Thanks To

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Outline

1. Background
2. The H295R Steroidogenesis system
   a) Performance criteria
   b) Model chemical evaluation
3. Preliminary inter-laboratory comparison
4. Conclusions
5. Future directions
H295R Cell Line

- Human female adrenocortical carcinoma
- Produces many steroid hormones
  - progestins
  - androgens & estrogens
  - Glucocorticoids & mineralocorticoids
- Expresses most of the important steroidogenic enzymes
  - CYP11A, CYP11B, CYP17, CYP19, CYP21
The cells maintain the capacity to synthesize most of the steroid hormones characteristic of three phenotypically distinct zones of the adult adrenal cortex

- Zona glomerulosa
- Zona fasciculata
- Zona reticularis
Effects on Steroidogenesis

- **At level of expression**
  - measure mRNA levels: RT-PCR

- **Effects on enzyme concentrations**
  - measure catalytic activities: selective substrates

- **Effects on metabolism of steroid hormones**
  - measure steroid hormone concentrations
Cholesterol

- CYP11A
  - Pregnenolone
    - 3β-HSD
      - Progesterone
        - CYP21
          - 11-Deoxycorticosterone
            - CYP11B2
              - Corticosterone
                - CYP11B2
                  - Aldosterone

Zona glomerulosa

- CYP17
  - 17α-OH-Pregnenolone
    - 3β-HSD
      - CYP17
        - 17α-OH-Progesterone
          - CYP21
            - 11-Deoxycorticisol
              - CYP11B1
                - Cortisol

Zona fasciculata

- CYP17
  - DHEA
    - 3β-HSD
      - CYP17
        - Androstenedione
          - CYP19
            - Estrone
              - 17β-HSD
                - Testosterone
                  - 17β-HSD
                    - 17β-estradiol

Zona reticularis

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Objectives

Develop and optimize a rapid screening test to determine effects of chemicals on sex steroid synthesis:

- Estrone
- Androstenedione
- Progesterone
- Testosterone
- Estradiol
- 17β-estradiol
Objectives
(cont’)

- Demonstrate the performance of the assay with known inhibitors and inducers of steroidogenesis
- Assess and quantify sources of variability in the assay to:
  - Establish performance criteria for large scale screening of chemicals
  - Demonstrate flexibility and transferability of the protocol to other laboratories prior to conducting ring tests
- Develop optimized protocol for inter-laboratory validation phase.
Goals

Establish an assay that will integrate possible effects on multiple parts of the steroidogenic pathway:

1. Steroidogenic signal transduction
2. Regulation of cholesterol transport by the STAR-Protein
3. Conversion of cholesterol to testosterone by:
   — P450SCC
   — 3β-HSD & 17β-HSD
   — P450C17
4. Androgen conversion to estrogen by CYP19 aromatase
Overall Approach

Initial Screening of P, T and E2 in H295R medium
- Compare performance of commercial ELISA kits
- Selection of ELISA kits

Optimization of cell culture methods
- Optimize culture and exposure conditions for optimal performance

Optimization of immunoassays
- Basal hormone production
- Precision, accuracy, linearity

Definition of performance quality criteria
- Response profiles/limits
- Cytotoxicity
- Compare to changes in gene expression

Validation of test system I (model compounds)
- Demonstrate transferability of test system

Validation of test system II (Inter-laboratory comparison)
H295R Cell Test Development
Time Series

- Progesterone
- Testosterone
- Estradiol

Testosterone & Progesterone [pg/ml]

Estradiol [pg/ml]

0 200 400 600 800 1000 1200

0 1000 2000 3000 4000 5000 6000 7000 8000

0 20 40 60 80

time (h)

0 1000 2000 3000 4000 5000 6000 7000 8000

0 200 400 600 800 1000 1200

0 20 40 60 80

time (h)
H295R Cell Test Development
Basal Hormone Production

Progestosterone
Testosterone
Estradiol

Experiment #

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Progestosterone [pg/ml]</th>
<th>Testosterone [pg/ml]</th>
<th>Estradiol [pg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7500 ± 1000</td>
<td>5000 ± 500</td>
<td>2500 ± 250</td>
</tr>
<tr>
<td>2</td>
<td>7000 ± 1000</td>
<td>5500 ± 550</td>
<td>2000 ± 200</td>
</tr>
<tr>
<td>3</td>
<td>8000 ± 1000</td>
<td>6000 ± 600</td>
<td>3000 ± 300</td>
</tr>
<tr>
<td>4</td>
<td>6500 ± 650</td>
<td>4500 ± 450</td>
<td>1500 ± 150</td>
</tr>
<tr>
<td>5</td>
<td>7000 ± 700</td>
<td>5000 ± 500</td>
<td>2000 ± 200</td>
</tr>
</tbody>
</table>
H295R Cell Test Development
Effects of Cell Passage

![Graph showing the effects of cell passage on progesterone, testosterone, and estradiol concentrations.](image)
H295R Cell Test Development
Effect of Solvent (0.1% DMSO)

% Blank

Progesterone  Testosterone  Estradiol
H295R Methods to Measure Effects on Hormone Production

Cells cultured in Petri Dish
Renew medium 2-3x weekly
Split cell when ~90% confluent

Trypsinize
Seed Plate (suppl. Medium)
Incubated for 24 hours
Replace Medium (suppl.)
Dose Cells
Incubate For 48 hrs

Extract Medium with ether
Collect Cells

Analyze for Hormone
ELISA, RIA, LC/MS

Freeze in Liquid N₂
(gene expression, enzyme analysis)
Model Chemicals

- **Prochloraz**
  - Imidiazol fungicide: Potent inhibitors of aromatase; Capable of affecting other P450 dependent enzymes

- **Aminoglutethimide**
  - Generation I aromatase inhibitor; Can also downregulate synthesis of cortisol and aldosterone

- **Forskolin**
  - General inducer: Stimulating adenylycyclase and increasing cAMP levels in adrenal cells

- **Ketoconazole**
  - Imidiazol fungicide: Inhibits p450 enzymes (24-hydroxylase, Cholesterol SCC and C-17,20 lyase)
Prior to initiation of exposure experiments, cytotoxicity of each chemical was assessed using the MTT assay.

Dose ranges for all subsequent exposures represent non-cytotoxic concentrations.
Model Chemical Exposure

Prochloraz

Prochloraz [μM]

relative change

-6.00
-5.00
-4.00
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
4.00
5.00
6.00

0.001 0.01 0.1 1 10

* = sign. P
+ = sign. T
@ = sign. E2

Progesterone
Testosterone
Estradiol
Model Chemical Exposure
Aminoglutethimide

* = sign. P
+ = sign. T
@ = sign. E2

relative change

Aminoglutethimide [µM]
Model Chemical Exposure

Forskolin

- Progesterone
- Testosterone
- Estradiol

* = sign. P
+ = sign. T
@ = sign. E2

Relative change vs. Forskolin [µM]

-1.00 -0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00
Model Chemical Exposure

**Ketoconazole** *(Preliminary Results)*

- **Progesterone**
- **Testosterone**
- **Estradiol**

Relative change vs. Ketoconazole concentration [µM]

- 0.001
- 0.01
- 0.1
- 1
- 10
Inter-Laboratory Comparison

- Participating Laboratories:
  - US Environmental Protection Agency
    Endocrinology Laboratory, U.S.A.
  - Chemicals Assessment Center
    Chemical Evaluation and Research Institute, Japan
  - Danish Institute for Food and Veterinary Research
    Department of Toxicology and Risk Assessment, Denmark
Inter-Laboratory Comparison (cont’)

- **Performance based comparison. Use of:**
  - same cells/same passages
  - different cell culture protocols/conditions
  - same seeding density
  - same acclimation and exposure protocols/conditions
  - different hormone detection methods
Phase I - General Test Performance

**Basal Hormone Production**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>US-EPA</th>
<th>MSU</th>
<th>CERI</th>
<th>DIFVR I</th>
<th>DIFVR II</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>10,000</td>
<td>1,000</td>
<td>100</td>
<td>10,000</td>
<td>100,000</td>
</tr>
<tr>
<td>T</td>
<td>1,000</td>
<td>100</td>
<td>10</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>A</td>
<td>100</td>
<td>10</td>
<td>1</td>
<td>100,000</td>
<td>10,000</td>
</tr>
<tr>
<td>E2</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>100</td>
<td>100,000</td>
</tr>
</tbody>
</table>

n.d. = below MDL
Phase I - General Test Performance
Hormone Detection Systems

Comparison CERI Medium

<table>
<thead>
<tr>
<th>Hormone</th>
<th>US-EPA</th>
<th>MSU</th>
<th>CERI</th>
<th>DIFVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Analytical Method Still Under Development
n.d. = below MDL
Phase I - General Test Performance
Comparison MSU Prochloraz Exposure

**Progesterone**

- **US-EPA**
- **MSU**
- **CERI**

**Estradiol**

- **US-EPA**
- **MSU**
- **CERI**

n.d. = below MDL
Phase I - General Test Performance

Comparison MSU Prochloraz Exposure

- **US-EPA**
- **MSU**
- **CERI**

### Androstenedione

<table>
<thead>
<tr>
<th>uM Prochloraz</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank SC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **pg/ml**

### Testosterone

<table>
<thead>
<tr>
<th>uM Prochloraz</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank SC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **pg/ml**

**n.d. = below MDL**
Some variation due to different hormone detection systems:

- Different antibody cross-reactivities (MSU P values explainable by cross-reaction with pregnenolone)
- Differences in clean-up/extraction procedures?
- Differences in sensitivity
Phase I - General Test Performance

Summary & Conclusions (cont’)

- Variation of basal hormone concentrations measured at different laboratories
  - Different medium composition:
    - Supplemented vs. non-supplemented medium
    - Use of antibiotics
Phase I - General Test Performance

Summary & Conclusions (cont’)

- Good reproducibility of results at each laboratory:
  - Low intra-assay variation
  - Low inter-assay variation
  - Good linearity
  - Good recovery of hormone spikes
Phase II - Model Chemicals

Progesterone (Preliminary Data)

- MSU
- US-EPA
- DIFVR
- CERI

% of SC (=100%)

0.001 0.01 0.1 1 10 uM Prochloraz

0.1 1 10 100 uM Aminoglutethimide

0 30 60 90 120 150 1800 uM Prochloraz

0 30 60 90 120 150 1800 uM Aminoglutethimide

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Phase II - Model Chemicals

Progesterone (Preliminary Data)

- MSU
- US-EPA
- DIFVR
- CERI

% of SC (=100%)

0 100 200 300 400

0.01 0.1 1 10

uM Forskolin

0.001 0.01 0.1 1 10 100

uM Ketoconazole

% of SC (=100%)

0 100 200 300 400 500

0 0.001 0.01 0.1 1 10 100

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Phase II - Model Chemicals

Testosterone (Preliminary Data)
Phase II - Model Chemicals

Testosterone (Preliminary Data)
Preliminary Inter-Lab Comparison

**Estradiol** *(Preliminary Data)*

![Graph showing the comparison of Estradiol levels across different laboratories and concentrations of Prochloraz and Aminoglutethimide.](image)
Phase II - Model Chemicals

Estradiol (Preliminary Data)
### Preliminary Inter-Lab Comparison

**Prochloraz (Progesterone)**

<table>
<thead>
<tr>
<th></th>
<th>MSU</th>
<th>US-EPA</th>
<th>DIFVR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y</strong></td>
<td>$1.4886x + 7.109$</td>
<td>$1.3304x + 6.4663$</td>
<td>$1.4694x + 6.6189$</td>
</tr>
<tr>
<td><strong>R²</strong></td>
<td>$0.9398$</td>
<td>$0.9543$</td>
<td>$0.9006$</td>
</tr>
<tr>
<td><strong>EC_{25}</strong></td>
<td>0.109 µM</td>
<td>0.254 µM</td>
<td>0.228 µM</td>
</tr>
<tr>
<td><strong>EC_{50}</strong></td>
<td>0.038 µM</td>
<td>0.079 µM</td>
<td>0.079 µM</td>
</tr>
</tbody>
</table>

**Graph:**
- **MSU**
  - $y = 1.3304x + 6.4663$, $R^2 = 0.9453$ (US-EPA)
  - $y = 1.4886x + 7.109$, $R^2 = 0.9398$ (MSU)
  - $y = 1.7616x + 6.9417$, $R^2 = 0.7307$ (DIFVR)
- **US-EPA**
  - $y = 1.3304x + 6.4663$, $R^2 = 0.9453$ (US-EPA)
- **DIFVR**
  - $y = 1.4886x + 7.109$, $R^2 = 0.9398$ (MSU)

**Legend:**
- MSU
- US-EPA
- DIFVR

**x-axis:** log uM Prochloraz
**y-axis:** % of SC (100%)
### Preliminary Inter-Lab Comparison

**Prochloraz** *(Testosterone)*

<table>
<thead>
<tr>
<th></th>
<th>MSU</th>
<th>US-EPA</th>
<th>DIFVR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y</strong></td>
<td>-0.509x + 4.655</td>
<td>-0.577x + 3.7121</td>
<td>-0.924x + 3.078</td>
</tr>
<tr>
<td><strong>R²</strong></td>
<td>0.9866</td>
<td>0.6976</td>
<td>0.9590</td>
</tr>
<tr>
<td><strong>EC&lt;sub&gt;25&lt;/sub&gt;</strong></td>
<td>4.437 µM</td>
<td>0.086 mM</td>
<td>0.045 µM</td>
</tr>
<tr>
<td><strong>EC&lt;sub&gt;50&lt;/sub&gt;</strong></td>
<td>0.210 µM</td>
<td>0.006 mM</td>
<td>0.008 µM</td>
</tr>
</tbody>
</table>

**Testosterone**

- **y = -0.5087x + 4.6547**
- **R² = 0.9866** (MSU)
- **y = -0.5786x + 3.7121**
- **R² = 0.6976** (US-EPA)
- **y = -0.9577x + 2.9672**
- **R² = 0.9937** (DIFVR)
## Preliminary Inter-Lab Comparison

**Prochloraz** *(Estradiol)*

<table>
<thead>
<tr>
<th></th>
<th>MSU</th>
<th>US-EPA</th>
<th>DIFVR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y</strong></td>
<td>-1.101x + 3.875</td>
<td>-1.169x + 3.302</td>
<td>-1.007x + 3.936</td>
</tr>
<tr>
<td><strong>R²</strong></td>
<td>0.9203</td>
<td>0.9540</td>
<td>0.9415</td>
</tr>
<tr>
<td><strong>EC_{25}</strong></td>
<td>0.396 µM</td>
<td>0.133 µM</td>
<td>0.411 µM</td>
</tr>
<tr>
<td><strong>EC_{50}</strong></td>
<td>0.095 µM</td>
<td>0.035 µM</td>
<td>0.088 µM</td>
</tr>
</tbody>
</table>
Phase II - Exposure to Model Chemicals

Summary & Conclusions

✧ Good reproducibility of dose-response profiles across laboratories:
  ➞ E2 production of cells exposed to all model chemicals
  ➞ P production of cells exposed to all model chemicals with the exception of one lab in the ketoconazole experiment
Phase II - Exposure to Model Chemicals

Summary & Conclusions

- Different dose-response profiles for T production of cells exposed to Aminoglutethimide and Forskolin:
  - Need to evaluate different cell exposure and hormone detection methods
  - Need to assess effects on other androgens (Androstenedione)
Conclusions

- H295R test system:
  - Rapid and easy to use
  - Constitutive basal production of estradiol, testosterone and progesterone
  - Can measure both increase and decrease of hormone production over several orders of magnitude
  - Can determine changes in hormone production with high precision and accuracy
  - Reproducible
Conclusions (cont’)

**H295R test system:**

- Flexible - can be tailored to identify effects at multiple biological levels in the same system:
  - Gene expression
  - Catalytic enzyme activities
  - Hormone production
- Has the potential to identify multiple mechanisms of action
- Significant reduction of whole animal tests
H295R test system:

✓ Cost effective:
  
  – ELISA: approx. 200 US$/sample/hormone\(^a\) + approx. 2 person hrs/sample/hormone\(^a\)
  
  – Cell culture and exposure: between 0.05 (48 well plate) and 0.15 (6 well plate) person hrs/sample

✓ Rapid and economic screen of chemicals for their potential to alter Steroidogenesis (priority setting, Tier 1 screening)

\(^a\) Calculation based on of triplicate measures of 6 different doses + solvent control per sample (chemical)
Conclusion II

- Results can be related to other endpoints
  - Pre-screening with certain model compounds resulted in responses that correlated with earlier studies on changes in expression patterns of steroidogenic genes
- Preliminary tests show great promise regarding the transferability of this test system for P and E2
- Need to address variation in responses of T concentrations in media
  - Measurement of alternative androgen endpoints such as androstenedione (currently under-way)
Conclusion II

Data compares well to in vivo results from rat and fish studies:

- Ankley et al. 2005: Prochloraz suppresses plasma estrogen and androgen concentrations in female and male fathead minnows, respectively.
- Vinggaard et al. 2005: Prochloraz suppresses testicular testosterone production and increases testicular progesterone production in rat offspring.
- Monteiro et al. 2000: Aminoglutethimide increases androstenedione and decreases estradiol in the flounder; Ketoconazole decreases androgen and estradiol concentrations.
Future Directions

- Extend hormone analyses to other steroids:
  - Estrone (under-way)
  - Androstenedione (under-way)
  - Cholesterol

- Confirm hypothesized mode of action by measuring actual enzyme activities (e.g., aromatase)
Future Directions

- Identify sources for inter-laboratory variability of basal hormone concentrations
- Identify causalities for different T patterns observed at different laboratories
Future Directions

- Establish optimized H295R test protocol
- Validation of H295R steroidogenesis test system in extended inter-laboratory trials
  - Use of optimized and standardized protocol
  - Reduced number of endpoints (2 hormones)
  - Larger number of participating laboratories (10 - 20)
Future Directions

- Establish exposure profiles (dose response/time response) for model compounds with other modes of action
- Apply test system to selected priority substances
Future Directions

- Validate transferability of test system (currently underway)
  - Compare to ex vivo and in vivo data
    - Xenopus laevis - MSU (ex vivo)
    - Fathead minnow - US-EPA lab Duluth (in vivo and ex vivo)
    - Minced testis assay
    - Uterotrophic assay


Thank You!

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H295R cell line

- Derived from the NCI-H295 pluripotent adrenocortical carcinoma cell line (Gazdar, et al. 1990) from a carcinoma of the adrenal cortex that arose in a 48 y.o. black female.

- Modified cells retain the ability to produce aldosterone, cortisol and C19 steroids (adrenal androgens).
Future Directions

睑 Validate transferability of test system (currently underway)

✓ Within the same laboratory (completed)
✓ Between different laboratories (underway)
Model Chemical Exposure
**Vinclozolin**

- **Progesterone**
- **Testosterone**
- **Estradiol**

<table>
<thead>
<tr>
<th>Relative Change</th>
<th>Vinclozolin [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>-4.00</td>
<td>0.1</td>
</tr>
<tr>
<td>-3.00</td>
<td>1</td>
</tr>
<tr>
<td>-2.00</td>
<td>10</td>
</tr>
<tr>
<td>-1.00</td>
<td>100</td>
</tr>
<tr>
<td>0.00</td>
<td>1000</td>
</tr>
</tbody>
</table>

* = sign. P
+ = sign. T
@ = sign. E2

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