APPLIED PHARMACOLOGY AND TOXICOLOGY, INC.

Consulting & Research Services

Are Key Characteristics Valid for Identifying Endocrine Hazards?

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Current Consensus Approach for EDCs: Weight of Evidence (WoE)

- Developed from a long tradition of WoE approaches used in:
  - Clinical medicine
  - Chemical carcinogenesis
  - Genetic toxicology
  - Reproductive toxicology
  - Developmental toxicology
  - Ecotoxicology

- Important elements of a WoE analysis:
  - Process for literature / data search
  - Process for data quality evaluation
  - Criteria for data selection
    - Criteria for judging relevance of data
    - Criteria for judging strength of results
    - Process of integration of information
  - Guidelines and criteria for reaching conclusions

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Criticisms of the WoE Approach

From a Scientific Perspective, Some Have Said:

- Evaluation of cause-effect between an endocrine MoA and an adverse effect not rigorous enough.
- Relies on judgement, not definitive evidence; subjective, not objective.
- No way to determine whether the result is correct or incorrect.

From a Precautionary Perspective, Some Have Said:

- Requires too much evidence to link an adverse effect to an endocrine MoA.
- Conclusions may fail to identify many EDCs and so will not be protective.
- Resource-intensive and slow; will assess too few chemicals over long time-frame.

What is the “Key Characteristic” (KC) Concept / Approach ?

- KC Concept: chemicals that produce certain types of adverse effects exhibit common “key characteristics” that can be used as distinguishing features.
- Examples
  1. Carcinogens (Smith et al., 2017)
  2. Male reproductive toxicants (Luderer et al., 2019)
  3. Female reproductive toxicants (Arzuaga et al., 2019)
  4. Endocrine Disruptors (La Merrill et al., 2020)

- Concept formulated during ”expert workshops” of selected participants
  - (e.g., La Merrill et al. 2020)
- Concepts applied in recent publications
  - Agrochemicals
  - Glyphosate
  - Etc. . . . see bibliography

Publications on the KC Approach Through December 2020

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13. Smith MT, Guyton KZ, Kleinstreuer N, Borrel A, Cardenas A, Chiu WA, Felsher DW, Gibbons CF, Goodson WH, Houck KA, Kane A, La Merrill MA, Lebrec H, Lowe L, McHale CM, Minocherhomji S, Rieswijk L, Sandy MS, Sone H, Wang A, Zhang L, Zeise L, Fielden M. 2020. The Key Characteristics of Carcinogens: Relationship to the Hallmarks of Cancer, Relevant Biomarkers, and Assays to Measure Them. *Cancer Epidemiol Biomarkers Prev*.
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Why Evaluate Whether the KC Approach is Valid ?

A. The approach is used instead of a rigorous weight of evidence evaluation.

"The KC [key characteristics] approach is a way to start organizing evidence so that it can be more readily evaluated by experts in the field to judge whether the evidence is strong and clear [for reproductive toxicity]" (Gail Prins)

"Another strength of the approach," Prins says, *"is that investigators do not need to know the mechanism of action. "For example, if it has been determined that a chemical causes more sperm cells to die, one does not need to know first what triggered that event— what receptor was involved, whether it was direct on the sperm or indirect by first affecting another system that controls sperm formation, et cetera,"* [emphasis added]

B. The approach would appear to have numerous technical flaws . . .

Are “Key Characteristics” Valid for Identifying Endocrine Hazards?

Borgert CJ, Farmer, DR, Freeman E, Klaunig JE, Boyd JW, Burgoon LD, (in preparation)

Factors Necessary for Valid Method (Borgert et al., in preparation)	**La Merrill et al., 2020**
Apply Consensus Definition of EDC adopted by IPCS and WHO ?	No formal definition; appear to define EDC as chemical that exhibits KCs
Empirical validation of endpoints and methods ?	No formal validation. Known positive and negative controls not included in method development.
Evaluation of mechanistic potency and mass action (fundamentals of receptor biology)	Does not consider strength of chemical via endocrine MoA
Evaluated dose-dependency of mechanisms and adverse effects ?	Does not consider whether dose-response for adverse effects is consistent with MoA
Rule out more plausible alternative modes of action ? (e.g., systemic toxicity at doses above kinetic threshold)	Does not consider whether alternative MoAs are more plausible in affecting endpoint
Use standardized interpretive criteria ?	No criteria for evaluating whether a chemical exhibits KC
Apply KCs in a transparent manner ?	No guidance for evaluating KCs; approach said to be flexible according to goals.

Hypothesis-Driven Weight-of-Evidence Framework

Borgert et al., 2011. *Regul Toxicol Pharmacol*. 61: 185-191.

Hypotheses Evaluated in Original EDSP

- ***Estrogen Agonist***
- ***Estrogen Antagonist***
- ***Androgen Agonist***
- ***Androgen Antagonist***
- ***Thyroid Inhibition***
- ***Steroidogenesis***

Methodology Applicable to Any Mode of Action

- Formulate MoA hypothesis
- Identify endpoints relevant to the hypothesis and weight each according to its relevance for evaluating the hypothesis
- Conduct a Systematic Literature Search and Selection for data on the endpoints
- Evaluate strength of response in each endpoint; consider study types and doses
- Formulate WoE determination

Hypothesis-Driven Relevance Ranking of Endpoints

Borgert et al., 2014. *Birth Defects Res & Dev Reprod Toxicol*. 101: 90-113

Rank 1 Endpoints

- *Specific and sensitive for the hypothesis.*
- *Can be interpreted without clarification from other endpoints*
- *Rarely confounded by non-specific activity.*
- *In vivo measurements only, (in vitro rarely identifies a biological effect).*

Rank 2 Endpoints

- *Specific and sensitive for the hypothesis less informative than Rank 1*
- *Often subject to confounding influences or other modes of action.*
- *Include both in vitro and in vivo data.*

Rank 3 Endpoints

- *Relevant for the hypothesis but only as corroboration of Rank 1 and 2.*
- *Not specific for a particular hypothesis / MoA*
- *Include some in vitro and many apical in vivo endpoints*

Estrogen Agonist Hypothesis

Rank 1 Endpoints

- Fish Screening Assay

  Vitellogenin: increased ♂
- Uterotrophic Assay

  Increased uterine weight (wet/blotted)

Estrogen Agonist Hypothesis

Rank 2 Endpoints

- Estrogen Receptor Transactivation Assay
  - Stimulation of estrogen receptor agonism
- Fish Screening Assay
  - Secondary Sexual Characteristics: decreased tubercle score: ♂
  - Gonad Histopathology: ♂
  - Behavior: ♂
- Uterotrophic Assay
  - Conversion to Estrous-Supplemental
- Pubertal Male Assay
  - Testes Weight
  - Testes Histopathology: atrophy

Estrogen Agonist Hypothesis

Rank 2 Endpoints

- Pubertal Female Assay
  - Age & Weight @ vaginal opening
  - Uterus Weight
  - Ovaries Weight
  - Uterus Histopathology
  - Ovary Histopathology
  - Age at first estrous

Estrogen Agonist Hypothesis

Rank 3 Endpoints

- Amphibian Metamorphosis Assay
  - Asynchronous Development
  - Delayed Development
- Estrogen Receptor Binding Assay
  - Competitive binding affinity
- Steroidogenesis Assay
  - Estradiol Levels
- Pubertal Female Assay
  - Growth
  - Estrous Cyclicity
- Pubertal Male Assay
  - Growth
  - Ventral Prostate Weight
  - Epididymides Histopathology

Estrogen Agonist Hypothesis

Rank 3 Endpoints

- Fish Screening Assay
  - Fecundity
  - Estradiol
  - Testosterone
  - Gonad Somatic Index: decreased ♂; increased ♀
  - Behavior: ♀
  - Fertilization Success: ♂ and ♀

Hypothesis-Driven Relevance Ranking of Endpoints

Borgert et al., 2014. *Birth Defects Res & Dev Reprod Toxicol*. 101: 90-113

A PATTERN of endpoints relevant to a specific MoA that are affected by a chemical, and the strength of response in those endpoints, can provide evidence of a potential endocrine MoA.

Weight of Evidence approaches apply such a rationale.

A random set of responses in various endpoints sensitive to various hormones, as results from the Key Characteristic Approach, does not provide evidence of a potential endocrine MoA.

Table1. Estrogen Agonist Hypothesis; Guideline Toxicity Studies					
Rank	Assay	Endpoint(s)	Expected Response	Respond to Styrene	No Response to Styrene
1	FSTRA	Vitellogenin	↑♂		
	Uterotrophic	Uterus Weight (Blotted or Wet)	↑		[21]
	Uterotrophic	Conversion to Estrus	↑		
	ERTA	Reporter Gene Activation	↑		[22]
	FSTRA	Behavior (sexual, mating)	Δ♂		
		Gonad histophathology	Δ♂		
		Tubercle score	↓♂		
	Female Pubertal	Age and body weight at vaginal opening	↓		
		Age at first estrus	↓		
		Ovary histopathology	Δ		
		Ovary weight	↓		
	Male Pubertal	Testis histopathology (atrophy)	Δ		
		Testis weight	↓		

2	Repeat Dose Toxicity	Epididymis histopathology	Δ		[1c,m][15c,r] [18c,r][19s,m,r]
		Epididymis weight	↓		[20n,s,r]
		Mammary histopathology	Δ		[1c,m] [15c,r]
		Ovary histopathology	Δ		[1c,m][2c,r] [15c,r] [18c,r][19s,m,r]
		Ovary weight	↓		[1c,m][2c,r] [15c,r][19s,m,r]
		Prostate weight	↓		
		Seminal vesicle weight	↓		
		Testis histopathology (atrophy)	↑	[3,n,r?] [20s,r↑]	[1c,m][2c,r][11s, m] [15c,r][18c,r] [19s,m,r]
		Testis weight	↓	[3,n,r↑] [9s,r↓]	[1c,m][2c,r] [10s,r] [11s,m] [15c,r] [18c,r][19s,m,r] [20n,s,r]
		Uterus histopathology	Δ		[1c,m][2c,r] [15c,r] [18c,r][19s,m,r]

Table1. Estrogen Agonist Hypothesis; Guideline Toxicity Studies

Rank	Assay	Endpoint(s)	Expected Response	Respond to Styrene	No Response to Styrene
2	Repeat Dose Toxicity	Uterus weight	↑		[2c,r][19s,m]
		Vagina histopathology	Δ		[19s,m,r]
	Developmental Toxicity	Corpora lutea	↓		
		Post-implantation loss	↑	[16m↑,h↑]	[12r,rb]
		Pre-implantation loss	↑		
	Reproductive Toxicity	Anogenital distance	Δ♂,♀		
		Corpora lutea	↓		[4F_1]
		Epididymis histopathology (atrophy)	Δ		
		Epididymis weight	↓		
		Estrous cyclicity	Δ		[4F_0,F_1]
		Fertility	↓♂,♀		[2F_0,F_1,F_2] [4F_0,F_1]
		Gestational length	↓♀		[4F_0,F_1]
		Implantations	↓		[4F_0,F_1]
		Litter size	↓		[2F_0,F_1,F_2] [4F_0,F_1]
		Mammary histopathology	Δ♀,♂		
		Mating index	↓♂,♀		[4F_0,F_1]
		Ovarian follicle count in offspring	Δ		[4F_1]
		Ovary histopathology	Δ		[2F_1,F_2] [4 F_1]
		Ovary weight in offspring	↓		[2F_1,F_2]

	Reproductive Toxicity	Ovarian follicle count in offspring	Δ		[4F_1]
		Ovary histopathology	Δ		[2F_1,F_2] [4 F_1]
		Ovary weight in offspring	↓		[2F_1,F_2]
		Prostate histopathology (atrophy)	Δ		
		Prostate Weight	↓		
		Seminal vesicle weight	↓		
		Sperm count	↓		[4F_0,F_1]
		Testis histopathology (atrophy)	Δ		[2F_1,F_2]
		Testis weight (absolute)	↓		[2F_1,F_2] [4F_2]
		Time to mating	↑♂,♀		[4F_0,F_1]
		Time to preputial separation	↑♂		
		Time to vaginal patency	↓		[5F_2]
		Uterus histopathology	Δ		[2F_1,F_2] [4F_1]

Table1. Estrogen Agonist Hypothesis; Guideline Toxicity Studies

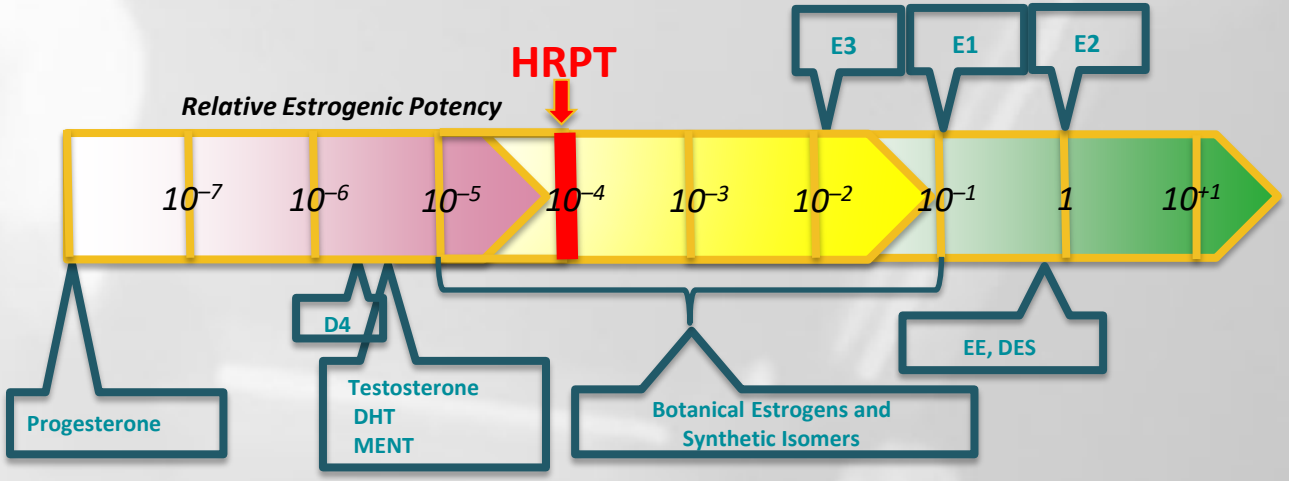
Rank	Assay	Endpoint(s)	Expected Response	Respond to Styrene	No Response to Styrene
2	Reproductive Toxicity	Uterus weight in offspring	↑		[4F$_2$]
		Vagina histopathology	Δ		
3	ERBA	Displacement of Estradiol	↑		[21] [22]
	FSTRA	Behavior	Δ		
		Estradiol level	↓♀		
		Fecundity	↓		
		Fertilization success	↓♀		
		Follicular Atresia	↑		
		Gonad- somatic Index	↓♂, ↑♀		
		Testosterone level	↓♂		
	Female Pubertal	Estrous cyclicity	↑		
		Growth	↑		
	Male Pubertal	Epididymis histopathology	Δ		
		Growth	Δ♂		
		Ventral prostate weight	Δ♂		
	Steroidogenesis	Estradiol level	↑		
	Repeat Dose Toxicity	Gross pathology	Δ♂,♀	[18c,r]	[1c,m] [15c,r]

♂= males; ♀=females; ↑-increase relative to controls; ↓-decrease relative to controls; ?-altered but not as expected. Numbers correspond to numbered citations in Reference List; r =rat; m =mouse; rb-rabbit; s = subchronic; c =chronic; n = non-guideline; F_0=F_0 generation; F_1 = F_1 generation; F_{-2} = F_2 generation; b=bolus; i=infusion; h=Chinese hamster; H=Human

Table1. Estrogen Agonist Hypothesis; Guideline Toxicity Studies					
Rank	Assay	Endpoint(s)	Expected Response	Respond to Styrene	No Response to Styrene
1	FSTRA	Vitellogenin	↑♂		
	Uterotrophic	Uterus Weight (Blotted or Wet)	↑		[21]
	Uterotrophic	Conversion to Estrus	↑		
	ERTA	Reporter Gene Activation	↑		[22]
	FSTRA	Behavior (sexual, mating)	Δ♂		
		Gonad histophathology	Δ♂		
		Tubercle score	↓♂		
	Female Pubertal	Age and body weight at vaginal opening	↓		
		Age at first estrus	↓		
		Ovary histopathology	Δ		
		Ovary weight	↓		
	Male Pubertal	Testis histopathology (atrophy)	Δ		
		Testis weight	↓		

Figure 1: Human-Relevant Potency-Threshold (HRPT) for the ERα-Agonist MoA

Borgert CJ, Matthews JC, Baker SP (2018) *Archives of Toxicology, 92: 1685-1702.*



Receptor Occupancy Calculations

D4 vs 17β-Estradiol

at Human Serum Levels

D4 Concentration	Endogenous Ligand	Endogenous Ligand Concentration	Endogenous Ligand Receptor Occupancy	D4 Receptor Occupancy
1.00E-06	0	0.00E+00	0.0000%	2.2000%
1.00E-05	0	0.00E+00	0.0000%	18.5000%
0.00E+00	E2	7.34E-10	76.9000%	0.0000%
0.00E+00	E2	1.84E-09	89.3000%	0.0000%
1.00E-06	E2	7.34E-10	76.5000%	0.5000%
1.00E-05	E2	7.34E-10	73.1000%	5.0000%
1.00E-06	E2	1.84E-09	89.1000%	0.2000%
1.00E-05	E2	1.84E-09	87.2000%	2.4000%

Receptor Occupancy Calculations

D4 vs Individual Endogenous Ligands

at Human Serum Levels

D4 Concentration	Endogenous Ligand	Endogenous Ligand Concentration	Endogenous Ligand Receptor Occupancy	D4 Receptor Occupancy
0.00E+00	ADIOL	1.10E-09	19.6000%	0.0000%
1.00E-06	ADIOL	1.10E-09	19.3000%	1.8000%
1.00E-05	ADIOL	1.10E-09	16.6000%	15.4000%
0.00E+00	ADIOL	1.80E-09	28.6000%	0.0000%
1.00E-06	ADIOL	1.80E-09	28.1000%	1.6000%
1.00E-05	ADIOL	1.80E-09	24.6000%	14.0000%
0.00E+00	DHEA	7.00E-09	0.6000%	0.0000%
1.00E-06	DHEA	7.00E-09	0.6000%	2.2000%
1.00E-05	DHEA	7.00E-09	0.5000%	18.4200%
0.00E+00	DHEA	1.90E-08	1.7000%	0.0000%
1.00E-06	DHEA	1.90E-08	1.6600%	2.2000%
1.00E-05	DHEA	1.90E-08	1.3900%	18.3000%

Receptor Occupancy Calculations
D4 vs Human Physiological Estrogens:
E1, E2, E3, Adiol, DHEA, DHEAS

D4 Concentration	Endogenous Ligand	Endogenous Ligand Receptor Occupancy	D4 Receptor Occupancy
1.00E-06	0	0.0000%	2.2000%
1.00E-05	0	0.0000%	18.5000%
0.00E+00	Mixture -- Mid-point Concentrations	94.6000%	0.0000%
1.00E-06	Mixture -- Mid-point Concentrations	94.5000%	0.1000%
1.00E-05	Mixture -- Mid-point Concentrations	93.4000%	1.2000%
0.00E+00	Mixture -- Minimal Concentrations	48.1000%	0.0000%
1.00E-06	Mixture -- Minimal Concentrations	47.5000%	0.6000%
1.00E-05	Mixture -- Minimal Concentrations	43.0000%	5.1000%

Conclusions

The KCs for EDCs were developed based on KCs for carcinogens.

Flaws in the KC approach for carcinogens have not been addressed or corrected and were repeated in the KCs for EDCs.

The KC approach requires less data and fewer resources than the WoE approach but lacks the basic elements of rigor and reproducibility that should be a standard requirement for regulatory science.

U.S. House of Representatives, Subcommittee on Energy and Environment, Committee on Energy and Commerce. 2010. Endocrine Disrupting Chemicals in Drinking Water: Risks to Human Health and the Environment. Available: http://energycommerce.house.gov/hearings/hearingdetail.

It is necessary to optimize both scientific rigor and timely evaluations;

- **Increasing speed at the expense of rigor, as the KC approach does, is not useful.**