Update on Research Using *in vitro* and Computer-based Tools for Screening Potential Estrogenic Activity

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Quantitative Structure-Activity Relationships

Assumptions

• A chemical’s structure imparts properties

• A group of chemicals that produce the same biological activity (toxicity; adverse effect) have something similar about their chemistry (structure)

• Goal is to quantify ‘structural similarity’ imparting biological activity; identify which other chemicals may be ‘similar’ with the assumption that an untested chemical may produce the same activity

Chemical similarity is defined in the context of biological similarity

• Robustness Depends on:
  – Well-defined biological system; Well-characterized chemistry
  – Well-defined application –
    • Risk context - What’s the question being asked - problem definition
QSAR Assumption

Δ Chemical Structure/Property → Δ Dose Metric (kinetics/metabolism) → Δ Endpoint Potency

Toxic potency is correlated to chemical concentration at the site of action
-C. Hansch

Well-defined system (chemistry and biology)
Well-Defined Biological System
(What do you know and what are you assuming)

• Is the chemical administered what you thought it was
  – Impurities

• Metabolism
  – Is the system used for collection of empirical data capable of xenobiotic metabolism?
  – Is what you’re measuring due to parent chemical or to a metabolite?

• Kinetics
  – What do you understand about the chemical kinetics within the system?
  – Is the chemical in solution
    • Bound and unavailable
    • Loss to hydrolysis

Has chemical form and/or concentration been measured in the biological system upon which the QSAR is based
QSAR Approach

• QSAR is an approach to help think about, hypothesize, and investigate, in a systematic manner how a chemical is most likely to interact with a biological system and what adverse effect might be the consequence of that interaction.

• QSAR depends upon a well-defined biological system.

• QSAR for large diverse chemical inventories is an iterative process.

• How QSAR used depends upon the regulatory context:
  – Defining the regulatory domain is non-trivial; identify the exact chemicals and verify structures.
  – Defining the regulatory question is essential; regulatory acceptance criteria are dependent upon the use.
Risk Context

Development and use of a QSAR in regulatory risk assessment requires clear problem definition

- The purpose of the QSAR application must be well-defined (e.g., priority setting for testing, and chemical-specific risk assessment are two very different purposes – different acceptance criteria)

- The chemicals of regulatory concern must be defined to establish an appropriate training set for QSAR development and/or to assess appropriateness of QSAR application
  - Regulatory Domain
  - Applicability Domain of QSAR (dependent on Training Set)

A QSAR can only be as good as the underlying toxicological understanding and data it is based upon

- Toxicological activity is assessed based on a well-defined endpoint in a well-defined assay
  - e.g., chemical dosimetry –
  - if you assume parent chemical is responsible for biological activity but in fact a metabolite produced toxicity, then you’re working from wrong structure
  - If you assume chemical was 100% available in your system but in fact 80% was loss due to volatility, or binding to glassware, unavailable in vehicle administered, etc then your concentration may have to be corrected
Today’s Research Update: Developing the Tools to move EPA toward the New Paradigm

- Use screening and priority setting to focus on the most plausible toxicological potential for chemical or group of chemicals, not all possible adverse outcomes.

- Challenge of implementing FQPA
  - Endocrine Disruptors - How to prioritize and efficiently test a large number of chemicals while still carrying out existing chemical (new and old) evaluation programs

- Hypothesis-driven approach
Food Quality Protection Act –
Need to prioritize *in vivo* testing options for classes of compounds where ‘endocrine data’ is lacking:
- Inert ingredients used in formulations of pesticides used on crops
- Antimicrobial active ingredient pesticides

Prioritize -
- Based on effect endpoint(s) in combination with existing exposure estimates
- Use QSARs to estimate potential for ‘estrogenic activity’ for untested inerts and antimicrobial pesticides
Research Focus:

• **Adverse outcome pathway:**
  – Reproductive impairment through the ER-mediated pathway

• **Chemicals:**
  – Inert ingredients
  – Antimicrobials

• **Hypothesis-driven approach:**
  – Chemicals that have similar activity will have similar structure; quantifying the structural similarity will allow extrapolation across chemicals
Research Approach:

- Test a ‘representative’ chemicals *in vitro* to extrapolated potential for activity to untested.

- Chemical Class Approach based on mechanism:
  - What types of chemicals can interact with the ER and which ones can’t.

- *in vitro* assays:
  - ER binding displacement
  - ER-mediated gene activation
Adverse Outcome Pathway
ER-mediated Reproductive Impairment
Measurements made across levels of biological organization

In vitro Assay focus area

In vivo

MOLECULAR Target
CELLULAR Response
TISSUE/ORGAN
INDIVIDUAL
POPULATION

Inerts; Antimicrobial Chemicals

Receptor Binding
ER Binding

Liver Cell Protein Expression

Liver Altered proteins, hormones;

Gonad Ova-testis

Sex reversal;
Altered behavior;

Repro.

Skewed Sex Ratios;

Yr Class

Toxicity Pathway

Adverse Outcome Pathway

↑ Vitellogenin (egg protein transported to ovary)

↓ Skewed Sex Ratios;
↓ Yr Class

Skewed Sex Ratios;
↓ Yr Class
Defining the Problem: *Prioritizing estrogenic potential of Food Use Inert Ingredients*

Inert chemicals in Pesticides used on Food Crops
The 2004 List included:

893 entries = 393 discrete chemicals + 500 non-discrete substances
(44% discrete : 56% non-discrete)

393 discrete chemicals include:
- 366 organics (93%)
- 24 inorganics (6%)
- 3 organometallics (1%)

500 non-discrete substances include:
- 147 polymers of mixed chain length
- 170 mixtures
- 183 undefined substances
Defining the Problem:
Prioritizing Estrogenic Potential of Antimicrobial Pesticides

Antimicrobials and Sanitizers
List included:

\[ 299 = 211 \text{ discrete chemicals} + 88 \text{ non-discrete substances} \]
(71% discrete : 29% non-discrete)

211 discrete chemicals include:
- 153 organics (72%)
- 52 inorganics (25%)
- 6 organometallics-acyclic (3%)

88 non-discrete substances include:
- 25 polymers of mixed chain length
- 35 mixtures
- 28 undefined substances
Data Example - primary *In vitro* assay used:

**Estrogen Receptor Binding Displacement Assay**

**Test Chemicals:**
- Negative response
- Positive response

**Positive Control:** Estradiol

**Log Concentration (M)**

**[3H]-E2 Binding (%)**
Data example – Confirmatory *in vitro* Assay:
Gene Activation

![Graphs showing gene activation assay results with different concentrations of chemicals and controls.](image-url)
Research Approach:

• Test a few ‘representative’ chemicals in *vitro* to extrapolate to others

• Chemical Class Approach based on mechanism:
  – What types of chemicals can interact with the ER and which ones can’t
    • chemicals selected to investigate mechanisms of binding the ER
    • chemicals selected to cover classes found on list
### Homologous Series

**Alkylphenols**

<table>
<thead>
<tr>
<th>Compound (C)</th>
<th>Molecular Structure</th>
<th>Log Kow</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0</td>
<td><img src="image" alt="Structure of C0" /></td>
<td>1.50 msrd</td>
</tr>
<tr>
<td>C1</td>
<td><img src="image" alt="Structure of C1" /></td>
<td>1.97 msrd</td>
</tr>
<tr>
<td>C2</td>
<td><img src="image" alt="Structure of C2" /></td>
<td>2.47 msrd</td>
</tr>
<tr>
<td>C3</td>
<td><img src="image" alt="Structure of C3" /></td>
<td>3.20 msrd</td>
</tr>
<tr>
<td>C4</td>
<td><img src="image" alt="Structure of C4" /></td>
<td>3.65 msrd</td>
</tr>
<tr>
<td>C5</td>
<td><img src="image" alt="Structure of C5" /></td>
<td>4.06 msrd</td>
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<tr>
<td>C6</td>
<td><img src="image" alt="Structure of C6" /></td>
<td>4.62 calc</td>
</tr>
<tr>
<td>C7</td>
<td><img src="image" alt="Structure of C7" /></td>
<td>4.15 msrd</td>
</tr>
<tr>
<td>C8</td>
<td><img src="image" alt="Structure of C8" /></td>
<td>5.68 calc</td>
</tr>
<tr>
<td>C9</td>
<td><img src="image" alt="Structure of C9" /></td>
<td>5.76 msrd</td>
</tr>
</tbody>
</table>
### Alkylphenols

<table>
<thead>
<tr>
<th>( \text{Log Kow} )</th>
<th>( \text{C0} )</th>
<th>( \text{C1} )</th>
<th>( \text{C2} )</th>
<th>( \text{C3} )</th>
<th>( \text{C4} )</th>
<th>( \text{C5} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.50 msrd</td>
<td>( \text{H}<em>2\text{C}-\text{C}</em>{10}\text{H}_2\text{OH} )</td>
<td>( \text{H}<em>2\text{C}-\text{C}</em>{10}\text{H}_2\text{OH} )</td>
<td>( \text{H}<em>3\text{C}-\text{C}</em>{10}\text{H}_2\text{OH} )</td>
<td>( \text{H}<em>3\text{C}-\text{C}</em>{10}\text{H}_2\text{OH} )</td>
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</tr>
<tr>
<td>3.20 msrd</td>
<td>( \text{H}<em>2\text{C}-\text{C}</em>{10}\text{H}_2\text{OH} )</td>
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</tr>
<tr>
<td>3.65 msrd</td>
<td>( \text{H}<em>2\text{C}-\text{C}</em>{10}\text{H}_2\text{OH} )</td>
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</tr>
<tr>
<td>4.06 msrd</td>
<td>( \text{H}<em>2\text{C}-\text{C}</em>{10}\text{H}_2\text{OH} )</td>
<td>( \text{H}<em>2\text{C}-\text{C}</em>{10}\text{H}_2\text{OH} )</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>( \text{C6} )</th>
<th>( \text{C7} )</th>
<th>( \text{C8} )</th>
<th>( \text{C9} )</th>
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<td>( \text{C7} )</td>
<td>( \text{C8} )</td>
<td>( \text{C9} )</td>
</tr>
<tr>
<td>4.62 calc</td>
<td>4.15 msrd</td>
<td>5.68 calc</td>
<td>5.76 msrd</td>
</tr>
<tr>
<td>4.36 clog</td>
<td>4.89 clog</td>
<td>5.16 clog</td>
<td>6.61 clog</td>
</tr>
<tr>
<td>6.61 clog</td>
<td>7.91 msrd</td>
<td>5.35 clog</td>
<td>6.01 clog</td>
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</tbody>
</table>
# Alkylphenols – (p-branched chain)

rtER tested chemicals - Training Set

<table>
<thead>
<tr>
<th>C0</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
</tr>
<tr>
<td>Log Kow = 1.50 msrd</td>
<td>1.97 msrd</td>
<td>2.47 msrd</td>
<td>3.20 msrd</td>
<td>3.65 msrd</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C3</th>
<th>C4</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
</tr>
<tr>
<td>2.90 msrd</td>
<td>3.31 msrd</td>
<td>3.32 msrd</td>
<td>3.83 msrd</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C6</th>
<th>C7</th>
<th>C8</th>
<th>C9</th>
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<td><img src="image.png" alt="Structure" /></td>
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<td><img src="image.png" alt="Structure" /></td>
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</tr>
<tr>
<td>4.62 calc</td>
<td>4.15 msrd</td>
<td>5.68 calc</td>
<td>5.76 msrd</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C6</th>
<th>C7</th>
<th>C8</th>
<th>C10</th>
<th>C12</th>
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</thead>
<tbody>
<tr>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
<td><img src="image.png" alt="Structure" /></td>
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</tr>
<tr>
<td>4.36 clog</td>
<td>4.89 clog</td>
<td>5.16 clog</td>
<td>6.61 clog</td>
<td>7.91 msrd</td>
</tr>
</tbody>
</table>

Log Kow = 1.50 msrd
= Inventory chemical in the Alkylphenols group
# Alkylanilines – (p-n chain)

## rtER tested chemicals - Training Set

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>msrd</th>
<th>clog</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Chemical 1" /></td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td><img src="image2" alt="Chemical 2" /></td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td><img src="image3" alt="Chemical 3" /></td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td><img src="image4" alt="Chemical 4" /></td>
<td>2.40</td>
<td></td>
</tr>
<tr>
<td><img src="image5" alt="Chemical 5" /></td>
<td>3.05</td>
<td></td>
</tr>
<tr>
<td><img src="image6" alt="Chemical 6" /></td>
<td>3.39</td>
<td>4.06</td>
</tr>
<tr>
<td><img src="image7" alt="Chemical 7" /></td>
<td>3.93</td>
<td>5.12</td>
</tr>
</tbody>
</table>
Figure 5. Relationship between Log Kow and RBA for alkylanilines.
Rainbow Trout ER binding Affinity vs. Log Kow
RBA = relative binding affinity compared to Estradiol at 100%
### Log$K_{ow}$ Cutoffs vary with Chemical Subgroups

<table>
<thead>
<tr>
<th>Chemical Subgroups</th>
<th>C0</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>p,n-Alkyl Phenols</strong></td>
<td><img src="image1" alt="Structure" /></td>
<td><img src="image2" alt="Structure" /></td>
<td><img src="image3" alt="Structure" /></td>
<td><img src="image4" alt="Structure" /></td>
<td><img src="image5" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td>Log$K_{ow}$=1.50 m</td>
<td>1.97 m</td>
<td>2.47 m</td>
<td>3.20 m</td>
<td>3.65 m</td>
</tr>
<tr>
<td><strong>p,n-Alkyl Anilines</strong></td>
<td><img src="image6" alt="Structure" /></td>
<td><img src="image7" alt="Structure" /></td>
<td><img src="image8" alt="Structure" /></td>
<td><img src="image9" alt="Structure" /></td>
<td><img src="image10" alt="Structure" /></td>
</tr>
<tr>
<td></td>
<td>0.90 m</td>
<td>1.39 m</td>
<td>1.96 m</td>
<td>2.40 m</td>
<td>3.05 m</td>
</tr>
<tr>
<td><strong>p,n-Alkyl Chloro benzenes</strong></td>
<td><img src="image11" alt="Structure" /></td>
<td><img src="image12" alt="Structure" /></td>
<td><img src="image13" alt="Structure" /></td>
<td><img src="image14" alt="Structure" /></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.84 m</td>
<td>3.88 c</td>
<td>4.41 c</td>
<td>4.94 c</td>
<td></td>
</tr>
<tr>
<td><strong>p,n-Alkyl Cyclo hexanols</strong></td>
<td><img src="image15" alt="Structure" /></td>
<td><img src="image16" alt="Structure" /></td>
<td><img src="image17" alt="Structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.23 m</td>
<td>2.32 c</td>
<td>3.37 c</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Many chemical groups were found to be ER “Inactive”

46 diverse chemicals tested of LogK_{ow} < 1.3 were all non-binders
### Alkylaromatic sulfonic acids

**rtER tested chemicals - Training Set**

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Physical Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Chemical 1" /></td>
<td>-0.62 msrd</td>
</tr>
<tr>
<td><img src="image2.png" alt="Chemical 2" /></td>
<td>0.38 clog</td>
</tr>
<tr>
<td><img src="image3.png" alt="Chemical 3" /></td>
<td>0.63 msrd</td>
</tr>
<tr>
<td><img src="image4.png" alt="Chemical 4" /></td>
<td>0.92 clog</td>
</tr>
<tr>
<td><img src="image5.png" alt="Chemical 5" /></td>
<td>3.56 clog</td>
</tr>
<tr>
<td><img src="image6.png" alt="Chemical 6" /></td>
<td>5.67 clog</td>
</tr>
</tbody>
</table>

**Inventory**

<table>
<thead>
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<tr>
<td><img src="image1.png" alt="Chemical 1" /></td>
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</tr>
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<td>5.67 clog</td>
</tr>
</tbody>
</table>

and other variations & salts of structures shown here
Results:

Chemical has Low Potential for Activity if:
- Belongs to a group where testing showed no evidence of ER interaction (RBA < 0.00001);
- LogKow <1.3, or meets other group-specific LogKow cutoffs

General characteristics of these chemicals:
- Acyclic (e.g., no benzene rings)
- Cyclic, but does not contain a likely H-bonding group;

RBA = relative binding affinity; (a ratio of measured chemical affinity for the ER relative to 17-beta-Estradiol = 100%)
Log Kow = log of octanol/water partition coefficient (also known as Log P); is an indicator of lipophilicity
Results:

**Chemical has Higher Potential for Activity if:**
- Belongs to chemical group with evidence of ER interaction, \((RBA > 0.00001)\), and:
- \(\log K_{ow} > 1.3\), and < any chemical group-specific high \(\log K_{ow}\) cutoff

**General characteristics of these chemicals:**
- Contains at least one cycle (e.g., benzene ring);
- Contains a possible H-bonding group;
### Food Use Inerts, and Antimicrobials

<table>
<thead>
<tr>
<th>Category</th>
<th>Count</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Food Use Inerts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Chemicals</td>
<td>393</td>
<td></td>
</tr>
<tr>
<td>Lower Probability</td>
<td>378</td>
<td>(96%)</td>
</tr>
<tr>
<td>Higher Probability</td>
<td>15</td>
<td>(4%)</td>
</tr>
<tr>
<td><strong>Antimicrobials</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Chemicals</td>
<td>211</td>
<td></td>
</tr>
<tr>
<td>Lower Probability</td>
<td>196</td>
<td>(93%)</td>
</tr>
<tr>
<td>Higher Probability</td>
<td>15</td>
<td>(7%)</td>
</tr>
</tbody>
</table>
High Potency Chemicals

Estradiol
Ethinyl Estradiol

ER Binders

Log(Kow)

Log(RBA)

Inerts
Antimicrobials
Adverse Outcome Pathway
ER-mediated Reproductive Impairment
Measurements made across levels of biological organization

Inert; Antimicrobial Chemicals

Receptor Binding
ER Binding

Liver Cell Protein Expression

Liver Altered proteins, hormones;
Gonad Ova-testis

Sex reversal;
Altered behavior;
Repro.

Skewed Sex Ratios;
Yr Class

MOLECULAR Target
CELLULAR Response
TISSUE/ORGAN
INDIVIDUAL
POPULATION

In vivo

In vitro Assay

QSAR focus area

Skewed Sex Ratios; Yr Class

Liver Cell Protein Expression

↑ Vitellogenin (egg protein transported to ovary)
Developing an Approach and Tools to move EPA toward the new paradigm

Summary

• **Hypothesis-driven** approach
  – Adverse Outcome pathway (*in vitro* linked to *in vivo*)
  – Strategic chemical selection and testing to cover types of chemicals found on the list that needed prioritizing
  – Mechanistic hypothesis (LogKow; low affinity binding types)

• Derived a QSAR-based Decision Support System that can be applied to next chemical list, and expanded where needed (chemical classes not yet tested)

• Developed **priority setting** tool to focus on the 4 to 7% of chemicals with plausible toxicological potential for an important **adverse outcome**.