A Comparison of ToxCast Test Results with *In Vivo* and Other *In Vitro* Endpoints for Neuro, Endocrine, and Developmental Toxicities:

A Case Study Using Endosulfan and Methidathion

Marilyn Silva, Ph.D., D.A.B.T.

Department of Pesticide Regulation
California Environmental Protection Agency
Sacramento, CA

Disclaimer

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Office of the Secretary
Gina Solomon, M.D., M.P.H. (Deputy Secretary for Science and Health)

Department of Pesticide Regulation (DPR), CalEPA
Marilyn Silva, Ph.D., D.A.B.T.
Carolyn Lewis, M.S., D.A.B.T.
Eric Kwok, Ph.D., D.A.B.T.

Office of Environmental Health Hazard Assessment (OEHHA), CalEPA
Nathalie Pham, Ph.D.
Shoba Iyer, Ph.D.
Lauren Zeise, Ph.D. (Acting Director)

QUESTION

Are the ***in vivo*** endpoints for endosulfan and methidathion concordant with the ***in vitro*** high throughput screening (HTS) results from the U.S.EPA's Toxicity Forecaster (ToxCast)?

In other words, do ToxCast results reflect what we already know or add to what we know from the animal assays?

Answer: yes and no!

THIS MATTERS BECAUSE…

- The mission of DPR is to protect human health and the environment
- We must use any tools available to accomplish this goal.
- Pesticide databases usually lack mode of action (MOA).
- ToxCast

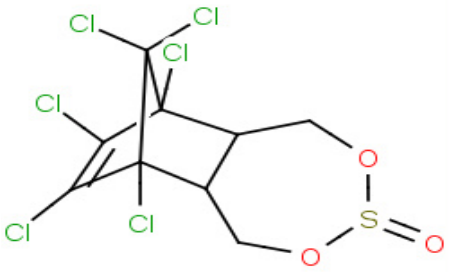
  --answers mechanistic questions

  --provides weight-of-evidence and reduces uncertainty in hazard identification.

Endosulfan and Methidathion

SELECTED BECAUSE

- Different modes of action (MOAs)
- Risk assessments completed by DPR
- They have large databases (registrant-submitted and open literature).
- Well-established (well-documented) *in vivo* effects



CAS: 115-29-7

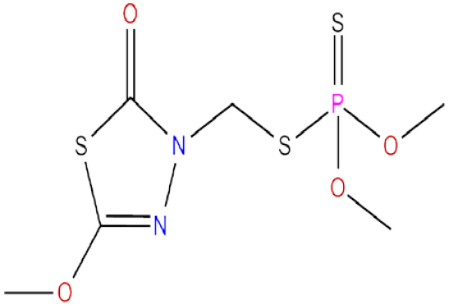
ENDOSULFAN (organochlorine)

Neurotoxic:

- Inhibits (noncompetitive) ɣ-Aminobutyric acid (GABA)
- Inhibits dopamine active transporter (DAT)

Endocrine disruption:

- Estrogenic or androgenic interference



CAS: 950-37-8

METHIDATHION (organophosphate)

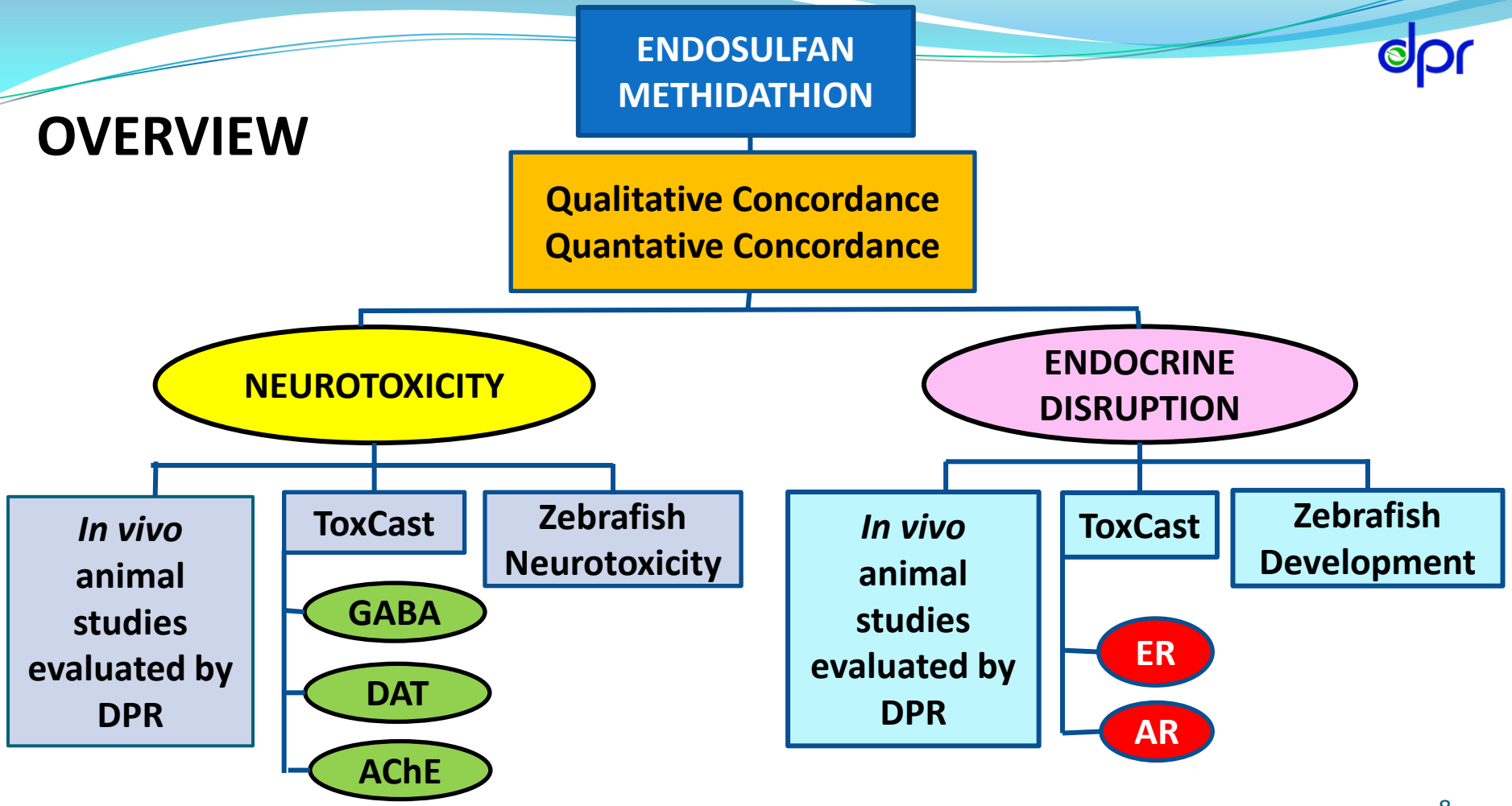
Neurotoxic:

- Inhibits acetylcholinesterase (AChE)

Systemic:

- Liver toxicity (carcinogenicity)
- Reproductive interference (**MOA unknown**)

OVERVIEW


ENDOSULFAN
METHIDATHION
Qualitative Concordance
Quantative Concordance
NEUROTOXICITY
ENDOCRINE DISRUPTION
In vivo animal studies evaluated by DPR
ToxCast
Zebrafish Neurotoxicity
GABA
DAT
AChE
In vivo animal studies evaluated by DPR
ToxCast
Zebrafish Development
ER
AR

TOXCAST PHASE II VENDORS

- Biochemical (Cell-free)

– NVS: Novascreen (PerkinElmer)

- Cell-Based

– ACEA: ACEA Biosciences, Inc.
– ATG: Attagene
– OT: OdysseyThera
– Tox21/NCGC: NIH/NCATS (NIH National Center for Advancing Translational Sciences); NCGC (NIH Chemical Genomics Center)

- Zebrafish

--Stephanie Padilla (National Health and Environmental Effects Research Lab)
--Robert Tanguay (Oregon State University)
--Stanley et al. (2009)

EPA iCSS ToxCast Dashboard: Efficient method of data extraction

Home

Choose a view: Assays / Chemicals

Database: prod_dashboard_v2

Dashboard: v2

Chemicals - 3

CASRN.. | endosulfan

RN	Chemical Name
29-7	Endosulfan
98-8	Endosulfan I
-07-8	Endosulfan sulfate

Assays - 1081

Assay Endpoint Name.. | Gene Symbol..

Actives - MC Only | All Tested

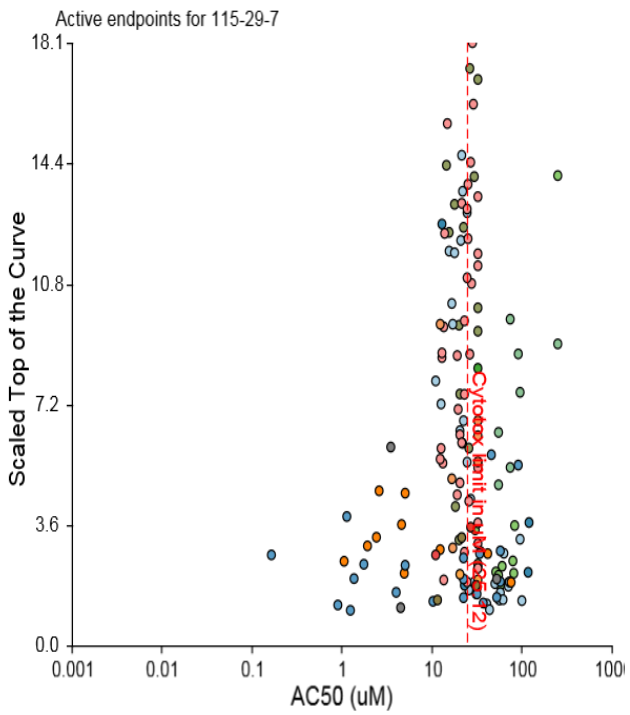
Assay Component Endpoint Name	Gene Symbol
ACEA_T47D_80hr_Negative	
ACEA_T47D_80hr_Positive	ESR1
APR_HepG2_CellCycleArrest_1h_dn	
APR_HepG2_CellCycleArrest_1h_up	
APR_HepG2_CellLoss_1h_dn	
APR_HepG2_CellLoss_1h_up	
APR_HepG2_MicrotubuleCSK_1h_dn	
APR_HepG2_MicrotubuleCSK_1h_up	
APR_HepG2_MitoMass_1h_dn	
APR_HepG2_MitoMass_1h_up	
APR_HepG2_MitoMembPot_1h_dn	

Filters - 0

Chemical Summary | Assay Summary | Bioactivity | Help

Start Tutorial - Chemical Tab

Save Chart | Preview Chart



- background measurement
- cell adhesion molecules
- cell cycle
- cell morphology
- cyp
- cytokine
- dna binding
- gpcr
- growth factor
- hydrolase
- ion channel
- kinase
- lyase

Quantitative Methods:

- **Lowest-observed-effect-levels (LOEL) from animal studies.**
- ***In Vitro* to *In Vivo* Extrapolation (IVIVE; Wetmore et al. 2013)**

--Translate ToxCast AC50s to oral equivalent doses (OEDs)

--One compartment pharmacokinetic model using rat metabolism and plasma binding

--OEDs (mg/kg/day) compared to rat LOELs (mg/kg/day)

Zebrafish Assays

Zebrafish embryos were treated with endosulfan and methidathion by two methods:

- **Chorion intact** (Padilla et al., 2012)
- **Chorion removed** (Truong et al., 2014)

Then assessed:

- **<u>Qualitatively:</u> Neurobehavioral and developmental effects**
- **<u>Quantitatively:</u> OEDs were calculated**

NEUROTOXICITY Results

IN VIVO ENDPOINTS

Endosulfan (Silva, 2009):

- **CRITICAL EFFECT:**
  GABA-ergic Clinical Signs (sensitivity to noise and light, convulsions)
  LOEL*: 1.8-2 mg/kg/d (pregnant rabbit; dog)
- **SECONDARY EFFECTS:**
  DAT inhibition: ↓ learning; ↑ foot-shock fighting behavior and ↓ hippocampal DAT
  LOEL: 1.0 mg/kg/d (weanling rats)
- **AChE inhibition:** ↓ plasma and RBC ChE
  LOEL: 16.6 mg/kg/d (rats).

Methidathion (Lewis, 2008)

- **CRITICAL EFFECT:**
  AChE inhibition (Cholinergic signs: salivation, diarrhea, tremors;↓AChE RBC & brain)
  LOEL: 0.62-2.2 mg/kg/d (monkey, rats)

*LOEL: LOWEST dose at which the effects described occur.

ToxCast Neurotoxicity Assays (Cell-free) from Novascreen (NVS)

Assay Names	Tissue Membrane	Target Gene
Receptor Binding Assays		
NVS_LGIC_bGABARa1	Bovine Cortical	$GABA_AR1$
NVS_LGIC_bGABARa5	Bovine Hippocampal	$GABA_AR5$
NVS_LGIC_rGABARa6	Rat Cerebellar	$GABA_AR6$
NVS_LGIC_bGABAR	Bovine Cerebellar	$GABA_AR$
NVS_LGIC_rGABAR	Rat Whole Brain	$GABA_AR$
NVS_TR_gDAT	Guinea Pig Striatum	DAT
NVS_TR_hDAT	Human recombinant	DAT
Regulation of Catalytic Activity		
NVS_ENZ_rAChE	Rat Whole Brain	AChE
NVS_ENZ_hAChE	Human recombinant	AChE

Endosulfan binds noncompetitively to the **$GABA_AR\beta3$ subunit *in vivo* which was not tested**

QUALITATIVE Concordance for Neurotoxicity Endpoints

Receptor	Test	Endosulfan	Methidathion
GABA	ToxCast	Inactive	Inactive
	Associated *in vivo* toxicity	Positive	Negative
	Concordance	**No**	Yes
DAT	ToxCast	Active	Inactive
	Associated *in vivo* toxicity	Positive	Negative
	Concordance	Yes	Yes
AChE	ToxCast	Inactive	Inactive
	Associated *in vivo* toxicity	Positive	Positive
	Concordance	**No**	**No**

All neurotoxicity assays are cell free and lack metabolic activation which may be necessary for activity.

QUANTITATIVE Concordance for Neurotoxicity Endpoints

Parameter	Endosulfan (mg/kg/d)	Methidathion (mg/kg/d)
In Vivo LOEL	1.0 (DAT Inhibition)	0.62 -2.2 (AChE Inhibition)
OED ToxCast	0.18 - 0.33 (DAT inhibition)	--
OED Zebrafish	Intact Chorion 0.038 (abnormal touch response & swim behavior)	Not evaluated for neurotoxicity

Problems Identified with ToxCast Neurotoxicity Assays

- **The critical endpoints were not characterized by the ToxCast assays**

- **Cross-validation by more than one assay vendor is needed**

ENDOCRINE DISRUPTION:

Unlike Neurotoxicity, U.S. EPA Proposed Criteria for Determining Activity in the Estrogen or Androgen Receptor Pathways

U.S. EPA CRITERIA FOR ACTIVITY

Activity is shown in a pathway when:

- There are ≥ **5 active assays** per pathway

Area under the curve (AUC) must be >0.1 to be active

- AUC is a **measure of activity** in a pathway **compared to a reference compound**
- **Range 0-1, where 1= reference** (e.g.,17β-estradiol)

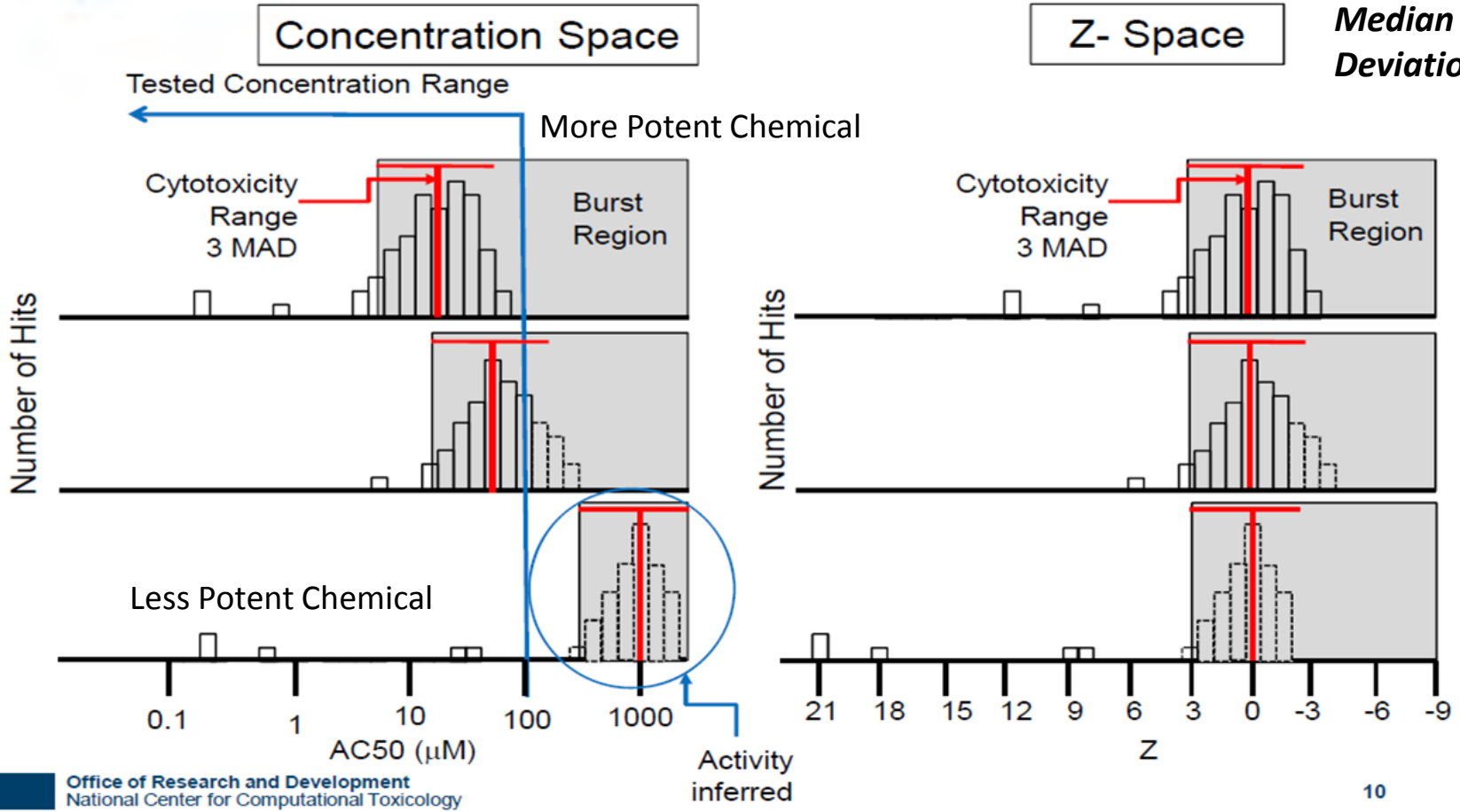
Chemical-receptor interaction is indicated by:

- **Gene score:** Specificity of a chemical for a gene
  Gene Score > 7 = specificity
- **Z-score:** Indicates whether **true activity** or cytotoxicity has occurred
  Z-score > 3 = true active; <3 = ? cytotoxicity

Most chemicals display a "burst" of activity near cytotoxity concentration

FutureTox II; Judson, , 2014

Median Absolute Deviation (MAD)



ESTROGEN RECEPTOR
Results

IN VIVO ENDPOINTS IN FEMALES

Endosulfan ***(Silva, 2009):***

Female Rats: ↑uterine ER down regulation; ↑uterotropic activity; disrupted estrus cycles.

LOEL = 6.0 mg/kg/d

Methidathion ***(Lewis, 2008):***

Female Rats: Poor maternal care (pups cool to touch, starving, weak, or lethargic); ↓ovarian weights in F_0 and F_1 females

LOEL = 2.5 mg/kg/d

ESTROGEN RECEPTOR SIGNALING PATHWAY

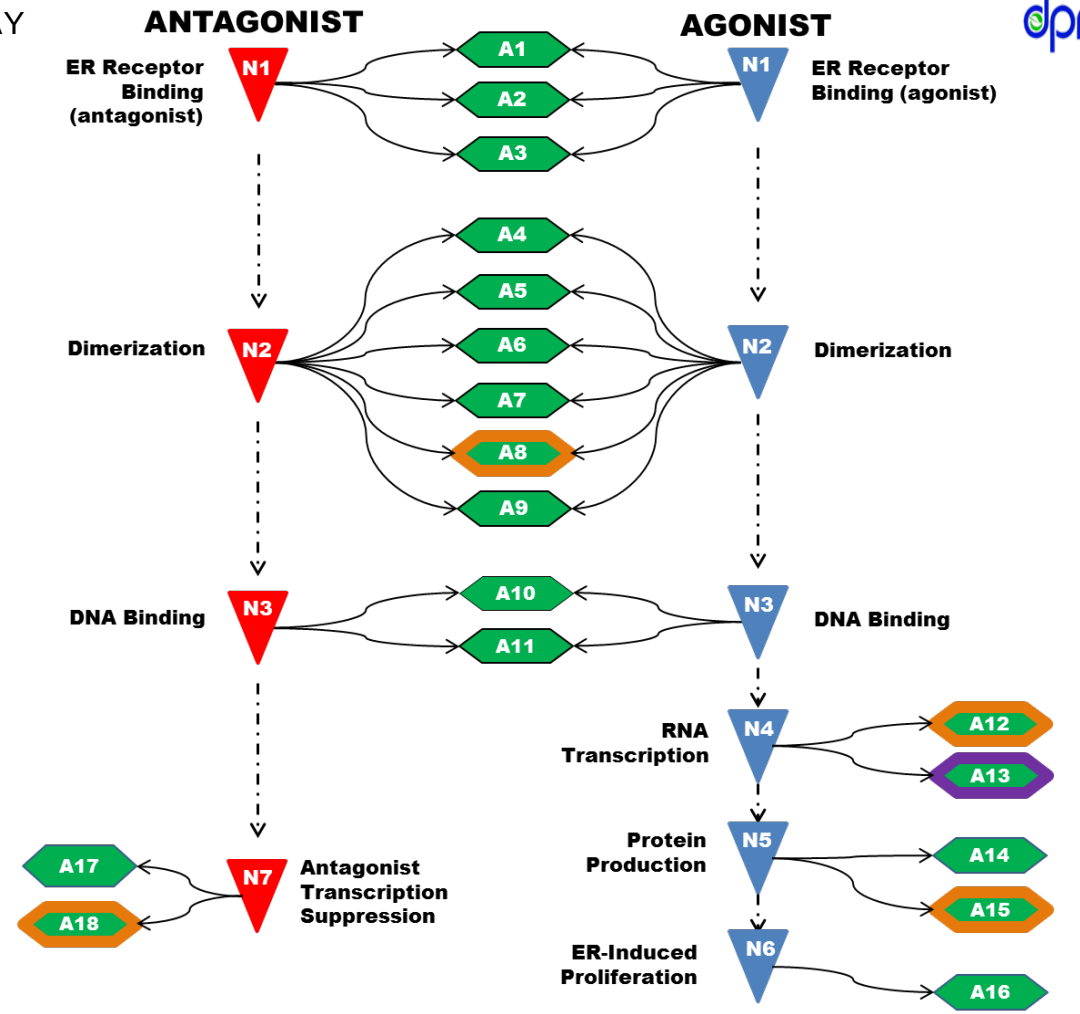
(adapted from FIFRA SAP presentation: USEPA, 2014)

Orange: endosulfan

Purple: endosulfan & methidathion

A1	NVS_NR_bER
A2	NVS_NR_hER
A3	NVS_NR_mERa
A4	OT_ER_ERaERa_0480
A5	OT_ER_ERaERa_1440
A6	OT_ER_ERaERb_0480
A7	OT_ER_ERaERb_1440
A8	OT_ER_ERbERb_0480
A9	OT_ER_ERbERb_1440
A10	OT_ER_EREGFP_0120
A11	OT_ER_EREGFP_0480
A12	ATG_ERa_TRANS_up *
A13	ATG_ERE_CIS_up* *
A14	Tox21_ERa_BLA_Agonist_ratio
A15	Tox21_ERa_LUC_BG1_Agonist
A16	ACEA_T47D_80hr_Positive
A17	Tox21_ERa_BLA_Antagonist_ratio
A18	Tox21_ERa_LUC_BG1_Antagonist

* Z-score > 3



Concordance for Estrogen Pathway

Test	Endosulfan				Methidathion			
Qualitative								
ToxCast	5 assays	AUC*	Z-Score	Gene Score	5 assays	AUC*	Z-Score	Gene Score
	4/18	0.034	2/4	16.25	1/18	0	1/1	2.6
In vivo estrogenic	Positive				Negative			
Concordance	No	No	Yes/No	Yes	Yes	Yes	NO	Yes
Quantitative (mg/kg/d)								
ToxCast OED	0.2 to 0.82				170			
Estrogenic LOEL	6.0				2.5 to 5.0			
Concordance	Yes				No			

*Data available on http://actor.epa.gov/edsp21

ANDROGEN RECEPTOR Results

IN VIVO ENDPOINTS IN MALES

Endosulfan:

Human male: Delayed sexual maturation.
Male Rat: Affected sperm morphology, motility or function.
LOEL = 1.0 mg/kg/d

Methidathion:

Male Rats: ↓mating activity
LOEL = 2.2 mg/kg/d

ANDROGEN RECEPTOR SIGNALING PATHWAY

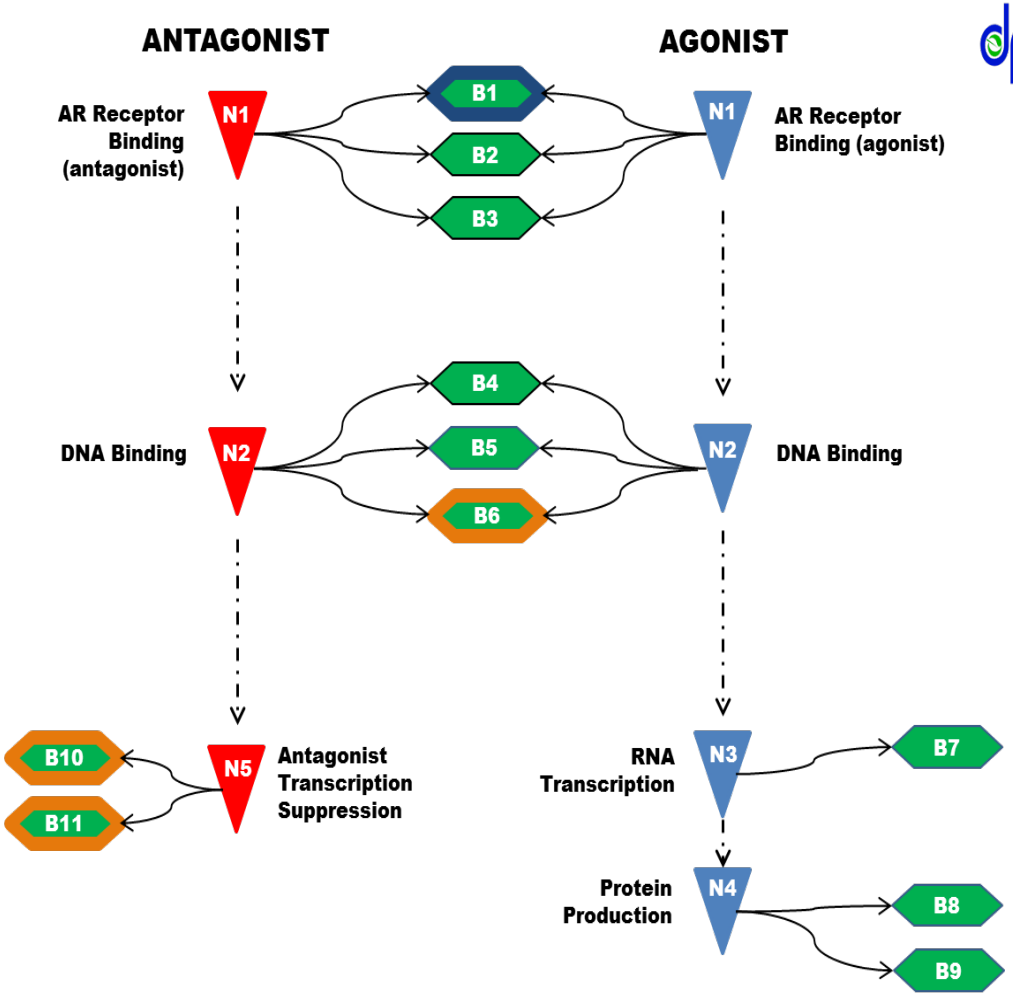
(adapted from FIFRA SAP presentation: USEPA, 2014)

Orange: endosulfan
Blue: methidathion

B1 NVS_NR_hAR **
B2 NVS_NR_cAR*
B3 NVS_NR_rAR
B4 OT_AR_ARE_LUC_AG_1440*
B5 OT_AR_ARSRC1_0480
B6 OT_AR_ARSRC1_0960
B7 ATG_AR_TRANS_up
B8 Tox21_AR_BLA_Agonist_ratio
B9 Tox21_AR_LUC_MADAKB2_Agonist
B10 Tox21_AR_BLA_Antagonist_ratio
B11 Tox21_AR_LUC_MADAKB2_Antagonist

***Assays have been discontinued for use in defining steps of the AR pathway**

** Z-score > 3



Concordance for Androgen Pathway

Test	Endosulfan				Methidathion			
Qualitative								
ToxCast	5 assays	AUC*	Z-Score	Gene Score	5 assays	AUC*	Z-Score	Gene Score
	3/11	0.028	0/3	16.25	1/11	0	1/1	2.6
In vivo androgenic	Positive				Negative			
Concordance	No	No	NO	Yes	Yes	Yes	NO	Yes
Quantitative (mg/kg/d)								
ToxCast OED	0.35 to 0.9				84			
Androgenic LOEL	1.0				2.5 to 5.0			
Concordance	Yes				No			

DEVELOPMENTAL EFFECTS IN ZEBRAFISH
Results

Concordance for Zebrafish Developmental Toxicity

Test	Endosulfan	Methidathion
Qualitative		
Zebrafish	Strong Active	Active
Developmental Toxicity	Positive	Negative
Concordance	**Yes**	**No**
Quantitative (mg/kg/d)		
Zebrafish OED	Chorion Intact 0.002 Chorion Removed 0.038	Chorion Intact 0.016 Chorion Removed 174
Developmental LOEL	1.0 (lowest LOEL male rat)	2.2
Concordance	**No**	**No**

Conclusions

Possible explanations for lack of concordance between ToxCast and in vivo assays include:

- Lack of metabolic activation
- Assays for **MOA** may not relate to what is occurring *in vivo*
- Limited biological coverage for pathways (e.g. neurotoxicity)
- Unknown factors in assay design (cross-validation)

Zebrafish (things to consider):

- A good model for predicting qualitative but not quantitative toxicity.
- Unknown effects of chorion
- Internal exposure to embryos is still uncertain.

Advantages of ToxCast

- Many pesticides and their presumptive metabolites have been tested in ToxCast and the **data are easily accessed**.
- The ToxCast program is an **evolving process, with frequent updates.**
- **Provides mechanistic information not available in other studies.**
- Human exposure data and ToxCast data can potentially be used to facilitate **chemical prioritization and uncertainty in risk assessment.**
- ToxCast assays may have potential for predicting acute effects and minimizing animal use.

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THANK YOU

CONTACT INFORMATION:
Human Health Assessment Branch,
Department of Pesticide Regulation,
California Environmental Protection Agency
Marilyn.silva@cdpr.ca.gov
Birth Defects Research (Part B) 104(2):71–89

REFERENCES

California DPR Risk Characterization Documents (RCD) and Open Literature

- Endosulfan (Silva &Gammon, 2009; Silva, 2008); Methidathion (Lewis, 2007); ToxCast (BDRB:1–19, 2015)

U.S.EPA ToxCast Assay Data Sources

- **Interactive Chemical Safety for Sustainability (iCSS) Dashboard:** actor.epa.gov/dashboard2/ and epa.gov/ncct/toxcast/data.html
- **Endocrine Disruptor Screening Program Dashboard**: **EDSP http://actor.epa.gov/edsp21**
- **Federal Insecticide, Fungicide and Rodenticide Act Scientific Advisory Panel:** Integrated Endocrine Activity and Exposure-based Prioritization and Screening (regulations.gov; Docket #: EPA-HQOPP-2014-0614; Judson, personal communication).
- **Zebrafish:** ToxCast (Padilla et al., 2012 and personal communication; Truong et al., 2014);open literature (Stanley et al., 2009).

Oral Equivalent Dose: Perkins et al. 2013; Wetmore et al. 2013

Z-score (chemical, assay) = (log AC50: chemical, assay) – (median[logAC50 chemical, cytotox])
Global cytotoxicity Median Absolute Deviation (MAD)

Global MAD: median of the MAD of the logAC50 (cytotoxicity) distributions across all chemicals.

Gene Score: Endosulfan ER: (−logAC50s of **active** ER assays) = 25.74 (logAC50).
(−logAC50s of active assays)/(total no. of assays for that gene: 18) = 1.43 = x;
Gene Score = 10^x = 26.93.