Gaining Confidence in Replacing Animal Tests: A Case Study of the Endocrine Disruption Program at the US EPA

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The views expressed in this presentation are those of the author and do not necessarily reflect the views or policies of the U.S. EPA
Conflict of Interest Statement

- The author has no conflicts of interest
Diethylstilbesterol – DES

- Synthetic estrogen
- From about 1940 to 1971, DES was given to pregnant women in the mistaken belief it would reduce the risk of pregnancy complications and losses
- Shown to cause reproductive organ cancers and other reproductive problems in women exposed in utero
- Time from exposure to adverse effect was 20 years or more
- “Chemical time bomb”
As a result, Congress passed laws leading to the formation of the EDSP (1996)
- FIFRA (Pesticide regulation)
- SWDA (Drinking water regulations)
- Requires all pesticide ingredients and potential drinking water contaminants to be tested for potential to be endocrine disruptors

Original regulations focused on estrogen mimics

Subsequent work by EPA EDSTAC expanded scope to androgen and thyroid (1998)
EDSP is a 2 Tier Testing Program

- **Tier 1**
  - “Screening”: Hazard assessment
  - 11 *in vitro* and *in vivo* assays
  - Assays development and validation finalized in 2009
  - Test orders for initial 73 chemicals issued in 2009
  - Tests run in starting in 2012
  - Evaluation of results of 52 tested chemicals in 2015
    - 19 years after law passage

- **Tier 2**
  - “Testing”: Quantitative risk assessment
  - No test orders issued to date
## EDSP Tier 1 battery

### Complementary Modes of Action Among Screening Assays in the EDSP Tier 1 Battery

<table>
<thead>
<tr>
<th>Screening Assays</th>
<th>Receptor Binding</th>
<th>Steroidogenesis</th>
<th>HPG&lt;sup&gt;3&lt;/sup&gt; Axis</th>
<th>HPT&lt;sup&gt;3&lt;/sup&gt; Axis</th>
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<tbody>
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<td></td>
<td>E&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Anti-E</td>
<td>A&lt;sup&gt;2&lt;/sup&gt;</td>
<td>E&lt;sup&gt;2&lt;/sup&gt;</td>
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<tr>
<td>In vitro</td>
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<td>ER Binding&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>ER&lt;sub&gt;α&lt;/sub&gt; Transcriptional Activation</td>
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<td>AR Binding&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>Steroidogenesis H295R</td>
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<td>Aromatase Recombinant</td>
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<tr>
<td>In vivo</td>
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<tr>
<td>Uterotrophic</td>
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<td>Hershberger</td>
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<tr>
<td>Pubertal Male</td>
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<tr>
<td>Pubertal Female</td>
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<td>Amphibian Metamorphosis</td>
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<td>Fish Short-term Reproduction (male &amp; female)</td>
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<sup>1</sup> Indicates the presence of a specific mode of action for the given screening assay.
Issues with EDSP Tier 1

- Total EDSP Universe is ~10,000 chemicals
  - Cost of Tier 1: ~$1M / chemical
  - Throughput: 50 chemicals / year
- Implications: $B’s, 100-200 years to complete
- Need new approaches
  - Prioritize chemicals
  - Replace some or all low-throughput Tier 1 assays
- Origin of EDSP21 Program (2012)
ToxCast and Tox21
High-Throughput Toxicology

- Started in 2007
- EPA, NTP, NCATS, FDA
- Screening up to 10,000 chemicals (Tox21 library)
- ToxCast has ~3000 chemicals in up to 800 *in vitro* assays
  - Cell-based (cell lines, primary cells)
  - Model organisms (zebrafish, *c. elegans*)
  - Organotypic systems
- EDSP21 is one example of Tox21 data and models
Tox21 Aims to Perform High-Throughput Risk Assessment

Semi-quantitative

*In Vitro to In Vivo*
- Hazard
- Exposure
- Uncertainty

Potential Exposure: ExpoCast

Potential Hazard: *In Vitro* + HTTK

mg/kg BW/day

Low Priority  Medium Priority  High Priority
**In Vitro Estrogen Receptor Model**
Combines results from multiple in vitro assays

- Use multiple assays per pathway
  - Different technologies
  - Different points in pathway

- No assay is perfect
  - Assay Interference
  - Noise

- Use model to integrate assays

- Evaluate model against reference chemicals

- Methodology being applied to other pathways

Major theme – all assays have false positives and negative

Assays cluster by technology, suggesting technology-specific non-ER bioactivity

Much of this “noise” is reproducible
- “assay interference”
- Result of interaction of chemical with complex biology in the assay

EDSP chemical universe is structurally diverse
- Solvents
- Surfactants
- Intentionally cytotoxic compounds
- Metals
- Inorganics
- Pesticides
- Drugs
In Vitro Validation Against Reference Chemicals

Agonist Score (R1) vs. Reference Activity Class

Active
- Relaxofene
- 4-Hydroxytamoxifen (E/Z)
- Tamoxifen citrate
- Tamoxifen
- Di-n-butyl phthalate
- Dicofol
- Kepone
- Diethylstilbestrol (DES)
- 17beta-Estradiol
- Genistein
- Bisphenol A
- Butylbenzyl phthalate
- Chrysin
- p,p’-DDE
- Progestrone
- Diethylhexyl phthalate

Inactive
- Chloramphenicol
- Ethylparaben
- p,p’-DDE
- p-n-Nonylphenol
- Fenarimol
- Di-n-butyl phthalate
- Haloperidol
- Spironolactone
- Reserpine
- Procymidine
- Phenobarbital Sodium
- Linuron
- Ketoconazole
- Hydroxyflutamide
- Flutamide
- Cycloheximide
- Corticosterone
- Atazanavir
- Dicofol

Very Weak
- 17alpha-Estradiol
- 17beta-Estradiol
- Ethylhexyl phthalate
- Estrone
- Genistein
- Bisphenol B
- Bisphenol A
- Daidzein
- Di-n-butyl phthalate
- 4-tert-Octylphenol
- 4-Cumylphenol
- 5alpha-Dihydrotestosterone
- p,p’-DDT
- 17alpha-Methyltestosterone
- Apigenin
- Methoxychlor
- Kaempferol
- Butylbenzyl phthalate
- Kepone
- Chrysin
- Ethylparaben
- p,p’-DDE
- p-n-Nonylphenol
- Fenarimol
- Di-n-butyl phthalate
- Haloperidol
- Spironolactone
- Reserpine
- Procymidine
- Phenobarbital Sodium
- Linuron
- Ketoconazole
- Hydroxyflutamide
- Flutamide
- Cycloheximide
- Corticosterone
- Atazanavir
- Dicofol
Validate the *in vitro* ER model against the *in vivo* version: the Uterotrophic Assay

**In vitro hER activity:**
- Human Breast
- Human Ovary
- Human Uterus
- Human Cervix
- Human Liver
- Human ER (cell free)

**ER-Bioactivity**
- Rat or Mouse uterus (guideline uterotrophic)

Human Relevance
Identifying Uterotrophic Reference Chemicals from the Literature

Literature Searches: 1800 Chemicals

Data Review: 700 Papers, 42 Descriptors, x2

Uterotrophic Database
98 Chemicals
442 GL uterotrophic bioassays

In Vivo ER Reference Chemicals
30 Active, 13 Inactive

Uterotrophic Assay Outline
Requirement for report to be “guideline-like”

- Remove ovaries: intrinsic source of estrogen & driver of uterine growth
- Test a chemical’s ability to restore uterine growth through ER activity

- Closely follows EPA and OECD guidelines
  1. Adult rats or mice: Ovariectomy: 6-8 weeks of age; treatment: 14+ days post-surgery (rats), 7 days (mice)
  2. Immature rats: start dosing between PND 18 and PND 21, and finish by PND 25
  3. Control groups: 3 animal min
  4. Test groups: 5 animal min; minimum of 2 test groups
  5. Oral gavage, subcutaneous injection, and intraperitoneal injection
  6. Dosing over a minimum of 3 days
  7. Necropsy: 18–36 hours after the last dose
Adding Tier 1 / List 1 chemicals to the Literature DB:
81 Guideline Chemicals

Uterotrophic Literature “Guideline-Like” studies: Chemicals with more than one consistent test

+ EDSP List 1 Uterotrophic “Guideline” Studies

Uterotrophic Reference Chemicals: 30 Active, 51 Inactive
### In Vitro Activity vs. Uterotrophic Outcomes

#### ER Agonist Model AUC

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<table>
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<tr>
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<tbody>
<tr>
<td>True Positive</td>
<td>29</td>
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<tr>
<td>True Negative</td>
<td>50</td>
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<tr>
<td>False Positive</td>
<td>1</td>
</tr>
<tr>
<td>False Negative</td>
<td>1</td>
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<tr>
<td><strong>Accuracy</strong></td>
<td>0.97</td>
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<tr>
<td><strong>Sensitivity</strong></td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>0.98</td>
</tr>
</tbody>
</table>
In vivo guideline studies have the same types of uncertainty as in vitro:
26% of chemicals have at least one positive and one negative study.
EPA Moves to Use ER Model in EDSP

- Federal Register Notice (FRN) June 2015
  - Based on 3 FIFRA Scientific Advisory Panels
  - Publications on ER Model, Uterotrophic Database, Comparison

- “The approach incorporates validated high throughput assays and a computational model and, based on current research, can serve as an alternative for some of the current assays in the Endocrine Disruptor Screening Program (EDSP) Tier 1 battery.”

- “Use of these alternative methods will accelerate the pace of screening, decrease costs, and reduce animal testing. In addition, this approach advances the goal of providing sensitive, specific, quantitative, and efficient screening using alternative test methods to some assays in the Tier 1 battery to protect human health and the environment.”

- Multiple public comments
Goal: To make EDSP-related data easily available to all stakeholders
- Assay-by-assays concentration-response plots
- Model scores – AUC agonist and antagonist
- ER QSAR calls
- Other relevant data

http://actor.epa.gov/edsp21
Summary

- EDSP21 ER model is the first successful example of using ToxCast/Tox21 data and models to replace low-throughput animal tests with high-throughput in vitro assays.

- Requires strong collaboration
  - Assay developers, modelers, assay validators, regulators.

- Next steps
  - Androgen receptor: aim to replace Hershberger.
  - Steroidogenesis: Move from low to high-throughput in vitro.
  - Thyroid – assay development and testing underway for several targets (THR, TPO, deiodinases, ...).
Key Publications

- **Estrogen Receptor *In Vitro* Model**

- **Uterotrophic Database**

- ***In Vitro* to *In Vivo* Comparison**

- **Federal Register Notice**
  - https://federalregister.gov/a/2015-15182
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