Eminent Toxicologist Lecture Series

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Society of Toxicology
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We Are Not Rodents: Environmental Toxicants and the Role of Human Studies

Society of Toxicology
Metabolism Affects Disposition and Distribution of Environmental Chemicals

- Exposure and Absorption at Portals of Entry
- Distribution to Organs and Tissues
  - Metabolism to More Toxic Metabolites
  - Metabolism to Less Toxic Metabolites
  - Metabolism to Conjugation Products
- Excretion
- Redistribution to Organs and Tissues
- Interaction with Macromolecules (Protein, DNA, RNA, Receptors, etc.)
- Toxic Effects
  - Genetic, Carcinogenic, Reproductive, Neurotoxic, etc.
- Turnover and Repair

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Natural Environments

Agricultural Ecosystems

Occupational (Work) Environments

Toxicant Exposure & Toxicant Action

Domestic & Urban Environments

Military Installations & Deployments

Superfund Sites

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The Exposome

The almost infinite variety of exposure scenarios presents a challenge for molecular epidemiology if molecular biomarkers of exposure and/or effect are to be linked to actual exposure. This has led to the exposome concept; an attempt to characterize all lifetime exposures that utilizes data from exposure sciences, epidemiology and other sciences. First advanced by Wild (2005) this concept has received recent support from the National Academy of Science (2010).
The Path to Human Studies

- Pesticide metabolism in non-human mammalian species.
- Characterization of insect cytochrome P450.
- Cytochrome P450 and insecticide resistance.
- Molecular biology of insect cytochrome P450.
- Interactions based on inhibition in non-human mammalian species.
- Interactions based on induction in non-human mammalian species.
- Inhibition and induction by methylenedioxyphenyl compounds.
Methylenedioxyphenyl Chemicals

- Biologically active secondary plant chemicals (e.g., safrole, isosafrole) and widely used commercial synergists (e.g., piperonyl butoxide).
- It had previously been shown that they affected P450-dependent oxidations in both insects and mammals.
- Two aspects were of interest to us and others and both became controversial; mechanism of inhibitory action and induction in mammals.
Monooxygenation of methylenedioxyphenyl compounds

Catechol

Carbene

Complexes with Fe^{2+} of cytochrome P450 to form metabolite inhibitory complex
Induction by MDP Chemicals

Following a long series of studies (and publications) involving Nancy Adams, Y-C Chui, Jon Cook, Pat Levi, Margaret Lewandowski, Doug-Young Ryu and myself, we concluded that MDP chemicals induced CYP1A1 via the AhR but induced CYP1A2 by a non-AhR dependent mechanism. This was largely ignored or disparaged until Frank Gonzalez produced the first Ah knockout mouse. To our relief (and gratification) piperonyl butoxide and acenaphthylene did not induce CYP1A1 in knockout mice but did, *mirabilis dictu*, induce both CYP1A2 and 1B1.

Human Studies Contribute to Human Health Risk Assessment and PBPK Modeling

**Past and Current.** Exposure and Epidemiology

**Current.** Defining human variation

Showing the potential for human-specific interactions.

Defining populations or individuals at increased risk.

Facilitating new molecular approaches to human health risk assessment.

Showing the potential for human-specific interactions.

Providing insight into uncertainty factors.
In Vitro Human Toxicant Metabolic Interactions

- Enzymes involved in toxicant metabolism
- Isoforms and polymorphic variants
- Variation between individuals
- Xenobiotic-xenobiotic interactions
- Xenobiotic-endogenous metabolite interactions
- Enzyme induction in human hepatocytes
- Cytotoxicity
Human Metabolism: Some Substrates Investigated

**Agrochemicals**
- Chloroacetamide herbicides
- Chlorpyrifos
- Carbaryl
- Carbofuran

**Repellents**
- DEET

**Diesel Fuel and Diesel Fuel Components**
- Nonane
- Naphthalene

Investigations may include human variation, isoform identification, effect of SNPs and metabolic interactions.
Chlorpyrifos Metabolism

Chlorpyrifos

CYP

Chlorpyrifos-oxon

S +

Diethyl phosphorodithioate

3,5,6-trichloro-2-pyridinol

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Isoform Specificity for Carbaryl Metabolism

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Chlorpyrifos Inhibition of Carbaryl Metabolism In Vitro

[Graph showing inhibition of carbaryl metabolism by chlorpyrifos with different concentrations.]

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Inhibition of Testosterone Metabolism by Pesticides
Effects on *In Vitro* Testosterone Metabolism

- 6β-hydroxytestosterone accounts for approximately 86% of all testosterone metabolites produced by human liver microsomes.

- CYPs 3A4 and 3A5 account for most of the 6β-hydroxytestosterone formed.

- Preincubation of CYP3A4 with chlorpyrifos (2 μM) resulted in 98% inhibition of major testosterone metabolite production (100 μM testosterone substrate concentration).
### Examples of Metabolic Interactions in Humans Based on Inhibition

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6</td>
<td>Carbaryl</td>
<td>Chlorpyrifos</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Fipronil</td>
<td>Chlorpyrifos</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Nonane</td>
<td>Chlorpyrifos</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>Chlorpyrifos, Fonofos, Phorate</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Estradiol</td>
<td>Chlorpyrifos, Fonofos, Permethrin, Deltamethrin</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Estradiol</td>
<td>Chlorpyrifos, Fonofos, Carbaryl, Naphthalene</td>
</tr>
</tbody>
</table>
Importance of CYP2B6

• Although there is normally more CYP3A4 than CYP2B6 in human liver, CYP2B6 has the lowest Km and is more effective

• Foxenberg et al.,(2008) reported Km constants for Chlorpyrifos oxon production.
  – CYP3A4 27µM
  – CYP2B6 0.81µM
CYP2B6 and CPS Toxicity

- CYP2B6 activity predicted Chlorpyrifos oxon production.
- CYP2B6 inhibition reduced Chlorpyrifos oxon production.
- CYP2B6*6/*6 samples could produce Chlorpyrifos oxon in large amounts.
- Individuals with high CYP2B6 could be at greater risk of chlorpyrifos poisoning.
The induction of xenobiotic-metabolizing enzymes has been studied extensively in surrogate animals for several decades, since the landmark review of Alan Conney and it is now clear that most induction involves one or more nuclear receptors. However, since an intact cell with functional sub-cellular organelles is required for experimental work, studies of induction in humans are more recent, and studies involving environmental chemicals even more so. Examples of chemicals investigated for induction and cytotoxicity in human hepatocytes include: Fipronil, Chlorpyrifos, Endosulfan.
Endosulfan—*In Vivo* Effects

1. Reduces sleep time in wild type mice.
2. Does not reduce sleep time in PXR-null mice.
3. Reduces sleep time in humanized PXR mice.
Increased Metabolism and Reduction in Sleep Time

Adapted from JBC 276: 37739-42.
Effects of Endosulfan on Human Hepatocytes

1. Is cytotoxic.
2. Induces CYP2B6 and CYP3A4.
3. Induction is PXR-dependent.
Endosulfan-a Increases CYP2B6 Levels in Human Hepatocytes

A

B

Endosulfan-α

CYP2B6

Actin

Fold Increase

Ctl  Rif  PB  0.1  1  5  10  50

uM

Treatments

* * * * * *
Pregnane X Receptor (PXR)

- Orphan member of the nuclear receptor family
- Xenosensor of toxic substances; binds xenobiotics and endogenous compounds and induces xenobiotic metabolizing enzyme gene expression
- Highly expressed in liver and intestine

Handschen (2003)

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PXR Mechanism of Action

Hepatocyte

Cytoplasm

Endoplasmic Reticulum

CYP3A4

Endogenous steroids
Pharmaceuticals
Xenobiotics

CYP3A4 mRNA

mRNA

PXR Mechanism of Action

Xenobiotic

RXR

CYP3A4

Hepatocyte

RXR

PXR-RE

CYP3A4

Endoplasmic Reticulum

Pharmaceuticals

Xenobiotics
Fipronil

- Fipronil is a member of the phenylpyrazole class of insecticides.
- Non-competitive GABA receptor Cl channel blocker.
Effect of Fipronil on CYP mRNA levels in Human Hepatocytes
Effect of Fipronil on CYP3A4 Protein and Activity in Human Hepatocytes

Con  Rif  0.1  0.5  1.0  5.0  10

Effect of Fipronil on CYP3A4 Protein and Activity in Human Hepatocytes

CYP3A4 protein
Testosterone hydroxylation

Induction ratio relative to controls

Control  0.1 uM  0.5 uM  1 uM  5 uM  10 uM  25 uM

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CYP3A4-Luc Induction in HepG2 Cells after Chlorpyrifos Exposure

Fold CYP3A4 Induction

Chlorpyrifos (uM)

Control 1 10 50

+pSG5-hPXR

Con 1 10 50

+pSG5

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### Fipronil Cytotoxicity: Human Hepatocytes and HepG2 Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Threshold dose</th>
<th></th>
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<tbody>
<tr>
<td><strong>Adenylate kinase release</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HepG2</td>
<td>&lt;0.5μM</td>
<td></td>
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<tr>
<td>Hepatocytes</td>
<td>&lt;25μM</td>
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<tr>
<td><strong>Caspase 3/7 activity</strong></td>
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<tr>
<td>HepG2</td>
<td>&lt;0.1μM</td>
<td></td>
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<tr>
<td>Hepatocytes</td>
<td>&lt;25μM</td>
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</tbody>
</table>
Chlorpyrifos Cytotoxicity

Adenylate Kinase Activity

Chlorpyrifos (uM)

Con 1 3.12 12.5 25 50 100

24 h Mean
Chlorpyrifos Cytotoxicity

Caspase 3/7 Activity

Chlorpyrifos (uM)

Con 1 3.12 12.5 25 50 100

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Red = CPS
Cyan = CON
Identification of Chlorpyrifos Regulated Genes in Human Hepatocytes by Microarray

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
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<tbody>
<tr>
<td>alcohol dehydrogenase 1B</td>
<td>3.59</td>
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<tr>
<td>glycine-N-acyltransferase</td>
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<tr>
<td>transferrin</td>
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<tr>
<td>Cytochrome P450, family 1, subfamily A, polypeptide 2</td>
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<td>Insulin-like growth factor binding protein 1</td>
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<tr>
<td>Uridine phosphorylase 1</td>
<td>2.48</td>
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<tr>
<td>Heat shock protein70 kDa family member 13</td>
<td>-2.00</td>
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<tr>
<td>Kyoto Encyclopedia of Genes and Genomes (KEGG)</td>
<td>Number of Regulated Genes in Pathway</td>
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<tr>
<td>---------------------------------------------------------------</td>
<td>--------------------------------------</td>
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<tr>
<td>Retinol metabolism</td>
<td>7</td>
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<tr>
<td>Metabolism of xenobiotics by CYPs</td>
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<td>Porphyrin and chlorophyll metabolism</td>
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<tr>
<td>Long-term potentiation</td>
<td>5</td>
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<td>MAPK signaling pathway</td>
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<tr>
<td>Drug metabolism enzymes</td>
<td>5</td>
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<tr>
<td>Regulation of actin cytoskeleton</td>
<td>9</td>
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</table>
What is the impact of DEET on global gene expression in primary human hepatocytes after a 72 h exposure?
Volcano Plot of Comparisons between DEET and Media Only Data

172 messages up or down regulated
Location of affected messages

↑ Up-regulated by DEET
↓ Down-regulated by DEET

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<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence Description</th>
<th>Seq. Length</th>
<th>#Hits</th>
<th>min. eValue</th>
<th>mean Similarity</th>
<th>#GOs</th>
<th>expression with DEET</th>
<th>Chromosome</th>
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<td>NR_001278.1</td>
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<td>NR_003610.1</td>
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<td>nuRNA, wap protein family homolog 2-like; WAP protein family 5 homolog pseudogene (WASH5P)</td>
<td>1137</td>
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<td>nuRNA, histone demethylease uty-like; uncharacterized LOC10199986 (LOC10199986)</td>
<td>2453</td>
<td>20</td>
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<td>NR_036350.1</td>
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<td>nuRNA, endogenous retrovirus group K13, member 1 (ERVK13-1)</td>
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<td>n/a</td>
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<td>nuRNA, metastasis-associated lung adenocarcinoma transcript 1 (non-protein coding)(MALAT1)</td>
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<td>n/a</td>
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</tbody>
</table>
What is the impact of fipronil mixtures with DEET on primary human hepatocytes?
172 genes differentially expressed (p-value of 0.05)

3703 genes differentially expressed (p-value of 0.05)
Mixture:
Greater than
Additive Effect

Additive Total  3,875

DEET + Fipronil vs Media
5146 genes differentially expressed (p-value of 0.05)
Number of Genes Affected per Chromosome

- DEET
- Fipronil
- DEET plus Fipronil
Percent Affected Genes per Chromosome

CHROMOSOME

PERCENT AFFECTED GENES

0.0% 2.0% 4.0% 6.0% 8.0% 10.0% 12.0% 14.0% 16.0%

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X

DEET
Fipronil
DEET plus Fipronil