxidation of both ethanol and retinol was inhibited (Reynier, 1969), indicating that pyrazole inhibits ADH. Pyrazole is a ring structure found in many pesticides and drugs. In subsequent work, 4-methylpyrazole was used in a number of *in vitro* studies to block ADH (Galli et al., 2001). In one study, 4-methylpyrazole ameliorated the toxic effect of retinol on embryonic mice (Collins et al., 1992). 4-methylpyrazole is also known as fomepizole and is available as a drug treatment for methanol and ethylene glycol overdose (Thanacoody et al., 2016).

### *3.1.7 Retinal dehydrogenase (ALDH, RALDH)*

In humans, the ALDH superfamily contains 19 isoenzymes expressed in different tissues at different times and accept a variety of substrates. While it is thought each member of the family has a preferred substrate, each will accept other substrates with lesser affinity (Koppaka et al., 2012). In the developing embryo, ALDH1A2 (also known as RALDH2 for retinal dehydrogenase) is the major retinal dehydrogenase responsible for the conversion of retinal to ATRA during early gestation. ALDH1A1 (RALDH1) and ALDH1A3 (RALDH3) come into play later during facial morphogenesis (Metzler and Sandell, 2016). In general, ALDH enzymes break down compounds from the aldehyde form to the less toxic acid form. Table 5 below (adapted from Koppaka et al. 2012) lists ALDH family members with relevance to retinoids.

**Tab. 5: Subset of aldehyde dehydrogenases (Adapted from (Koppaka et al., 2012))**

| Gene | Preferred substrate |
|---|---|
| ALDH1A1 | Retinal |
| ALDH1A2 | Retinal |
| ALDH1A3 | Retinal |
| ALDH1B1 | Retinal and acetaldehyde |
| ALDH2 | Acetaldehyde |
| ALDH8A1 | Retinal |

The records in PDB for ALDH1A1, ALDH1A2, and ALDH1A3 reflect the search for new chemical entities specific for these enzymes (Table S9[1]). Morgan and Hurley developed an assay that identifies selective inhibitors of ALDH1A1 including two distinct chemical classes (Morgan and Hurley, 2015). The structure of ALDH1A2 was studied using crystallography and binding studies were performed against a range of compounds, including WIN18,446 (Chen et al., 2018). PDB contains one record for ALDH1A3 describing its structure bound to ATRA (Moretti et al., 2016).

The 72 assays deposited in ChEMBL are summarized in Table S10[1]. Many of the studies are motivated by the need to modulate the retinoid pathway for disease treatment. Specific inhibitors have been sought for ALDH1A1, ALDH1A1, and ALDH1A3 for treatment of Parkinson's disease, obesity, and cataracts, and various types of cancer (Huddle et al., 2018; Morgan and Hurley, 2015; Quattrini et al., 2020). The ChEMBL collection includes high throughput assays conducted by the National Center for Advancing Translational Sciences (NCATS). Originally deposited in PubChem, over 220,000 chemicals were evaluated for inhibition of ALDH1A1 activity in an *in vitro* assay (Yasgar et al., 2017).

The links between the retinoid pathway and ethanol are strong, both in the literature about embryonic development and in the literature about treatments for alcohol abuse. Ethanol is known to be toxic to the developing fetus. With ethanol, the first oxidation step transforms alcohol to acetaldehyde, the compound acted upon by ALDH in the second oxidation step to form acetic acid. When this second oxidation is blocked by inhibition of ALDH or by genetic variations in ALDH2 that render it incapable of performing the transformation, acetaldehyde builds up and causes alcohol flushing syndrome in humans, a condition characterized by flushing, shakiness, nausea, and tachycardia. The drug disulfiram (trade name Antabuse) was designed to inhibit ALDH and cause alcohol flushing syndrome with the goal of causing patients to avoid drinking (Bell and Smith, 1949) (Jacobsen and Larsen, 1949). The search for new alcoholism treatments has energized research into ALDH to identify compounds which specifically target certain isoforms (Koppaka et al., 2012). Disulfiram has been shown to inhibit the retinal dehydrogenase ALDH1A1 as well as ALDH2 (Jin et al., 2018; Kim et al., 2017). When signs of alcohol flushing syndrome are observed following (non-ethanol) chemical exposures, physicians and researchers have learned to suspect ALDH inhibition is involved (Sharma et al., 2009; Plouvier et al., 1982; Garnier et al., 1992; Finulli and Magistretti, 1961).

A link between disulfiram and cancer treatments has also been established, resulting in an increase in publications describing the drug as a cancer therapeutic by itself (Liu et al., 2012; Lu et al., 2021; Zhang et al., 2020), and as an adjunct therapy for chemotherapeutics (Kast and Belda-Iniesta, 2009). The activity of ALDH is understood to protect cancer cells from the effects of therapeutics and contributes to drug resistance. As an adjunct therapy, disulfiram blocks ALDH, making the cancer cells less likely to develop resistance to treatment (Wang et al., 2018; Raha et al., 2014). This line of inquiry has contributed to the testing of compounds in ALDH assays to search for other drugs to use for cancer patients (Thomas et al., 2016).

Koppaka et al. have reviewed inhibitors of ALDH (Koppaka et al., 2012). Table 6 summarizes their findings on significant inhibitors of the retinal dehydrogenases from the literature. A number of the compounds require metabolism to be active and the manuscript should be consulted for more detail.

**Tab. 6: Known inhibitors of aldehyde dehydrogenases (Adapted from (Koppaka et al., 2012))**

| Chemical inhibitor |
|---|
| Ampal |
| Benomyl |
| Citral |
| Chloral hydrate |
| Chlorpromamide analogs |
| Coprine |
| Cyanamide |
| Daidzin |
| CVT-10216 |
| DEAB |
| Disulfiram |
| Gossypol |
| Kynurenine |
| Molinate |
| Nitroglycerin |
| Pargyline |

A number of pesticides in the thiocarbamate family share chemical structural features with disulfiram and its metabolites and have also been shown to inhibit ALDH. Quistad et al. tested a set of thiocarbamate pesticides in *in vitro* ALDH binding assays and *in vivo* assays measuring the effects on acetaldehyde levels in mice (Quistad et al., 1994). They found some of the pesticides had activity similar to the ALDH inhibitor disulfiram. The publication has the full list of chemicals tested and their percent inhibition of liver ALDH. Among the most potent inhibitors were EPTC, thiobencarb, pebulate, vernolate, and molinate. Experiments testing molinate and its metabolites for their relative inhibitory potency in mouse and human *in vitro* models indicated

molinate sulfone was the most potent in ALDH inhibition (Allen et al., 2010). These experimental results are supported by the observation that agricultural workers exposed to these compounds develop an intolerance to alcohol drinking (Quistad et al., 1994).

DEAB (Diethylaminobenzaldehyde) is a chemical that is used as a control in the Aldefluor assay (Stemcell Technologies, Inc.) to test for aldehyde activity associated with cancer stem cells. Its selectivity against the ALDH family members has been studied to establish its substrate profile (Morgan et al., 2015) revealing that it is a substrate for ALDH3A1, ALDH1A1, ALDH1A3, ALDH1B1, and ALDH5A1.

The natural product citral has been known for many years to be an aldehyde dehydrogenase inhibitor and has routinely been used to block ATRA synthesis in the laboratory (Xu et al., 2018; Kikonyogo et al., 1999).

Win18,446 is shown liganded to ALDH1A2 in the PDB records. This chemical, also known as bisdiamine, was long known to be a spermatogenesis inhibitor (Kar et al., 1966). Further studies linked Win18,446 to other reproductive and adverse developmental effects (Oster et al., 1974; Momma et al., 1990; Singh and Dominic, 1995). Mey et al. showed that this compound, along with three others, caused congenital diaphragmatic hernias in rats and the authors were able to establish *in vitro* inhibition of retinal dehydrogenase (Mey et al., 2003). Other chemicals with similar activity were the herbicide nitrofen, 4-biphenyl carboxylic acid (BPCA), and SB-210661.

#### *3.1.8 Retinoic Acid Receptor alpha (RARa)*

The structure of RARa has been studied extensively. PDB has data on RARa that include the protein complexed with agonists, antagonists, and RXR (Table S11[1]). ChEMBL contains over 250 assays for RARa binding and functional assays (Table S12[1]). A review of the PubMed publications behind the depositions to ChEMBL shows that most are studies of newly synthesized compounds designed with the goal of optimizing compound activity. The natural ligand, ATRA, is commonly used as a reference compound. The ToxCast/Tox21 battery of assays includes three assays testing for RARa ligand binding and/or reporter gene transactivation (Table S17[1]). Selected chemicals of interest are discussed below.

Am580 and Am80 (tamibarotene) were synthesized in 1988 as synthetic retinoids and found to have potent activity as RARa agonists (Kagechika et al., 1988). These chemicals have been tested in many assays and studied as treatments in a number of diseases. BMS493 is an inverse agonist of RARa (Germain et al., 2009). AGN 193109 was identified as a potent RARa antagonist in 1995 (Johnson et al., 1995). Since then, it has been referenced in over 30 publications where it is studied for use in disease treatment and as a reference compound for RAR binding assays. Developmental *in vivo* assays demonstrate that it causes craniofacial abnormalities (Kochhar et al., 1998). AGN 193109 has been referred to as an inverse agonist (Thacher et al., 1999) and has been shown to bind RARb as well as RARg (Agarwal et al., 1996). The compound is commercially available from many vendors. ALRT 1550 was identified as a novel agonist of RARa in 1996 (Zhang et al., 1996). It has received some attention as a potential treatment for cancer (e.g., (Hu et al., 2002). The compound is also available commercially.

#### *3.1.9 Retinoic Acid Receptor beta (RARb)*

RARb has 12 records in PDB (Table S13[1]). The early records reflect research into understanding the receptor and its endogenous ligand; later, synthesized compounds are tested for their potential as pharmacologically useful ligands. ChEMBL contains over 200 assays for RAR beta. These are summarized in Table S14[1]. The assays include agonist, antagonist, binding, and activation modes. ToxCast includes one applicable RARb assay. The assay is described in Table S18[1] and the results are summarized in Table S17[1]. Selected chemicals are discussed below.

Benzo[a]pyrene diol epoxide (BPDE) is a metabolite of benzo[a]pyrene, a combustion by-product of a number of processes including cigarette smoking. This commercially available compound is thought to inhibit RARb through suppression of the RARb promoter (Song and Xu, 2001). BMS453 is a RARb-specific agonist and a RARa antagonist (Chen et al., 2001). CD 2019 is a RARb-specific agonist. In a study of the relative teratogenicity of three specific RAR agonists, CD 2019 was the RARb agonist used, while AM 580 was the RARa agonist, and CD 437 was the RARg agonist (Elmazar et al., 1996). LE 135 is a RARb antagonist and has been shown to affect the chondrogenic pathway in development (Li et al., 2011).

#### *3.1.10 Retinoic Acid Receptor gamma (RARg)*

RARg, similarly to RARa and RARb, has received attention as a drug target. Researchers look for compounds specific to RARg and often look for compounds that do not resemble retinoids in order to avoid the side effects associated with this structural family. RARg has 11 records in PDB (Table S15[1]), most of which were compounds synthesized as potential drug candidates. There are 13 assay records in ChEMBL (Table S16[1]) that include agonist, antagonist, and binding functionality. ToxCast has one assay for RARg specifically: ATG_RARg_TRANS_up. The top results in terms of highest potency for this assay are in Table S17[1]. Selected chemicals are discussed below.

BMS 961 is a selective RARg agonist (Klaholz et al., 1998). Identified as a ligand in 1998, the compound has been used as a test chemical in limb development assays (Galdones and Hales, 2008) and is available commercially. CD1530 has been used in several studies as a specific RARg agonist. It has also been shown to inhibit CYP26A1 with a potency similar to ketoconazole (Thatcher et al., 2011). Trifarotene (CD5789) is a recently synthesized RARg selective agonist that has been approved for the treatment of acne (Thoreau et al., 2018). Because the compound has undergone clinical trials, there are safety data available (Blume-Peytavi et al., 2020; Tan et al., 2019). CD 437 has been used to induce apoptosis in a number of cancer cell types since its identification in 1995 (Shao et al., 1995) and was one of the three compounds studied for teratogenic effects (Elmazar et al., 1996).

*3.1.11 Collection of data on retinoid pathways*

Several existing assays test activity of the retinoid pathway as a whole. These are generally reporter assays that measure some effect downstream from receptor binding such as gene transcription. In a mouse pluripotent P19 cell model, Chen and Reese tested a reporter assay designed to measure levels of Hoxa1 expression, an important gene in development whose transcription levels are controlled by retinol (Chen and Reese, 2013). These authors tested the assay using chemicals known to perturb various steps in the retinoid pathway (Table 7). In a subsequent publication, the authors performed the same assay on a set of phthalate esters (Chen and Reese, 2016). In more recent work, Chen, Reese and colleagues performed a RARE reporter assay in C3RL4 mouse cell line using the Tox21 high-throughput screening platform and tested over 1000 chemicals (Chen et al., 2016b; Attene-Ramos et al., 2013). This assay was then implemented as part of the ToxCast/Tox21 platform, and while the data have not been analyzed and published yet, they can be accessed under the names TOX21_RAR_LUC_Agonist and TOX21_RAR_LUC_Antagonist. Another assay in ToxCast that measures the effects on the retinoid pathway is ATG_DR5_CIS_up, which monitors DR5 (direct repeats of 5 nucleotides) for RAR/RXR transactivation. This assay uses fluorescence intensity to indicate induction of RARE, a cis acting reporter response element responsive to RARa, RARb and RARg.

The top results in terms of highest potency for the TOX21_RAR_LUC agonist and antagonist assays and the DR5 assay are in Table S17[1]. The ToxCast DR5 results show effects for some organochlorine pesticides such as endrin, dieldrin, endosulfan, and chlordane, in addition to known retinoids. Lemaire et al. performed extensive assays on these compounds with the goals of delineating their activity at RAR alpha, beta, and gamma (Lemaire et al., 2005). They suggest that these compounds may disrupt the retinoid receptor by working as low-affinity agonists on RAR alpha, beta, and gamma and hypothesize that prolonged exposure to these compounds may contribute to teratogenicity.

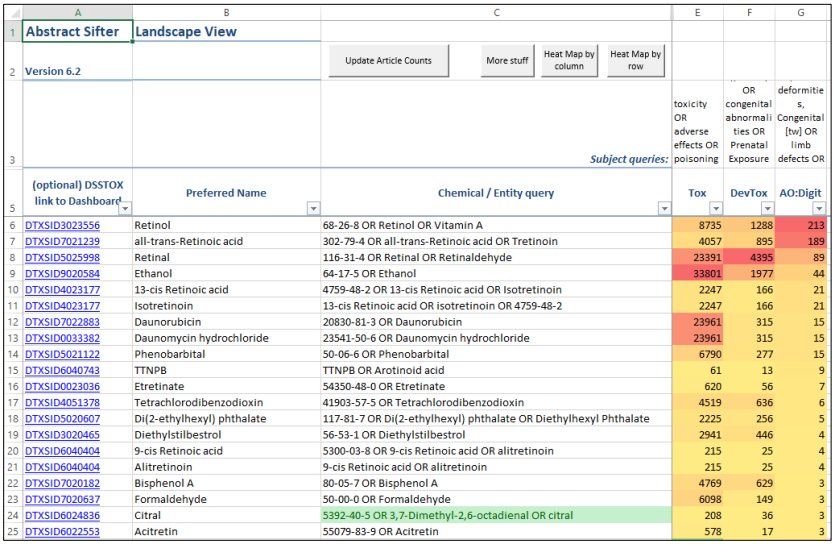
**Tab. 7: Summary of results from published retinoid pathway assays**
The publications should be consulted for potency, measurements, and assay details.

| Chen et al., 2016b | | Chen and Reese, 2013 |
|---|---|---|
| **Agonists** | **Antagonists** | **Active** |
| 1,10-Phenanthroline monohydrate | 5-Azacytidine | Ethanol |
| 10058-F4 | Amoxapine | 4-Methylpyrazole |
| 13-cis-retinoic acid | Amsacrine hydrochloride | Geraniol |
| 4-Aminoazobenzene | Auranofin | 3,7-Dimethyloctan-1-ol |
| AC-55649 | Bay 11-7085 | Citronellol |
| BF-170 hydrochloride | BAY 61-3606 hydrochloride hydrate | Citral |
| DFB | Brefeldin A from *Penicillium brefeldianum* | Citronellal |
| GW9662 | Camptothecin | Bisdiamine |
| K114 | CGP-74514A hydrochloride | (DEAB) 4-(Diethylamino)benzaldehyde |
| Kenpaullone | CGP-7930 | Nitrofen |
| Niclosamide | Desipramine hydrochloride | Metam-sodium |
| PD 98,059 | Dilazep hydrochloride | Thiram |
| Retinoic acid (ATRA) | D-ribofuranosylbenzimidazole | 4-Nonylphenol |
| Retinoic acid p-hydroxyanilide | Emetine dihydrochloride hydrate | Diethylstilbestrol |
| Rhodblock 6 | H-8 dihydrochloride | Bisphenol A |
| Rutaecarpine | Idarubicin hydrochloride | Genistein |
| SB 204741 | LY-294,002 hydrochloride | Dibutyl phthalate |
| SB 206553 hydrochloride | Mifepristone | Dipentyl phthalate |
| SB-366791 | Mitoxantrone | Di(2-ethylhexyl) phthalate |
| SCH 58261 | MNS | |
| SIB 1757 | Parthenolide | |
| SIB 1893 | PD-166285 hydrate | |
| SU 4312 | Spironolactone | |
| TTNPB | Stattic | |
| Tyrphostin AG 494 | Topotecan hydrochloride hydrate | |

**3.2 Literature exploration and citral case study**

*3.2.1 Literature exploration using Abstract Sifter*

Approximately 280 putative reference chemicals (referred to as Refchemset) were identified in this work. Results of queries on the Landscape sheet show that, of the chemicals in Refchemset, ethanol, daunorubicin and retinal are associated with the highest



| | A | B | C | E | F | G |
|---|---|---|---|---|---|---|
| 1 | **Abstract Sifter** | **Landscape View** | | | | |
| 2 | **Version 6.2** | | Update Article Counts / More stuff / Heat Map by column / Heat Map by row | | | |
| 3 | | | ***Subject queries:*** | toxicity OR adverse effects OR poisoning | OR congenital abnormalities OR Prenatal Exposure | deformities, Congenital [tw] OR limb defects OR |
| 5 | **(optional) DSSTOX link to Dashboard** | **Preferred Name** | **Chemical / Entity query** | **Tox** | **DevTox** | **AO:Digit** |
| 6 | DTXSID3023556 | Retinol | 68-26-8 OR Retinol OR Vitamin A | 8735 | 1288 | 213 |
| 7 | DTXSID7021239 | all-trans-Retinoic acid | 302-79-4 OR all-trans-Retinoic acid OR Tretinoin | 4057 | 895 | 189 |
| 8 | DTXSID5025998 | Retinal | 116-31-4 OR Retinal OR Retinaldehyde | 23391 | 4395 | 89 |
| 9 | DTXSID9020584 | Ethanol | 64-17-5 OR Ethanol | 33801 | 1977 | 44 |
| 10 | DTXSID4023177 | 13-cis Retinoic acid | 4759-48-2 OR 13-cis Retinoic acid OR Isotretinoin | 2247 | 166 | 21 |
| 11 | DTXSID4023177 | Isotretinoin | 13-cis Retinoic acid OR isotretinoin OR 4759-48-2 | 2247 | 166 | 21 |
| 12 | DTXSID7022883 | Daunorubicin | 20830-81-3 OR Daunorubicin | 23961 | 315 | 15 |
| 13 | DTXSID0033382 | Daunomycin hydrochloride | 23541-50-6 OR Daunomycin hydrochloride | 23961 | 315 | 15 |
| 14 | DTXSID5021122 | Phenobarbital | 50-06-6 OR Phenobarbital | 6790 | 277 | 15 |
| 15 | DTXSID6040743 | TTNPB | TTNPB OR Arotinoid acid | 61 | 13 | 9 |
| 16 | DTXSID0023036 | Etretinate | 54350-48-0 OR Etretinate | 620 | 56 | 7 |
| 17 | DTXSID4051378 | Tetrachlorodibenzodioxin | 41903-57-5 OR Tetrachlorodibenzodioxin | 4519 | 636 | 6 |
| 18 | DTXSID5020607 | Di(2-ethylhexyl) phthalate | 117-81-7 OR Di(2-ethylhexyl) phthalate OR Diethylhexyl Phthalate | 2225 | 256 | 5 |
| 19 | DTXSID3020465 | Diethylstilbestrol | 56-53-1 OR Diethylstilbestrol | 2941 | 446 | 4 |
| 20 | DTXSID6040404 | 9-cis Retinoic acid | 5300-03-8 OR 9-cis Retinoic acid OR alitretinoin | 215 | 25 | 4 |
| 21 | DTXSID6040404 | Alitretinoin | 9-cis Retinoic acid OR alitretinoin | 215 | 25 | 4 |
| 22 | DTXSID7020182 | Bisphenol A | 80-05-7 OR Bisphenol A | 4769 | 629 | 3 |
| 23 | DTXSID7020637 | Formaldehyde | 50-00-0 OR Formaldehyde | 6098 | 149 | 3 |
| 24 | DTXSID6024836 | Citral | 5392-40-5 OR 3,7-Dimethyl-2,6-octadienal OR citral | 208 | 36 | 3 |
| 25 | DTXSID6022553 | Acitretin | 55079-83-9 OR Acitretin | 578 | 17 | 3 |

**Fig. 3: Landscape sheet from the Abstract Sifter Excel application showing literature counts for reference chemicals**
The numbers represent citation counts returned from PubMed when a query built from the chemical phrase in Column C is appended with "AND" to the subject matter queries terms in Column 3. Note: query results indicate co-occurrence of terms and publications should be consulted for results.

number of articles describing toxicity (column E), while retinal has the most citations connecting it to developmental toxicity (Figure 3). Around 180 (approximately 64%) of the chemicals in Refchemset have some connections to citations about developmental toxicity. When the queries are structured to focus on one developmental adverse outcome associated with retinoid disruption – limb defects – we find approximately 58 chemicals have some supporting literature.

### *3.2.2 Citral case study*

In two studies to describe the toxicity of citral, and the first ones to describe the chemical's effect on embryonic chicks, citral caused malformations in the chick limbs when applied early in undifferentiated tissue (Abramovici et al., 1973; Abramovici et al., 1980). A study in 1996 used citral to block endogenous ATRA production in the chick embryo and found that citral caused truncated cartilage elements (Tanaka et al., 1996). In a study in zebrafish and mummichog, dosing with excessive ATRA resulted in duplicated fins; citral administration reversed this effect and even caused loss of pectoral fins (Vandersea et al., 1998). In dose-ranging developmental studies in pregnant rats, researchers found that oral doses over 60 mg/kg caused minor skeletal abnormalities and growth retardation (Nogueira et al., 1995). In an inhalation study in pregnant rats, only high maternally toxic doses caused defects, including hypoplastic bones (Gaworski et al., 1992). Citral has significant evidence linking it to the adverse outcome in our limb defect AOP in Figure 2.

Next, we looked for literature linking citral to the other key events in the AOP. RALDH activity comes downstream of several targets discussed here and it affects levels of ATRA. The key event following RALDH inhibition is a deficiency or down-regulation of ATRA. There is ample literature describing this activity of citral (Connor, 1988); this effect is often the rationale for using the chemical in experiments.

The next step in the AOP describes disrupted FGF signaling. Citral's activity with respect to FGF during development is described in studies of facial bone development, not limb development. In studies on the developing chick jaw, Shimomura et al. determined that both ATRA and citral work through the regulation of FGF (Shimomura et al., 2015). In the developing chick face, Song et al. found that endogenous retinoids and citral act upstream of FGF8 and regulate the programmed cell death and morphogenesis of the face (Song et al., 2004).

The next key event in the AOP revolves around DHAND protein in the zone of polarizing activity (ZPA). A search of PubMed using citral and terms referring to DHAND and ZPA did not produce any citations. The next key event is Homeobox (Hox) gene disruption of retinoid pathway. Two publications describe citral's inhibitory effect (75% inhibition of Hoxa1 expression) in a Hox gene expression assay (Chen and Reese, 2013, 2016).

## 4 Discussion

This work demonstrates that there is a large amount of information regarding the effects of chemicals on gene and protein targets in the retinoid pathway, but the amount of information varies for each target. Figure 4 illustrates this data disparity. A target like retinol binding protein, for example, has many PDB and ChEMBL entries, while STRA8 and CYP26 have very few. Gene and protein targets encompassed in high throughput testing programs like ToxCast contribute significantly to expanding the target space

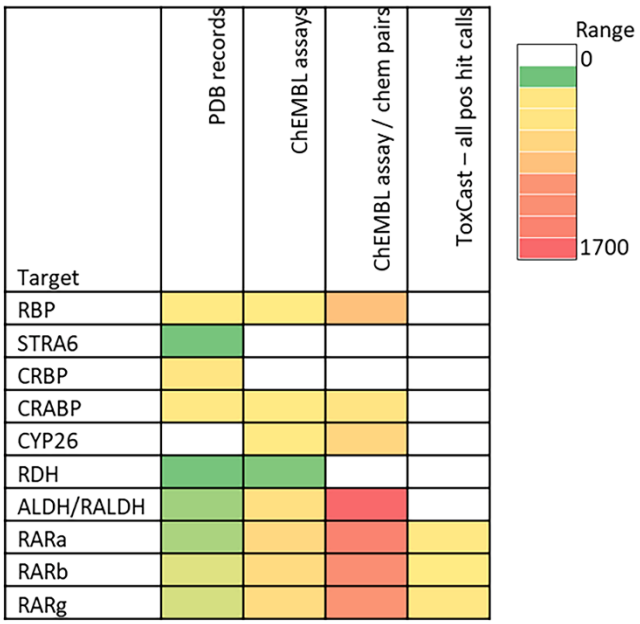



**Fig. 4: Indicator of data volume for each retinoid pathway target**
Depth of record counts noted in the top row are indicated by the color bar key.

and the chemical space, but gaps remain. These gaps are significant in the retinoid pathway: the retinoic acid receptors are the only retinoid targets directly tested in ToxCast.

This inconsistency in information volume among the retinoid targets is driven to some extent by the search for therapeutics. Retinoid pathway targets like CRABP receive attention from researchers when they are recognized as potential therapeutic targets, not when they could be involved in an adverse outcome. Binding data available through PDB, while useful for its specificity, is motivated mostly by pharmacological goals, yielding chemicals similar to each other in structure. When this is the case, only molecules that achieve some success in the development pipeline have follow-up studies. The information is useful, but patchy, and can weaken inferences to environmental chemicals. For example, pesticides have been tested against RAR receptors, RALDH, RDH, but not against RBP and CRABP.

The role of reference chemical metabolism is a challenge to assess consistently. Intact metabolic enzymes may be required to activate a chemical. Conversely, a chemical can be an excellent reference chemical for a cell-free biochemical assay, but not when it is metabolically inactivated in a cell-based assay. Indeed, a chemical can be an excellent reference chemical for a binding assay but have no evidence connecting it to any downstream outcome associated with the binding target.

The project of identifying reference chemicals has a circular aspect. A researcher needs reference chemicals to develop a robust assay but needs assays to identify active chemicals. One approach often employed is to test endogenous retinoids (e.g., retinol, retinal, ATRA, 9-cis-retinoic acid), then exogenous retinoids, and then transition into non-retinoids. This approach can be seen for instance in the study of RBP and CRABP.

High throughput *in vitro* testing has resulted in millions of data points describing chemical activity at the molecular level, but for most chemicals, any downstream effects in a cell, a tissue, an organ, or an organism are not known and can only be inferred. A chemical like citral has strong evidence linking it to its putative MIE (RALDH inhibition), but despite the long publication history, piecing together evidence that it participates in the key events of provisional AOP for limb defects caused by retinoid pathway disruption is challenged by lack of information, particularly observations of activity at the cellular and tissue level.

As the toxicology community performs fewer and fewer tests on animals, published reports of developmental and reproductive adverse outcomes will slow, even as the number of untested chemicals introduced to the marketplace grows. This data disconnect underscores the importance of developing assays that test the effects of chemicals on complex multi-system tissues such as organ-on-a-chip systems. If these systems can be designed to measure end points relevant to important AOPs, more light could be shed on candidate reference chemicals and strengthen the case not just that they have the molecular initiating event of interest, but that their effects are consistent – or not – with AOPs of interest. When such complex culture assays can be scaled up and run in high throughput, then candidate reference chemicals can be selected with the support of much stronger lines of evidence.

In this work, we have surveyed the data and literature for ten protein targets in the retinoid pathway and then assembled, discussed, and compiled in Excel a set of candidate reference chemicals for each target. Assembling this complex information into one place with links to the data and the literature will facilitate development of new testing programs, new *in vitro* assays, and other new approach methodologies for the retinoid system. These approaches depend on reference chemicals to calibrate the activity thresholds and establish confidence.

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## Conflict of interest

The authors declare no conflicts of interest.

## Data availability statement

The ToxCast data is available from the EPA Chemicals Dashboard[6].

[6] https://comptox.epa.gov/