Development and Pre-Validation of a H295R Cell Line Screening Test to Evaluate Toxicant-Induced Effects on Steroidogenesis

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www.entrix.com

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

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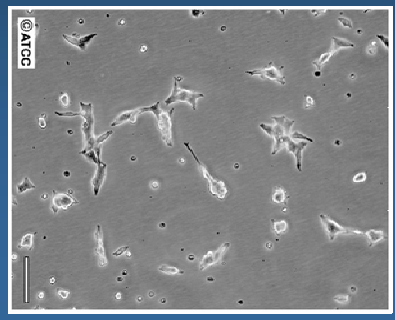
H295R Cell Line

- **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

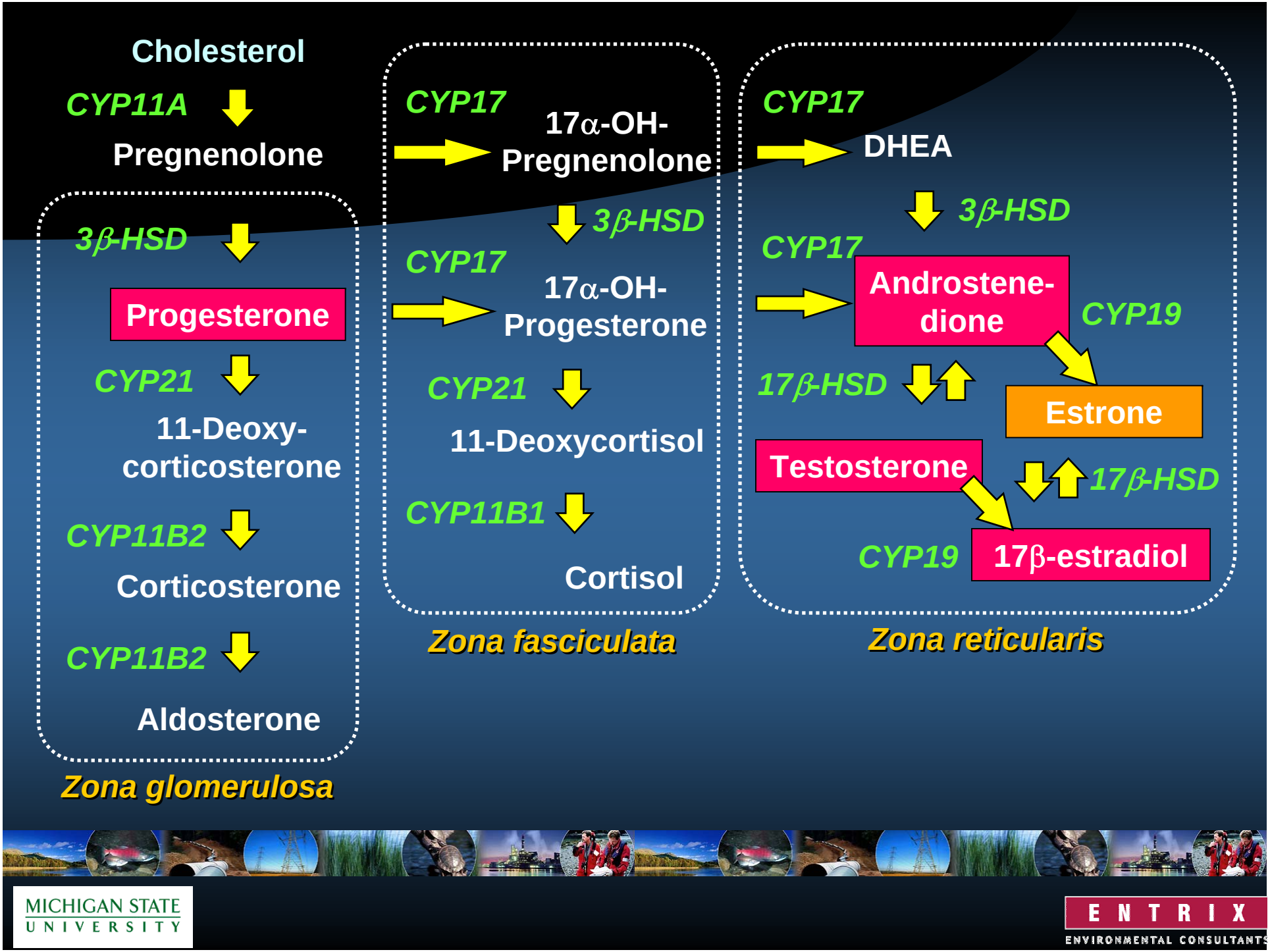


H295R Cell Line

- **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

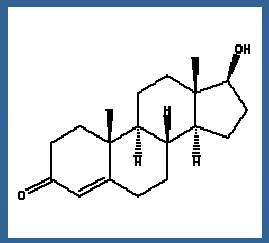
Effects on Steroidogenesis

- **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

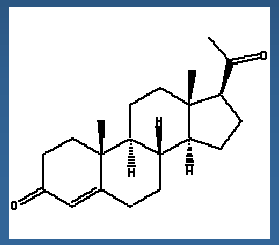

Cholesterol
CYP11A
Pregnenolone
3β-HSD
Progesterone
CYP21
11-Deoxy-corticosterone
CYP11B2
Corticosterone
CYP11B2
Aldosterone
Zona glomerulosa
CYP17
17α-OH-Pregnenolone
3β-HSD
CYP17
17α-OH-Progesterone
CYP21
11-Deoxycortisol
CYP11B1
Cortisol
Zona fasciculata
CYP17
DHEA
3β-HSD
CYP17
Androstene-dione
CYP19
17β-HSD
Estrone
Testosterone
17β-HSD
CYP19
17β-estradiol
Zona reticularis

Objectives

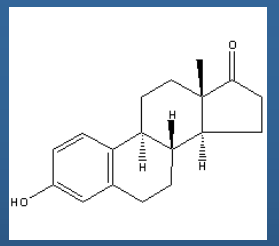
- **Develop and optimize a rapid screening test to determine effects of chemicals on sex steroid synthesis:**



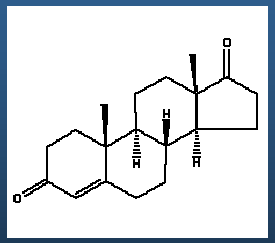
—Progesterone



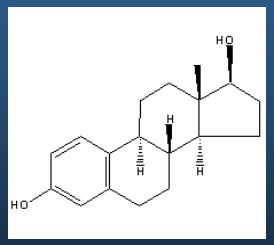
—Testosterone



—Estrone



—Androstenedione



—17β-estradiol

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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**

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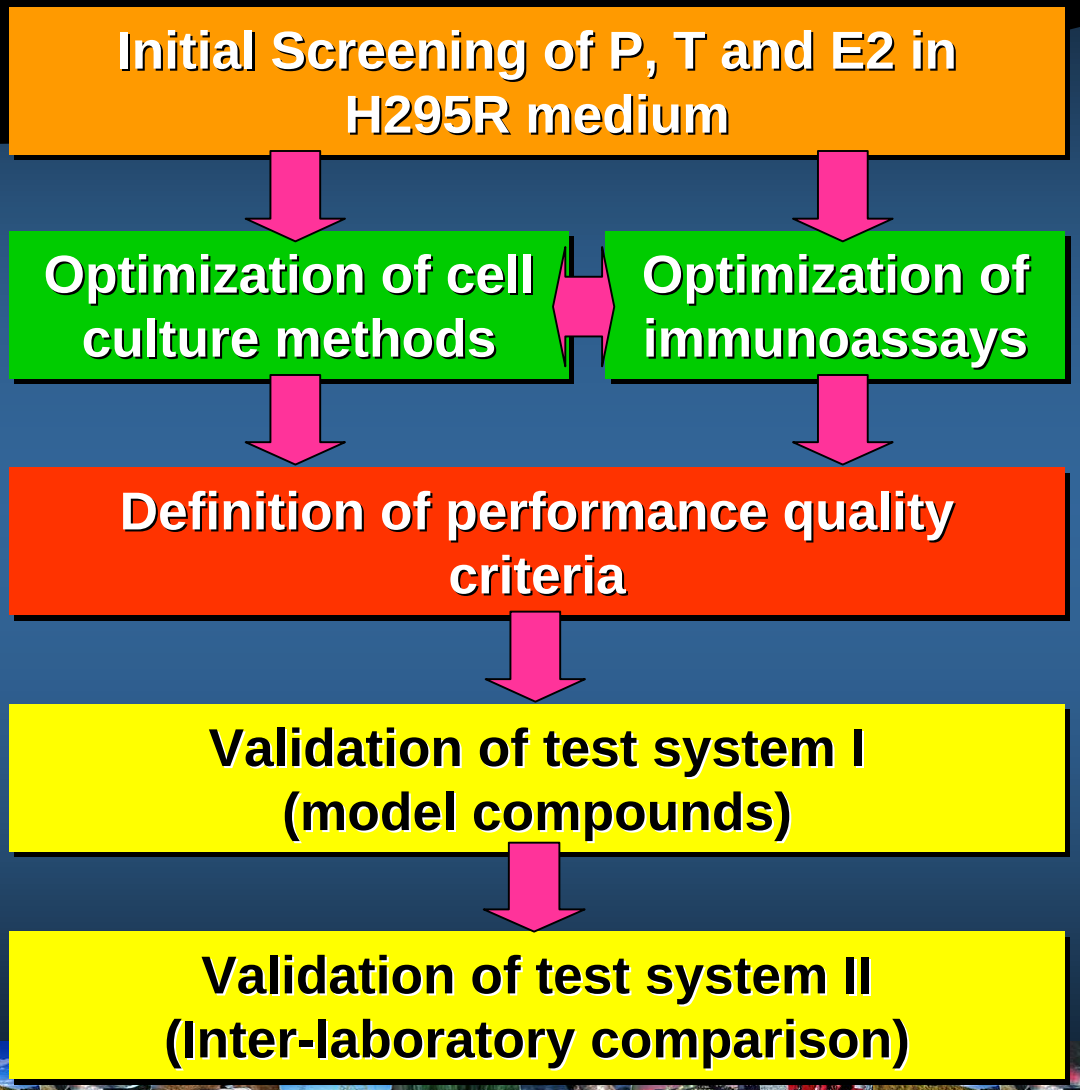
Overall Approach



- Compare performance of commercial ELISA kits
- Selection of ELISA kits

- Optimize culture and exposure conditions for optimal performance

- Basal hormone production
- Precision, accuracy, linearity

- Response profiles/limits
- Cytotoxicity
- Compare to changes in gene expression

- Demonstrate transferability of test system

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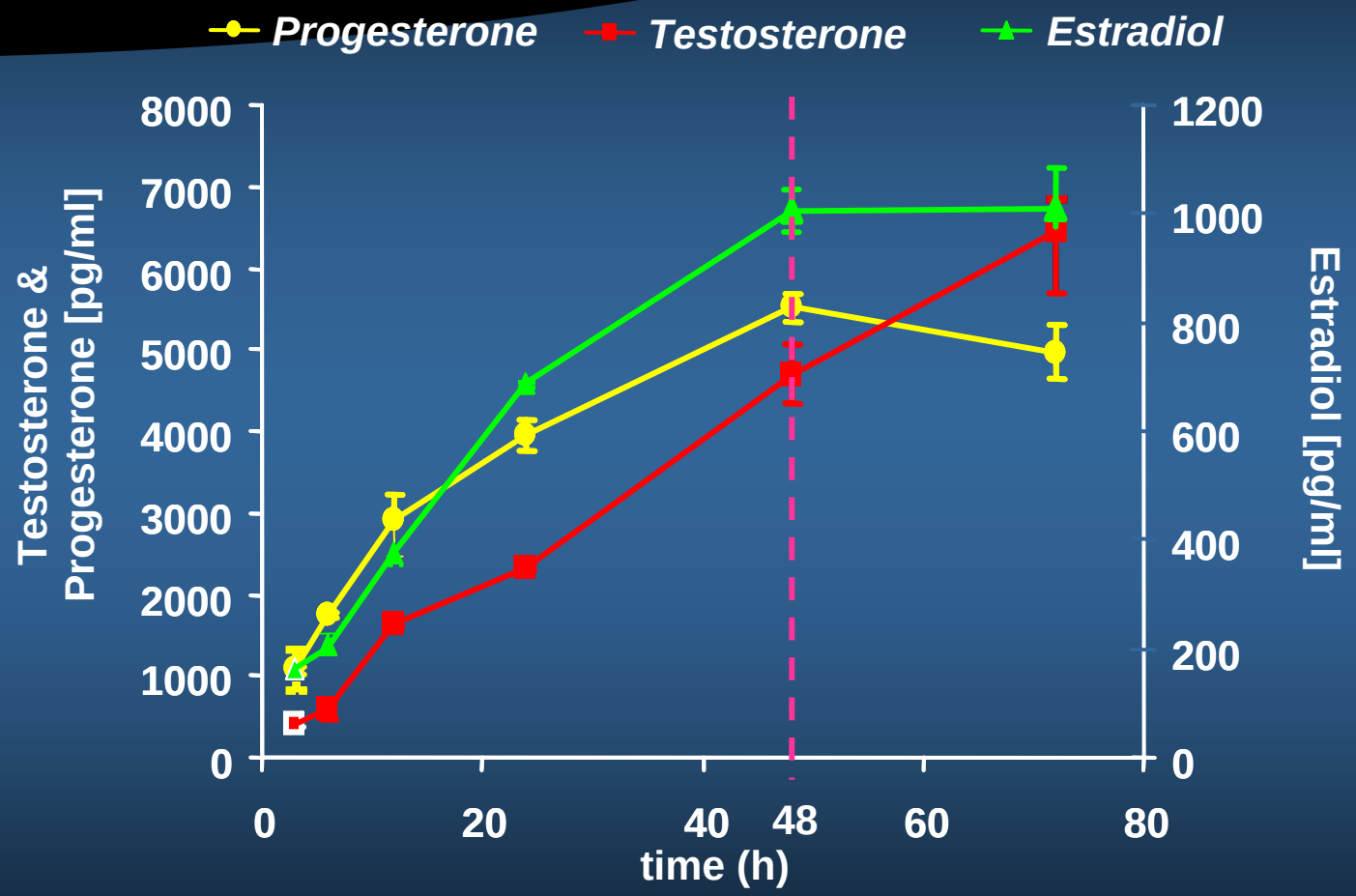
H295R Cell Test Development
Time Series



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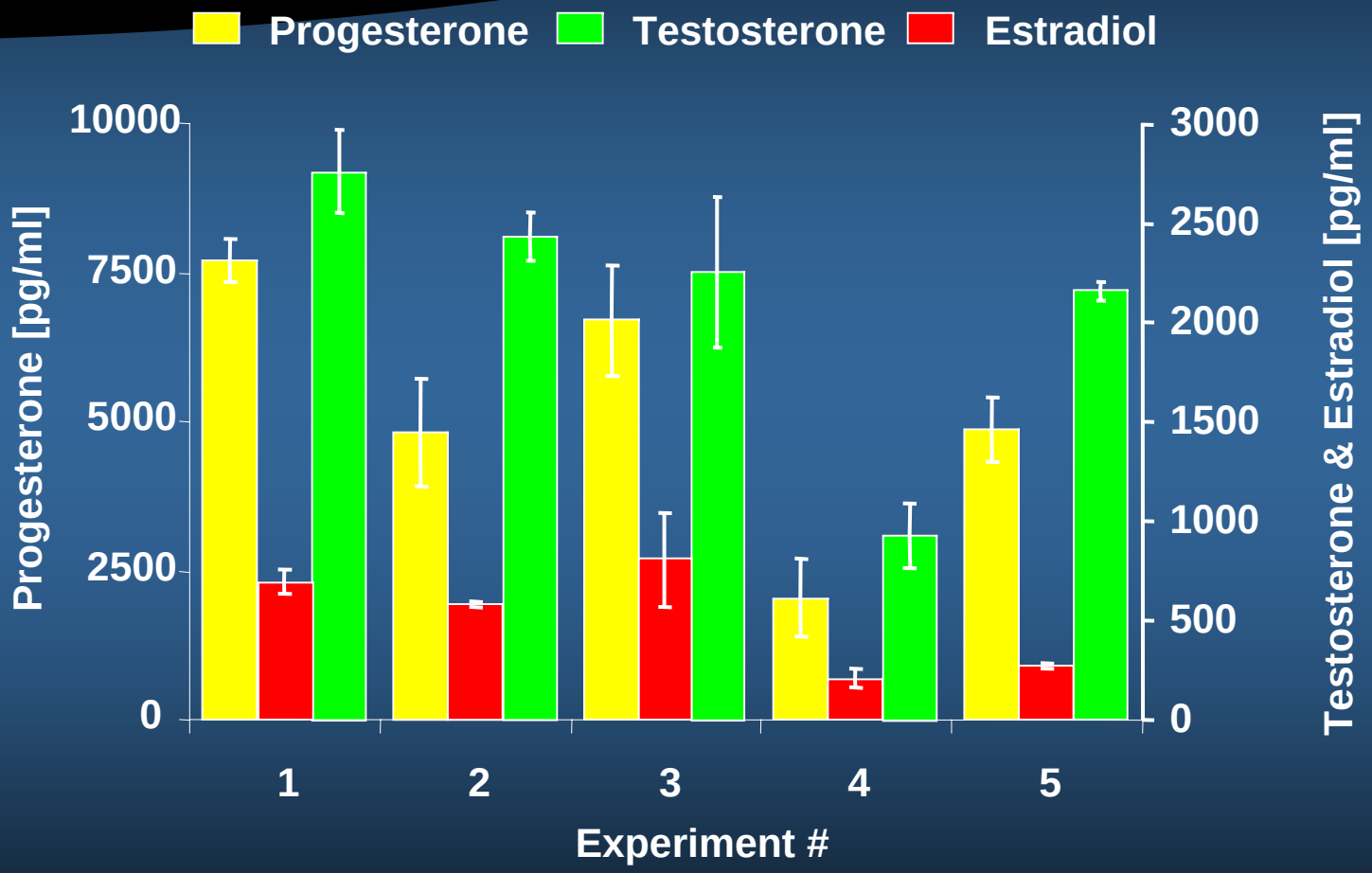
H295R Cell Test Development
Basal Hormone Production



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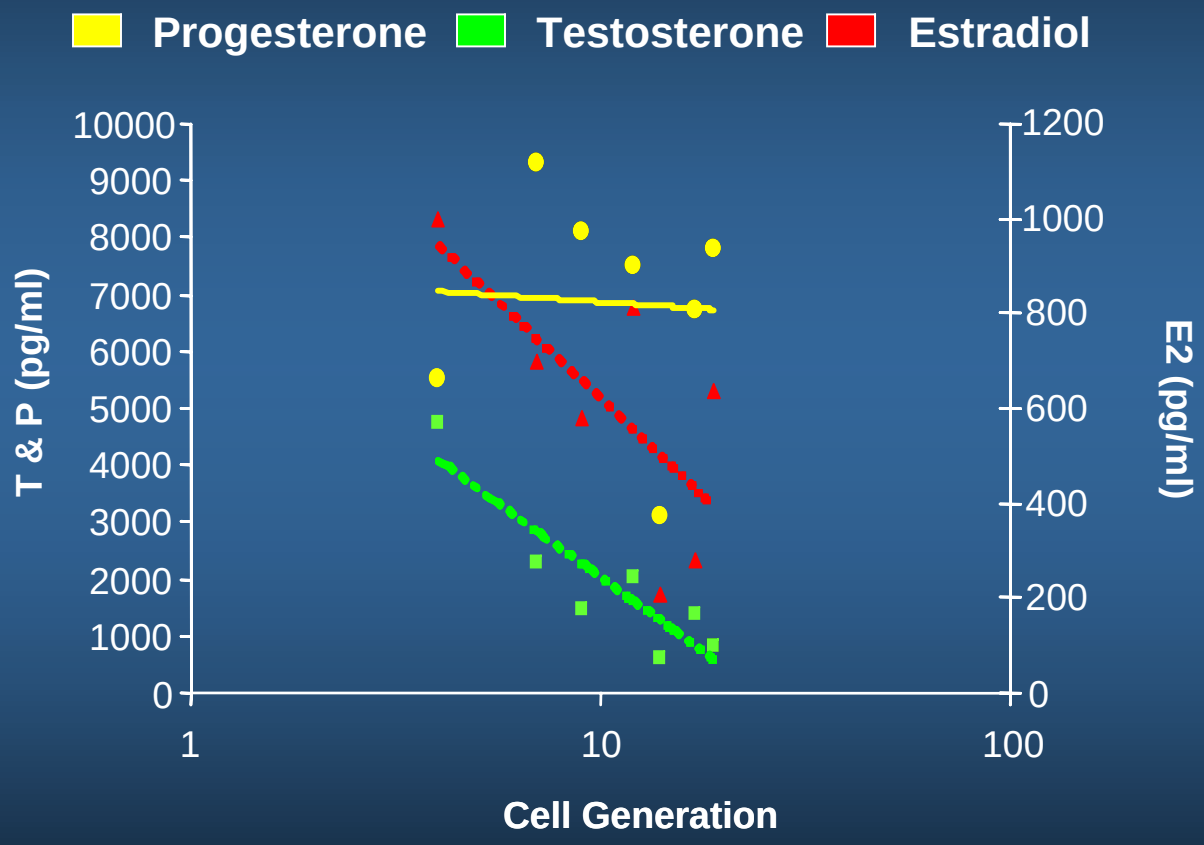
H295R Cell Test Development
Effects of Cell Passage



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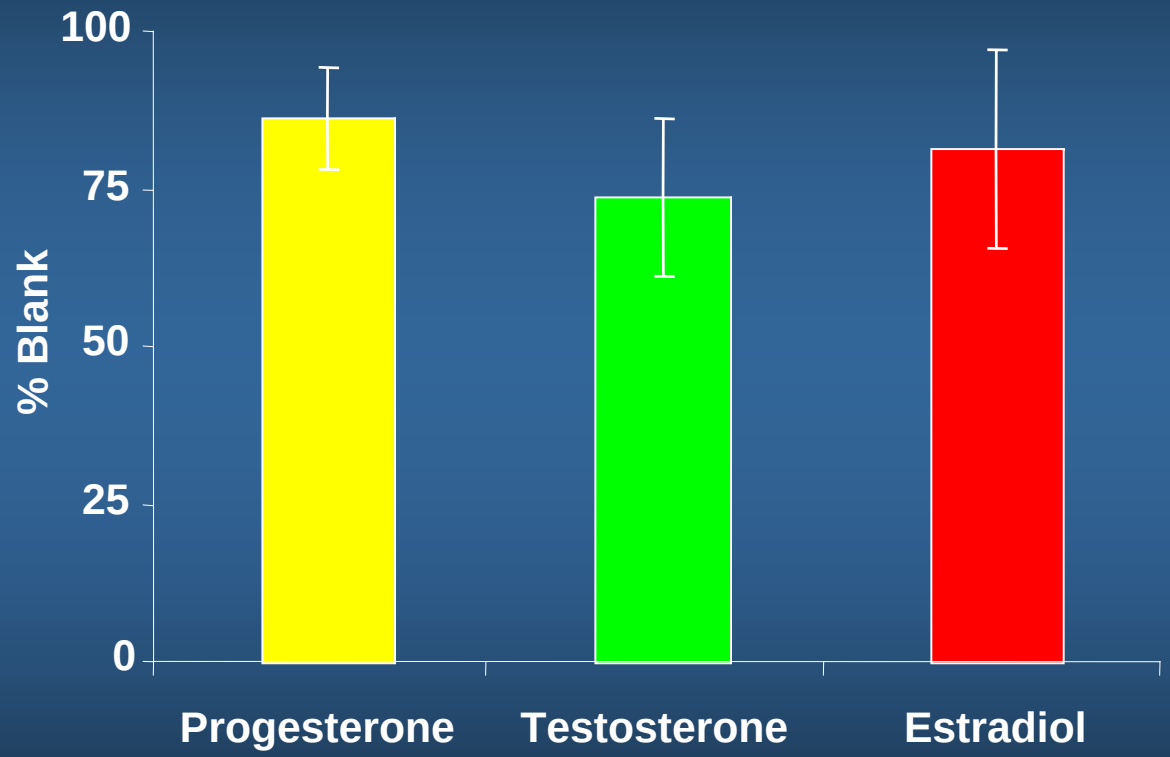
H295R Cell Test Development
Effect of Solvent (0.1% DMSO)



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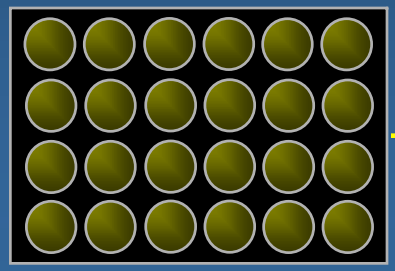
H295R Methods to Measure Effects on Hormone Production

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

Trypsinize

Seed Plate
(suppl. Medium)

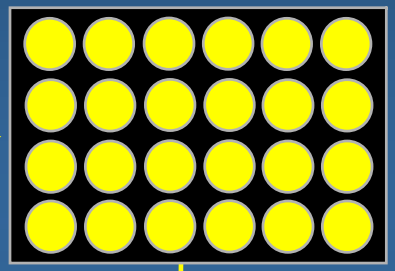
Incubated for 24 hours



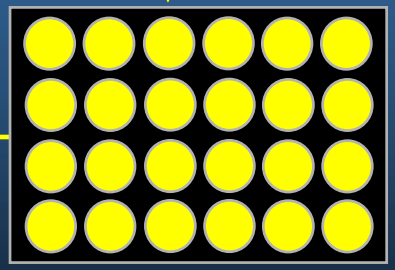
Replace

Medium
(suppl.)

Dose Cells

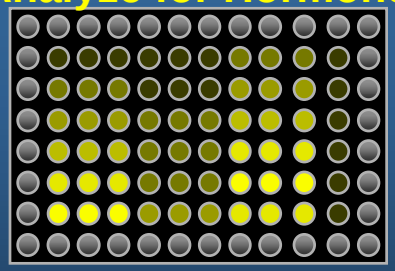


Incubate
For 48 hrs



Extract
Medium with
ether

Analyze for Hormone



ELISA, RIA, LC/MS

Collect Cells

Freeze in Liquid N_2

(gene expression, enzyme analysis)

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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 adenylcyclase and increasing cAMP levels in adrenal cells
- **Ketoconazole**
  - ✓ Imidiazol fungicide: Inhibits p450 enzymes (24-hydroxylase, Cholesterol SCC and C-17,20 lyase)

Cytotoxicity/Cell Viability

- 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

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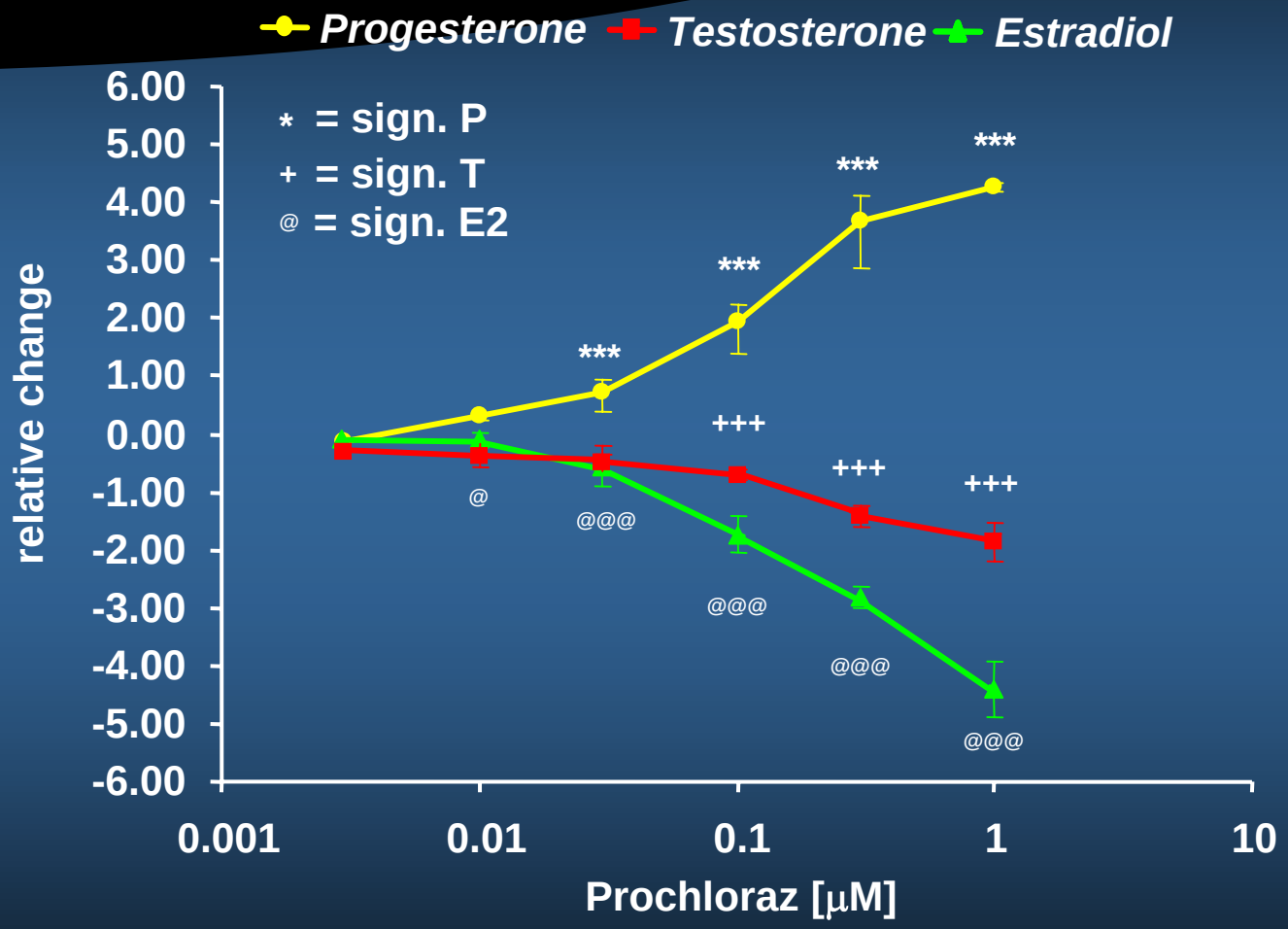
Model Chemical Exposure
Prochloraz



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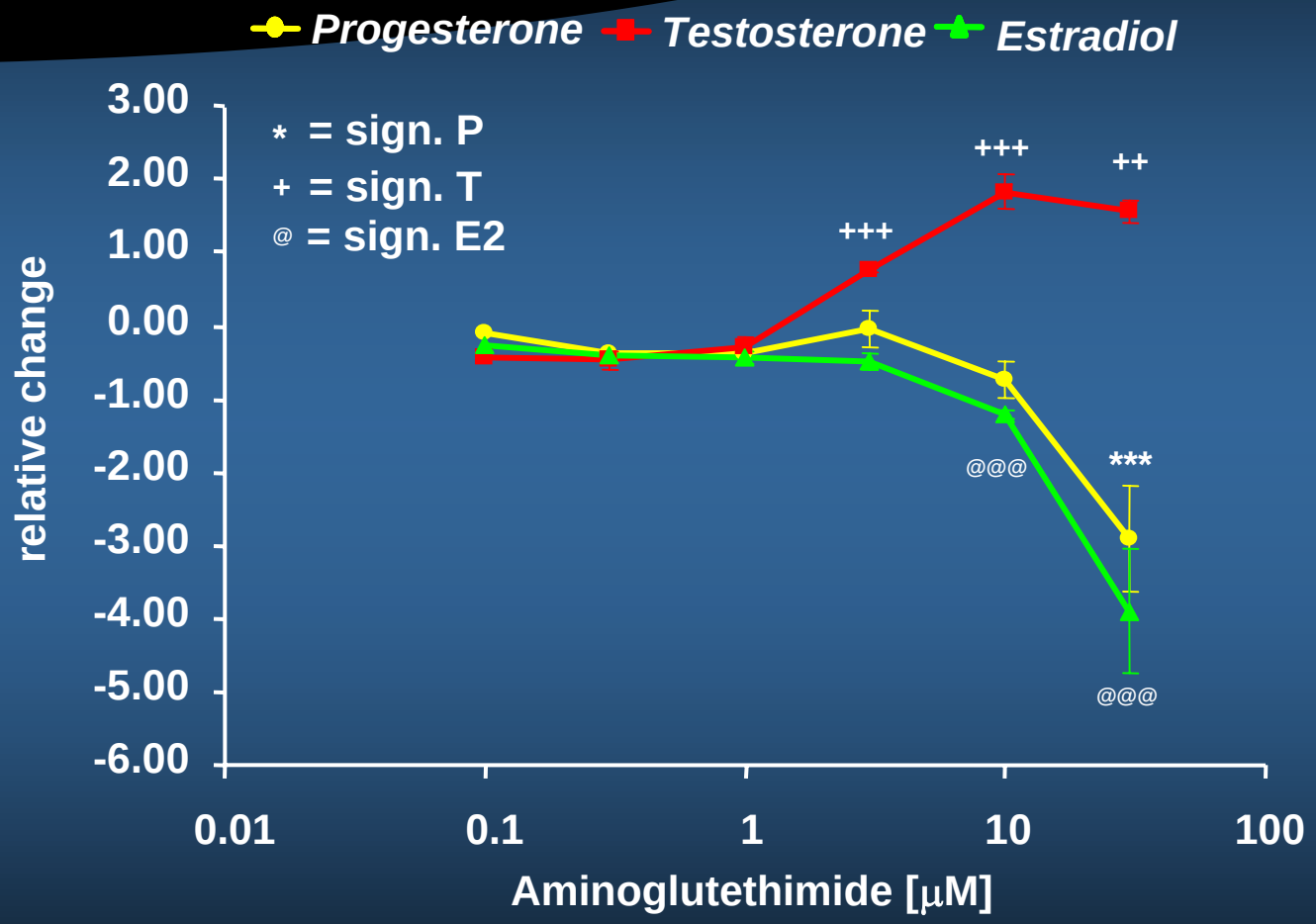
Model Chemical Exposure
Aminoglutethimide



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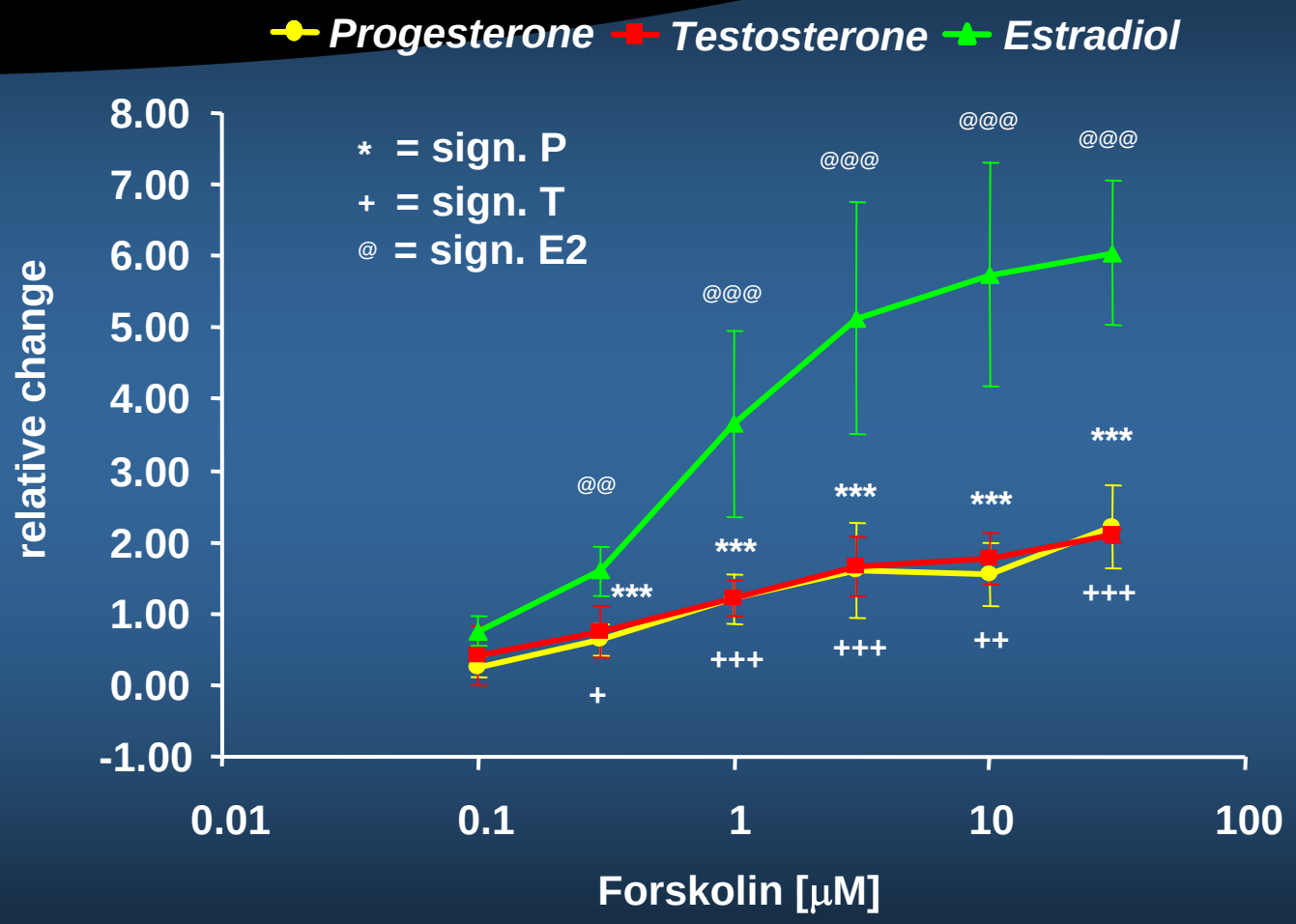
Model Chemical Exposure
Forskolin



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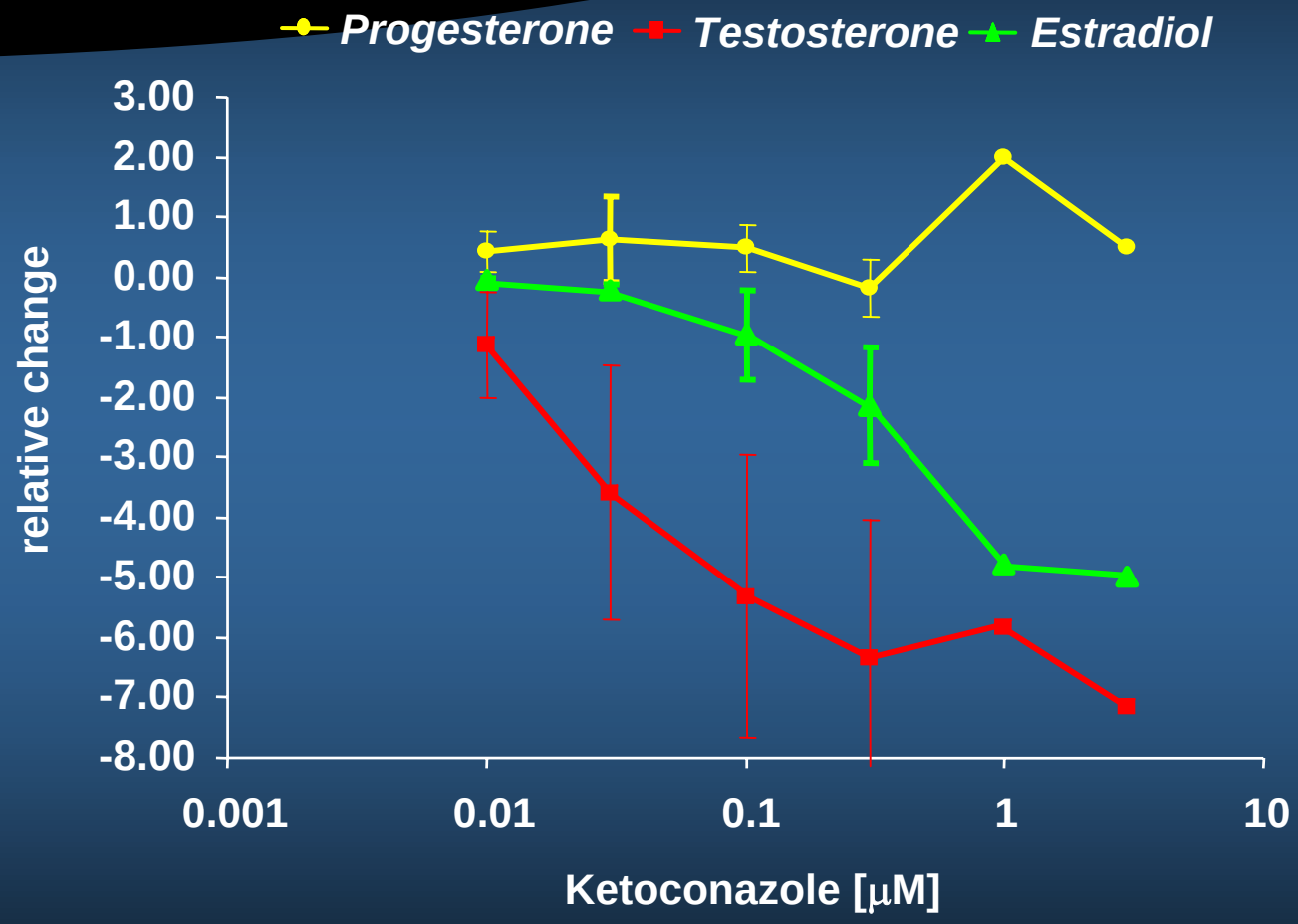
Model Chemical Exposure

Ketoconazole *(Preliminary Results)*



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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

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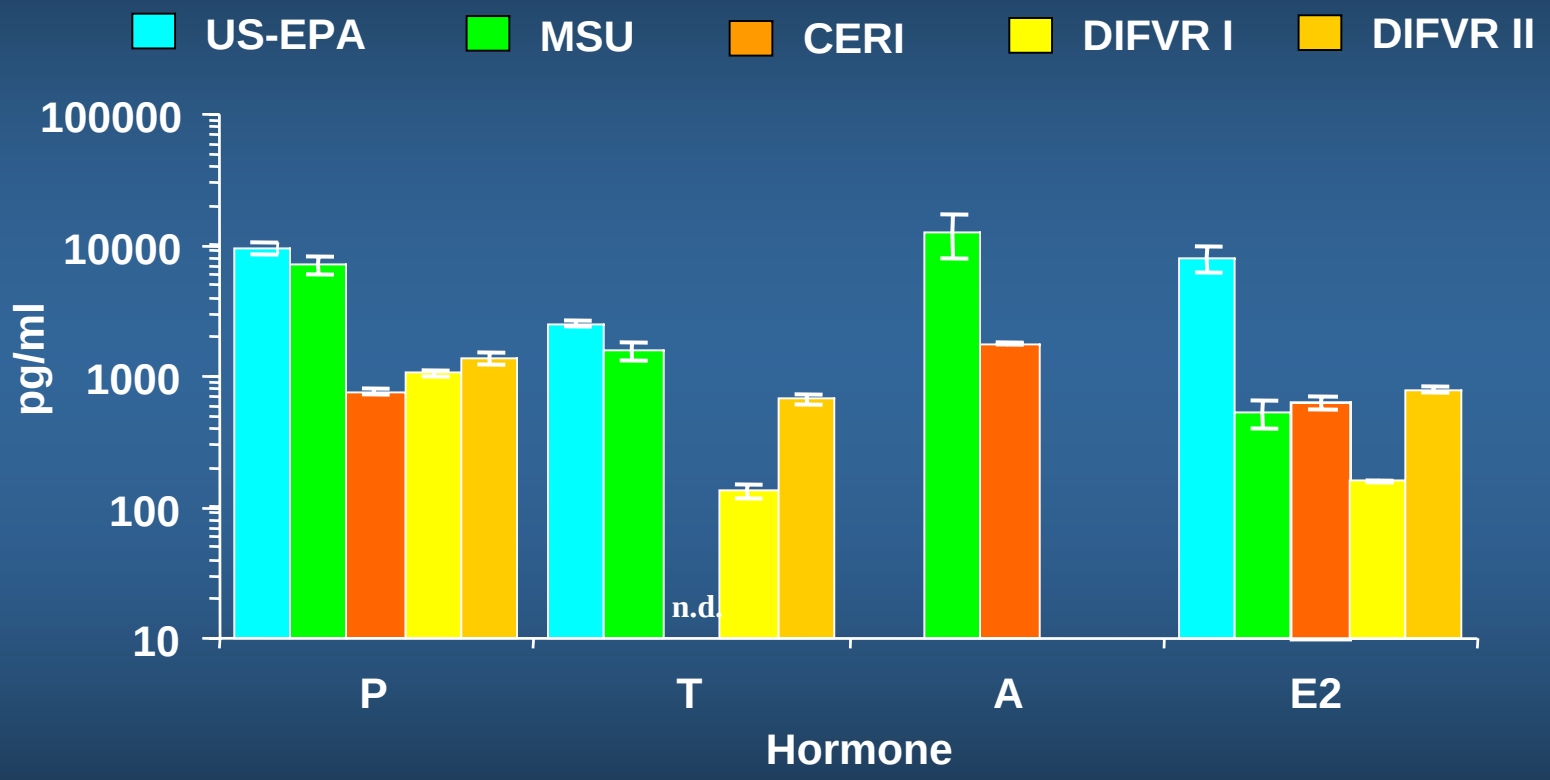
Inter-Laboratory Comparison
(cont')

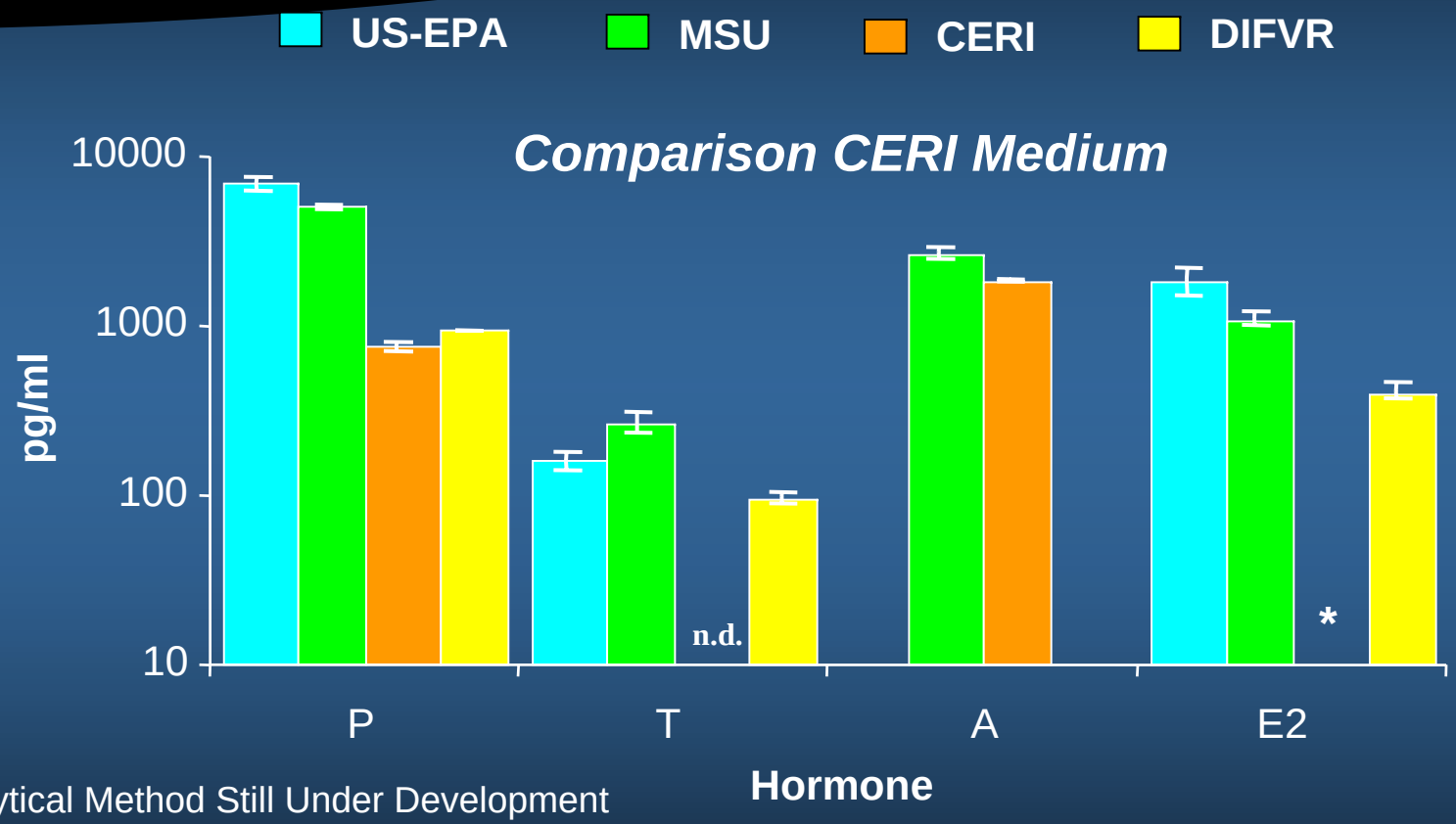
- **Performance based comparison. Use of:**
  - ✓same cells/same passages
  - ✓different cell culture protocols/conditions
  - ✓same seeding density
  - ✓same acclimation and exposure protocols/conditions
  - ✓different hormone detection methods

Phase I – General Test Performance
Basal Hormone Production



n.d. = below MDL

Phase I – General Test Performance
Hormone Detection Systems



*Analytical Method Still Under Development
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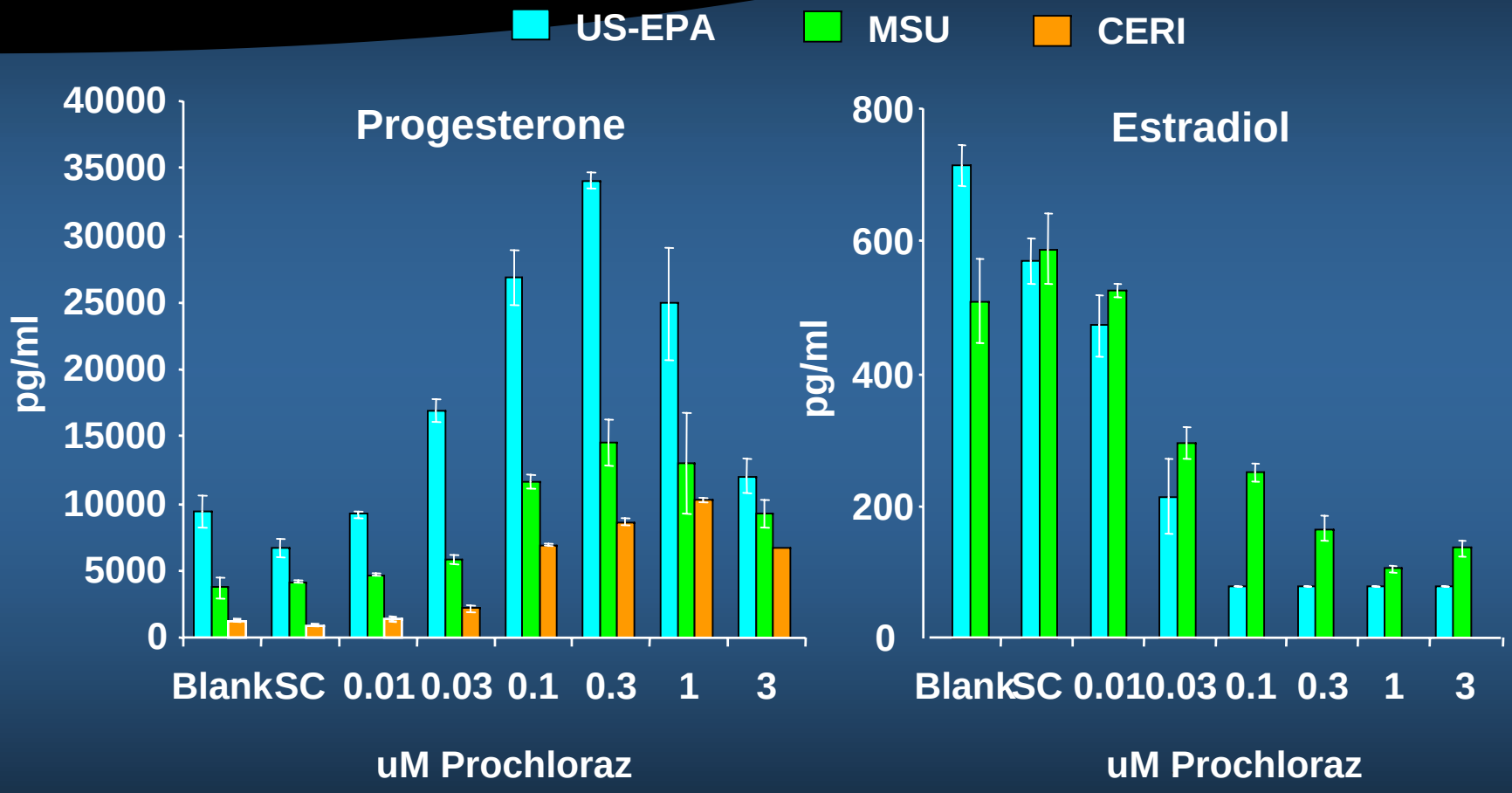
Phase I – General Test Performance
Comparison MSU Prochloraz Exposure



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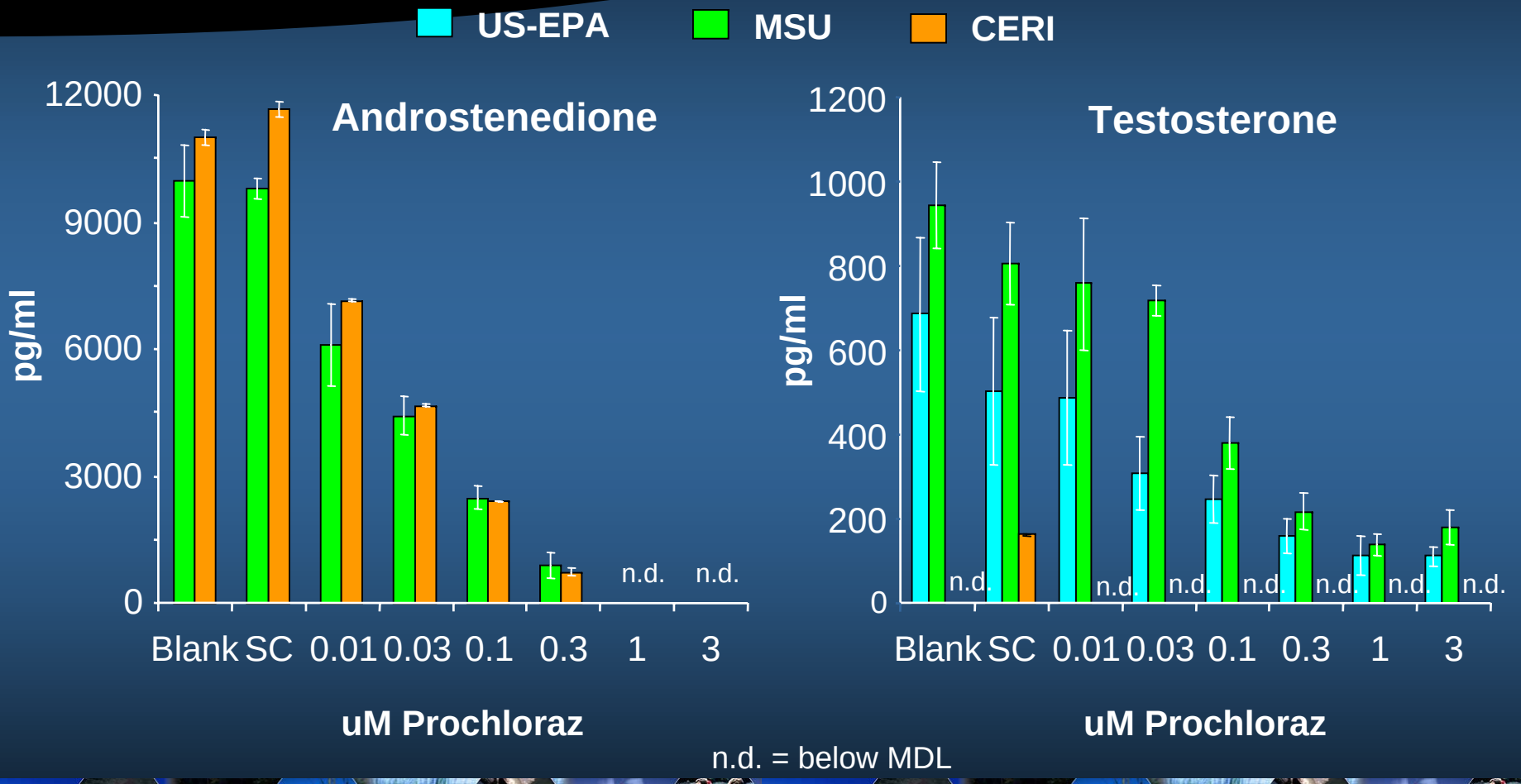
Phase I – General Test Performance
Comparison MSU Prochloraz Exposure



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Phase I – General Test Performance
Summary & Conclusions

- **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

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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

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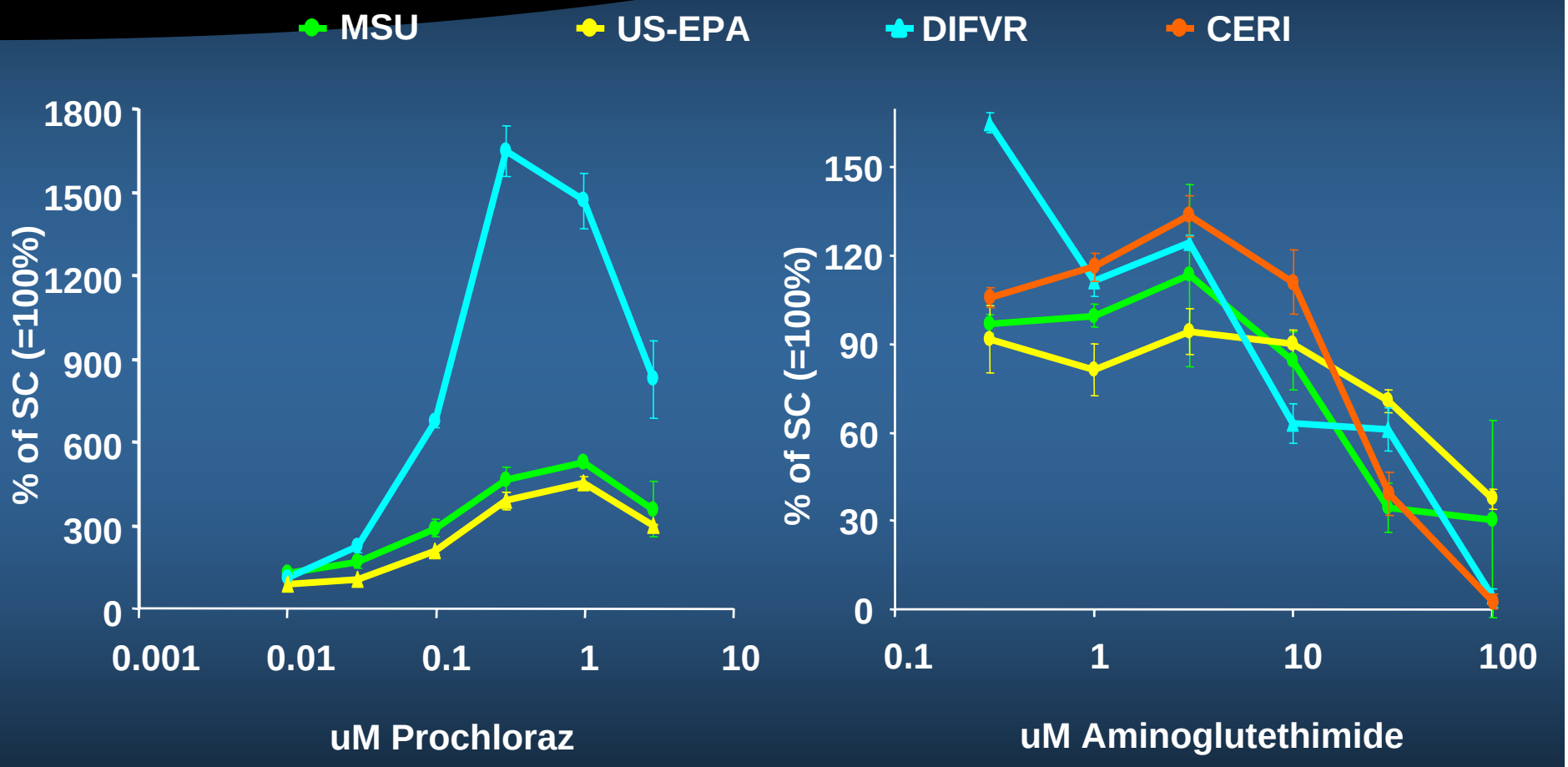
Phase II – Model Chemicals

Progesterone *(Preliminary Data)*



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Phase II – Model Chemicals

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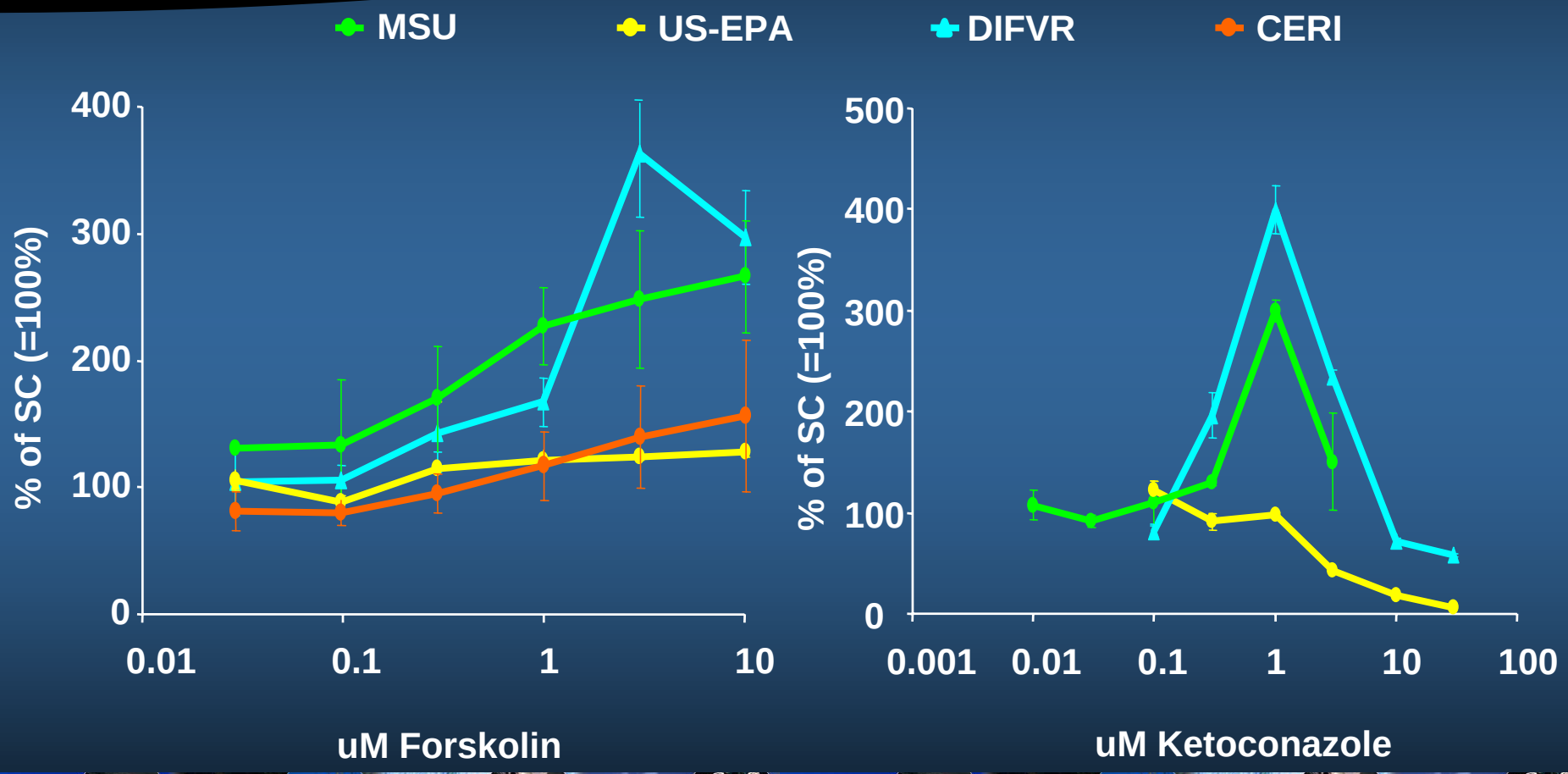

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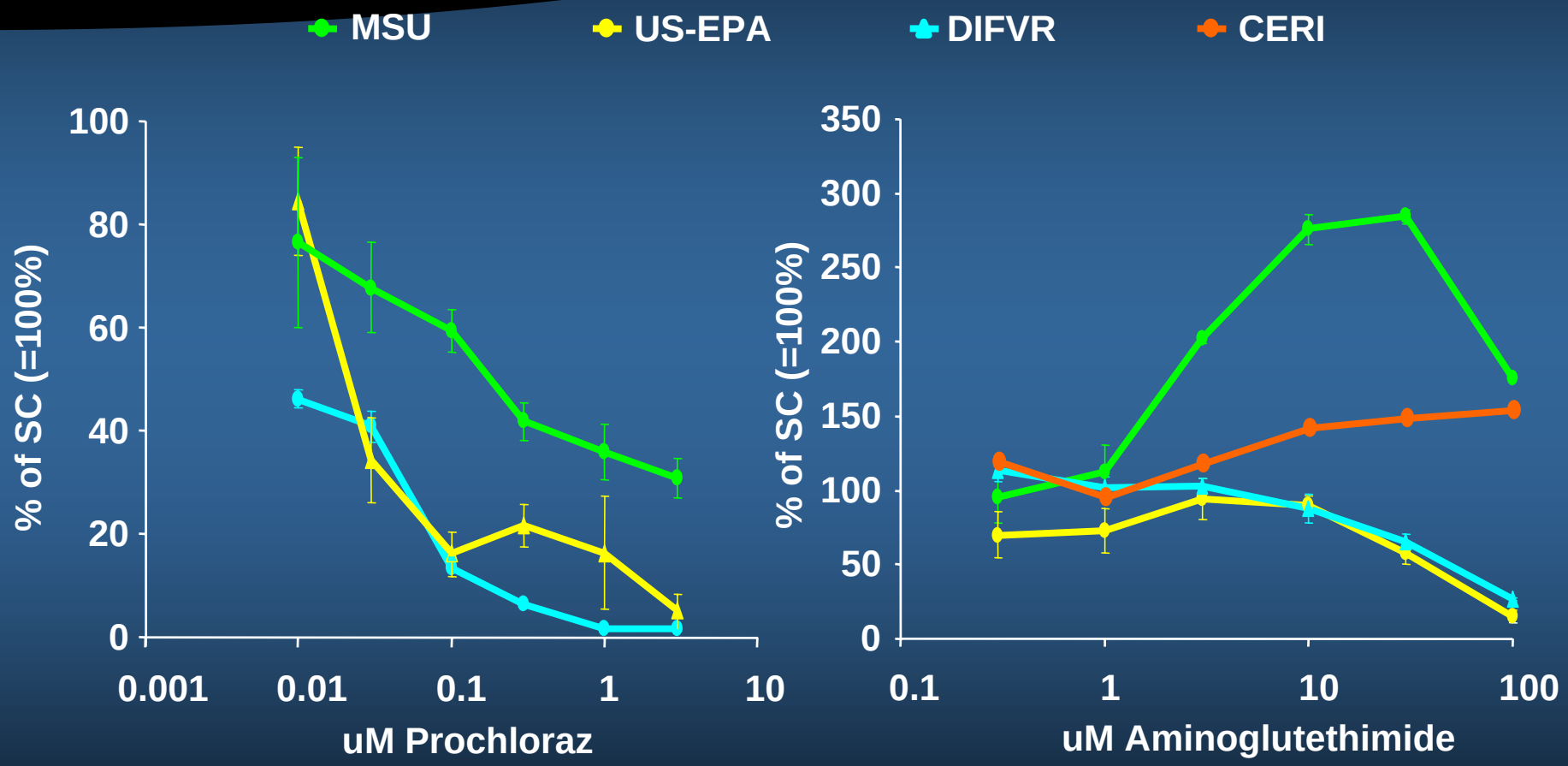
Phase II – Model Chemicals

Testosterone *(Preliminary Data)*



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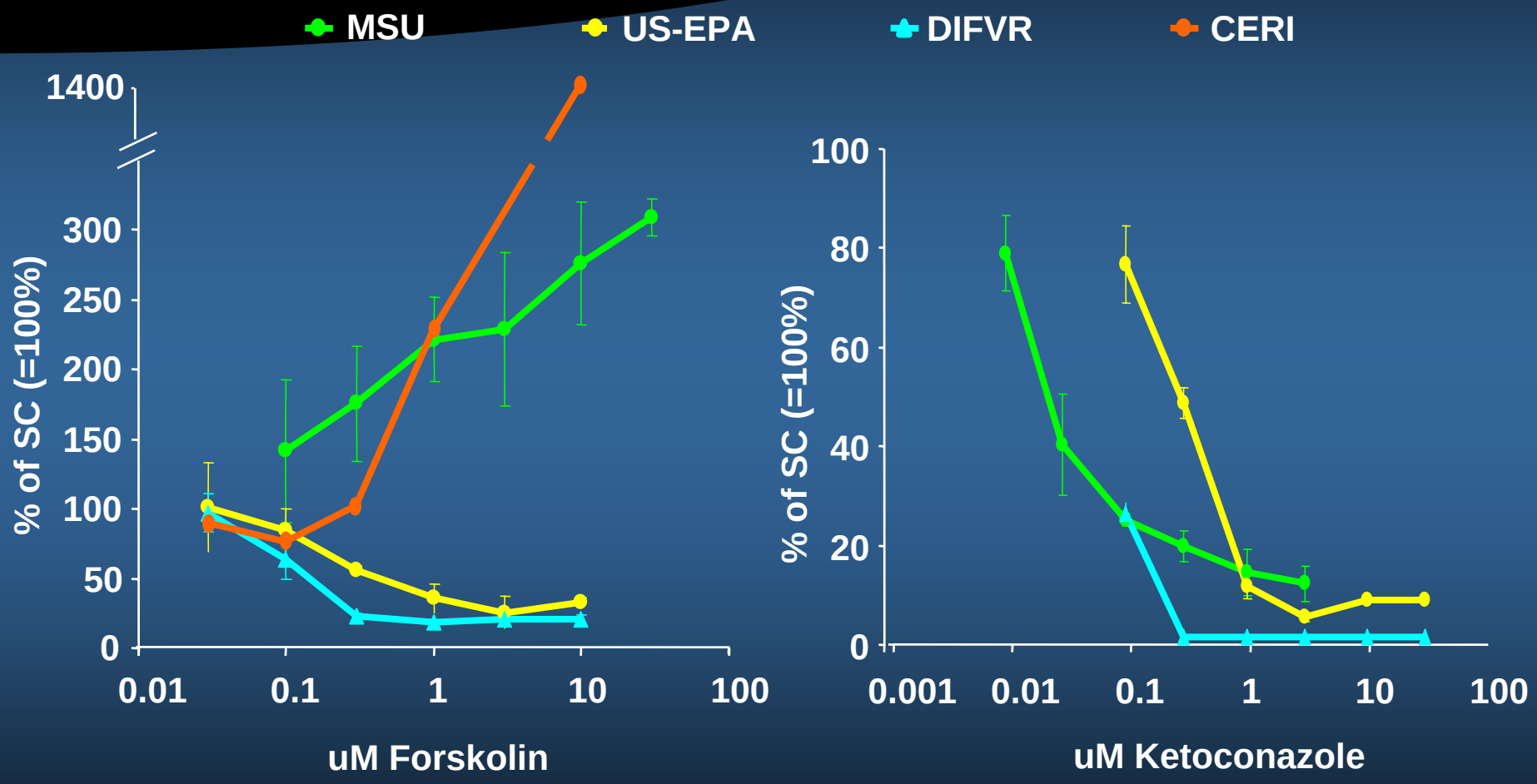
Phase II – Model Chemicals

Testosterone *(Preliminary Data)*



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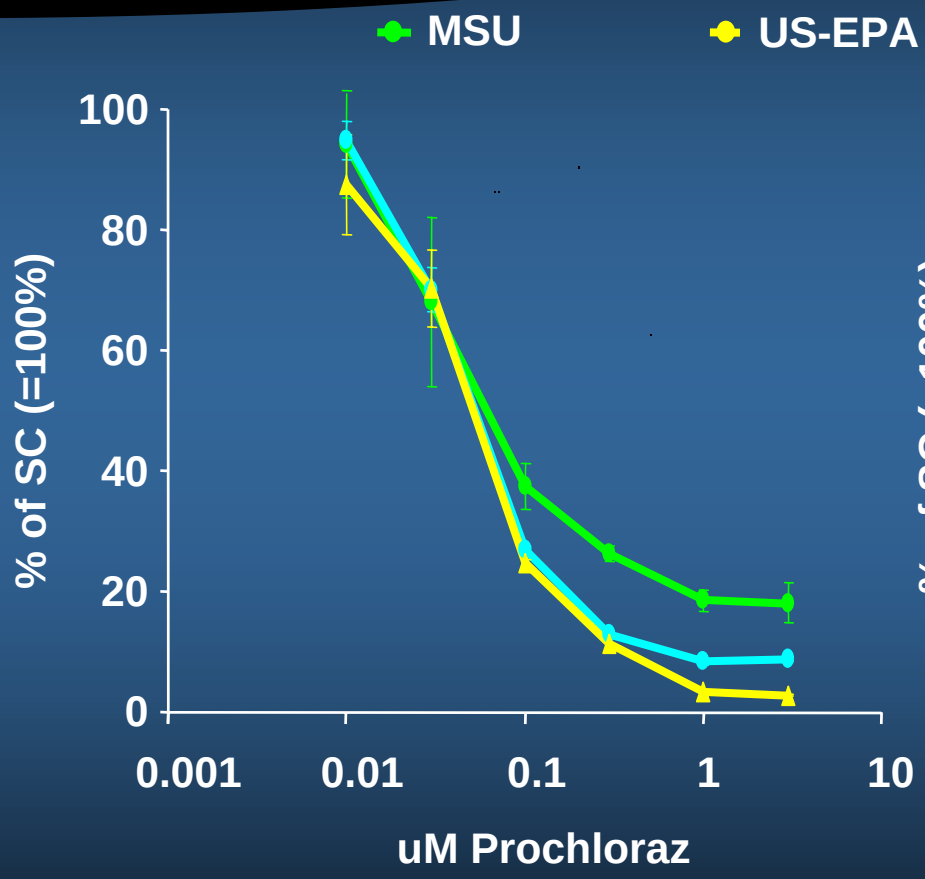
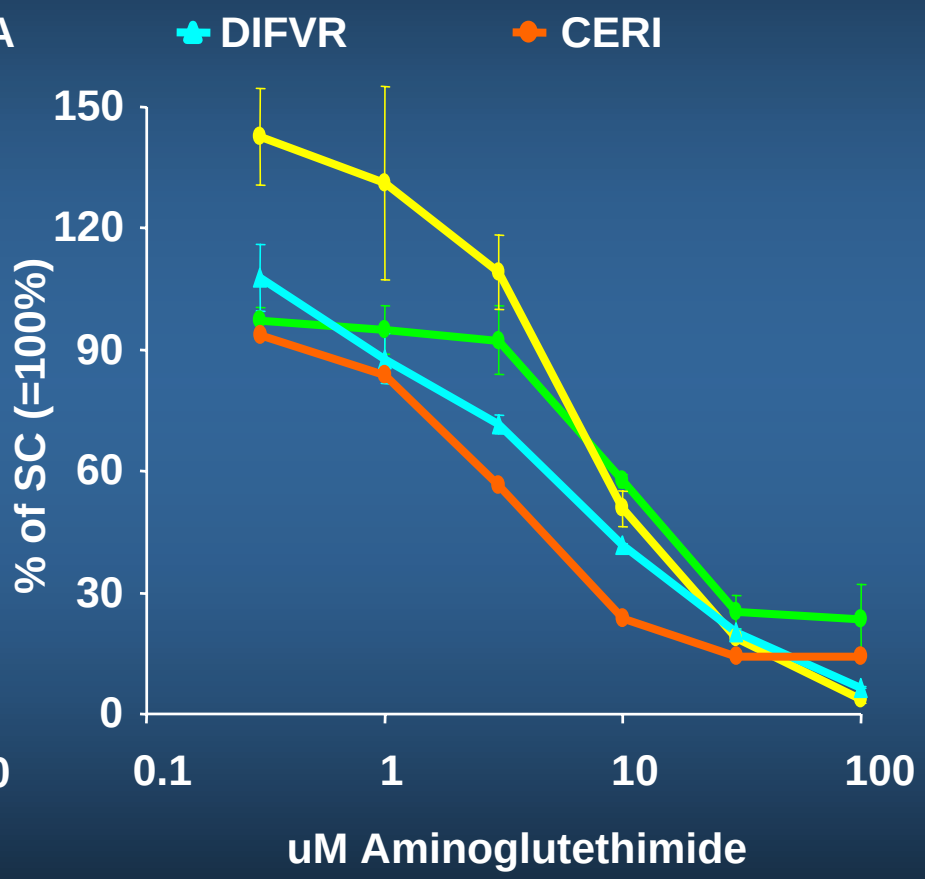
Preliminary Inter-Lab Comparison

Estradiol *(Preliminary Data)*





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Phase II – Model Chemicals

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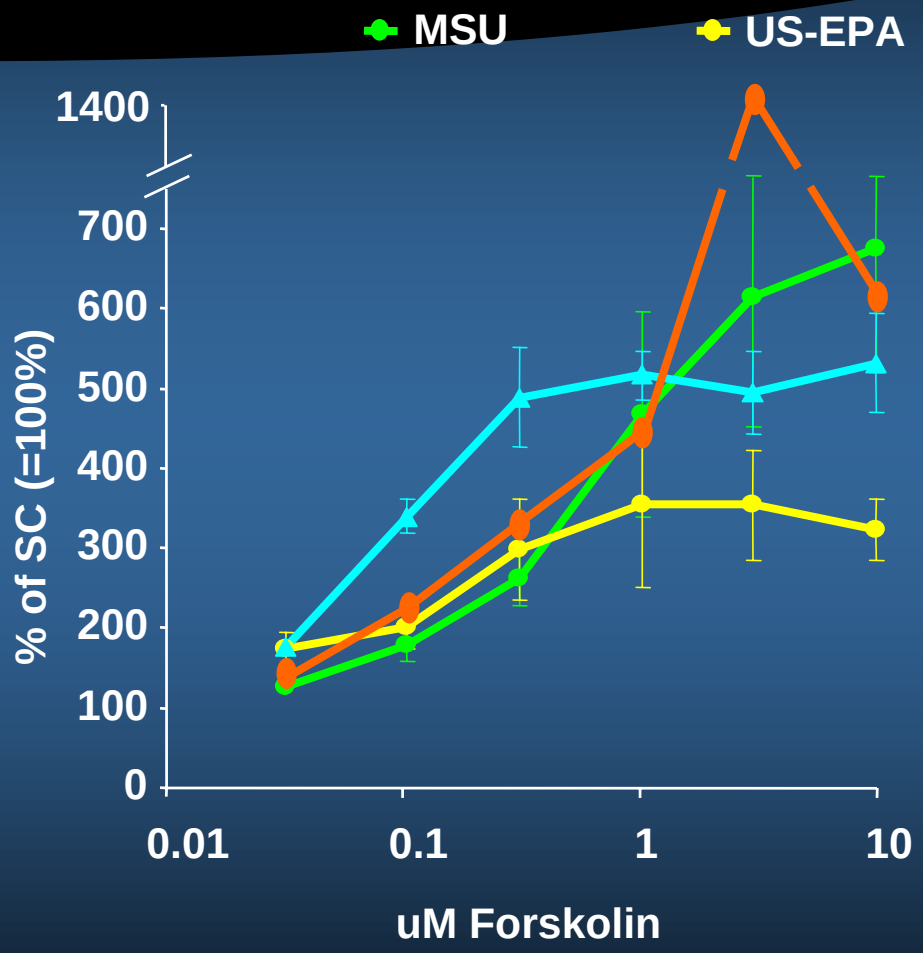

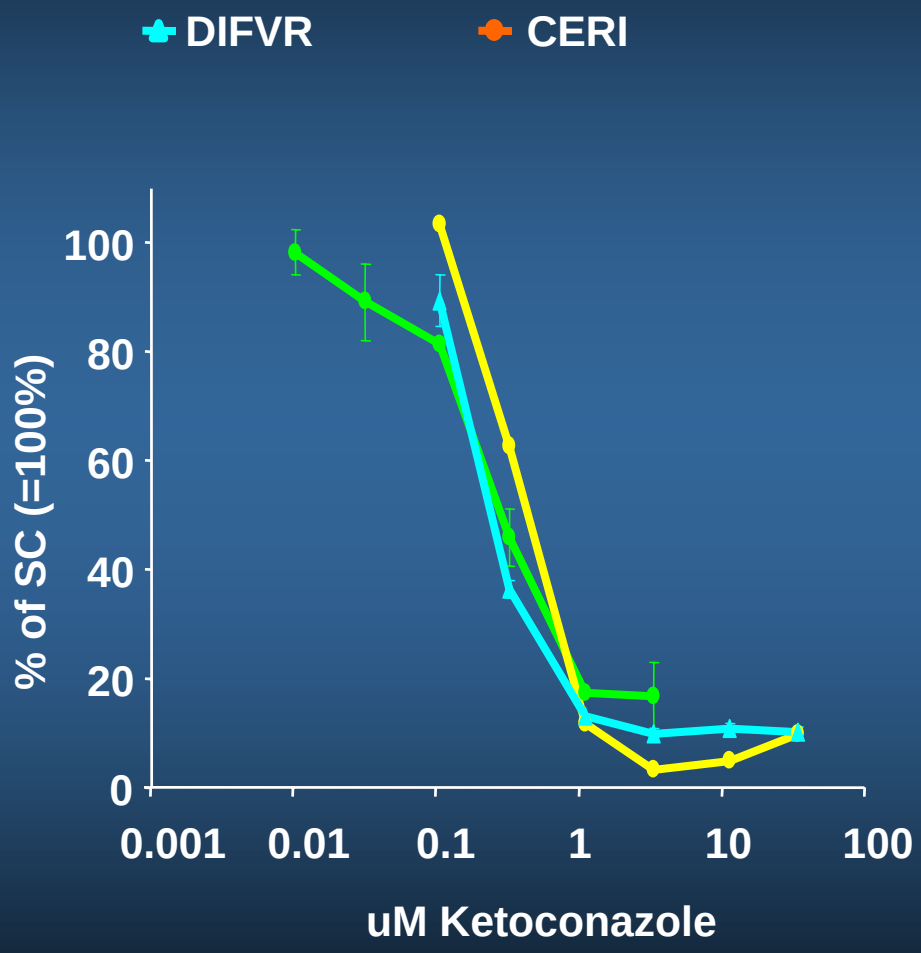

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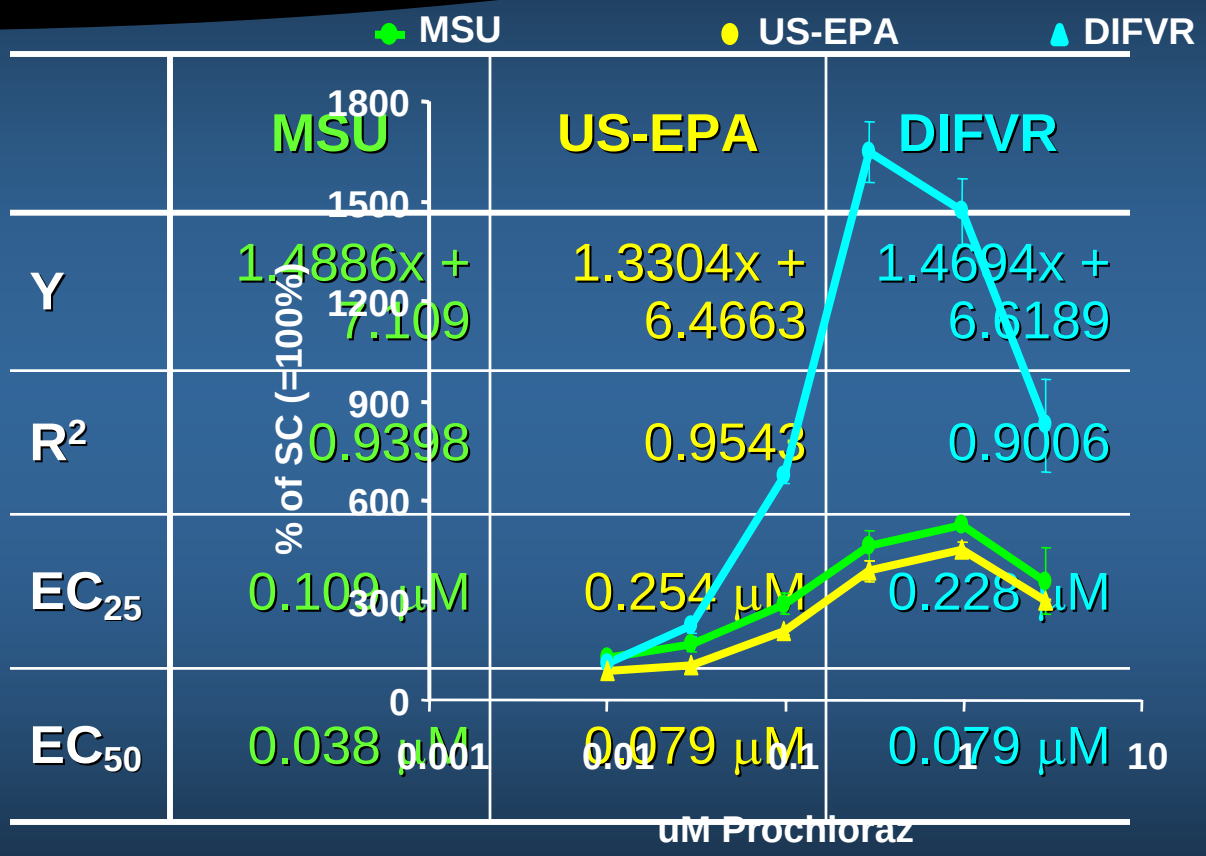
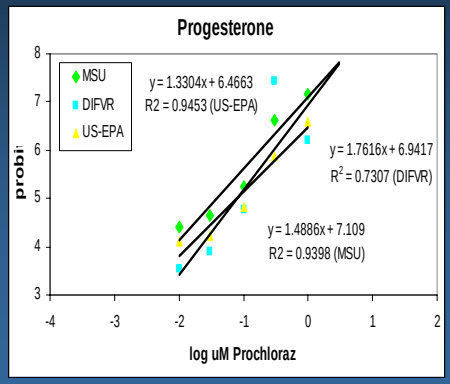
Preliminary Inter-Lab Comparison

Prochloraz *(Progesterone)*

	MSU	US-EPA	DIFVR
Y	1.4886x + 7.109	1.3304x + 6.4663	1.4694x + 6.6189
R^2	0.9398	0.9543	0.9006
EC_{25}	0.109 µM	0.254 µM	0.228 µM
EC_{50}	0.038 µM	0.079 µM	0.079 µM





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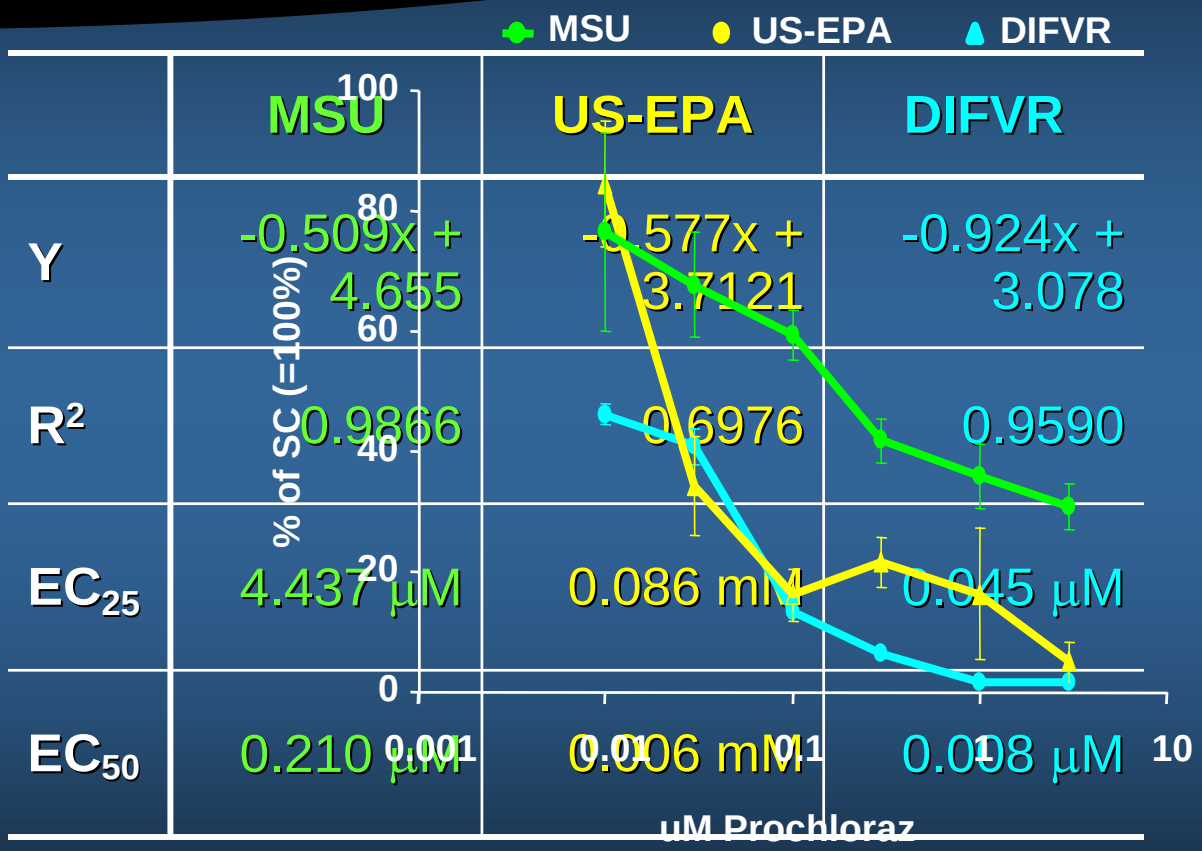
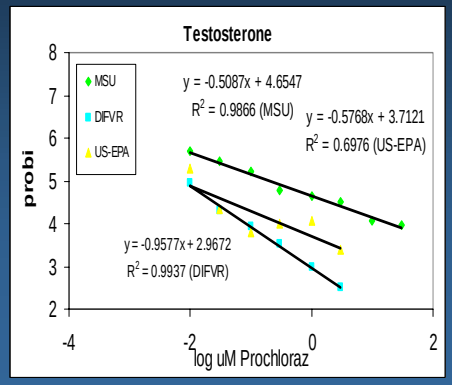
Prochloraz *(Testosterone)*

	MSU	US-EPA	DIFVR
Y	-0.509x + 4.655	-0.577x + 3.7121	-0.924x + 3.078
R^2	0.9866	0.6976	0.9590
EC_{25}	4.437 µM	0.086 mM	0.045 µM
EC_{50}	0.210 µM	0.606 mM	0.098 µM





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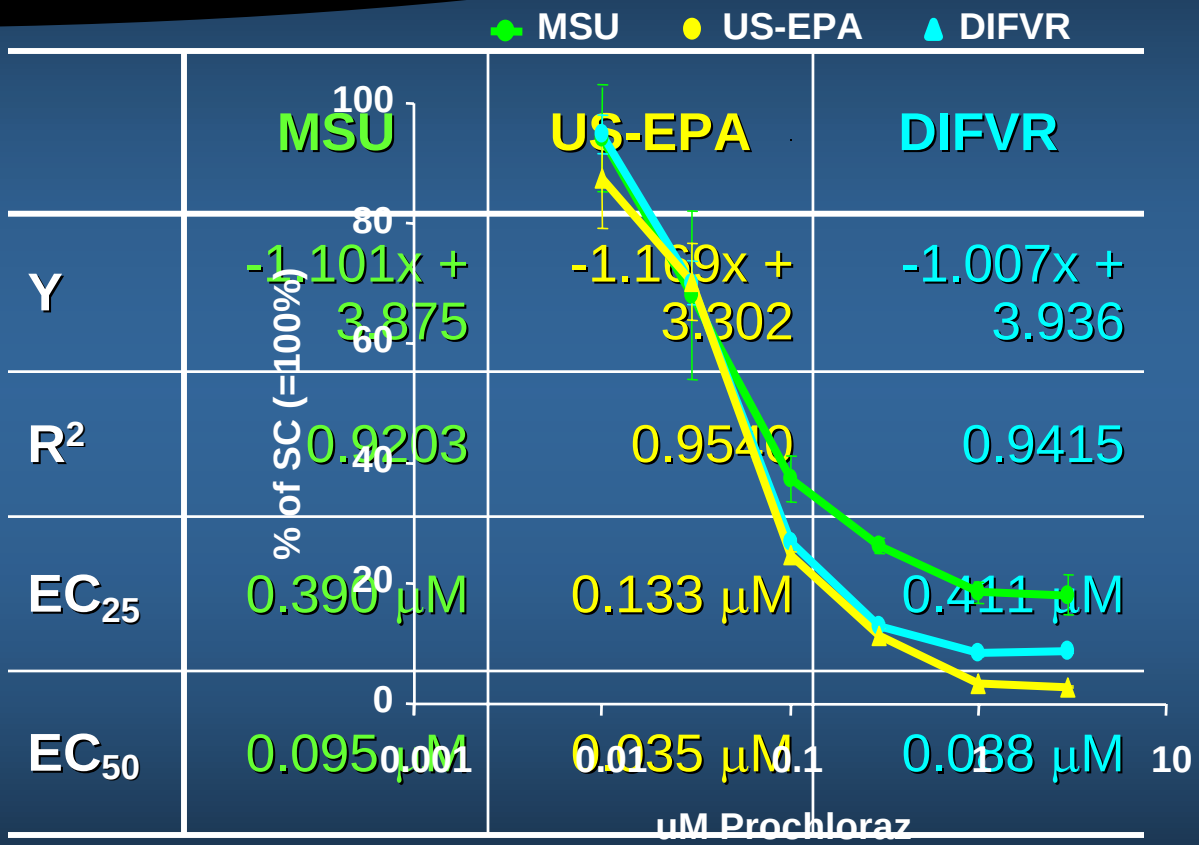
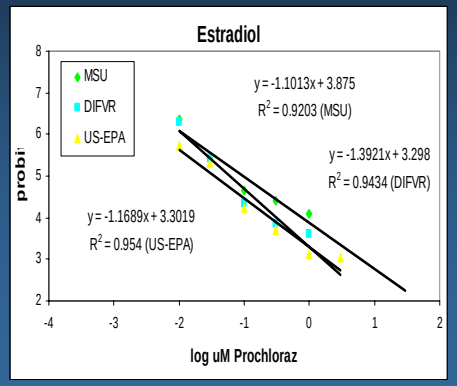
Preliminary Inter-Lab Comparison

Prochloraz *(Estradiol)*



	MSU	US-EPA	DIFVR
Y	-1.101x + 3.875	-1.169x + 3.302	-1.007x + 3.936
R^2	0.9203	0.9540	0.9415
EC_{25}	0.390 µM	0.133 µM	0.411 µM
EC_{50}	0.095 µM	0.035 µM	0.088 µM



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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

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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

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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

Conclusions
(cont')

- **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)

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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)**

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Future Directions

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

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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 particiating laboratories (10 - 20)

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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

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Publications

Hilscherova, K.; Jones, P. D.; Gracia, T.; Newsted, J. L.; Zhang, X.; Sanderson, J. T.; Yu, R. M. K.; Wu, R. S. S.; Giesy, J. P. Assessment of the Effects of Chemicals on the Expression of Ten Steroidogenic Genes in the H295R Cell Line Using Real-Time PCR. *Toxicol. Sci.* 2004, 81, 78-89.

Zhang, X, Yu, R, Jones, P.D., Newsted, J.L., Gracia, T., Hecker, M., Hilscherova, K., Sanderson, J.T., Wu, R., and Giesy, J.P. 2005. Quantitative RT-PCR Methods for Evaluating Toxicant-Induced Effects on Steroidogenesis Using the H295R Cell Line. *Environ. Sci. Technol.* 39: 2777-2785.

Hecker, M., Newsted, J.L., Murphy, M.B., Higley, E.B., Tompsett, A.R., Jones, P.D., and Giesy, J.P. 2005. Effects of Prochloraz, Aminoglutethimide, Forskolin and Ketoconazole on steroid hormone production human adrenocarcinoma cells (H295R) cells. *Environ. Sci. Technol.* , submitted for publication

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Gracia, T., Hilscherova, K., Jones, P.D., Newsted, J.L., Zhang, X., Hecker, M., Higley, E.B., Sanderson, J. T., Yu, R.M.K., Wu, R.S.S. and Giesy, J.P. 2005. Effects of Chemical Mixtures on the Expression of Ten Steroidogenic Genes in the H295R Cell Line. Comp. Physiol. Biochem. B, submitted for publication.

Blaha, L., Hilscherova, K., Mazurova, E., Hecker, M., Jones, P.D., Bradley, P., Gracia, T.R., Duris, Z., Holoubek, I. and Giesy J.P. Alteration of steroidogenesis in H295R cells by organic sediment contaminants and relationships to other endocrine disrupting effects. In prep.

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

- **Validate transferability of test system (currently underway)**
  - ✓ Within the same laboratory (completed)
  - ✓ Between different laboratories (underway)

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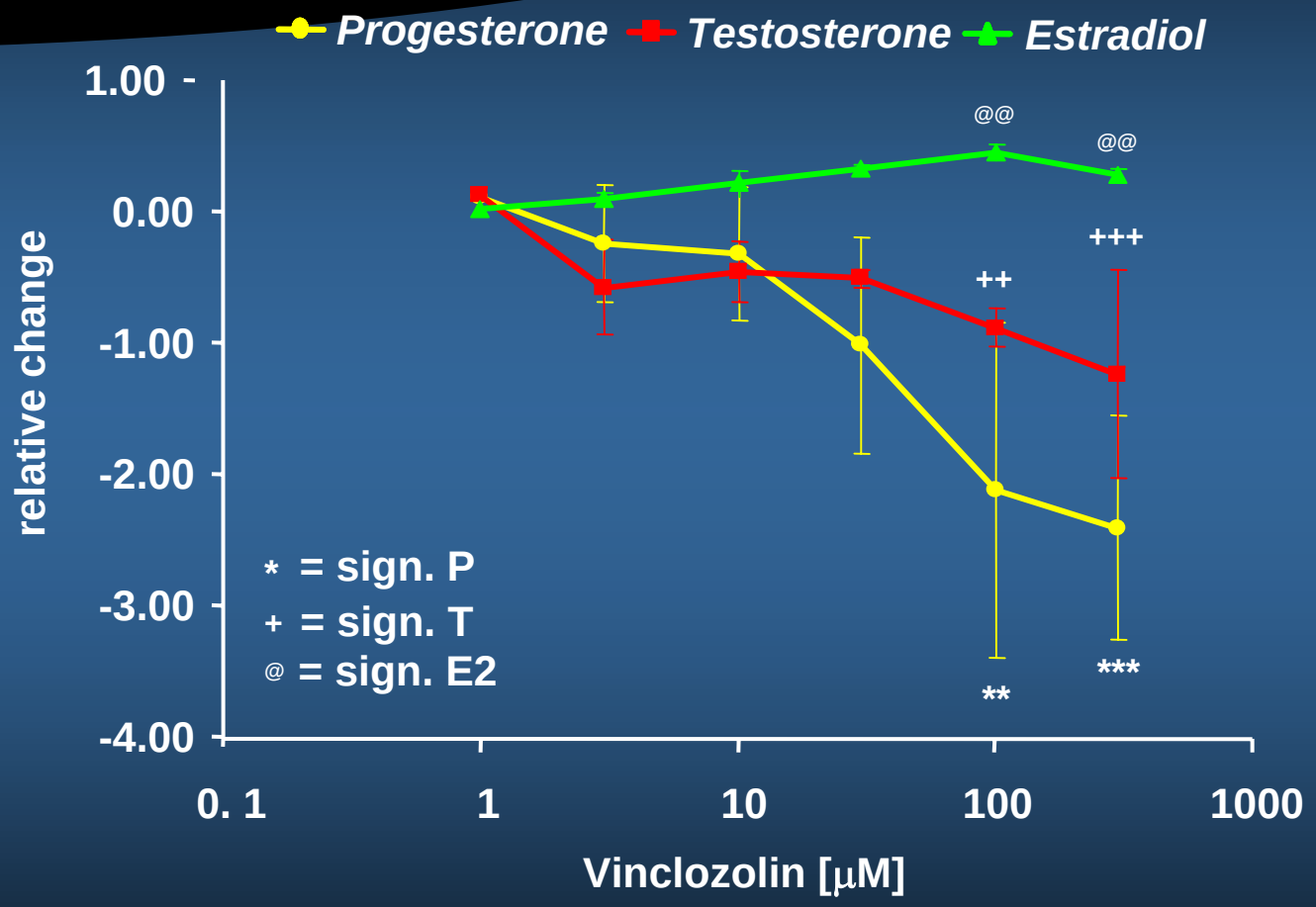
Model Chemical Exposure
Vinclozolin



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