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# Endocrine Disruptor Mechanisms: Beyond Receptor Binding

J.P. Giesy

M. Hecker, K. Hilscherova, X. Zhang,

J. Newsted, P.D. Jones, E. Higley

Michigan State University, Dept. Zoology  
National Food Safety and Toxicology Center  
Institute of Environmental Toxicology

R. Wu, M. Murphy, R. Yu

Dept. Biology and Chemistry  
City University of Hong Kong  
Kowloon, Hong Kong, SAR, PRC



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# ENDOCRINE DISRUPTION (ED)

*“...an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior.”*

**Kavlock et al., 1996. Research needs for the assessment of environmental effects of endocrine disruptors: a report of the USEPA-sponsored workshop**



# Endocrine Disruption

- **Major Functions of the Endocrine System:**
  1. Coordinate homeostasis in the body
  2. Allow communication among organs



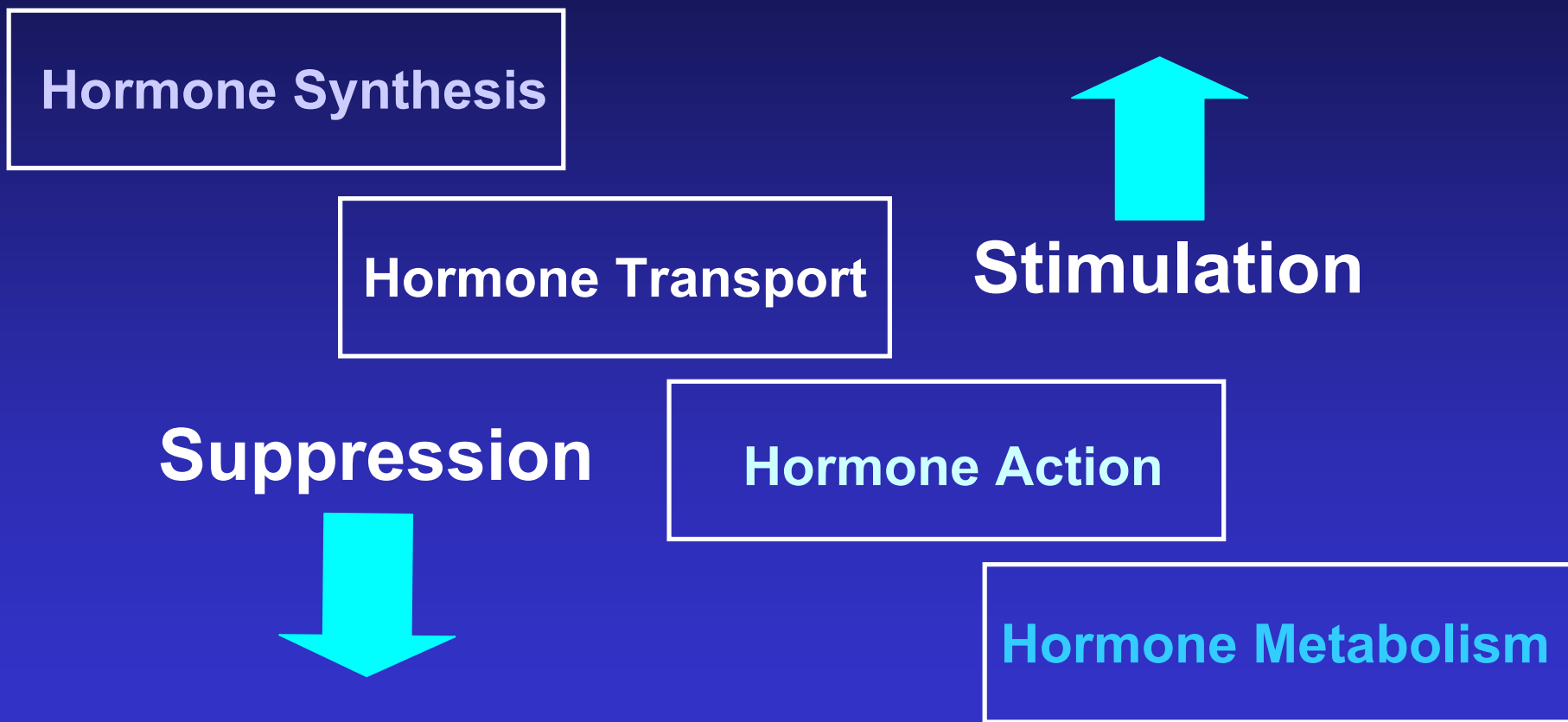
# Maintaining Homeostasis is a Complex Process

- Involves many signal transduction pathways
- Many alternative pathways
- Many enzymes involved
- Rate limiting steps unknown
- Cybernetic feedback loops



# Endocrine Disruption

## *Levels of EDC – Organism Interaction*



# ENDOCRINE DISRUPTION

- **Direct - (Mimics)**
  - Agonists
  - Antagonists
  - Partial Agonists
- **Environmental estrogens are only one mimic**
- **Indirect**
- **Induction of Enzymes that Directly or Indirectly Affect Hormone Concentrations**
- **Alteration of signal transduction pathways**





# Endocrine Disruption

- In the United States current attention is focused primarily on compounds that can affect steroid hormones
- Reauthorization of the *Clean Water Act*
- Food Safety Protection Act
- Endocrine Disrupter Screening and Testing Committee (EDSTAC)
  - Estrogen receptor (ER)
  - Androgen Receptor (AR)
  - Thyroid Receptor (TR)



# Endocrine Disruption & Food Safety (USA)

- **When setting new or reassessing existing tolerances and tolerance exemptions, EPA must also evaluate the potential for endocrine disruption. The law directs the Agency to use its authority to require specific tests and information on estrogenic effects for all pesticide chemical residues.**



# Endocrine Disruption & Food Safety (EU)

- Council Directive 96/22/EC of 29 April 1996 concerning the prohibition on the use of stock-farming of certain substances having a hormonal or thyrostatic action and of  $\beta$ -agonists (amended by Dir. 2004/73/EC)
- Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market:
  - *Endocrine disrupting activity: waiting for test methodology to be endorsed by OECD. In the meantime in case of suspicion of ED potential – fish full life cycle test.*



# Endocrine Disruptor Screening Program

Universe of chemicals - 87,000 chemicals:

- 900 pesticide active ingredients
- 2,500 other pesticide formulate ingredients
- 75,500 industrial chemicals
- 8,000 cosmetics, food additives and nutritional supplements

Initial focus on:

- pesticide actives
- high production volume inerts



# Proposed Tier 1 Screening Battery (EDSTAC)

- *In Vitro* Screens
  - ER Binding / Reporter Gene Assay
  - AR Binding / Reporter Gene Assay
  - Steroidogenesis Assay with minced testis
- *In Vivo* Screens
  - Rodent 3-day Uterotrophic Assay (sc)
  - Rodent 20-day Pubertal Female Assay with Thyroid
  - Rodent 5-7 day Hershberger Assay
  - Frog Metamorphosis Assay (FETAX)
  - Fish Reproduction Screening Assay
- Alternate assays have also been proposed



# Uncertainties and Concerns

1. **Exposure - outcome linkages**
  - are effects occurring in humans?
2. **Comparative toxicology**
  - extrapolations between species?
3. **Multiple mechanisms of action**
4. **Cumulative exposure/latency between exposure and outcome**



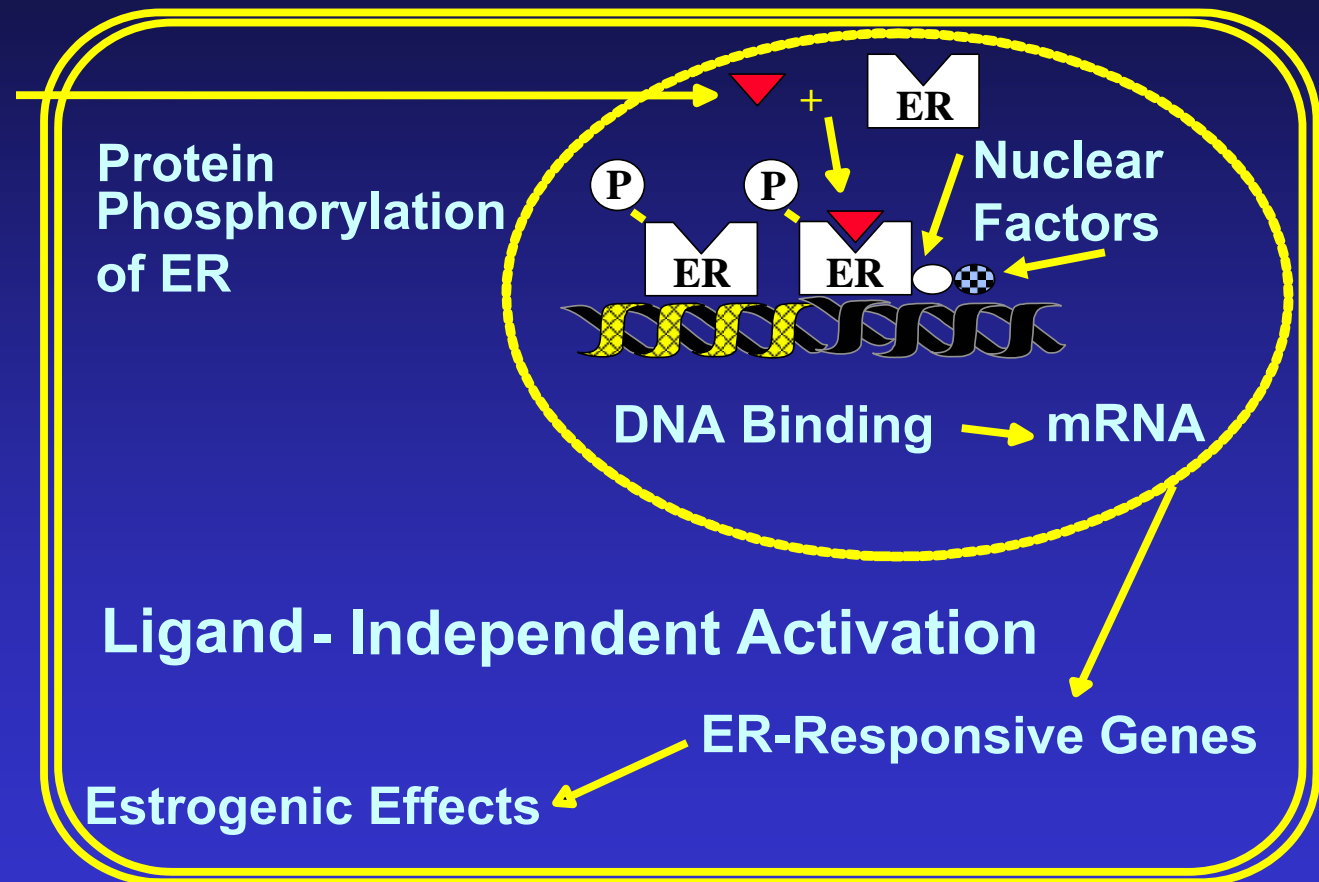
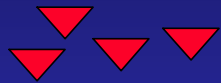
# Uncertainties and Concerns cont'd

5. **Cost - \$200,000 per substance for Tier 1**
6. **Animal welfare**
  - Estimate that 0.6 - 1 million animals would be used per 1,000 substances (1999, *Toxicol. Sci.* 52, 141-7)
7. **Do endocrine disruptors require special consideration in risk assessment?**
8. **Assay and test validation (Interagency Coordinating Committee for the Validation of Alternative Methods - ICCVAM)**



# Mechanism of Action for ER-Activation

Estrogen  
or xenoestrogen

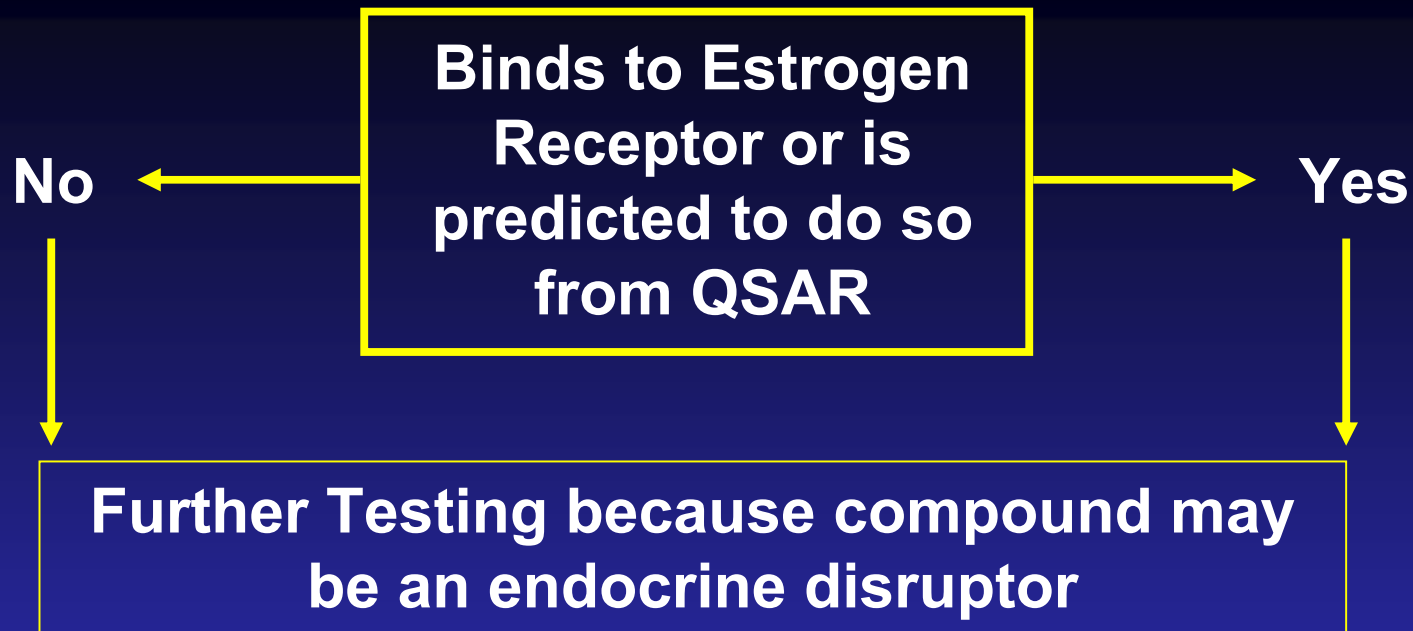




# Limitations of Screening Methods

- **If used in a sequential decision process**
  - False negatives
  - If negative in the binding assay, may still be positive as an endocrine disrupting compound





*The proposed sequential testing provides useful information for designing additional testing, but does not allow for a sorting of compounds and does not assist in prioritizing compounds for additional testing*



# Example: Triazine Herbicides

Do not bind to ER!

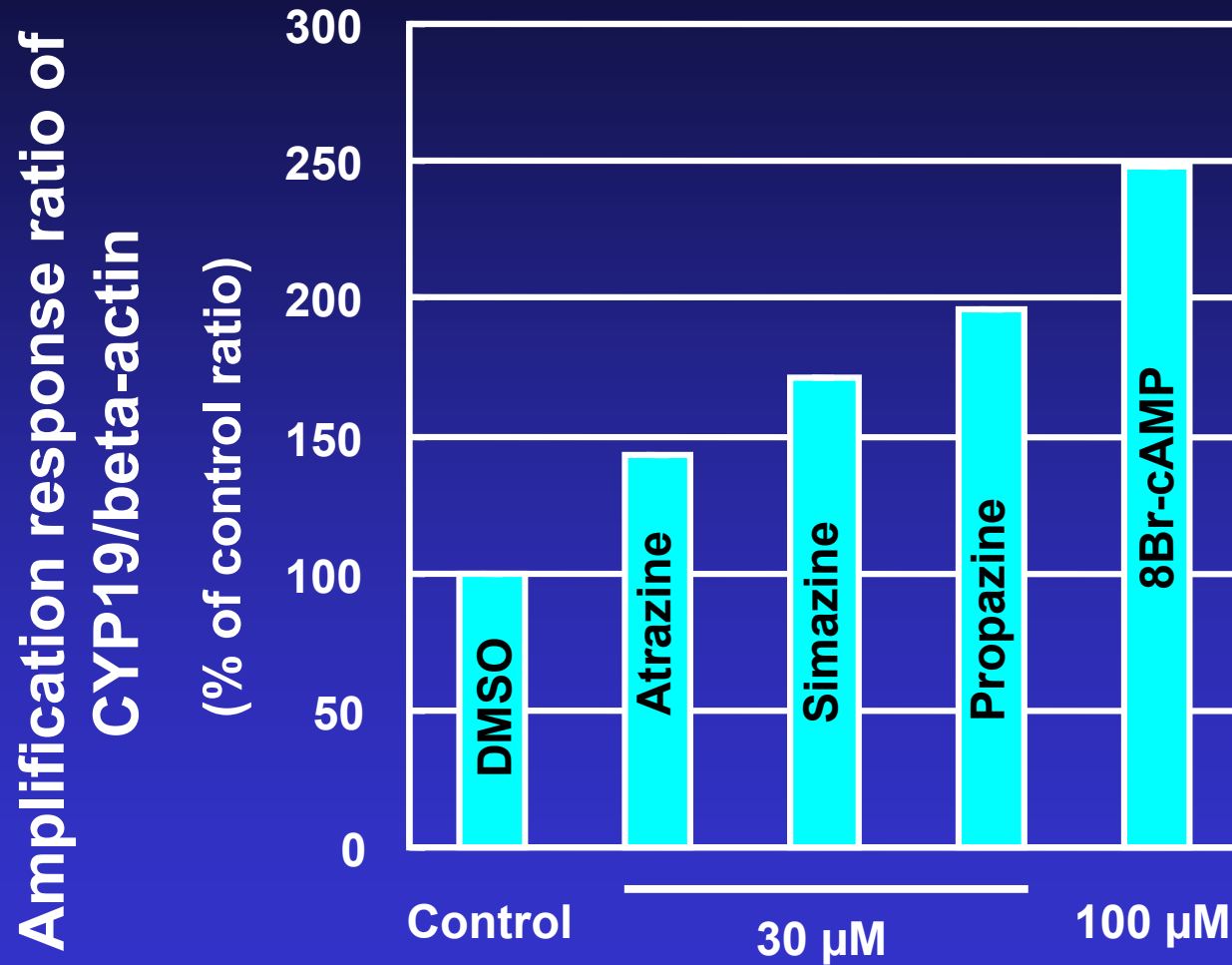


Results in estrogenic effect *in vitro*

Sanderson. J.T., W. Seinen, J.P. Giesy and M. van den Berg. 2000. 2-chloro-S-Triazine Herbicides Induce Aromatase (CYP-19) Activity in H295R Human Adrenocortical Carcinoma Cells: A Novel Mechanism for Estrogenicity. *Toxicol. Sci.* 54:121-127.



# Induction of CYP19 mRNA by triazines



# Effects of Other Compounds on Aromatase activity H295R cells

- Imidazole-type fungicides decrease aromatase activity. Competitive inhibitors
  - imazalil
  - prochloraz
  - difenoconazol
  - penconazole
  - Propiconazole
  - Diclobutrazole
  - Tricyclazole
  - Paclobutrazole
  - Nuarimol

the structurally similar fungicide Vinclozolin increases cAMP 150%, whereas forskolin increases cAMP 300%

Sanderson et al. 2001. *Organohalogen Compounds*. 53:10-13.

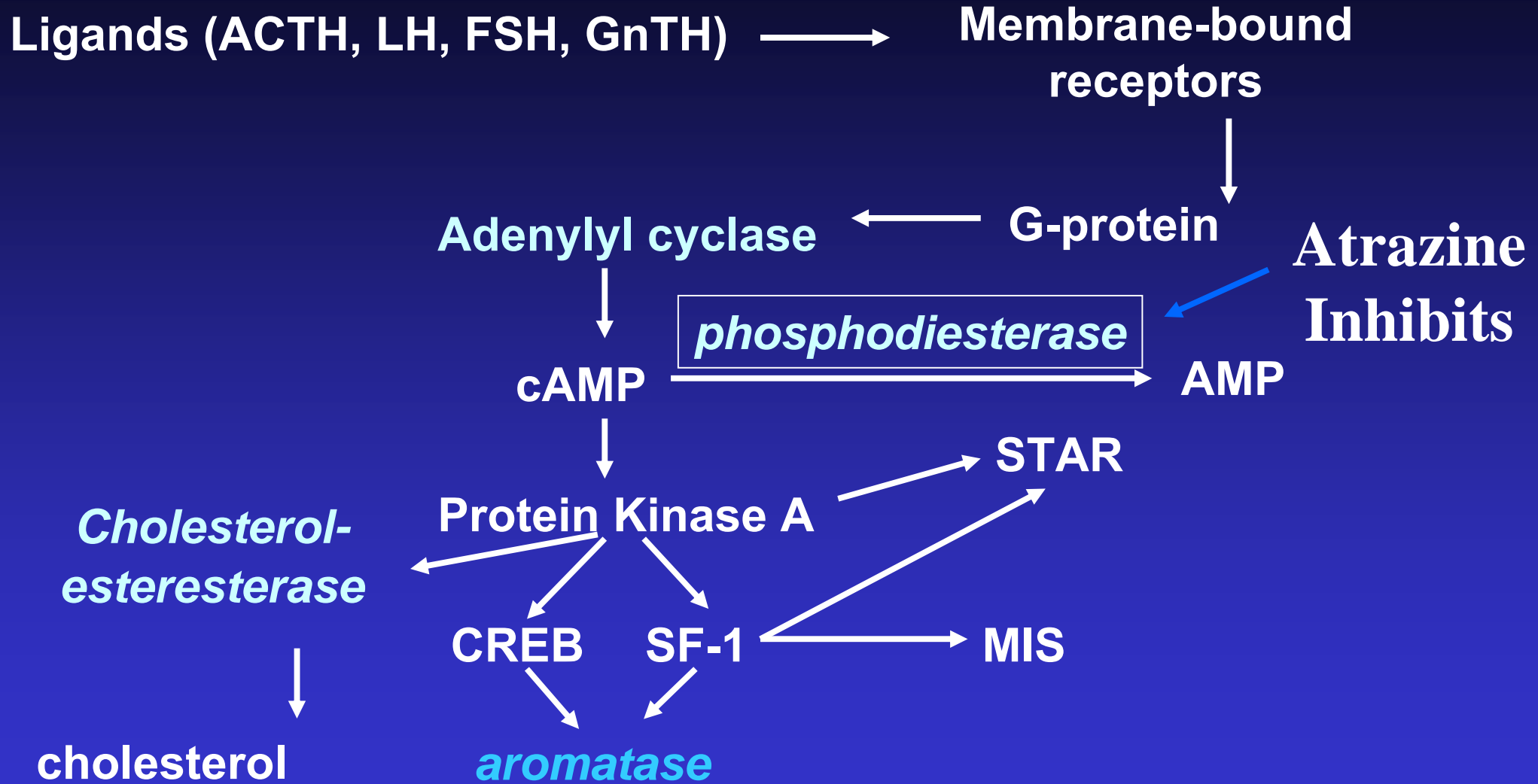


# Proposed Mechanisms of Action for Triazines

- Aromatase induction / promotion
  - via protein kinase A pathway
  - via steroidogenic pathways
- Inhibition of phosphodiesterase
- Results in less conversion of c-AMP to AMP so that AMP increases
- c-AMP increases signal transduction of Protein Kinase A
- Protein kinase A increases CREB and SF-1
- Aromatase m-RNA is up-regulated such that more aromatase is formed and aromatase activity increases



# Protein Kinase A Signaling Pathway



# *In vitro* Model System

- Needed a flexible assay system that would allow for rapid studies of mechanisms of action
- Needed to express major enzyme systems
- Needed to be stable so results are reproducible





# H295R cell line

- **Human female adrenocortical carcinoma**
- **Produces many steroid progestins, androgens & estrogens hormones**
  - glucocorticoids, mineralocorticoids
- **Express most of the important steroidogenic enzymes**
  - CYP11A, CYP11B, CYP17, CYP19, CYP21



# 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).

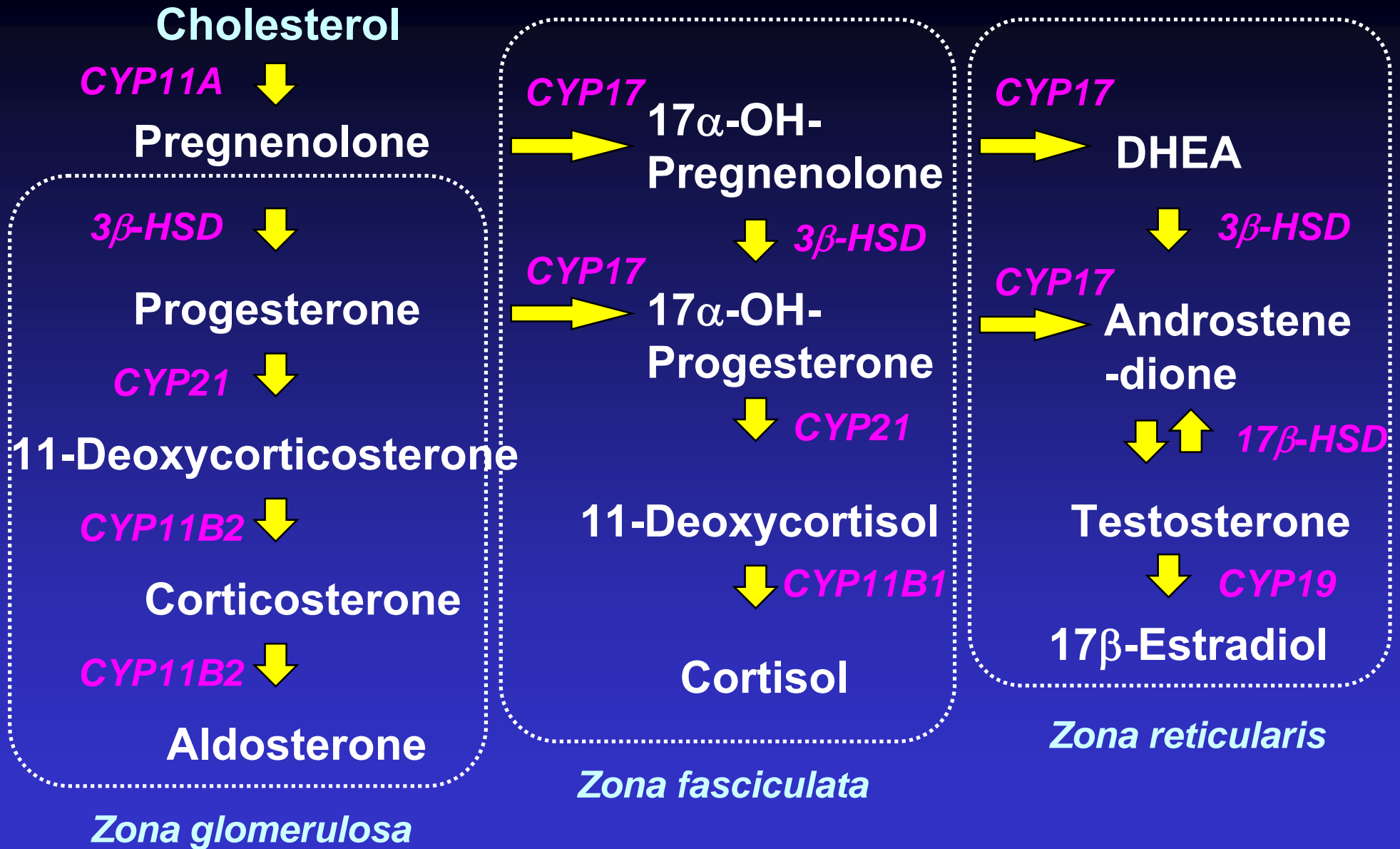


# H295R Cells

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
  - measure amount of enzyme present
- **Effects on enzyme concentrations**
  - measure catalytic activities: selective substrates
- **Effects on metabolism of steroid hormones**
  - measure steroid hormone concentrations



# Objectives

- **Develop methods to screen for effects on steroidogenic enzymes**
- **Develop and optimize a rapid screening test to determine effects of chemicals on sex steroid synthesis:**
  - Progesterone
  - Testosterone
  - 17 $\beta$ -estradiol
- **Demonstrate the performance of the assay with known inhibitors and inducers of steroidogenic pathways**



# Objectives (Cont.)

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
  - $3\beta$ HSD &  $17\beta$ -HSD
  - P450C17
4. Androgen conversion to estrogen by CYP19 aromatase



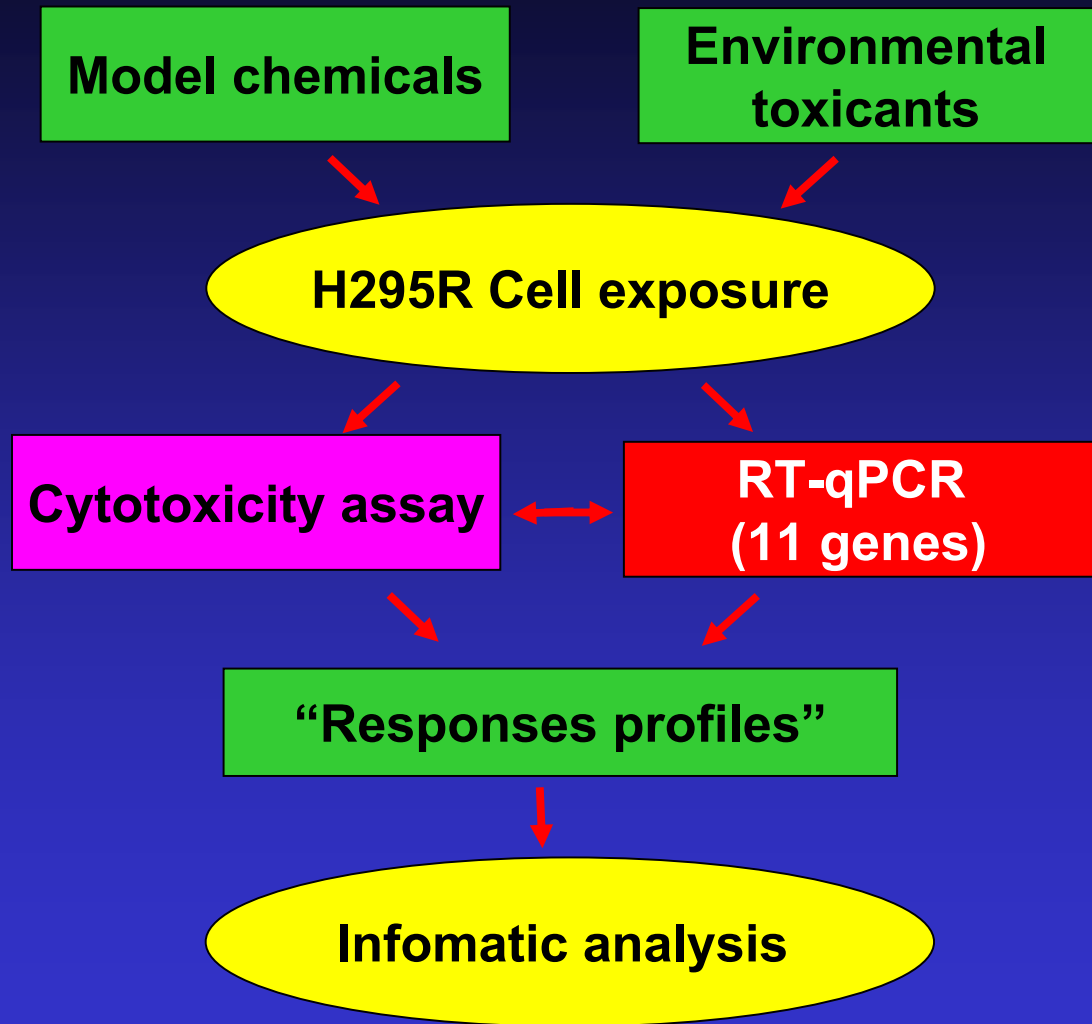
# Objectives (Cont)

- **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.**





# OVERALL APPROACH



Model chemicals: inhibitors & Inducers

Incubation time: 48 h

Gene will be monitored in dose range without causing cell death

The responses profiles will be used in infomatic analyses of the effects of the compounds screened.



# Real-time Reporters

To indicate the accumulation of PCR products

## Non-specific DNA binding dyes

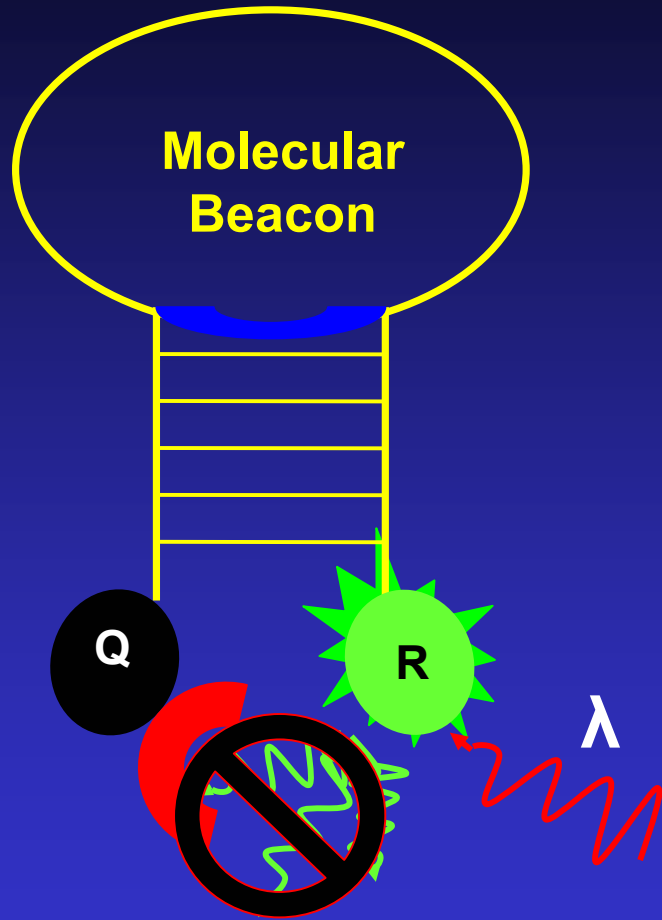
- 1) Ethidium Bromide (first reported by Higuchi, 1993)
- 2) SYBR® Green I

## Specific Hybridization Probes

- 1) TaqMan probes
- 2) Molecular beacons \*
- 3) others (dual-oligo FRET pairs)



# Molecular Beacons

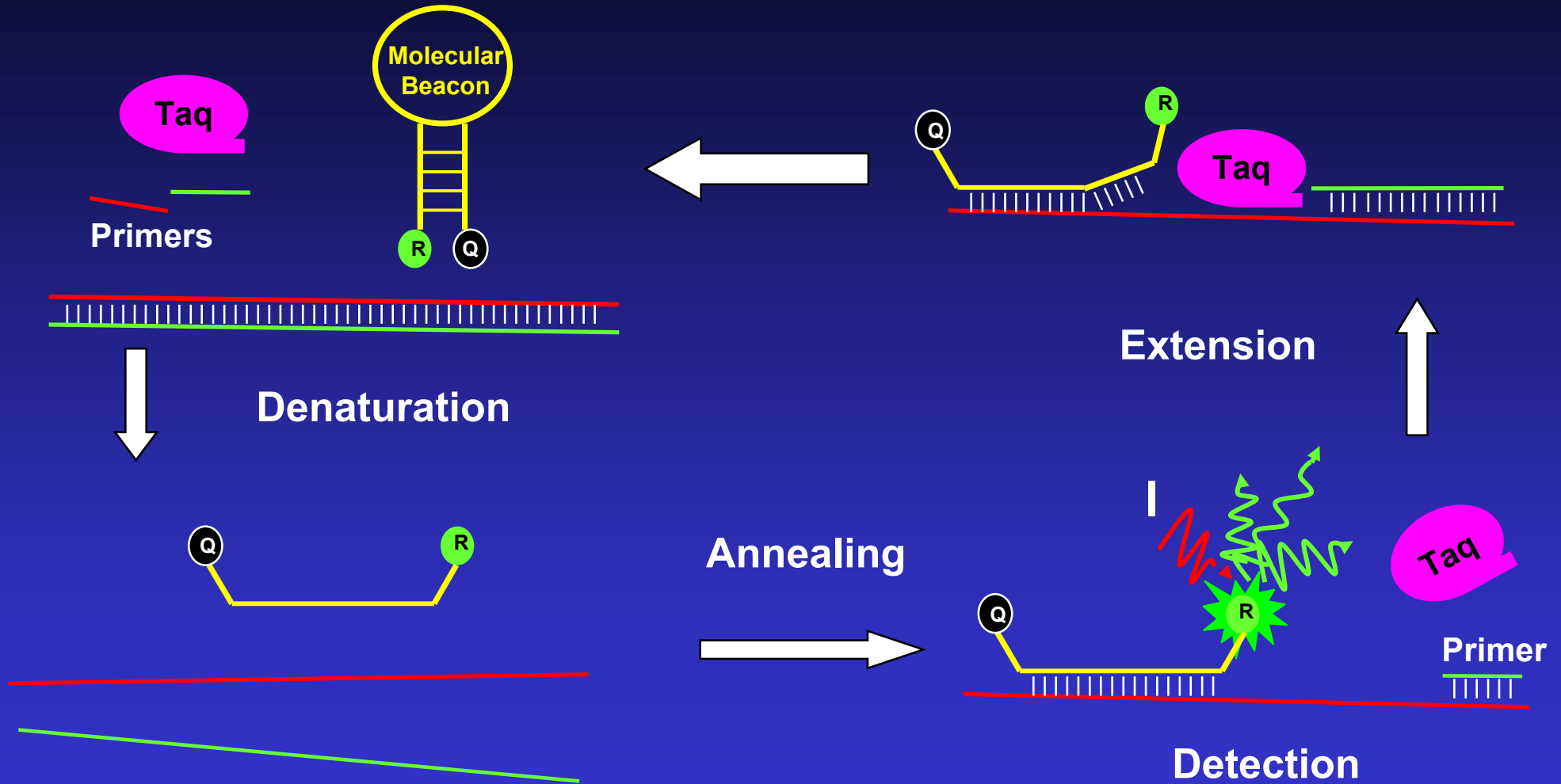


Reporters	Quenchers
FAM	DABCYL
TAMRA	BHQ-0
TET	BHQ-1
ROX	BHQ-2

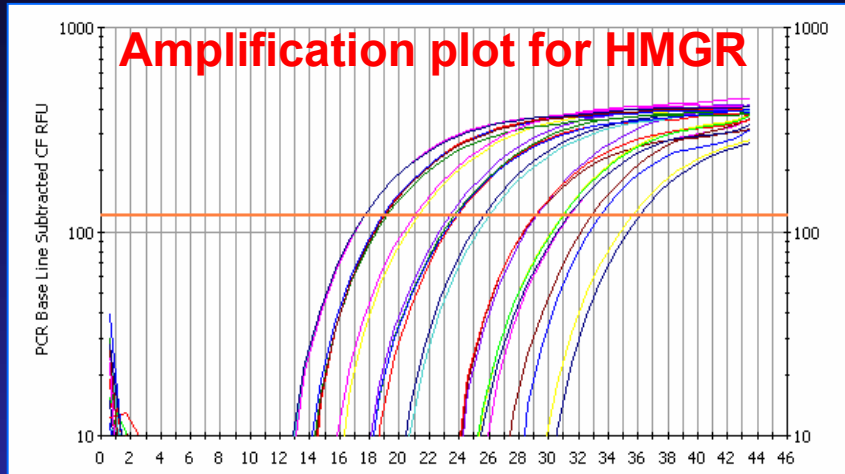
## Fluorescence Resonance Energy Transfer (FRET)



# Molecular Beacons



# Example of duplex PCR



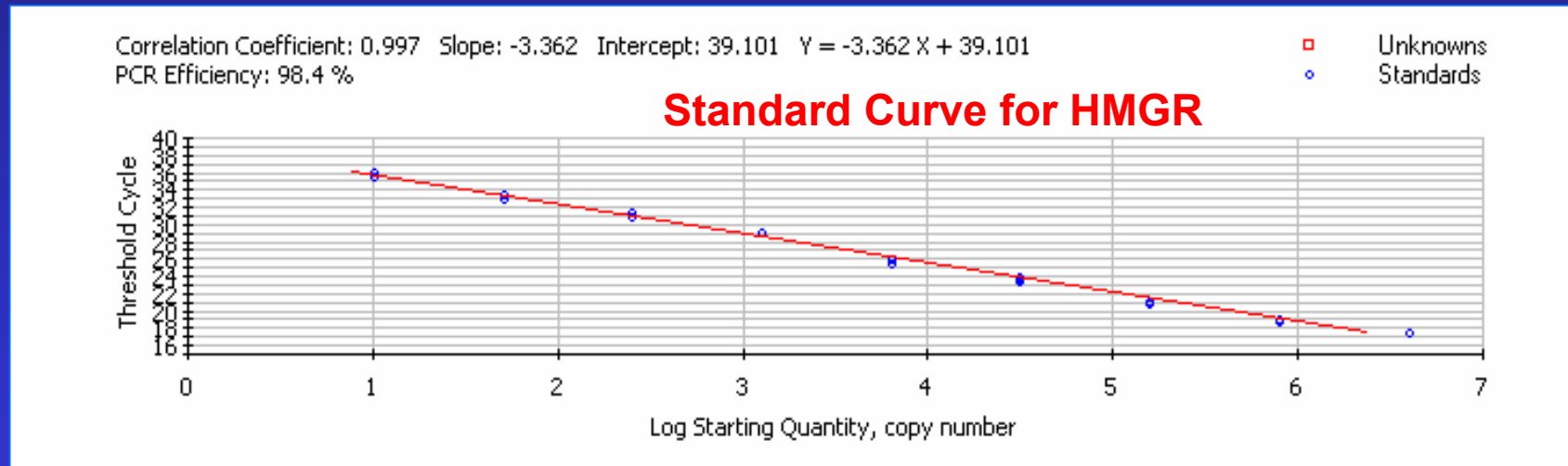
**HMGR +  $\beta$ -actin:**  
**HMGR:**

Diluted in a 5-fold manner

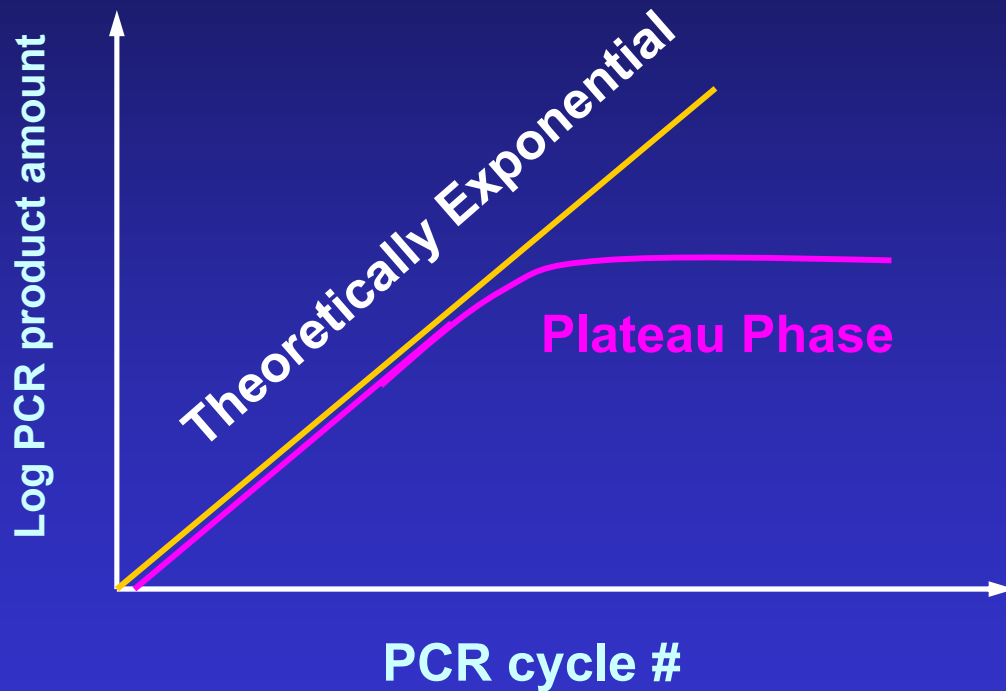
**$\beta$ -actin:**

Fixed at a concentration

Triplicate analyses



# Benefits of RT PCR (1)



Due to:

- Inhibitors of the polymerase
- Reagent limitation (primers)

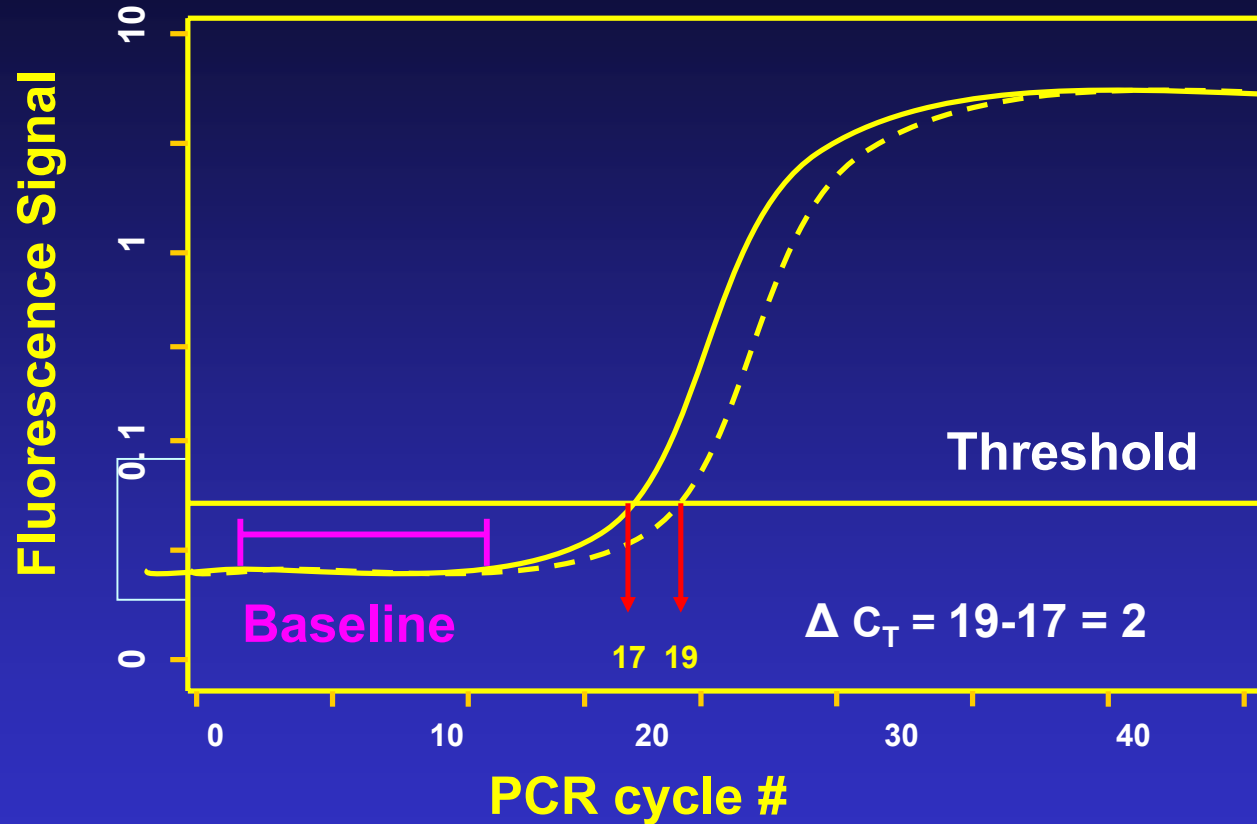
Eventually, “Plateau Phase”

To provide a more accurate and precise measurement, it is necessary to collect quantitative data in the exponential phase of amplification



# Benefits of RT PCR (2)

By the reliable detection of products generated during each cycle of PCR, Real-Time PCR allows us to analyze reactions during exponential phase



**More specifically, sensitively, and reproducibly**



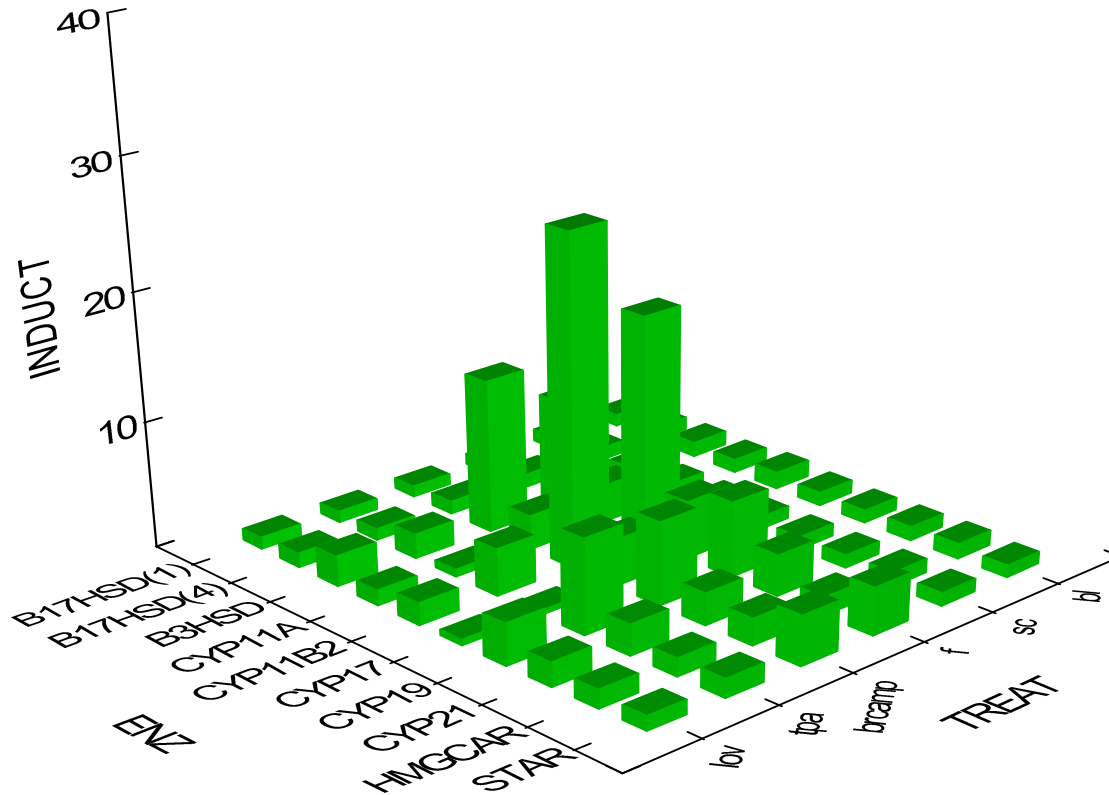
# Inducers of Steroidogenic Genes

Gene	Inducer	Reference	Notes
CYP11A	cAMP (cyclic adenosine monophosphate)	Payne et al., 1992	Not specific (3- $\beta$ HSD,CYP17); Cell testing
CYP11B1	ACTH	Vallée et al., 1995	Not specific (CYP11B1, CYP 17); Cell testing
CYP11B2	Angiotensin II	Holland et al., 1993	Not specific (CYP11A, STAR, CYP21, 3 $\beta$ HSD) (H295R)
CYP19	Prostaglandin (PG) E <sub>2</sub>	Zhao et al., 1997	Cell testing; increases intracellular cAMP levels
	8Br-cAMP, Vinclozolin	Sanderson et al., 2000	Cell testing
CYP21	forskolin	Vallée et al., 1995	Not specific ( CYP11A, CYP17, STAR, CYP11B1/ B2, 3 $\beta$ HSD); Cell testing
3 - $\beta$ HSD	dexamethasone	Feltus et al., 2002	Not specific (CYP19, StAR) (H295R)
StAR	MG132	Tajima et al., 2001	Proteasome inhibitor





# Exposure Results



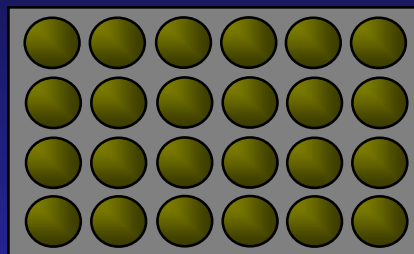
# H295R Methods to Measure Effects on Hormone Production

Cells cultured in Flask  
Renew media 2-3 x weekly  
Split cell when ~90% confluent



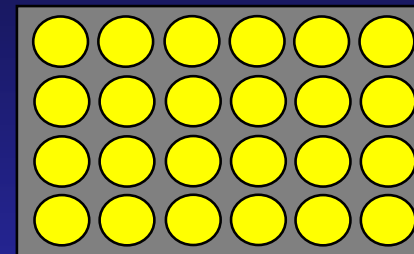
Trypsinize  
Seed Plate

Incubated for  
24 hours

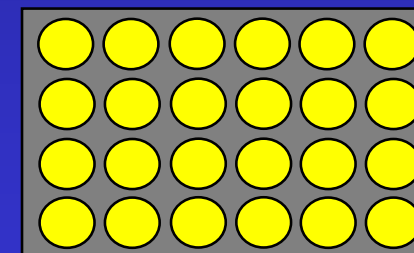


Replace  
Media

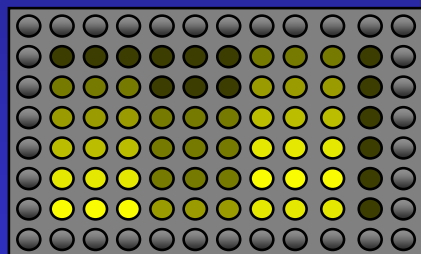
Dose Cells



Incubate  
For 48 hrs



Analyze for Hormone



ELISA, RIA, LC/MS

Extract Medium  
with ether

Collect Cells

Freeze in Liquid N<sub>2</sub>

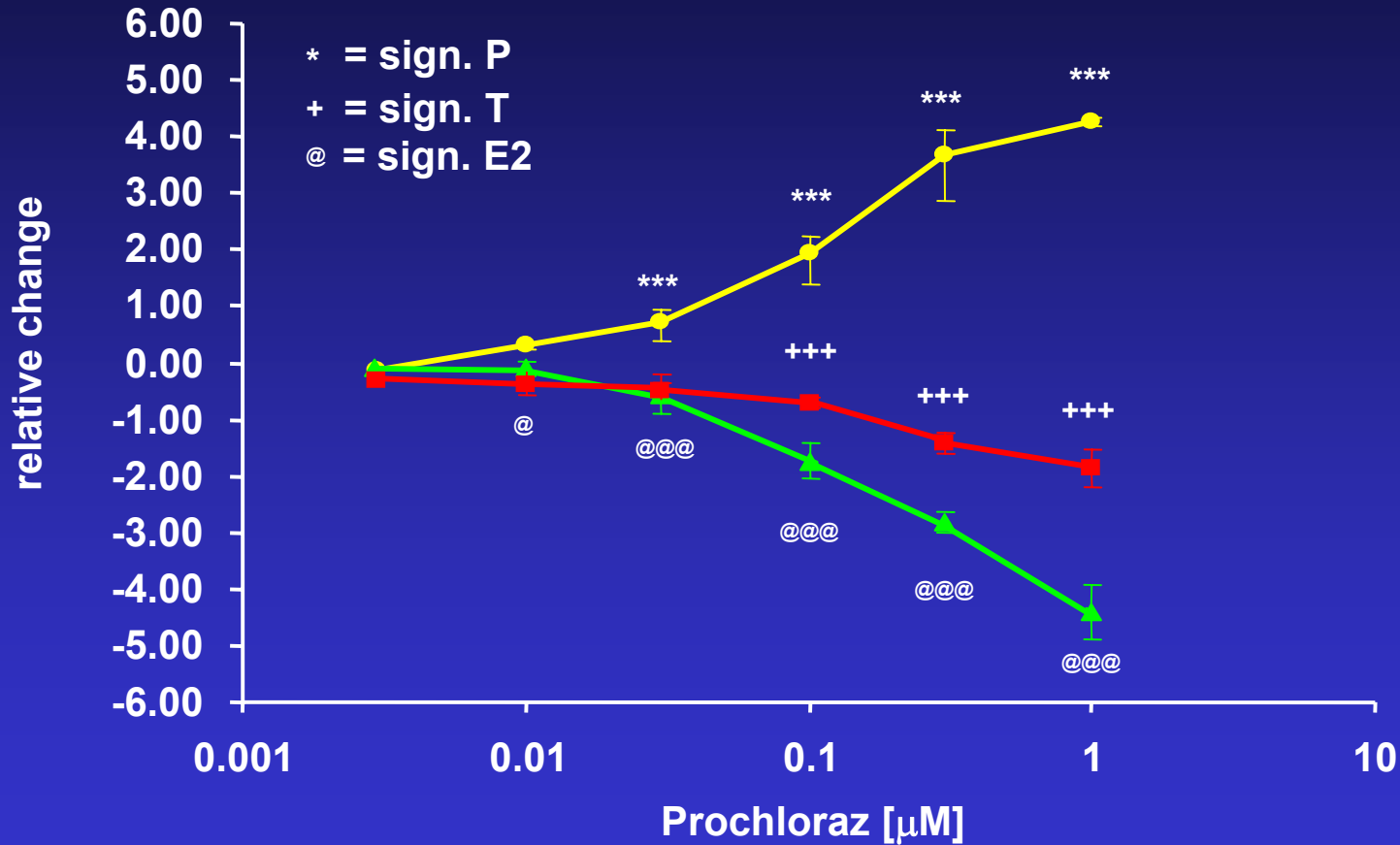
(gene expression, enzyme analysis)



# Model Chemical Exposure

## Prochloraz

● Progesterone    ■ Testosterone    ▲ Estradiol



# 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



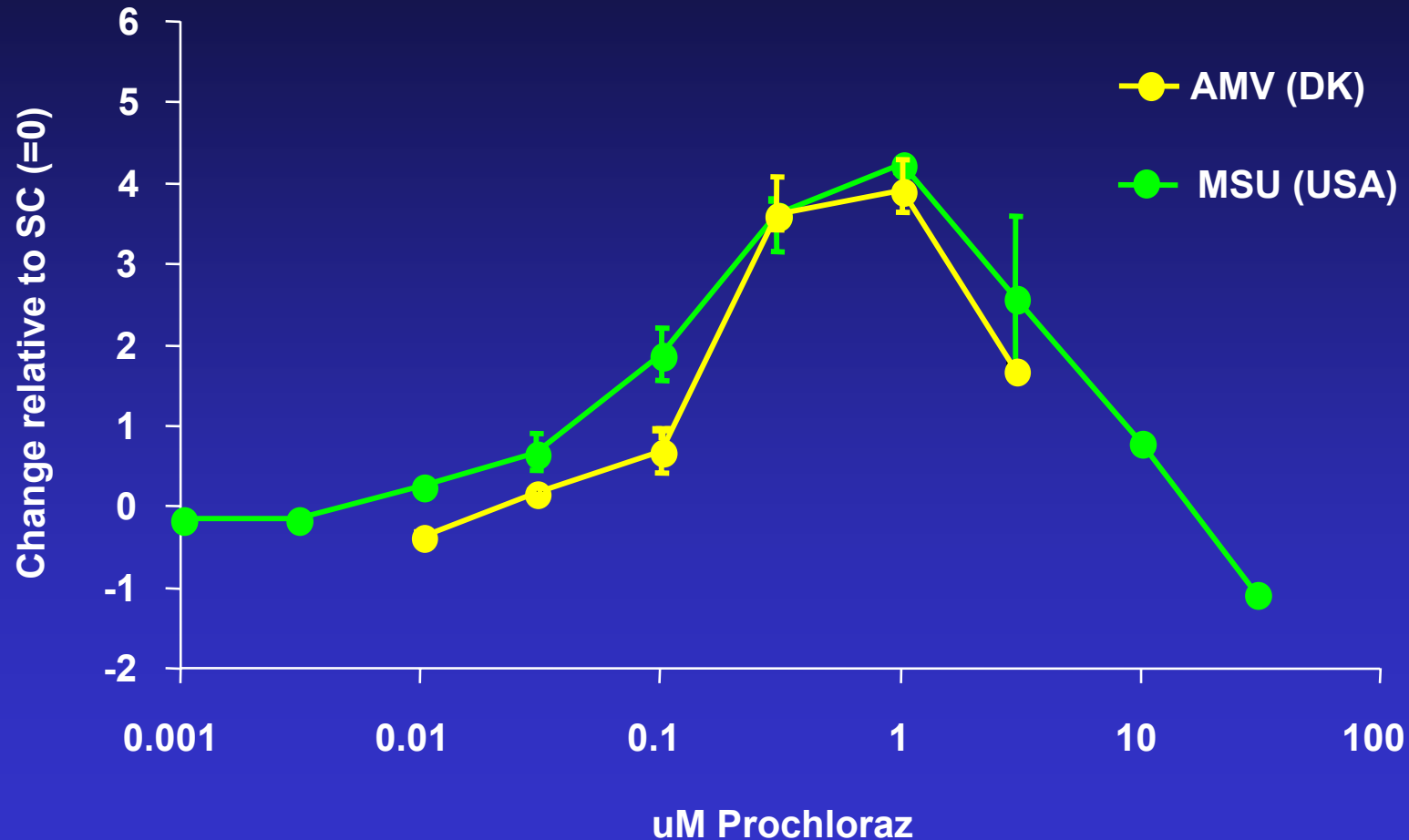
# Inter-laboratory Comparison

Performance based comparison. Use of:

- Same cells
- Different cell culture protocols/conditions
- Same seeding density
- Same acclimation and exposure protocols/conditions
- Different hormone detection methods

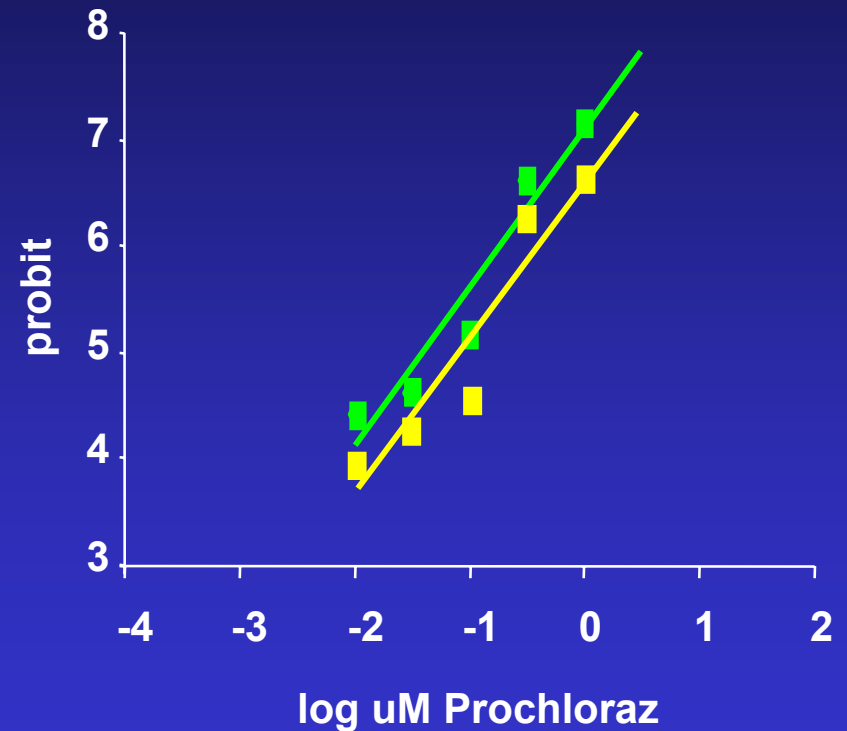


# Preliminary Inter Lab Comparison *Prochloraz (Progesterone)*



# Preliminary Inter Lab Comparison *Prochloraz (Progesterone)*

	MSU	AMV
y	$1.4886x + 7.109$	$1.4696x + 6.6189$
R <sup>2</sup>	0.9398	0.9006
EC25	0.228 mM	0.109 mM
EC50	0.079 mM	0.038 mM



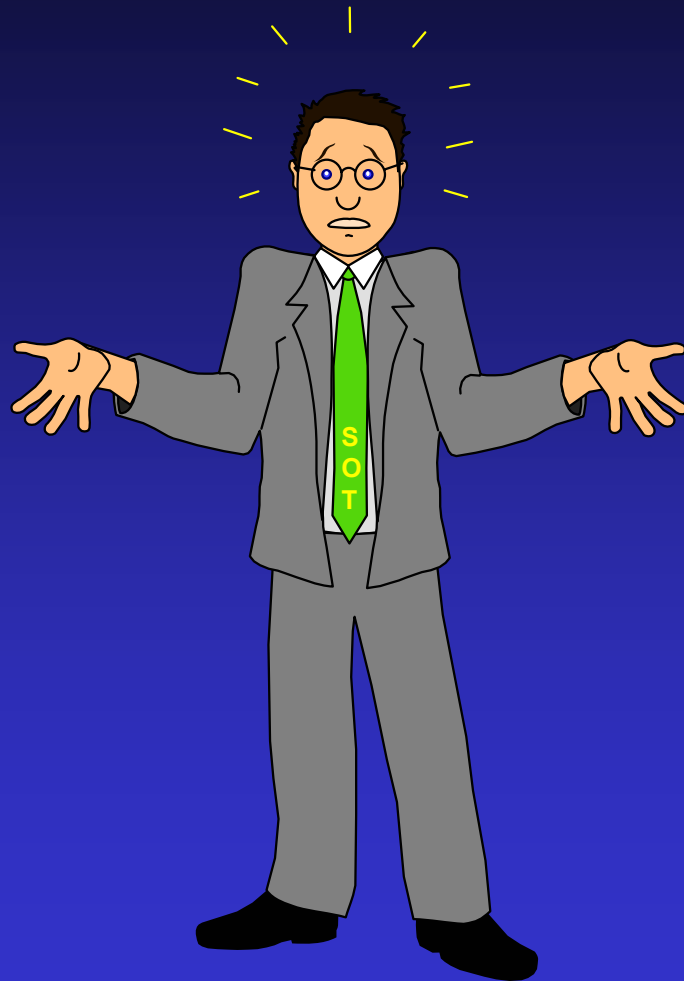
# Future Directions & Upcoming Events

- Present results during 2nd Meeting of the OECD Validation Management Group for Non-Animal Testing: 14-15 December 2005, Paris
- Good chance that this test system will be adopted into the OECD Chemical Testing Guidelines for Endocrine Disrupter Testing and Assessment
- ICCVAM Validation By NICETUM (NTP-NIH)

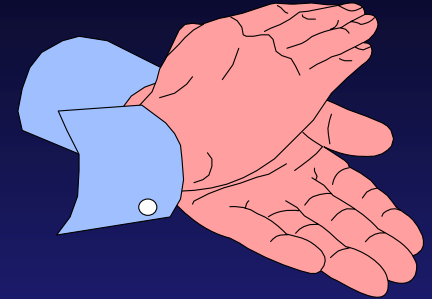




# Questions ????????



# Thank You



- **John P. Giesy**
- **Dept. Zoology**
- **Michigan State University**
- **East Lansing, Michigan, 48824, USA**
- **Tel: (517) 353-2000**
- **Fax: (517) 432-1984**
- **Email: [JGIESY@AOL.COM](mailto:JGIESY@AOL.COM)**
- **Web Site: <http://www.msu.edu/user/giesy>**

