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Perspectives on Development & Use of Screens & Tests for Endocrine Activity

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EPA's Legislative Mandates (August 1996)

- **Food Quality Protection Act (FQPA)**
 - Must screen pesticides (including inerts) for estrogenic effects that may affect human health
 - Must use appropriate validated test systems or other scientifically relevant information
 - Can include other endocrine effects
- **Safe Drinking Water Act (SDWA)**
 - Can screen drinking water contaminants to which substantial numbers of persons are exposed

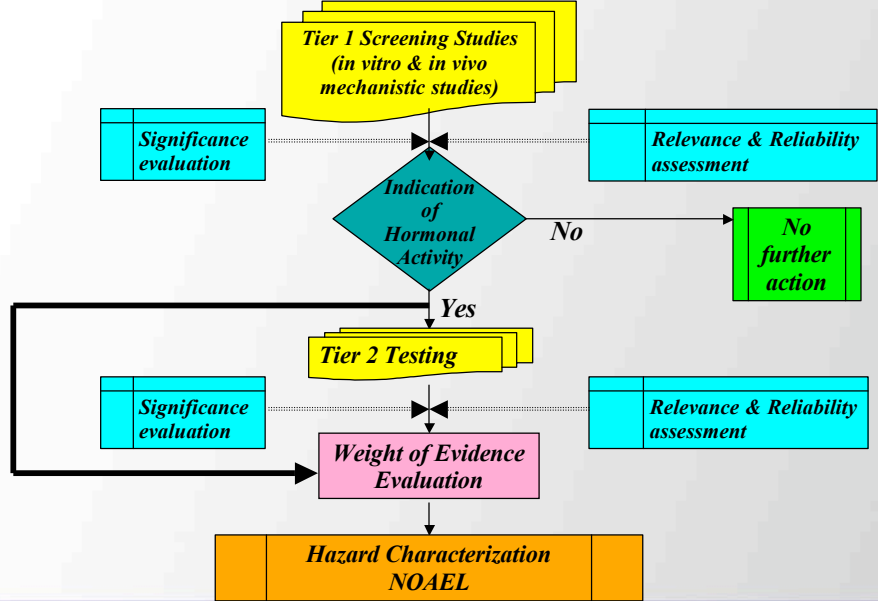
The Endocrine Disrupter Screening Program

- Tier 1 – to identify the potential of chemicals to interact with the estrogen, androgen and thyroid systems
- Tier 2 – to identify and characterize the adverse effects resulting from that interaction and the exposure required to produce them

Tiered Hierarchical Framework for Hormonally Active Substances

1. **Prioritization** of chemicals for screening and testing
2. **Screening assays (must be validated)** to identify substances with hormonal activity & prioritize for more definitive testing
3. **Tests (must be validated)** to characterize dose response of adverse effects mediated by the endocrine system

Hierarchical Approach for Hormonally Active Agents



Tier 1 Screening Assays:

- maximal sensitivity (minimize false negatives)
- range of vertebrate organisms
- detect relevant modes of action
- diverse and complementary endpoints for specific modes of action
- fast and cost-effective

Tier 2 Tests

- Protocols designed to evaluate **adverse health effects**
- Data to characterize nature, incidence, severity and **dose-response relationship of any adverse effects**
- Include **critical life stages, appropriate doses & administer by relevant route**
- **Results supersede screening assay results**
- ‘Gold standard’ – multigeneration reproduction toxicity test

Perspective: ER and AR Binding or Transcriptional Activation Assays

- Need systematic evaluation for inter-lab variability, sensitivity, reproducibility
- Recommend standardization & validation of a single methodology
- A single protocol ‘Gold Standard’ needed by which to compare alternatives & for validation of QSAR
- Validation - multiple labs, identical protocols, reference chemicals, GLP, data interpretation (pass / fail criteria)
- ICCVAM review - finding support this perspective

Perspective: Tier 1 *in vitro* Steroidogenesis Assay

- Confounding by cell toxicity is major limitation
- (Powlin *et al.* 1998) - correctly detected only 4 of 9 substances
- To date - standardization studies have not adequately addressed the cytotoxicity issue, the sliced testis assay validation has been discontinued, work on the H295R human adrenocortical carcinoma cell line continues
- It remains to be shown that the assay would be useful as a routine screen

Perspective: Tier 1 Uterotrophic & Hershberger

- Validation activities in progress via OECD (industry and research institute labs)
- Uterotrophic assay is essentially complete - Peer review finalized, Test Guidelines under development
- Hershberger assay is in last validation phase
- Industry supported chemical repository; approximately _ of the testing effort
- OECD program - will result in guidelines that are internationally harmonized and accepted (therefore there will be **mutual acceptance of data**)

Perspective: Low Priorities

Tier 1 Fish Gonadal Recrudescence Assay

Numerous practical difficulties - Not suitable as Tier 1 screen (endpoints affected by multiple factors that will be difficult to interpret)

Tier 1 Frog Metamorphosis Assay

This assay is **redundant** - other thyroid assays
Problems quantifying tadpole tail resorption; Prometamorphosis protocol under development
Lack of specificity (corticosterone and prolactin)

Perspective: Tier 1 - Pubertal Assays

- Considerable effort has been expended by EPA to assess performance

However --

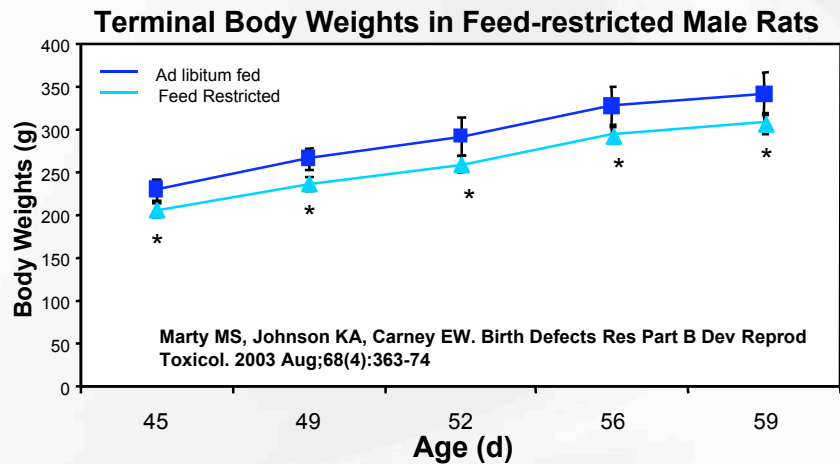
- Endpoints are apical (*i.e.*, can be influenced by systemic toxicological effects unrelated to hormonal activity)
- Dose selection is critical - many physiologic and toxicologic mechanisms can affect pubertal onset. Confounded in MTD is used.
- Inherent variability -- influences interpretation of "small" (< 2 days) changes in age at VP or PPS

Perspective: Tier 1 - Pubertal Assays (continued)

What has not been done & is needed:

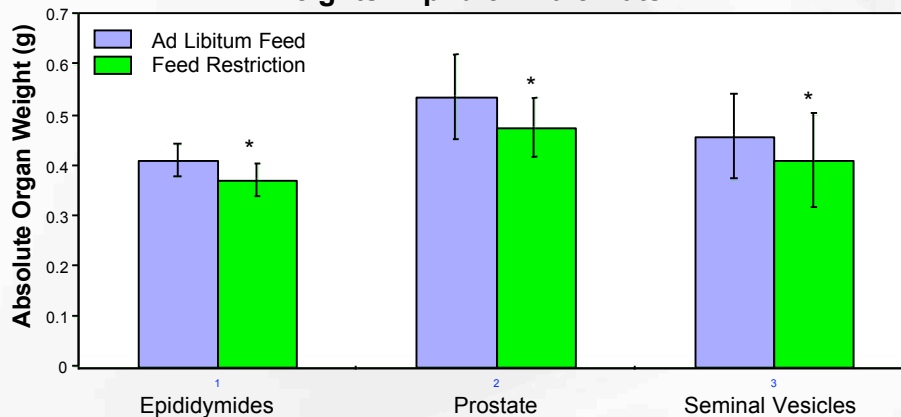
- Demonstration of dose-selection criteria
- “Negative controls” or “non-endocrine active” compounds run according to the dose selection criteria (& at MTD)
 - Are effects seen on these apical endpoints?
 - If so, what value is gained by the pubertals?
- What constitutes a “response” given the inherent variability of the endpoints?

Tier 1 - Pubertal Assays: Watch out for Confounding - Systemic Toxicity



Tier 1 - Pubertal Assays: Watch out for Confounding - Systemic Toxicity

Weights in pnd 52 Male Rats



Tier 1 Intact Adult Male Assay - Alternative to Pubertals

- Industry - preferred approach that can be incorporated in a Tier 1 battery, utilizes fewer animals and is more cost effective than the EDSTAC-recommended battery
- Systemic evaluation > 20 reference substances representing diverse endocrine mechanisms
 - Good sensitivity and specificity



Comparison of the EDSTAC-Recommended and Alternative Tier 1 Screening Batteries

Recommended Screening Battery	Alternate Screening Battery No. 1	Alternate Screening Battery No. 2
<i>In vitro</i> assays	<i>In vitro</i> assays	<i>In vitro</i> assays
ER binding / transactivation	ER binding / transactivation	ER binding / transactivation
AR binding / transactivation	AR binding / transactivation	AR binding / transactivation
Minced testis assay	Placental Aromatase	Placental Aromatase
<i>In vivo</i> mammalian assays	<i>In vivo</i> mammalian assays	<i>In vivo</i> mammalian assays
Uterotrophic assay (3-day)	Uterotrophic assay (5-day)	Uterotrophic assay (3-day)
Hershberger	Intact adult male	Pubertal male
Pubertal female		

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Comparison of the EDSTAC-Recommended and Alternative Tier 1 Screening Batteries

Assays	No. Animals	Median costs	Recommended T1S Battery	Alternative 1 T1S Battery	Alternative 2 T1S Battery
In Vitro					
Estrogen receptor binding		\$6,050	X	X	X
Androgen receptor binding		\$7,500	X	X	X
Steroidogenesis	10	\$6,850	X		
Placental armoatase		\$8,175		X**	
In Vivo					
Uterotrophic (OVX/Oral)***	36	\$14,500	X		
Hershberger	54	\$23,880	X		
Pubertal female	60	\$44,700	X		
Intact male	75	\$44,000		X	
Pubertal male	45	\$67,900			X
Total # of Animals			160	111	81
Median Estimates Total			\$103,480	\$80,225	\$95,950

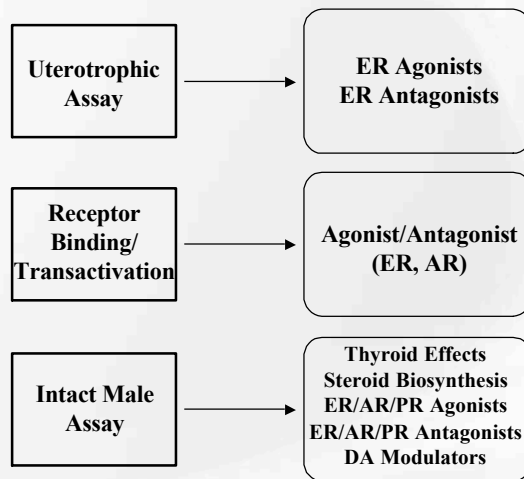
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Estimated Costs of Mammalian Tier 1 Battery

• ER Binding	~\$8,000	
• AR Binding	~\$8,000	
• Steroidogenesis	~\$15,000	
• Aromatase	~\$10,000	
• Uterotrophic	~\$20,000	Total
• Hershberger	~\$25,000	~\$126,000
• Female Pubertal	~\$55,000	
• Male Pubertal	~\$55,000	
• Intact male	~\$65,000	

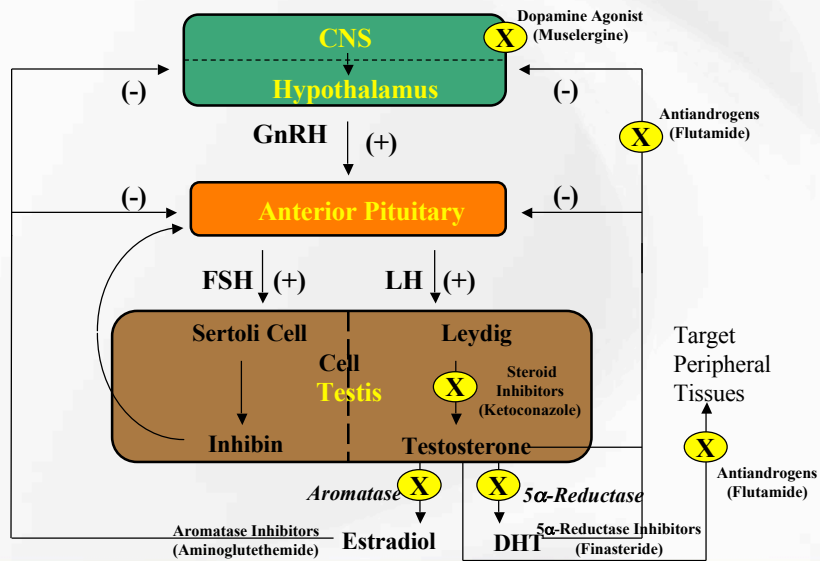
Tier 1 Battery



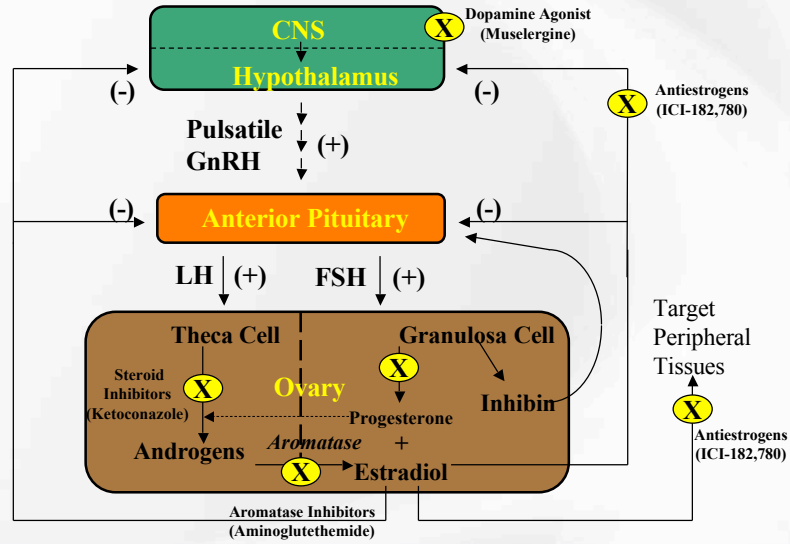
Advantages: Intact Male Assay

- Comprehensive mode-of-action screen
 - Capable of screening multiple modes of action (E, A, T)
 - Run in parallel: uterotrophic assay & *in vitro* binding assays
 - Redundancy of endpoints - focus on mode of action
 - Tier 1 - this assay provides mechanistic info that focuses direction of any further testing
- Design allows integration of new endpoints
 - Intact endocrine system
- Cost effective because it integrates
 - Intact male assay replaces three assays in EDSTAC battery
 - Uses fewer animals than EDSTAC battery

Hypothalamic-Pituitary-Testis Axis



Hypothalamic-Pituitary-Ovary Axis



Intact Male Assay: Fingerprint of Unknowns

Endocrine Activity	ASG Unit (% body weight)	Thyroid (% body weight)	Test. (ng/ml)	E2 (pg/ml)	PRL (ng/ml)	LH & FSH (ng/ml)	TSH (ng/ml)	T _d (µg/dl)
Receptor Agonists								
Estrogen Receptor	-/↓	-	↓	-/↓	↑	-/↓	-	-
Androgen Receptor	↑	-	↓	↓	↑	↓	-	-
Progesterone Receptor	↓	-	↓	↑	↓	↓	-	-
Receptor Antagonists								
Estrogen Receptor	-	-	-	-	-	-/↑	-	-
Androgen Receptor	-/↓	-	-/↑	↑	-	↑	-	-
Progesterone Receptor	↓	-	-	-	-	-/↑	-	-
Steroid Biosynthesis								
Testosterone Synth. Inhib.	↓	-	↓	-	-	↑	-	-
5α-Reductase	↓	-	-	-	↑	-/↑	-	-
Aromatase	↑	-	-	↓	-	-/↑	-	-
Thyroid-Active Compounds	-	↑	-/↓	-	-/↑	-	↑	↓
Prolactin Pathway								
D-2 Receptor Modulators	-	-	↓	-	-	-/↑	-	-
D-2 Agonist / Antagonist	-	-	-	-	↑	-	-	-
Broad Catechol Depletor	↑	-	↓	↓	-	↓	↓	↓



Substances Examined: 15 Day Intact Male Assay

Intact Male	Endocrine Active Compound	Endocrine Activity
+	17 β -Estradiol	Estrogen receptor agonist (full or potent)
+	Coumestrol	Estrogen receptor agonist (weak or partial)
+	ICI-182,780	Estrogen receptor antagonist
+	Testosterone	Androgen receptor agonist
+	Flutamide	Androgen receptor antagonist (full or potent)
+	<i>p,p'</i> -DDE	Androgen receptor antagonist (weak or partial)
+	Vinclozolin	Androgen receptor antagonist (weak or partial)
+	Cyproterone Acetate	Androgen receptor antagonist (weak or partial)
+	Linuron	Androgen receptor antagonist (weak or partial)
+	Progesterone	Progesterone receptor agonist
+	Mifepristone (RU 486)	Progesterone receptor antagonist
-	Apomorphine	D ₂ receptor agonist
+	Haloperidol	D ₂ receptor antagonist
+	Reserpine	Dopamine depletor (broad catecholamine depletion)
+	Phenobarbital	Thyroid hormone excretion enhancer
+	Oxazepam	Thyroid hormone excretion enhancer
+	Propylthiouracil	Thyroid hormone synthesis inhibitor
+	Propylimidazole-2-thione (PTI)	Thyroid hormone synthesis inhibitor
+	Finasteride	5 α -Reductase inhibitor
+	Ketoconazole	Testosterone biosynthesis inhibitor
+	Di-n-butyl phthalate	Steroid biosynthesis inhibitor (???)
+	Anastrozole	Aromatase inhibitor
+	Fedrozole	Aromatase inhibitor
+	Ammonium Perfluorooctanoate	Aromatase inhibitor

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Perspective: Alternative Battery 1 is Superior

Recommended Screening Battery	Alternate Screening Battery No. 1	Alternate Screening Battery No. 2
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Purpose of Tier 2 Tests: Reminder

- Protocols designed to evaluate adverse health effects
- Data to characterize nature, incidence, severity and dose-response relationship of any adverse effects
- Include critical life stages, appropriate doses & administer by relevant route
- Results supersede screening assay results
- ‘Gold standard’ – multigeneration reproduction toxicity test

Overview: 2-Gen Rat Repro Study

- Current guideline protocol considered the definitive mammalian reproduction study for human health risk assessment
- Guideline was recently revised -- extensively -- to increase sensitivity to detect effects mediated by the endocrine system
 - sperm parameters, estrous cycling, developmental markers, more extensive parental histopathology, brain, spleen and thymus weights of weanlings, oocyte counting
- 5 to 8 years to revise and harmonize internationally
1998:U.S. EPA 2001:OECD, Japan
- A globally harmonized protocol is critical:
 - Animals – Mutual Acceptance of Data
 - Resources – Avoid ambiguous status

Overview: 2-Gen Rat Repro Study

Additional endpoints have been suggested,

However -

- Any significant modifications must take into account international harmonization -- any changes to the guideline will affect it globally
- Already a complex study – need to consider logistics and value of new endpoints

Overview: 2-Gen Rat Repro Study

- Some modifications could be demonstrated and validated in a relatively short period of time, examples:
 - enhanced clinical observations
 - thyroid weight & histopathology
 - whole brain weight and brain histology
- Major modifications – would be problematic – EPA has considered many suggestions for changes, including increasing number of retained pups

Perspective: 2-Gen Rat Repro Study

- The existing multigenerational study is sensitive and effectively detects potential endocrine mediated adverse effects, including those that occur by estrogenic, androgenic, and thyroidogenic mechanisms, even those that occur at relatively low incidence.
- Recall that this is a multi-dose study (3 doses + control) and that the guidelines require the highest dose tested to induce some reproductive and/or systemic toxicity but not death or severe suffering
- Apical endpoints, adverse effects and dose response from Tier 2 are used for risk assessment
 - Tier1 assays do not evaluate adversity
 - Tier 2 results supersede Tier 1

EPA is asking: Should a myriad of additional procedures and endpoints be added to & required in the 2-Gen?

A number of these proposed additional endpoints appear to be unnecessarily **redundant**

- If considered, each new measurement should be demonstrated to add value by increasing sensitivity, specificity, or reliability without impairing ability to assess endpoints currently required

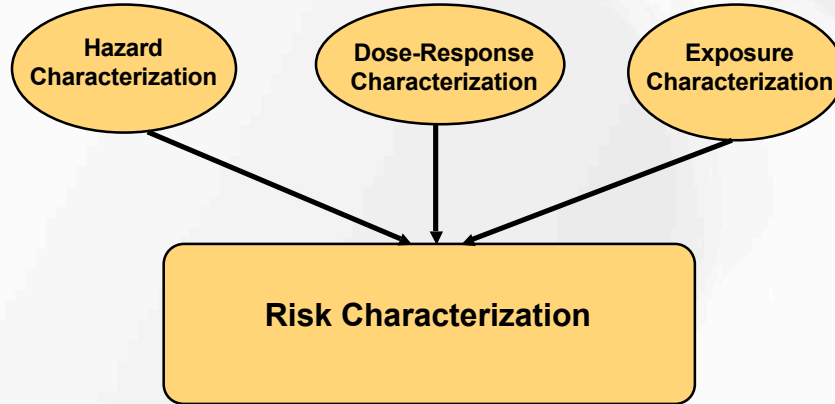
Concerns: Other Tier 2 Non-Mammalian Tests

- Fish - intermediate tier needed
- Fish - lack of specificity of endpoints (FFLC)
- Avian - inadequate triggers
- Mysid - inappropriate species - need to show plausible biological mechanism (such as estrogen specific receptor) otherwise inappropriate triggering criteria for the test

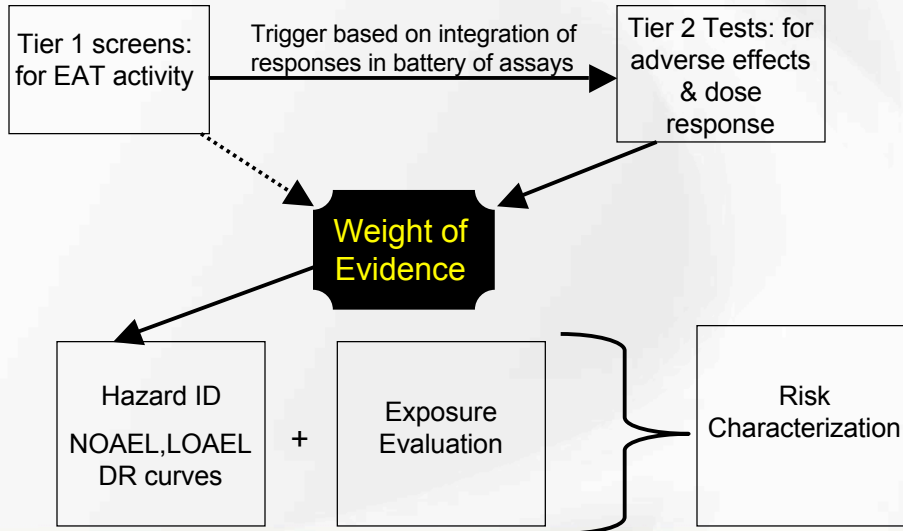
Hazard \neq Risk

- Screening assay data – insufficient for risk characterization
- Risk characterization requires integration of testing data & exposure assessment:
 - Understanding of the hazard data endpoints & inferences/ relevance to humans
 - Dose-response
 - Exposure
- For example -- cannot assess risk with only RBA, uterotrophic/Hershberger

Risk Assessment



Endocrine Screening & Testing



Hazard Identification & Characterization *Weight of Evidence Approach*

Integration- relevant studies- assessment of overall adequacy and concordance of the data set:

- longer-term **definitive tests outweigh** or supercede **screening assays**
- **higher quality studies** are given **more weight** than lower quality studies
- substances with **inadequate data** sets – are candidates for **additional evaluation** (case specific screening or testing)
- **lack of concordance** – base decisions on **preponderance of available data**
- **adequate & concordant data sets** - no additional testing, proceed to **risk assessment (& integrate exposure)** then risk management processes as appropriate

Implementation Timeline

- **Late 2007:** Mammalian screens (and possibly the wildlife screens) ready for use.
 - **Before this can occur, OMB must approve the program**
- **Late 2009/early 2010:** Tests validated



Priority Setting for Chemicals

- **EPA will require chemicals to be screened in batches**
- **First batch of chemicals – Strategy released in 3Q 2005 (Fed. Reg. Sept. 27)**
 - Prioritization and first list of chemicals based on exposure, not suspected endocrine disruption
 - Inert exposure based on HPV status (obviously not HPV as an inert)



EPA's Current Approach to Priority Setting

- First list of compounds to be tested is planned to be released approximately 1 year before screens are ready
 - Letter to NRDC suggested 1Q 2005
 - Will have a comment period
- Requirement for screens (and tests)
 - Approximately 4Q 2006

How Will Endocrine Testing Affect Inerts?

- EPA is developing data compensation and confidentiality rule, but little progress to date
- First list targeted for 50-100 pesticides and inerts
 - no decision on the split between pesticides and inerts
 - List likely to be out more than 1 year before anything can be done
- Important for inert and pesticide suppliers to work together for the good of both industries
 - Tier 1 screening studies estimated at ~\$250K/chemical. Tier 2 tests about \$1 million/chemical – protocols not well developed enough to predict accurately

Issues with Screening “Today”

- What kind of screening is supposedly being done by some now?
 - Literature
 - Modeling
 - *In vitro* / *in vivo*
- Studies are not validated
- Relevance has not been established
- Apical endpoints are not likely to establish mechanism