POSTER

A Comparison of ToxCast in Vitro Testing with In Vivo Endpoints for Endocrine and Developmental Toxicities: A Case Study Using Endosulfan and Methidathion

Abstract

USEPA's Toxicity Forecaster (ToxCastTM) program is of interest to the California Environmental Protection Agency (Cal/EPA) as a tool for chemical prioritization, understanding hazard traits, weighing product safety concerns, and risk assessment. In order to improve our understanding of the applicability, strengths, and limitations of the ToxCast high-throughput screening (HTS) assays, we conducted a case study in which we compared ToxCast HTS data with well-established toxicity endpoints from in vivo studies for 2 pesticides: endosulfan and methidathion. Both pesticides are primarily neurotoxins but endosulfan is also an endocrine disruptor via estrogenic and androgenic effects. They were selected because 1) they have large in vivo databases, 2) risk assessments on these compounds were completed recently by Cal/EPA, and 3) they were tested in ToxCast assays evaluating estrogenic and androgenic activities. We devised roadmaps for each pesticide denoting positive ToxCast assay activity in the estrogen receptor (ER) and androgen receptor (AR) pathways. HTS results indicated that endosulfan has weak agonistic (no antagonistist) effects and there were no active ER assays for methidathion. Neither compound is active for AR. An in vitro-in vivo extrapolation (IVIVE) method was used to convert ToxCast half-maximal activity concentrations (AC50: µM) to rat oral equivalent doses (OED: mg/kg/day). OEDs were compared against doses tested and toxicities observed in traditional in vivo studies evaluating the toxicities of these chemicals. Endosulfan OED for ER was ~9% of the in vivo lowest-observed-effect-level (LOEL). There was similarity between endosulfan ER-related OEDs to observed in vivo activities and pathways whereas there was no in vivo/in vitro correlation with the AR pathways tested. The lack of methidathion activity in the ER and AR assays indicates they have little impact on toxicity observed in the traditional bioassay database. Both pesticides had neurotoxic and developmental effects in the ToxCast zebrafish assay, but were not active in the limited other ToxCast assays examining neurotoxicity. We found the ToxCast zebrafish assay to be of particular value because it utilized a whole organism model and detected neurotoxicity and developmental effects for both pesticides.

INTRODUCTION

- **US EPA's Toxcast program produces high throughput screening results (HTS, including zebrafish [ZF] assays) to profile more than 1,000 chemicals in ~ 600 assays.**
- **CDPR and OEHHA explored ToxCast data to facilitate chemical risk assessments, hazard trait identification, and prioritization.**

- **The *in vivo* and *in vitro* assays submitted for pesticide registration are designed to identify adverse effects such as endocrine disruption, neurotoxicity, or cancer, and to establish advisory levels.**

- **We examined whether the well-established endpoints for two pesticides would coincide with results from biochemical pathway-specific HTS assays.**

- **Endosulfan (organochlorine) and methidathion (organophosphate) were selected because they have (1) a vast *in vivo* and *in vitro* database with well-defined major endpoints; (2) completed risk characterization documents; (3) numerous ToxCast assays including endocrine disruption (estrogen receptor (ER), androgen receptor (AR)), developmental (ZF), and neurotoxicity (AChE, GABA, and ZF).**

KNOWN TOXICITIES OF ENDOSULFAN AND METHIDATHION

ENDOSULFAN:

- **Functions primarily as a neurotoxin (GABA channel blocker).**

- **Secondary effect is endocrine disruption potentially via interaction with the ER (agonist) and AR (antagonist) as observed in *in vivo* and *in vitro* studies (Table 1).**

- **Developmental effects occurred at or below doses causing neurotoxicity.**

METHIDATHION:

- **Functions primarily a neurotoxin (AChE inhibition).**

- **Secondary effects are liver toxicity and ovarian toxicity (possibly from oxidative stress and/or endocrine disruption) (Table 1).**

- **Neurotoxicity (severe fasciculations and tremors) was observed at the same dose as the reproductive effects.**

Table 1. *In vivo* Results Related to Presumptive Endocrine Disruption from Endosulfan or Methidathion

Species/ sex/ cell type	Exposure info	Endpoint	LOEL (mg/kg/d)	References
ENDOSULFAN				
Rat Pup/M	S.C. PND 7-22	↓serum T; ↓ reproductive organ weights	4.5	Ahmad et al., 1993
Wistar Rat/M	Gavage 7 or 15d	Male hormone and aromatase effects[a]	2.5	Singh & Pandey, 1989
Wistar Rat/M	Gavage 15-30d	Male hormone and aromatase effects[b]	7.5	Singh & Pandey, 1990
Albino Rat	Gavage 30d	↑relative testes (body weight)	5	Dikshith et al., 1984
ITRC Rat/M	Gavage 15d	Effects on Male reproductive tract[c]	10	Gupta & Chandra, 1977
Druckery Rat	Gavage 90d	↓sperm, spermatid counts; ↑sperm abnormality	2.5	Sinha et al. 1997
Druckrey Rat/F	*Gavage GD 12-21*	↓sperm, spermatids, ↓M reproductive organ weights	*1*	*Sinha et al., 2001*
Wistar Rat/F	Gavage GD 15-LD 22	↑ testis wts; ↓ sperm production & % seminiferous tubules with complete spermatogenesis	1.5	Dalsenter et al., 2003
Wistar Rat/M	Gavage 6d/wk, 10wk	↓sperm production, & count; ↑# abnormal sperm	2.5	Zhu et al., 2000
Wistar Rat/F	Diet GD6 - PND 28	↓pup bodywt, preputial separation & bodywt gain	3.74	Gilmore et al., 2006
SD Rat/F	*S.C. PND 16-18*	*↑uterine estrogenicity; E2 agonist; ↑MCF-7 prolif*	*3*	*Wade et al., 1997*
Swiss Mice/F	Gavage 15d	↓corpus lutea & ↓healthy follicles, ↓ovary wt; ↓estrus cycles;↑atretic follicles & estrous duration	3	Hiremath & Kaliwal, 2002
SD Rat/F	Gavage GD 6-14	↓fetal bwt, %live fetuses & length;↑growth retardation, skeletal anomalies, ↑% resorptions	6	Fung, 1980
Albino Rat/F	Gavage GD 6-19	↑% fetal skeletal abnormalities	5	Gupta et al., 1978
Crl:COBS(CD)BR Rat	Diet 2 Gen	↓mean litter weight (F0 & F1)	6.55	Edwards et al., 1984
METHIDATHION				
CD Rat	1-gen, 2-litter	↓ mating index M	9.1	Salamon, 1986
CR1:CD BR Rat/F	2-gen. repro	↓ mating index M	2.2	Salamon, 1987
Wistar Rat/F	*Gav, 5 d/wk 4 wk*	*↓cycles (↓proestrus, estrus & metestrus; ↑ diestrus); ↓ healthy follicles & corpus lutea; ↑atretic follicles & granulosa cells with lipofuscin pigments; ↑ serum malonaldehyde*	*4.5*	*Güney et al., 2007*
CR1:CD BR Rat/F	2-gen. repro	↓ absolute & relative ovary weight (to body)	5	Salamon, 1987

a- ***Decreased:*** Testosterone, androstenedione, aromatase; 3β- & 17β-hydroxysteroid dehydrogenase; ***Increased:*** dihydroxytestosterone and androstanediol
b- ***Decreased:*** Serum testosterone, testicular testosterone, 3-hydroxysteroid-dehydrogenase, 17–hydroxysteroid-dehydrogenase
c- ***Decreased:*** Testes weight, seminiferous tubule degeneration, interstitial edema, spermatogenic elements in tubules
LOEL = Lowest-Observed-Effect-Level. Blue and Red italic indicate LOELs selected for male and female Oral Equivalent Dose (mg/kg/d) comparisons, respectively (Table 3). ***Blue background:*** effects in males; ***Pink:*** effects in females.

DATA HANDLING

- **Focus was on positive HTS assays (Figures 1a & 1b; Table 3).**

- **Cytotoxicity assays were used to define the region where effects observed from cell death/apoptosis began to interfere with true positive assay activity.**
    - **A median value was determined for all cytotoxicity assays (burst median; endosulfan = 4.6 uM) for each individual chemical.**
    - **When there were no positive cytotoxicity assays, 3uM was used as a default cytotoxicity median (default burst median).**

- **Z-score (# median absolute deviations (MADs)) from logAC_{50} to the burst median indicates specificity (should be: > 3 = true positive, <3 = potentially inactive)**

- ***In Vitro* to *In Vivo* Extrapolation (IVIVE) was used to convert the AC_{50} values (50% maximal activity) into a rat oral equivalent dose (OED), by using estimated pharmacokinetic data derived from linear regression and published *in vitro* hepatic metabolism and protein binding rat and human data.**

- **Comparisons were made between *in vivo* results (highlighted in Table 1) and OEDs derived from *in vitro* HTS data.**

- **Data from ZF embryos with the chorion intact (NHEERL; Padilla et al. 2012) as well as dechorionated embryos (Oregon State University; Tanguay et al. 2013; Truong et al., 2014) were compared to *in vivo* observations from rat studies.**

- **Padilla et al. (2012) used endosulfan as a mixture of the α- and β-isomers (usually ~70:30 ratio). Truong et al. (2014) tested the endosulfan-α-isomer (ENα) only.**

Table 2. ToxCast ERα and AR Vendors and Assay Descriptions[a]

Vendor	*Tissue*	*Cell Line/ Receptor*	*Assay Format*	*Biological Process Target*	*Detection Technology*
ACEA[b]	Human mammary	T47D	Real-Time Growth Kinetics	Cell proliferation	Electrode impedance
ATG	Human liver	HepG2	mRNA induction	Regulation of TF activity via nuclear receptor	Inducible reporter (mRNA)
NVS	Cell-free	Nuclear receptor	Receptor binding	Inhibition (activation) of enzyme activity; inhibition of ligand binding	Radioligand/ fluorescent receptor binding; enzyme-substrate intensity/mobility shift
OT	Human (cervix, kidney)Hamster	HeLa HEK293T CHO-1	Protein fragment complementation	Binding Reporter Dimerization (cofactor complex)	Luciferase or Fluorescent protein induction
Tox21	Human (kidney, ovarian)	HEK293T, BGI	Reporter gene protein product; transcription suppression	Steroid-nuclear receptor interaction	BLA or fluorescent protein induction
ZF[c]	*Danio rerio*	+/- Intact embryo	Embryonic Development	Neuro-, behavioral-, developmental toxicity	Visual

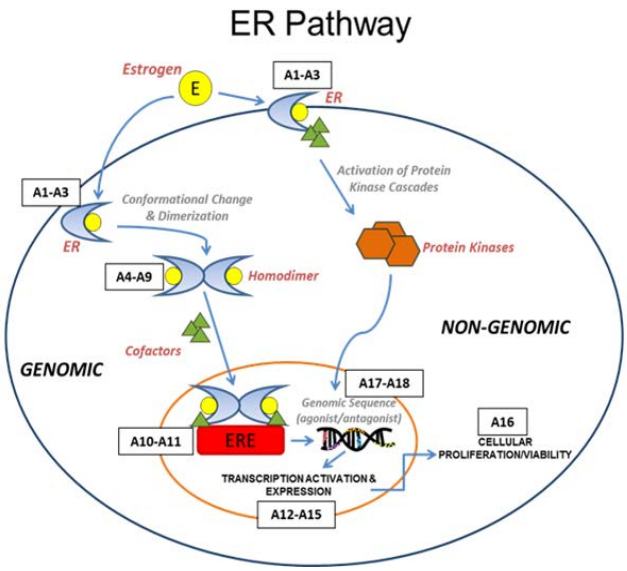
VENDORS: ACEA Biosciences, Inc. (ACEA), Attagene (ATG), NIH Chemical Genomics Center (Tox21), Novascreen (NVS) and OdysseyThera (USEPA 2013); Zebrafish (ZF: *Danio rerio*) HTS *in vivo* assays for ToxCast screening: NEERL ZF (intact embryos; Padilla et al. 2012) and embryos with the chorion removed (Tanguay et al. 2013).

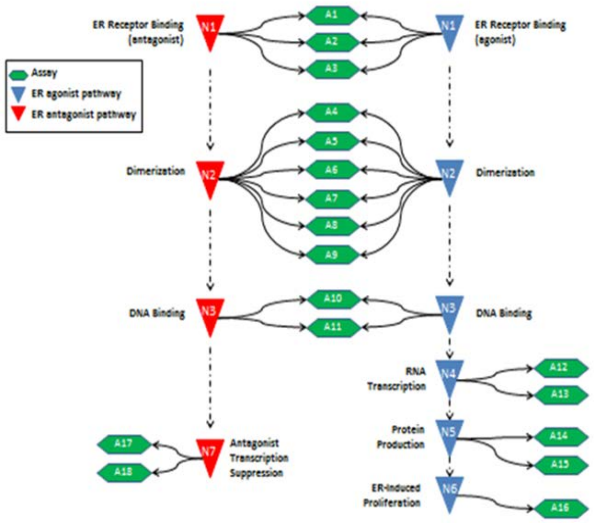
a- DMSO served as the negative control except where specifically designated (OT assays reported for AR).

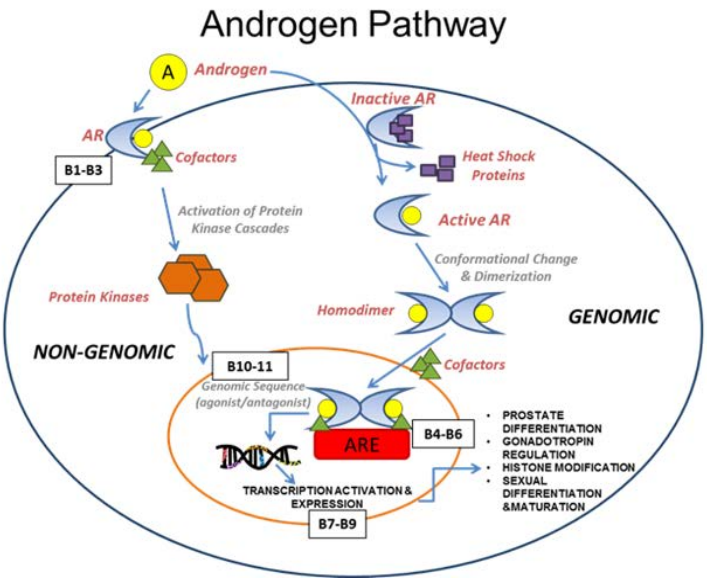
b- A negative control was not applicable to these assays since the AC_{50} values are reported as viability calculations

c- DMSO was the negative control for both Padilla et al. (2012) and Truong et al. (2014)

d- A positive control (trimethyltin chloride) was used only with ZF assays from Truong et al. (2014)







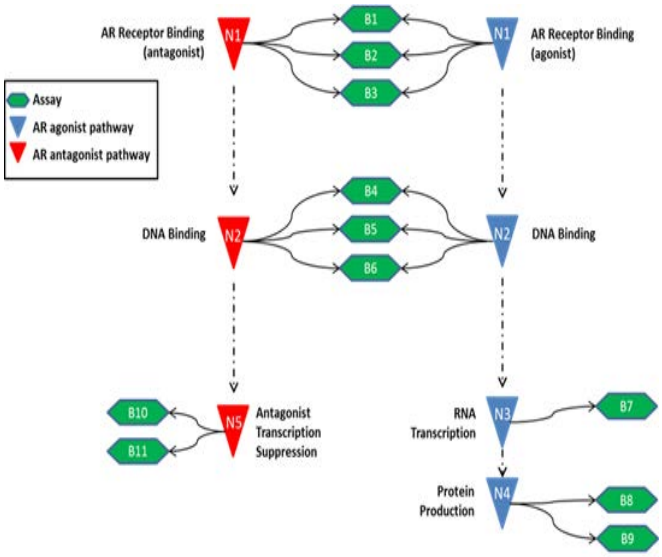


Figure 1a. ER and AR Signaling Pathways tested with ToxCast ER assays A1-A18 or B1-B11 (Table 3).

Figure 1b: Graphical representation of the network used in the *in vitro* analysis of the ER and AR (Table 3) pathways across assays and technology platforms (adapted from Judson et al. (2014). Colored arrow nodes represent "receptors" with which a chemical can directly interact.

RESULTS:

Associations between ER-related ToxCast Assays and Registrant-submitted/Open Literature Studies

ENDOSULFAN:

- **Endosulfan affects the ER as well as potentially ER-related estrogenic activity and fetal/pup development in animal studies (body weight, skeletal anomalies, resorptions, sperm abnormalities, delayed preputial separation, pup length) (Table 1).**

- **Pathways for ER assays are identified in Figures 1a and 1b.**

- **Determination of a "hit" or "true positive" or "active" response was comprised of 4 parameters: (1) slope (W); (2) potency (activity concentration at 50%: AC_{50} in µM); (3) upper (T, or EMAX) and 4) lower (B) asymptotes of the curve.**

- **Of the 18 ER-related assays performed in the ToxCast program (Table 3), 4 were "actives" (A8, A12, A14 and A15) as shown on the USEPA http://actor.epa.gov/dashboard.**

- **Z-scores >3 occurred in only 2 assays (A12 and A15), but the data support the in vivo/in vitro indications that endosulfan may be a weak agonist for ER. In this respect, the ToxCast data support the previously reported in vivo results.**

- **Endosulfan has minimal effect on the AR based on the ToxCast results, as opposed to the robust database for in vivo-related endocrine disrupting effects reported in male animals (TABLE 1 and references). No ToxCast AR assays were positive for endosulfan (Table 2).**

METHIDATHION:

- **Methidathion, tested in a two-generation rat reproduction study reported ↓absolute and relative ovarian weights in the absence of effects related to fertility, development or reproduction (Salamon, 1987). A subchronic gavage study in female rats resulted in ↓estrous cycles as well as other ovarian effects (Güney et al., 2007) (Table 1).**

- **No positive ToxCast ER or AR assays.**

NEUROTOXICITY:

- **NVS tested for GABA-receptor activity and for AChE with endosulfan and methidathion but there were no actives. However they were cell-free assays performed without metabolic activation.**

ID	ToxCast Assay Target[a, b]	Endosulfan		
		AC_{50} (µM)	Z-Score[b]	OED_c (mg/kg/d)
RECEPTOR BINDING				
NVS Nuclear Receptor Assays				
A1	ERa (bovine)	---	--	--
A2	ERa (human)	---	--	--
A3	ERa (mus)	---	--	--
DIMERIZATION				
OT ER Protein Complementation Assays				
A4	ERaERa (8h)	NT	--	--
A5	ERaERb (8h)	NT	--	--
A6	ERaERb (24h)	NT	--	--
A7	ERbERb (8h)	NT	--	--
A8	ERbERb (24h)	10.6	1.29	0.169
A9	EREGFR (2h)	NT	--	--
A10	EREGFR (8h)	---	---	--
A11	ERaERa (8h)	---	---	
RNA TRANSCRIPTION				
ATG Assays				
A12	ERa_TRANS	2.14	3.39	0.034
A13	ERE_CIS	---	--	--
PROTEIN PRODUCTION				
Tox21 ERa Assays				
A14	Agonist Ratio w/ β-lactamase	8.23	1.26	0.130

A15	Agonist Activity w/BG1 cells LUC reporter	2.42	3.23	0.038
ER-INDUCED PROLIFERATION				
ACEA RT Cell Growth Assays				
A16	T47D Cytotoxicity (80h)	33.9	0.24	0.54
ANTAGONIST (TRANSCRIPTION SUPPRESSION)				
Tox21 ERa Assays				
A17	Antagonist Ratio w/ β-lactamase	--	--	--
A18	Antagonist Activity w/BG1 cells LUC reporter	---	--	--
RECEPTOR BINDING				
NVS Nuclear Receptor Assays				
B1	AR (guinea pig)	---	---	---
B2	AR (human)	---	---	---
B3	AR (rat)	---	---	---
DNA BINDING				
OT AR Complementation Assays				
B4	ARARE w/ agonist activity LUC reporter (24h)	---	---	---
B5	ARSRC1 (8h)	---	---	---
B6	ARSRC1 (16h)	---	---	---
RNA TRANSCRIPTION				
ATG Assays				
B7	AR_TRANS	---	—	—
PROTEIN PRODUCTION				

Tox21 AR Assays				
B8	**Agonist Ratio w/ β-lactamase**[d]	---	—	—
B9	**Agonist Activity with MDAKB2 – LUC**	---	—	—
ANATGONIST (Transcription Suppression)				
Tox21 AR Assays				
B10	**Antagonist Ratio w/ β-lactamase**	---	---	---
B11	**Antagonist Activity with MDAKB2 cells LUC**	---	---	---

NT = Not tested; " — " = Not active

a- Adapted from Judson, et al. (2014). ER and AR assays used to determine the pathways in Figure 1a and 1b are numbered A1-A18 and B1-B11, respectively. They are divided according to their assay function as well as the platform (or vendor) generating the data.

b- Z-score indicates specificity: > 3 = true positive, <3 = potentially a false positive (Judson et al. 2014)

c- OED: Rest-CL: Oral.Equiv-(mg/kg)-Calculated; Predicted using the 10μM metabolic clearance rate from the rat model of Wetmore et al. (2012).

d-Data were expressed as the "ratio" of 460 nm/530 nm fluorescent emissions

Zebrafish HTS Results for Endosulfan and Methidathion (Table 4)

Padilla et al. (2012) Zebrafish Results:

- **Endosulfan showed an AC_{50} of ~1 μM (slope [W] = 0.5; R^2 = 0.9) and indicated that it was positive for unspecified malformations. The AC_{10} (0.012 μM) was within the control range. The EMAX score of 40 indicated that at peak concentrations (4 μM), endosulfan had the highest score possible for malformations.**

- **Methidathion had an AC_{50} of 45.1 µM (slope = 0.8; R2 = 0.9) indicating that it was weakly positive for unspecified malformations. The AC_{10} (2.1 µM) showed that methidathion induced malformations at the low dose. The EMAX score was 31.5 suggesting that methidathion was less toxic than endosulfan.**

Table 4. Endosulfan and Methidathion Toxicity Assays Using Zebrafish (Padilla et al. 2012)

Assay Name/Parameter	*AC50 (µM) or Score*
Endosulfan	
ZF_Total_Score_AC10[a] (AC_{10} uM)	0.012 µM
ZF_Total_Score_AC50[b] (AC_{50} uM)	1.0 µM
ZF_Total_Score_EMAX[c] (EMAX Toxicity Score)	40.0 (Toxicity Score/response)
ZF_Total_Score_R^2 (Goodness of fit)[d]	0.9 (R^2)
ZF_Total_Score_W[e] (Hillslope)	0.5
Methidathion	
ZF_Total_Score_AC10[a] (AC_{10})	2.1 µM
ZF_Total_Score_AC50[b] (AC_{50})	45.9 µM (potency)
ZF_Total_Score_EMAX[c] (EMAX Toxicity Score)	31.5 (Toxicity Score/response)
ZF_Total_Score_R2 (Goodness of fit)[d]	0.9 (R^2)
ZF_Total_Score_W[e] (Hillslope)	0.8

[a]- Concentration (µM) at 10% of maximum activity (AC_{10})

[b]- Chemical "potency" = AC_{50} (concentration at ½ maximal activity concentration)

[c]- EMAX Toxicity Score (response) =top asymptote of the sigmoidal fit (minimum cutoff = 6.5/one standard deviation above the mean of the vehicle control response)

[d]- Goodness-of-fit (R^2: minimum cutoff = 0.4).

[e]- Slope of the calculated Hill Model (W).

Truong et al. (2014) Zebrafish Results:

- **Endosulfan: Statistically significant increase in axis malformations at 0.064 µM.**

- **Methidathion: Statistically significant increase in axis, caudal fin, and truncated body malformations at 64 µM.**

Results highly correlated with "any ZF Endpoint" results for maternal or pregnancy studies with a LOEL (mg/kg/d) from the USEPA ToxRefdb (http://www.epa.gov/ncct/toxrefdb/) endpoints (Table 5) categorized as:

- **Rabbit: developmental/pregnancy-related, developmental/pregnancy-related with embryo or fetal loss, any maternal effects, maternal general effects, maternal general systemic effects, maternal pregnancy-related effects, maternal pregnancy-related effects, including pregnancy loss, prenatal loss.**
- **Rat: prenatal loss**

Table 5. Endosulfan and Methidathion are Highly Correlated with "any Zebrafish Endpoint" and Maternal or Pregnancy Studies with a LOEL (mg/kg/d) for each ToxRefdb Endpoint (Truong et al. 2014; *http://www.epa.gov/ncct/toxrefdb/*)

	Developmental Rabbit LOEL (mg/kg/d) from ToxRefdb								***Developmental Rat (mg/kg/d)***
Chemical Name	**Development/ Pregnancy Related**	**Development/ Pregnancy Related Embryo/Fetal Loss**	**Maternal**	**General Maternal**	**General Maternal-Systemic**	**Maternal-Pregnancy Related**	**Maternal Pregnancy Loss**	**Prenatal Loss**	**Prenatal Loss**
Endosulfan	-	--	2	2	2	2	2	6	5
Methidathion	-	--	1	1	1	-	--	-	6

a- ToxRefdb USEPA ToxRefdb (http://www.epa.gov/ncct/toxrefdb/) contains all the ToxCast-related *in vivo* studies related to tested compounds.

DISCUSSION

- **According to Judson et al. (2010), if a chemical to be considered active in a pathway, it has to show activity in at least five assays mapped to that particular pathway.**

ToxCast HTS Assays

It is evident that one of the problems with data interpretation when comparing *in vivo* results to *in vitro* results is generally the lack of focus on a specific endpoint via a specific pathway as it is designed with HTS. While there is a plethora of data relating endosulfan to endocrine effects in males and females, there is limited evidence for specific interactions with ER or AR. Most data show effects that are likely related but these data are also open to interpretation based on dose level where endocrine effects occur compared with dose levels for other effects (eg. body weight, food consumption, etc.). When an *in vivo* study is performed along with an *in vitro* study, doses and endpoints can potentially be compared, but even then there is a question of what is happening *in vivo* to the compound and which pathway is it taking to induce effects.

ENDOSULFAN:

- **OEDs from the HTS assays were expected to be lower than the LOEL from *in vivo* studies since they focus on a specific pathway without interference from whole animal metabolism or other upstream effects.**

- **The OED results are within the same range of the presumptive ER-related *in vivo* LOEL in the rat despite the fact that the *in vivo* mode of action (MOA) likelyn comprises many different pathways depending on dose, species, strain, dosing duration and other factors.**

- **The OED did not support the observed LOEL for presumptive effects to AR from endosulfan since none of the HTS assays was active**

METHIDATHION:

- **OED for methidathion is representative of what is occurring with the ER and/or AR *in vivo* as shown by the lack of response in the HTS ER and AR assays.**

- **The reproductive endpoints observed in female rats could be from general toxicity or oxidative stress rather than effects to the ER.**

- **Male mating index could have been affected by disruption in a pathway or via a mechanism not related to the AR.**

- **The weight-of-evidence is lacking for an association between endocrine disruption or ER-related effects for methidathion *in vivo* and this correlates with the lack of positive ToxCast assays**

ER Assay:

- **Based on the premise that a compound must be active in at least 5 assays for a given pathway to be considered active in that pathway; neither compound could be considered active.**
- **This is contrary to what is predicted for endosulfan based on *in vivo* data but agrees with methidathion *in vivo* predictions.**

AR Assay:

- **Neither compound is active in the AR pathway.**
- **Considering the many *in vivo* and *in vitro* assays showing endosulfan affecting developmental/reproductive parameters in male adults, fetuses, and the developing young (Table 1), it was expected that more AR assays would be positive.**
- **Results for methidathion in ToxCast are consistent with *in vivo* results.**

ZF Assays:

An advantage to the ZF assay is that it tests for neurotoxicity as well as developmental effects. Stanley et al. (2009) used ENα in the ZF model with the embryo chorion removed (similar to Truong et al. 2014), as a basis for elucidating the mechanism of neurotoxicity. Abnormal touch response (measure of neurotoxicity) in larvae (LC50 of 2.2 uM for response vs. ENα-isomer in treatment media) was higher than the AC50 (1.0 uM) observed with endosulfan-α + β by Padilla et al. (2012). This is likely due to use of the more toxic ENα- on a dechorionated embryo compared to the endosulfan α, β mixture on a chorionated embryo. Stanley et al (2009) focused on developmental neurotoxicity (DNT), whereas Padilla et al. (2012) tested all effects. The former test was useful for elucidating endosulfan's DNT by HTS, since other ToxCast assays relating to

endosulfan's neurotoxicity MOA (i.e. GABAa) were negative.

CONCLUSIONS:

a. The weight-of-evidence from *in vivo* and *in vitro* studies (not related to ToxCast, Table 1) indicate that endosulfan affects the ER and AR to the point of inducing developmental and reproductive toxicity; this is somewhat supported by ToxCast data for the ER. Endosulfan exhibited weak ER agonist activity in some assays and no AR activity in any assays.

b. ToxCast data, by lack of ER and AR activity in assays, support the *in vivo* findings for methidathion. Neither receptor showed ER or AR activity with methidathion.

c. Wetmore et al. (2013) model was used to convert AC_{50} values to mg/kg/d for *in vivo* mammalian toxicity. We concluded that the *in vivo* endosulfan studies produced effects that agree with the modeled predictions for ER but not for AR. The OED was close to the LOEL for ER effects for endosulfan.

d. NVS ER, AR, GABA and ACHE assays are cell-free (no metabolic activation). Endosulfan and methidathion are both metabolically activated as part of their MOA, which is likely the cause for the observed negative results.

e. ZF assays provided a good predictor of neurotoxic effects for endosulfan and methidathion. They were also good predictors of developmental toxicity, which was not tested in other ToxCast assays. Potency agreed strongly with what was observed in *in vivo* studies for these two compounds.

f. We conclude that currently ToxCast has limited use for prioritization due to the lack of assays that detect developmental and neurotoxicity; however, there was dose concordance on endocrine disruption. These limitations might be overcome with use of integrated whole animal systems (ZF) or by use of metabolic activation.

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