Using In vitro ToxCast Assays to Evaluate Mechanistic Plausibility and Build Confidence in the Selection of Analogues for Quantitative Read-Across: A Case Study on *p,p'*-Dichlorodiphenyldichloroethane

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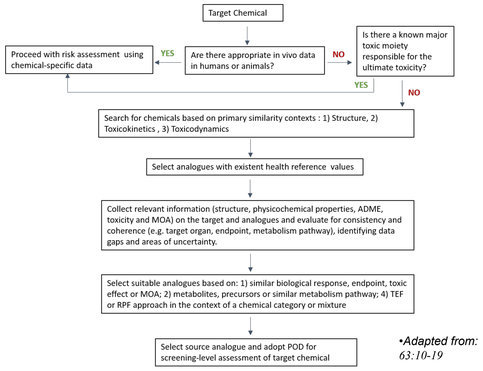
Overview

- Deriving human health reference values for environmental chemicals has traditionally relied on toxicity data from humans and/or experimental animals
- In the absence of in vivo toxicity data, new approach methodologies such as read-across can be used to fill data gaps for a target chemical using known information from a source analogue
- A read-across approach illustrated below (Figure 1) was applied to assist in screening-level assessment of noncancer oral toxicity for the target, *p,p'*-DDD, a data-poor chemical known to occur at contaminated sites in the U.S.

Figure 1: Read-across Approach



- Analogues were identified and evaluated for similarities in structure and physicochemical properties, toxicokinetics, and toxicodynamics (toxicity and in vitro bioactivity) with respect to the target chemical
- The primary focus of this investigation was to evaluate the integration of mechanistic evidence from in vitro high-throughput screening (HTS) assays from ToxCast in support of the similarity justification for the selection of analogues for quantitative read-across

•*Adapted from:* Wang et al., 2012, *Regul Toxicol Pharmacol 63:10-19*

Structural and Toxicity Similarity Comparisons

Identification of Structural Analogues of *p,p'*-DDD

Table 1. Structural Analogues of p,p'-DDD

	Target Chemical	Analogues[a]		
Name	p,p'-Dichlorodiphenyl dichloroethane (p,p'-DDD)	p,p'-Dichlorodiphenyl trichloroethane (p,p'-DDT)	p,p'-Dichlorodiphenyl dichloroethylene (p,p'-DDE)	p,p'-Dimethoxydiphenyl trichloroethane (Methoxychlor)
CASRN	72-54-8	50-29-3	72-55-9	72-43-5
Structure				
ChemIDplus similarity score (%)	100	77	67	65
DSSTox similarity score (%)	100	96	61	52

[a]Analogues represent a set of structurally similar chemicals identified using two publicly available similarity databases (ChemIDplus and DSSTOX) prefiltered on the basis of availability of health reference values for non-cancer oral toxicity from regulatory agencies, including ATSDR (2002a, b) and U.S. EPA (2017 b, c).

Putative Toxicity Targets for *p,p'*-DDD and Analogues Include the Liver and Reproductive System in Animals

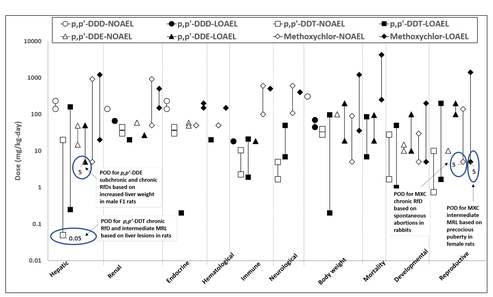


Figure 1. Comparison of Health Effects and Associated Effect Levels for Non-Cancer Oral Toxicity. Range of effect levels (no-observed-adverse-effect levels [NOAEL] and lowest-observed-adverse-effect levels [LOAEL]) for noncancer endpoints for the target and analogues from repeated-dose animal toxicity studies via oral administration reported by ATSDR (2002a, b) and U.S. EPA (2017 b, c). Circles note points-of-departure (PODs) used in the derivation of oral reference doses (RfDs) and minimal risk levels (MRLs) for these chemicals (ATSDR, 2002a,b; U.S. EPA 1987c, 1999, 2017a).

Bioactivity Similarity Comparisons Evaluating Mechanistic Plausibility for Liver and Reproductive Toxicity

p,p'-DDD and Analogues Exhibit Similarities in Cell-specific Responses and Target Gene Pathways in *In Vitro* ToxCast Assays Conducted in Human Liver Cells

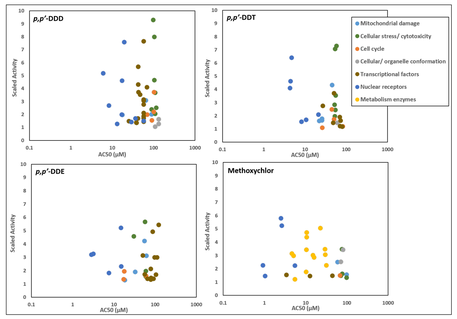


Figure 2. Bioactivity data for *p,p'*-DDD and Analogues in ToxCast Assays Conducted in Human Hepatoma HepG2 Cells and Primary Human Hepatocytes. Scatterplots show AC50 and scaled activity values for *p,p'*-DDD, *p,p'*-DDT, *p,p'*-DDE and methoxychlor from *in vitro* assays visualized according to the type of biological response or biological target. AC50 values refer to the concentration that elicits half maximal response and the scaled activity refers to the response value divided by the activity cutoff. Metabolism enzyme-related assays were conducted in human primary hepatocytes and all other *in vitro* assays were measured in HepG2 cells. Assays for which chemicals were inactive are not displayed. Data were sourced from the EPA's CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard) (U.S. EPA, 2017a).

(Lizarraga et al., 2019, *Regul Toxicol Pharmacol* 103:301-313)

p,p'-DDD and Analogues Exhibit Similar Estrogenic and Anti-Androgenic Activities in *In Vitro* ToxCast Assays and Model Predictions for the ER and AR Across Multiple Tissues and Cell lines

Table 2. ToxCast Bioactivity Summary and Model Prediction Scores (AUC values) for ER and AR activities[a]

	p,p'-DDD	p,p'-DDT	p,p'-DDE	Methoxychlor
ER assays				
Active/Total Assays (%)	7/18 (39)	11/18 (61)	8/18 (44)	14/18 (78)
AC50 values (μM)	Range = 14.0 - 32.4 Median = 18.7	Range = 3.3 - 59.8 Median = 6.1	Range = 3.5 - 46.2 Median = 16.5	Range = 0.9 - 44.2 Median = 4.6
Agonist activity AUC value (95% CI)[b]	0.0715 (0.0342-0.0738)	0.190 (0.181-0.231)	0.0679 (0.0614-0.0963)	0.254 (0.247-0.260)
Antagonist activity AUC value (95% CI)	0	0	0	0
AR assays				
Active/Total Assays (%)	4/11 (36)	3/11 (27)	4/11 (36)	3/11 (27)
AC50 values (μM)	Range = 31.0 - 62.8 Median = 44.8	Range = 17.8 - 72.0 Median = 47.0	Range = 7.0 - 58.7 Median = 29.6	Range = 29.3 - 40.8 Median = 34.2
Agonist activity AUC value (95% CI)	0	0	0	0
Antagonist activity AUC value (95% CI)	0.0973 (0.0649-0.124)	0.0642 (0.0318-0.108)	0.251 (0.234-0.291)	0.0429 (0.0364-0.0465)

[a]Data were sourced from Judson et al. (2015) and Kleinstreuer et al. (2016). [b] 95% CI for the ER activity model were sourced from a subsequent publication to the Judson et al., (2015) study (Watt and Judson, 2018).
Abbreviations: AUC = area under the curve score ranging from 0–1. An AUC value of 0 indicates that the chemical is inactive; CI = confidence interval.

(Lizarraga et al., 2019, *Regul Toxicol Pharmacol* 103:301-313)

p,p'-DDD and Analogues Exhibit Similar Upregulation of Steroid/Xenobiotic-sensing Nuclear Receptors in *In Vitro* ToxCast Assays Conducted in Hepatoma HepG2 Cells

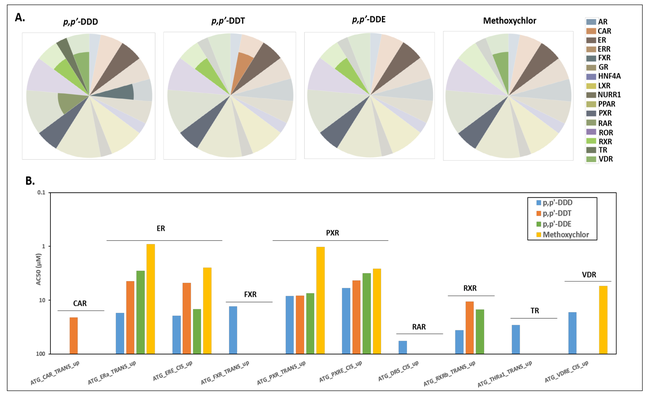


Figure 3. ToxCast Assays Evaluating Regulation of Nuclear Receptor Activity for *p,p'*-DDD and Analogues in Human Hepatoma HepG2 Cells. Panel A shows radar plots for *p,p'*-DDD, *p,p'*-DDT, *p,p'*-DDE and methoxychlor, summarizing active calls from nuclear receptor assays conducted in HepG2 cells and mapped to specific target genes. The shaded area of the pie slice represents the number of active assays as a proportion of total assays. The width of the slice refers to the proportion of assays within a given target gene. Bar graphs compare AC50 values (concentration at half maximal response) for active assays (panel B). The scale for the AC50 values is shown in reverse order to visualize the most sensitive nuclear receptor activities (the higher bar indicates a lower AC50 value). Data were sourced from the EPA's CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard) (U.S. EPA, 2017a).
Abbreviations: AR, androgen receptor [■]; CAR, constitutive androgen receptor [■]; ER, estrogen receptor [■]; ERR, estrogen-related receptor [■]; FXR, farnesoid X receptor [■]; GR, glucocorticoid receptor [■]; HNF4A, hepatocyte nuclear factors 4 alpha [■]; LXR, liver X receptor [■]; NURR1, nuclear receptor related-1 protein [■]; PPAR, peroxisome proliferator-activated receptor [■]; PXR, pregnane X receptor [■]; RAR, retinoid acid receptor [■]; ROR, RAR-related orphan receptor [■]; RXR, retinoid X receptor [■]; TR, thyroid hormone receptor [■]; VDR, vitamin D receptor [■].

(Lizarraga et al., 2019, *Regul Toxicol Pharmacol* 103:301-313)

Summary and Conclusion

- The current read-across approach relies on the evaluation and integration of evidence across three primary similarity contexts (structure, toxicokinetics and toxicodynamics) for the selection of a suitable source analogue for screening-level quantitative assessment of the target, *p,p'*-DDD (Table 3)
- Analysis of ToxCast assays reveal similarities between *p,p'*-DDD and analogues in *in vitro* responses related to mitochondrial damage, cellular stress/cytotoxicity and the upregulation of specific steroid/xenobiotic-sensing nuclear receptors (Figures 2 and 3) that are relevant to their mechanism of hepatotoxicity
- ToxCast assays and model predictions suggest that *p,p'*-DDD and analogues may act as ER agonists and AR antagonists (Table 2), coinciding with the estrogenic and anti-androgenic reproductive effects observed *in vivo*
- Coherence across *in vivo* toxicity and *in vitro* bioactivity similarity comparisons help reduce uncertainties associated with toxicity data gaps for the target
- These findings demonstrate the utility of integrating evidence from HTS data platforms to support mechanistic conclusions and increase confidence in the application of read-across in quantitative risk assessment

Evidence Integration

Table 3. Using Evidence Integration to Identify Suitable Source Analogues for Read-across

Similarity Context	Summary of Findings	Evidence Integration Conclusions
Structure and physicochemical properties	• *p,p'*-DDD and identified analogues (*p,p'*-DDT and *p,p'*-DDE and methoxychlor) demonstrate similarities in basic structural features (chlorinated diphenylalkane structure) • *p,p'*-DDT and *p,p'*-DDE also share key functional groups (*p,p'*-chlorine substituents) and physicochemical properties important for bioavailability (lipophilicity and low BCF values) with *p,p'*-DDD	• *p,p'*-DDT is selected as a suitable source analogue for the assessment of non-cancer oral toxicity of *p,p'*-DDD based largely on toxicokinetic similarities, with supportive information from *in vivo* toxicity testing, structural similarity evaluations and *in vitro* bioactivity from HTS assays
Toxicokinetics	• *p,p'*-DDT is a metabolic precursor of *p,p'*-DDD and both chemicals show similarities in toxicokinetics (Absorption, Distribution and Metabolism [ADME]) in humans and experimental animal models (preferential partitioning into fat, similar metabolism and excretion pathways and prolonged elimination rates) • Other analogues demonstrate differences in ADME in comparison to the target. *p,p'*-DDE is less metabolically active; methoxychlor is metabolized differently and appears to be less bioaccumulative	
Toxicodynamics	• Consistency and coherence across health effects in experimental animals for non-cancer oral toxicity among the analogues point to putative toxicity targets for *p,p'*-DDD (primarily liver and reproductive toxicity) • Similarities in *in vitro* bioactivity profiles from ToxCast assays between the target and analogues with respect to cell-specific responses and target gene pathways provide mechanistic plausibility for the liver and reproductive effects associated with this group of chemicals	

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