New and existing biodetectors at BioDetection Systems; state-of-the-art

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Contents

- **Intro basic CALUX Quantitative High Throughput Assays**
- **Applications in safety assessment**
- **Specific requirements for different applications**
- **Identification of nutraceuticals**

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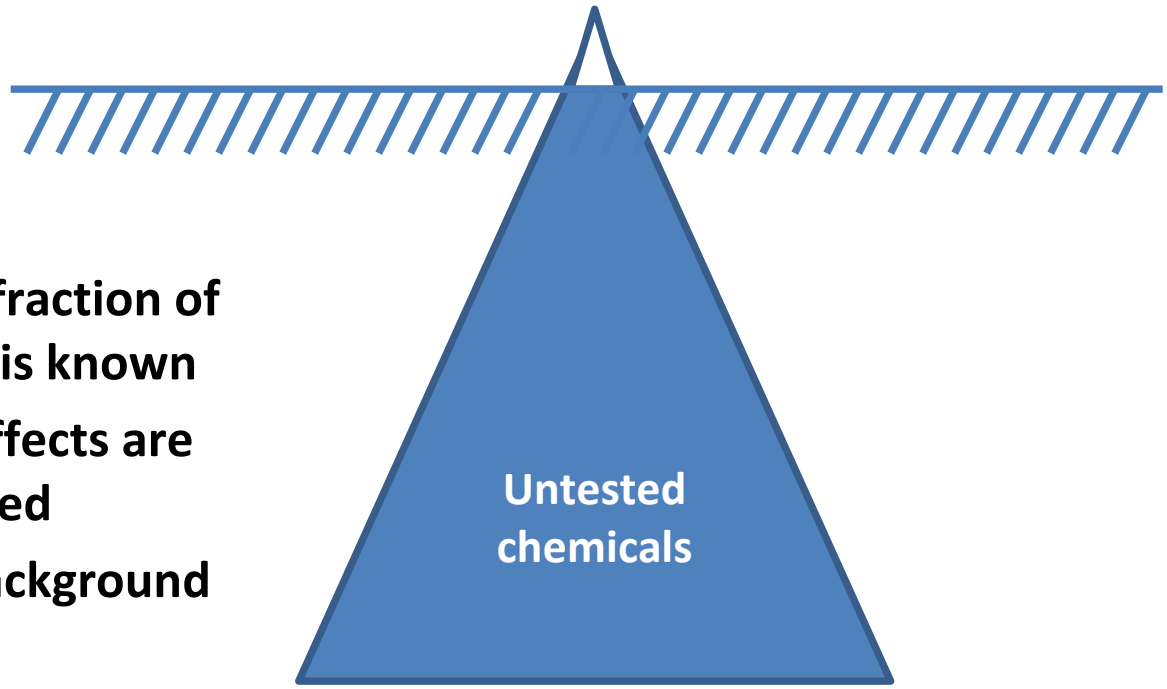
The challenge: mixtures

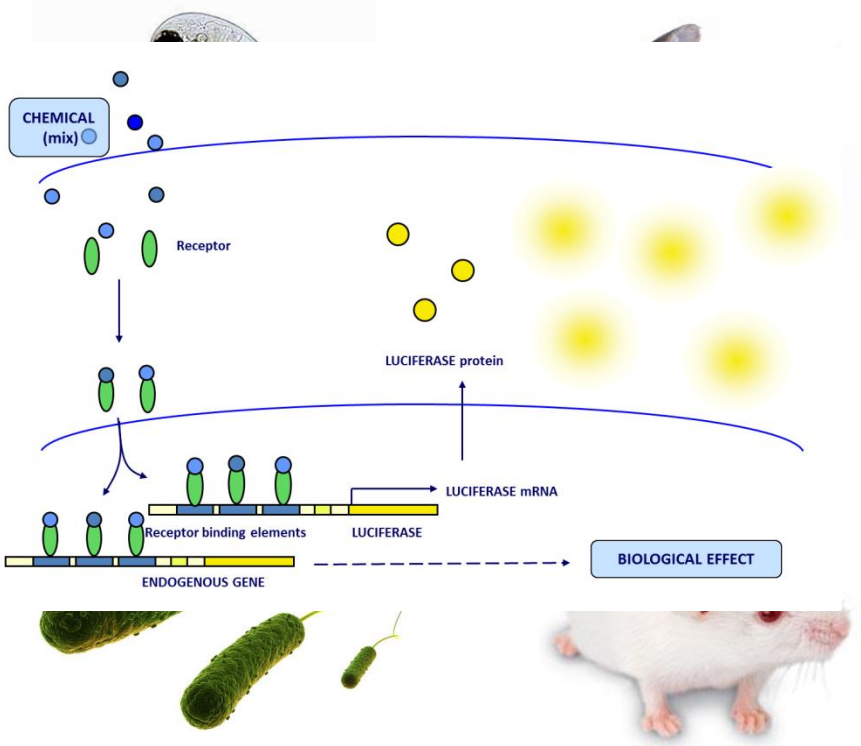


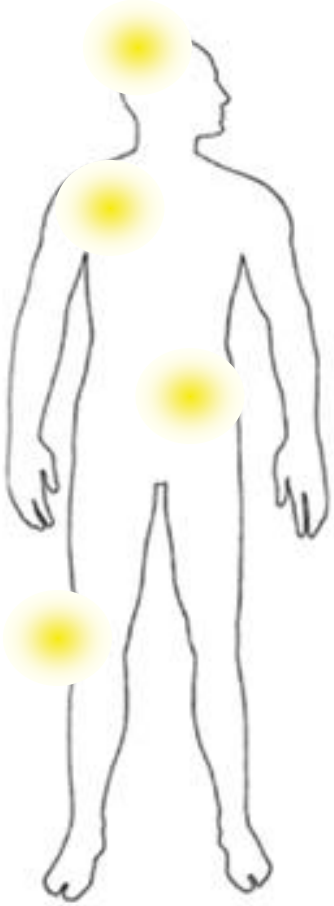
- **Effects of fraction of chemicals is known**
- **Mixture effects are not assessed**
- **Natural background**

- *"Tip of the iceberg" is measured*

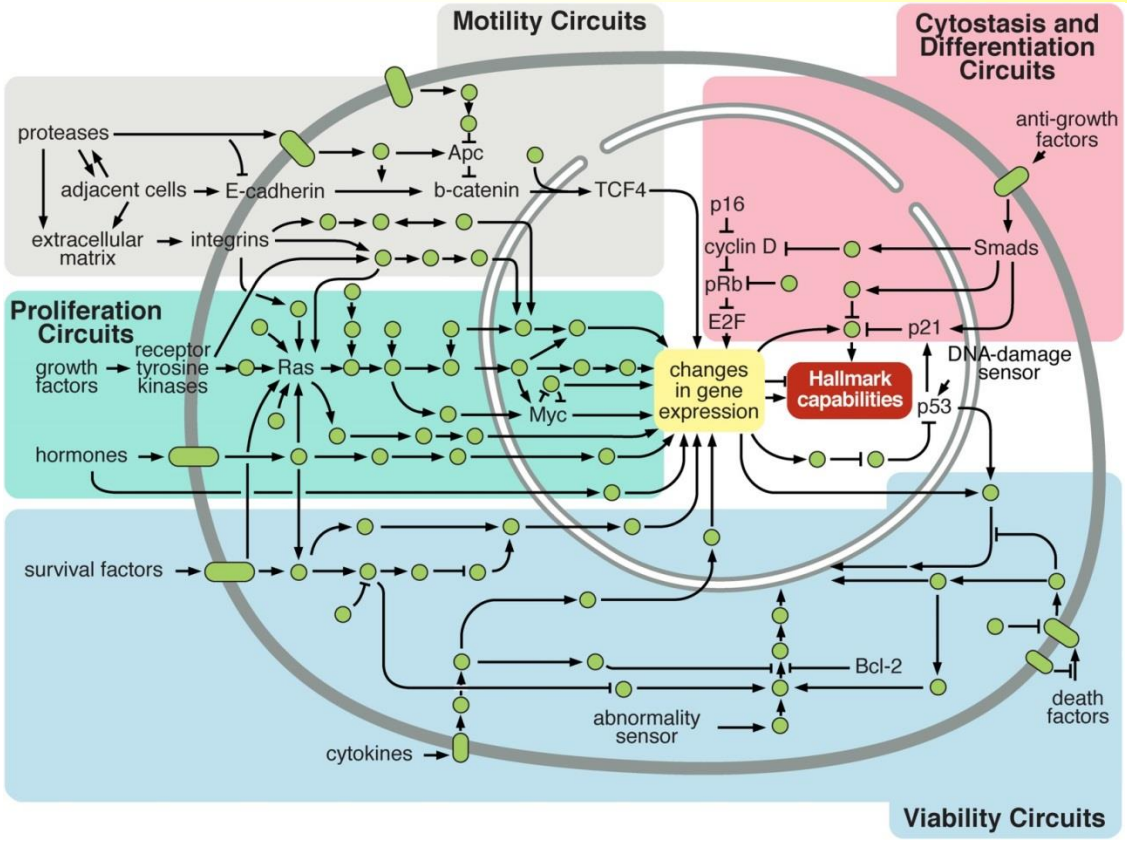
➢ *Fast, cheap and integrative methods needed: bioassays*

Measuring toxicity of chemicals/mixtures: bioassays are only option





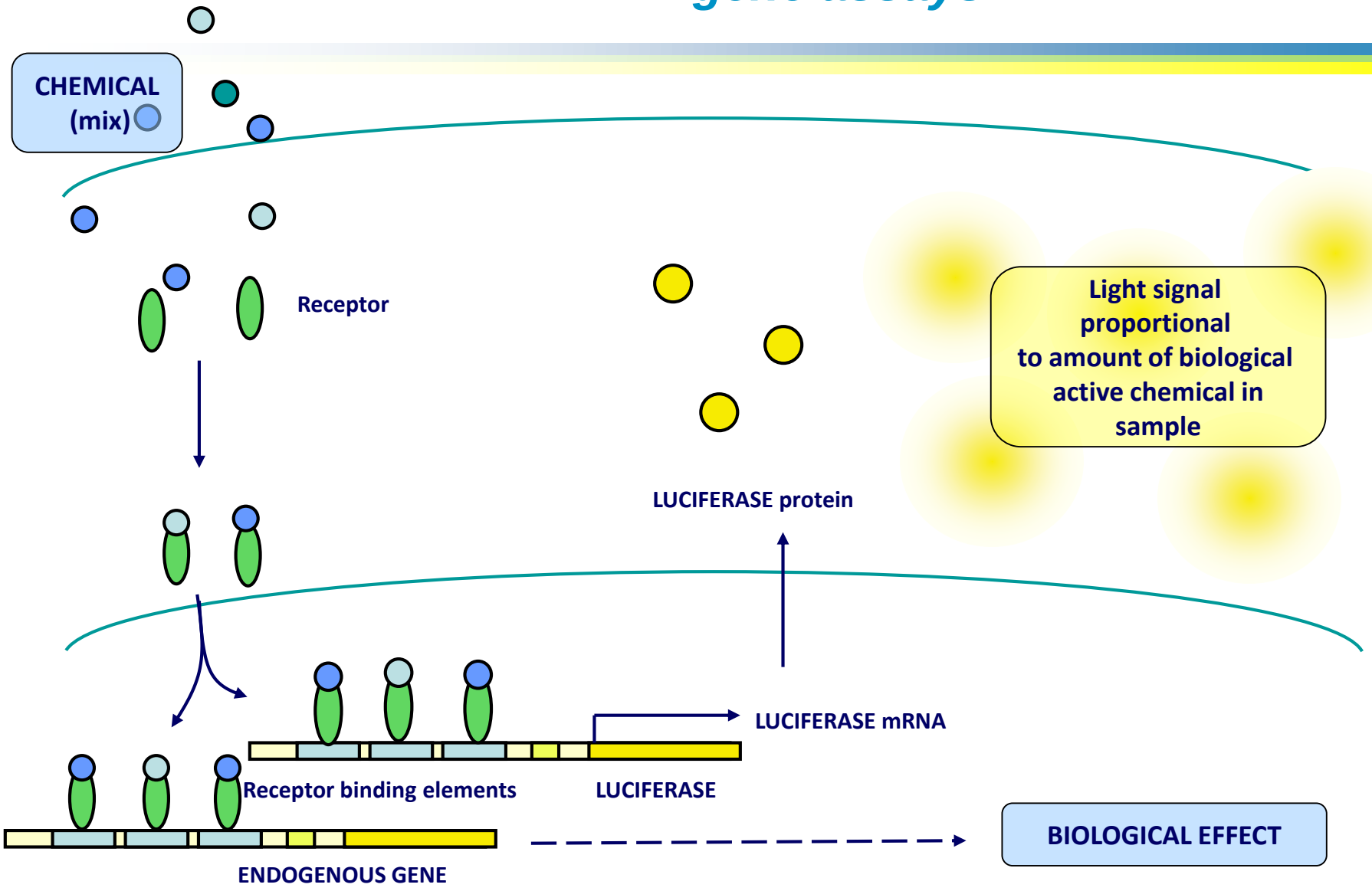
Opportunity: cellular pathway-based approach



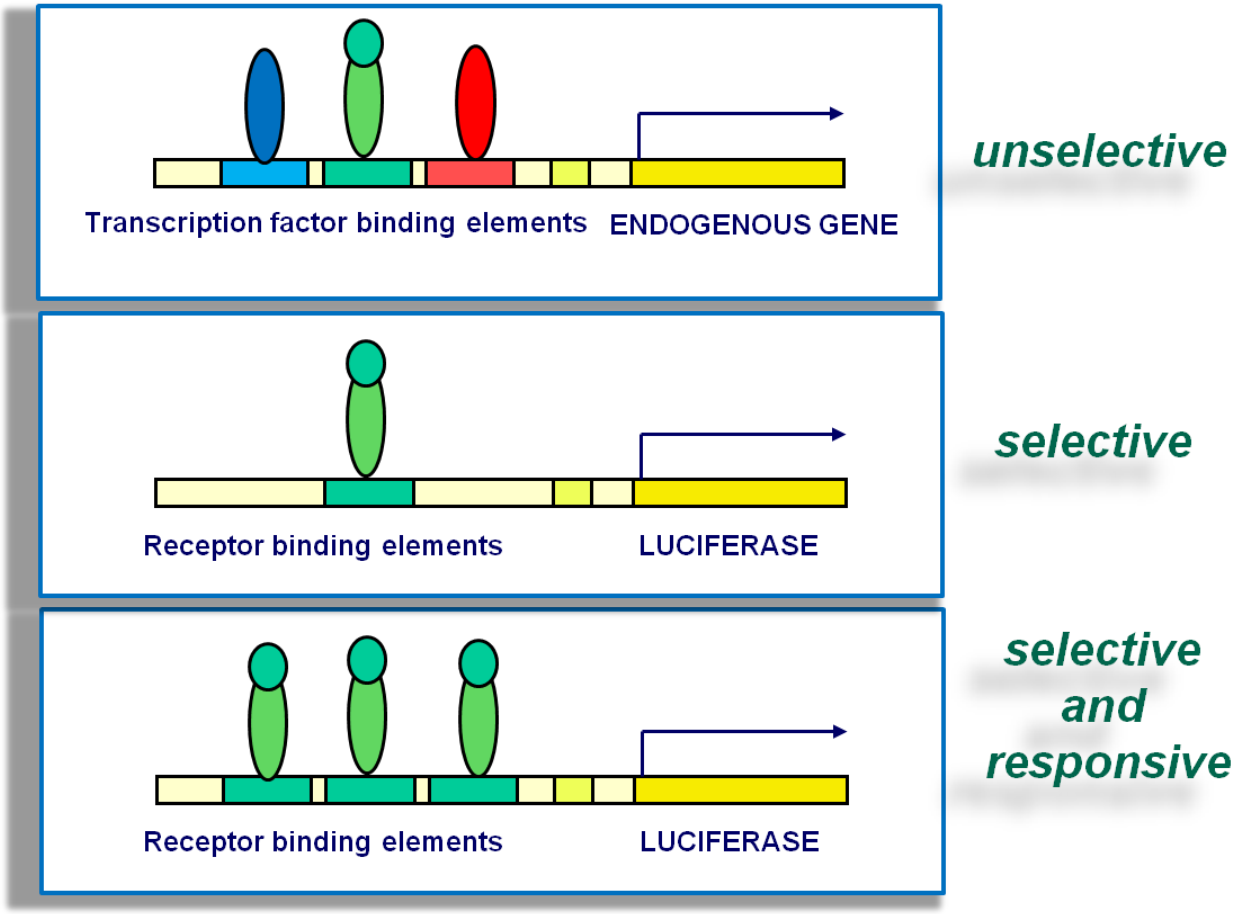
Hanahan and Weinberg 2011, Cell 144(5):646-74

- **Cancer diagnosis: pathway analysis is increasingly used in complementing and replacing conventional pathology**
- **Toxicology is next in line**

CALUX® mechanism (AOP)-based reporter gene assays



Use pathway selective assays for Adverse Outcome (AOP) linkage and to assess complex mixture effects



Legler et al (1999) Toxicological Sciences 48, 55-66.

CALUX High Throughput Screening panel

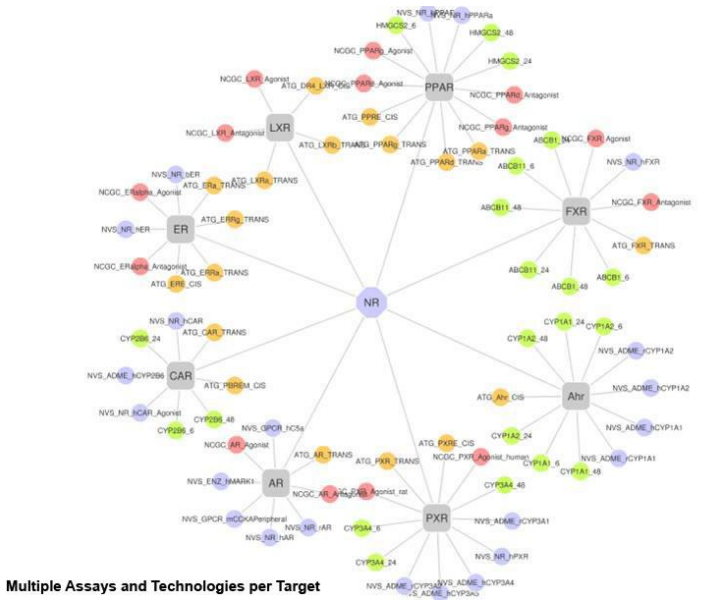
name	basal line	species	pathway	reference compound	key reference
DR CALUX	H4IIE/HepG2	Rat/human	dioxin receptor activation	2,3,7,8-TCDD	Van Vugt 2013/Buddin 2018
PAH CALUX	H4IIE	rat	dioxin receptor activation	benzo-a-pyrene	Pieterse 2013
ER CALUX	T47D	human	estrogen receptor activation	17β-estradiol	Legler 1999
ERalpha CALUX	U2OS	human	estrogen receptor α activation	17β-estradiol	Sonneveld 2005 OECD 2013
LXR CALUX	U2OS	human	LXR activation	GW3965	unpublished
kappaB CALUX	U2OS	human	NFκB pathway activation	TPA	Piersma 2013, Van der Burg 2013
P21 CALUX	U2OS	human	transcription of p21 inhibitor of cell cycle progression	actinomycin D	Piersma 2013, Van der Burg 2013
Nrf2 CALUX	U2OS	human	activation of the Nrf2 pathway	curcumin	Van der Linden 2014
P53 CALUX	U2OS	human	p53-dependent pathway activation	actinomycin D	Van der Linden 2014
genotox CALUX	U2OS	human	p53-dependent pathway activation +/-S9	cyclophosphamide	Van der Linden 2014
TCF CALUX	U2OS	human	wnt/TCF pathway activation	lithium chloride	Piersma 2013, Van der Burg 2013
AP1 CALUX	U2OS	human	AP1 pathway activation	TPA	Piersma 2013, Van der Burg 2013
HIF1alpha CALUX	U2OS	human	Hif1alpha pathway activation	cobaltous chloride	Piersma 2013, Van der Burg 2013
ER stress CALUX	U2OS	human	ERSE activation leading to endoplasmic reticulum stress	tunicamycin	Piersma 2013, Van der Burg 2013

- **Sensitive, selective, quantitative assays for major hormonal systems and cell signalling pathways**
- **Addresses major types of toxicity (general toxicity, genotoxicity/carcinogenicity, endocrine disruption, reproduction, developmental tox, etc)**
- **More than 50 assays (approx. 30 assays in regular use in panel)**
- **Data on >500 chemicals**

QA and validation

- OECD 2016: ERalpha CALUX for estrogenic/antiestrogenic EDCs included in TG455
- ECVAM (ongoing) AR CALUX validation and OECD guideline development for androgens/antiandrogens
- OECD (ongoing): Introducing metabolic steps in the ERα CALUX transactivation bioassay.
- In preparation: validation thyroid interference panel
- ISO (2016) standard for waste-water testing using ERalpha CALUX
- Establishment of normal- and trigger values
- ISO17025 accreditation

Why not more assays?



Multiple Assays and Technologies per Target

	1 CALUX	18 TOXCAST	REFERENCE
17alpha-Ethinylestradiol	-1,2E+01	1,0E+00	6
meso-Hexestrol	-1,2E+01	9,9E-01	6
17beta-Estradiol	-1,2E+01	9,4E-01	6
Diethylstilbestrol	-1,1E+01	9,4E-01	6
17alpha-Estradiol	-9,8E+00	1,1E+00	4
Estrone	-9,5E+00	8,1E-01	4
4-Octylphenol	-6,2E+00	1,2E-01	4
Genistein	-8,0E+00	5,4E-01	2
5alpha-Dihydrotestosterone	-7,5E+00	4,0E-01	2
Bisphenol A	-7,3E+00	4,5E-01	2
4-Cumylphenol	-7,0E+00	3,8E-01	2
o,p'-DDT	-6,9E+00	3,9E-01	2
Kepone	-6,6E+00	1,7E-01	2
Butyl benzyl phthalate	-6,4E+00	1,8E-01	1
Methoxychlor	-6,2E+00	2,5E-01	1
Kaempferol	-6,1E+00	2,5E-01	1
17-Methyltestosterone	-6,0E+00	5,0E-01	1
Fenarimol	-5,7E+00	1,1E-01	1
Ethylparaben	-5,4E+00	8,6E-02	1
p,p'-DDE	-5,3E+00	6,8E-02	1
Dicofol	-5,3E+00	0,0E+00	1
Dibutyl phthalate	-5,2E+00	2,7E-02	1
4-Nonylphenol	-5,1E+00	8,8E-02	1
Di(2-ethylhexyl) phthalate	-4,0E+00	0,0E+00	1
Atrazine	-4,5E+00	0,0E+00	0
Haloperidol	0,0E+00	6,0E-03	0
Spironolactone	0,0E+00	2,8E-04	0
Corticosterone	0,0E+00	5,6E-05	0
Flutamide	0,0E+00	0,0E+00	0
Procymidone	0,0E+00	0,0E+00	0
Linuron	0,0E+00	0,0E+00	0
Reserpine	0,0E+00	0,0E+00	0
Ketoconazole	0,0E+00	0,0E+00	0

- **Toxcast: many assays per pathway**
- **Our approach: One selective, validated assay with minimal false positives**

Van Vugt-Lussenburg et al. 2018, Reproductive Toxicology 75, 40-18

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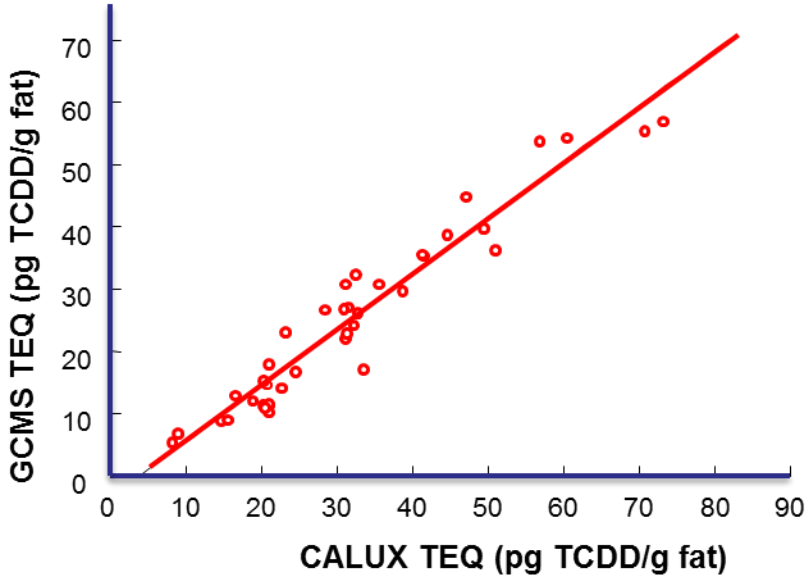
Environ-
ment
Food risk
& benefit
Pharma
BDS
Bio-based
economy
Chemical
safety

CALUX assays as analytical tool of mixtures



- **Indirect:** *Chemical analytical method*

•*Compound 1:*	•concentration 1	•x TEF1 =	•TEQ1
•*Compound 2:*	•concentration 2	•x TEF2 =	•TEQ2
•*Compound 3:*	•concentration 3	•x TEF3 =	•TEQ3
•*Compound n:*	•concentration n	•x TEFn =	•TEQn

•+

•*Total dioxin toxicity of mixture:* • SumTEQ

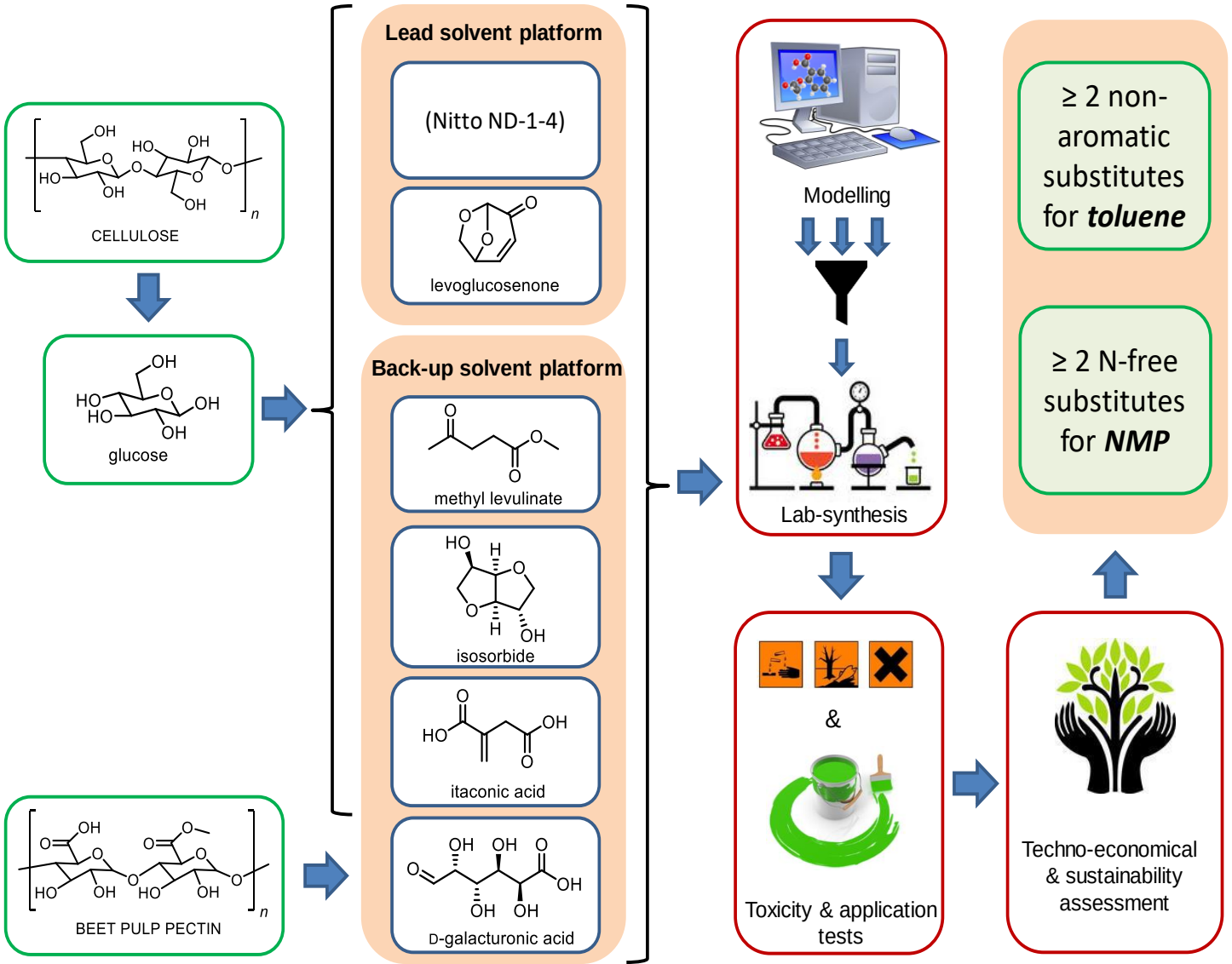
- **Direct:** *Biological (CALUX®) method*

•Direct measurement of TEQ value of sample

•≈

ReSolve
CREATING SAFE AND RENEWABLE SOLVENTS

Green Chemistry



Application in read across, safe design/green chemical identification

compound	Cytotox10%	Cytotox50%	ERa	ERa-anti	ERb	ERb-anti	AR	AR-anti	PR	PR-anti	GR	GR-anti	TRb	RAR	PPARa	PPARg	DR	PAH	Hif1a	TCF	AP1	ESRE	NFkB	Nrf2	p21	p53
bisphenol A	-4	-3.7	-7.3		-6.8			-6.8		-5.5		-4.5										-4.3				
Butyl benzyl phthalate	-3.9	-3.5	-5.7		-4.4			-6.1		-5.7							-3.7									
Di(n-hexyl)phthalate	-3.5		-5					-5		-5.5		-4.5					-4				-4.2					
Dibutylphthalate	-4.5	-4	-5.2					-5.5		-5.5																
Diethylphthalate	-3.5	-3.0	-4.3					-5		-4.3																
Diisobutyl phthalate	-4.5	-4	-5.7					-6		-6																
Nonylphenol	-4.9	-4.7	-5.1		-5.6			-6.5		-5.5												-4.6				
FDCA																										

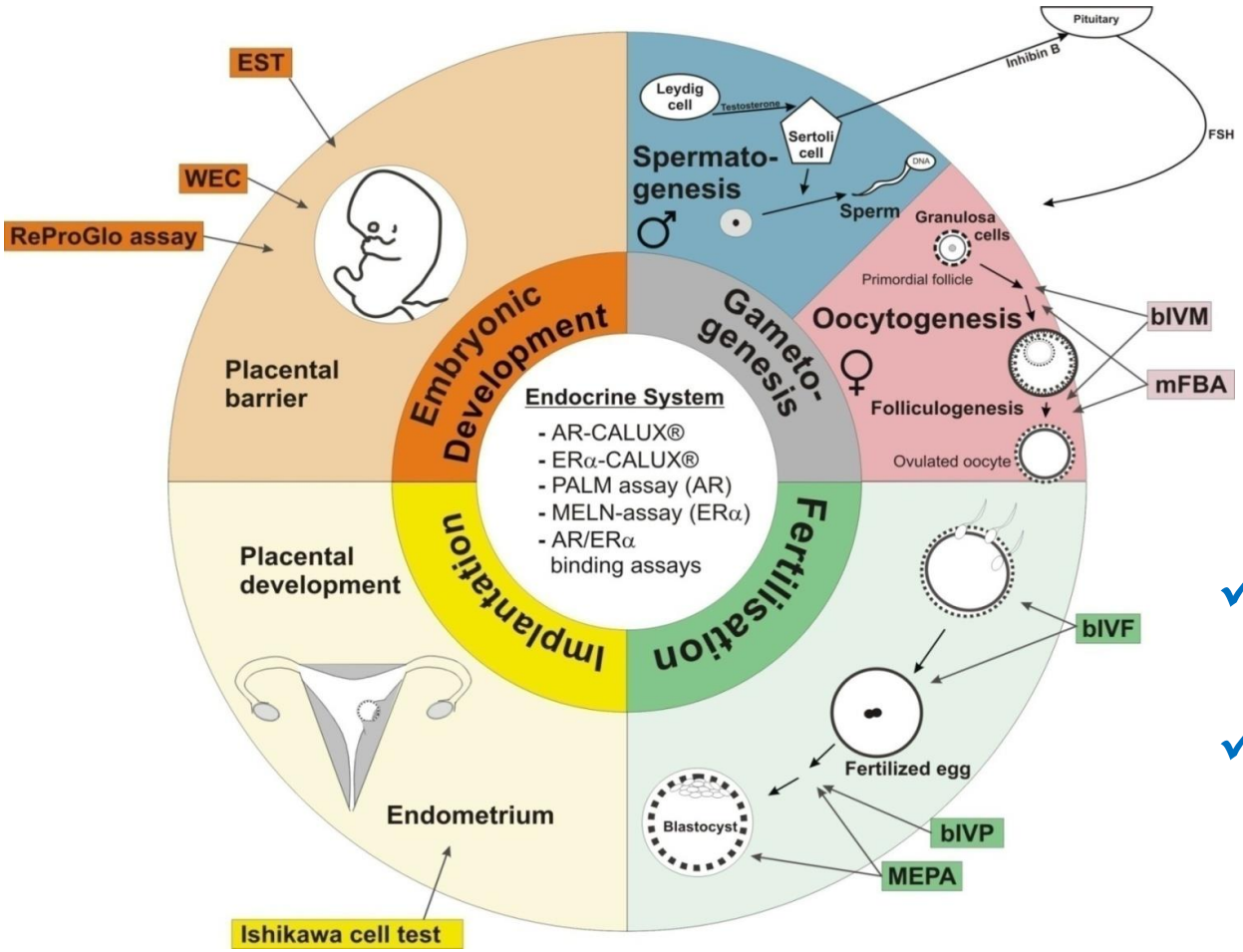
Case study: CALUX panel identifies FDCA as a potentially non-toxic alternative to current plastic ingredients/building blocks

Cases that show applicability to different chemical classess

Comparable "read-across" methods are increasingly used in chemical safety assessments; used in approx. 30% reproductive tox dossiers (100-1000TPA) in REACH (ECHA 2014)

Kroese et al., 2015 reproductive toxicology 55; Van Vugt-Lussenburg, in preparation

Can a test battery predict complex toxicity (reproductive toxicity)?



✓ ***ReProTect:*** *Yes*

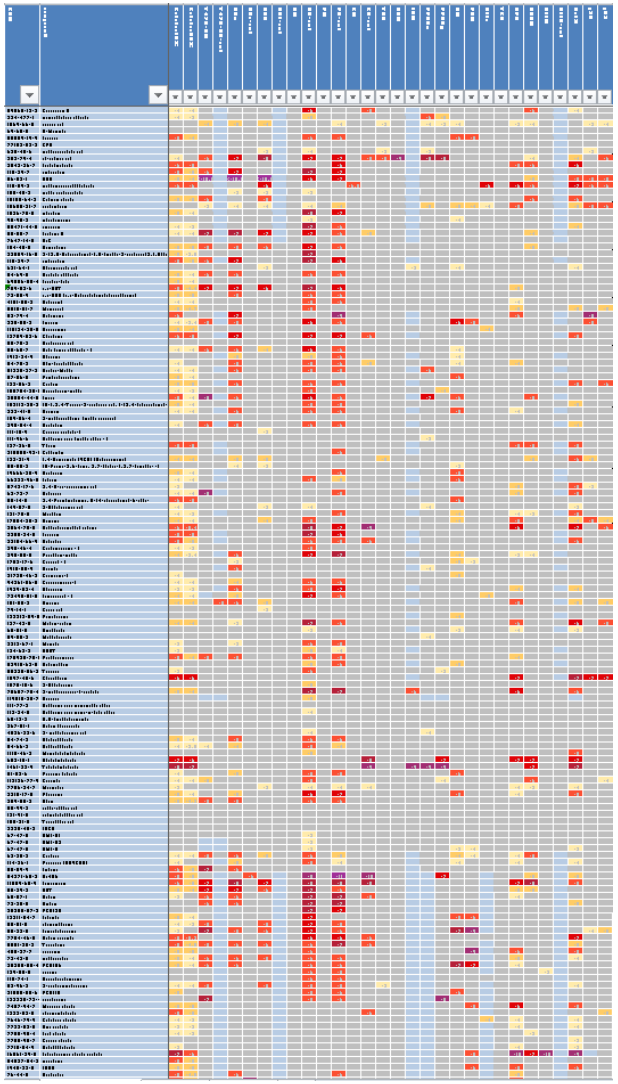
✓ ***ChemScreen:*** *Yes*

Schenk et al. 2010 Reproductive Toxicology 30, 200-218; Piersma et al. 2013. Reproductive Toxicology

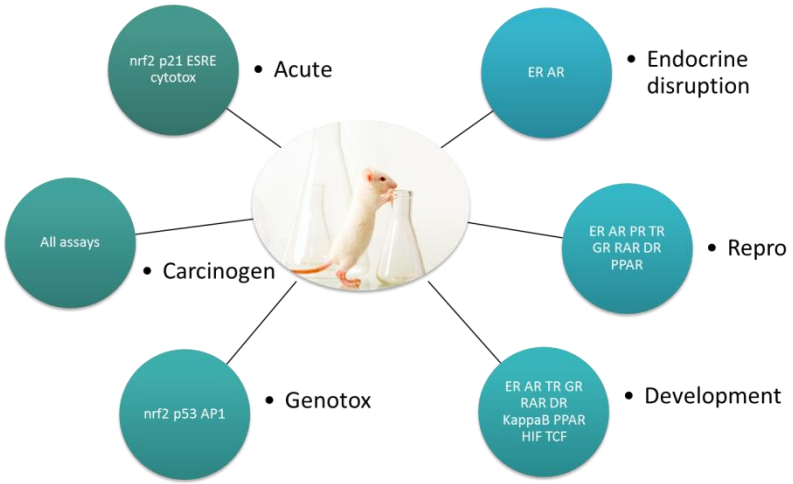
Can HTS pathway-based assays be used to predict toxicity?

tests

compounds



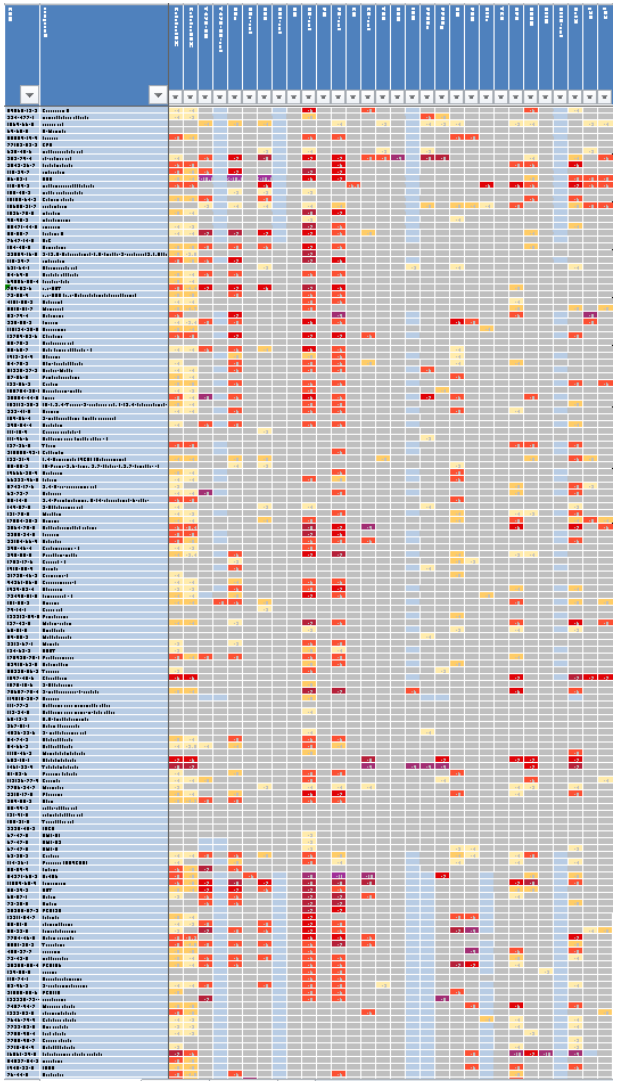
?

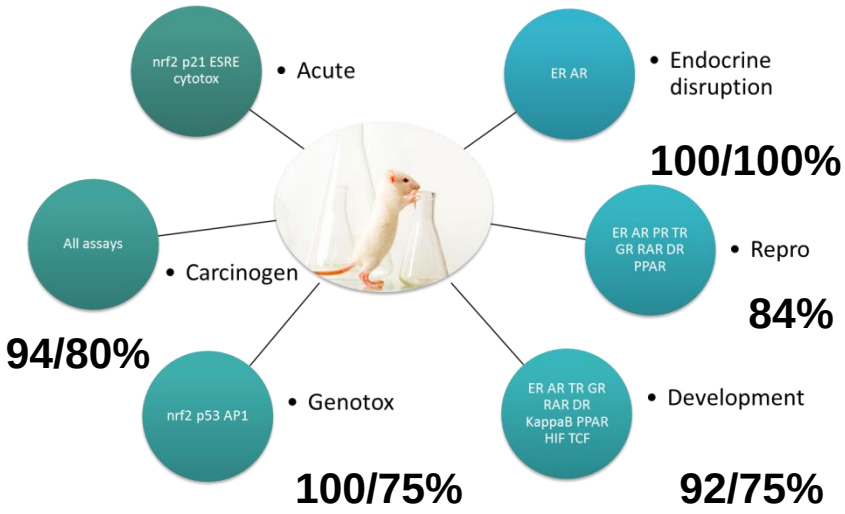


Various validation studies: predictivity panel/subsets

tests

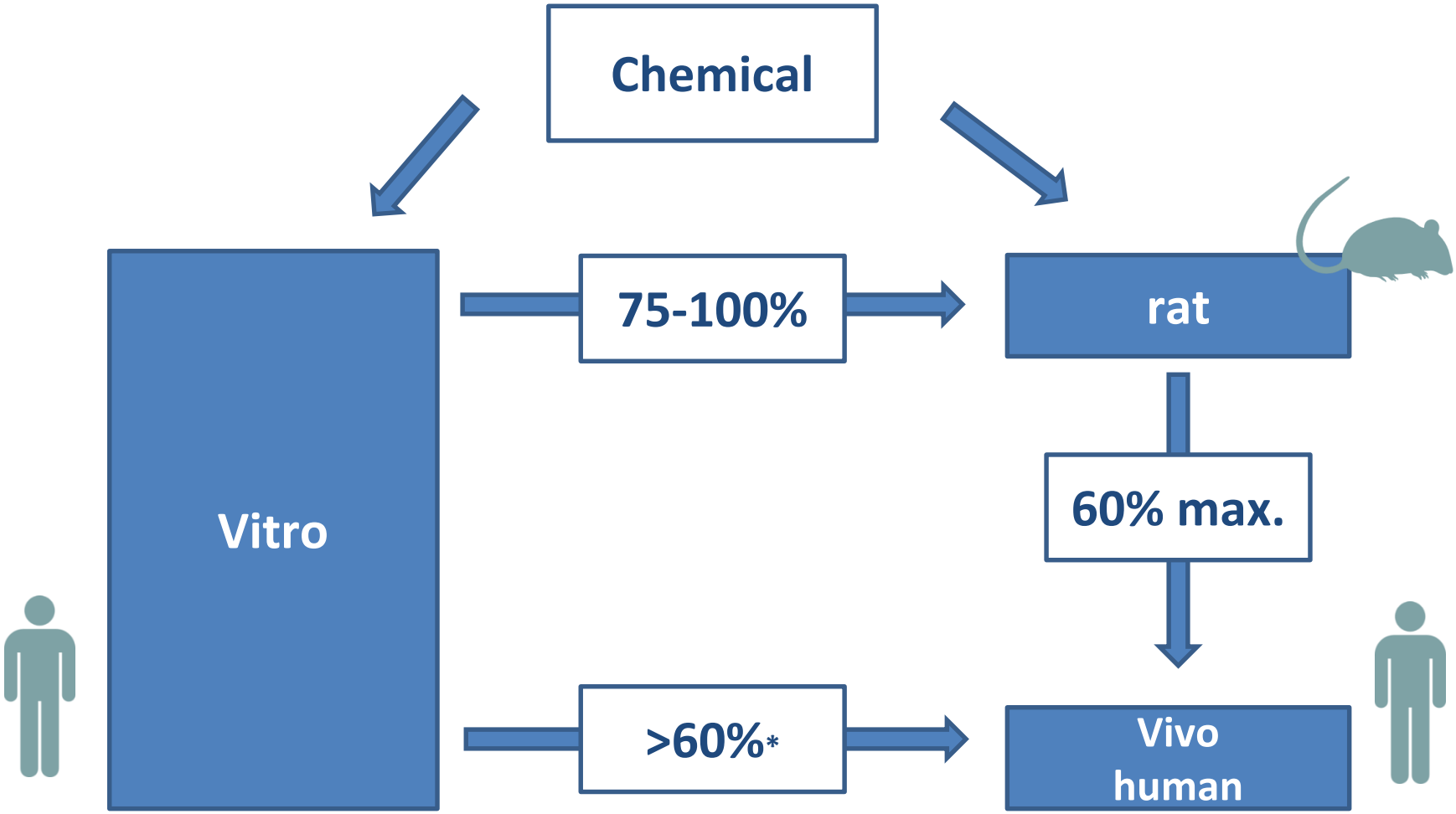
compounds





Predictions range between 75-100% (published data/in press)

Validation: what level of predictivity to expect?



*Hartung T. Nature 2009, 460:208-212.

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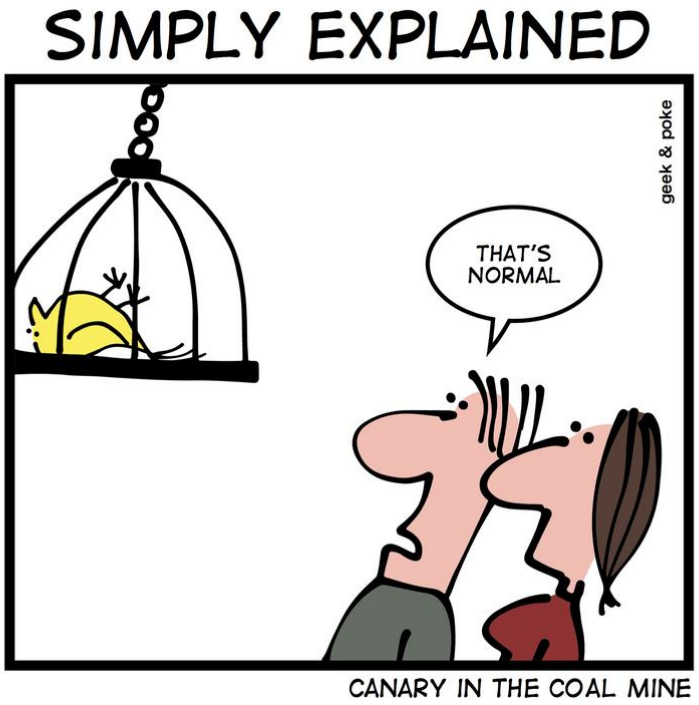
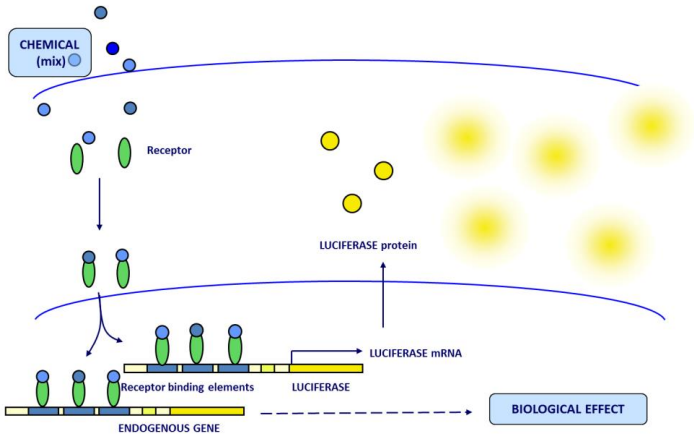
Risk assessment : extrapolation and interpretation of data

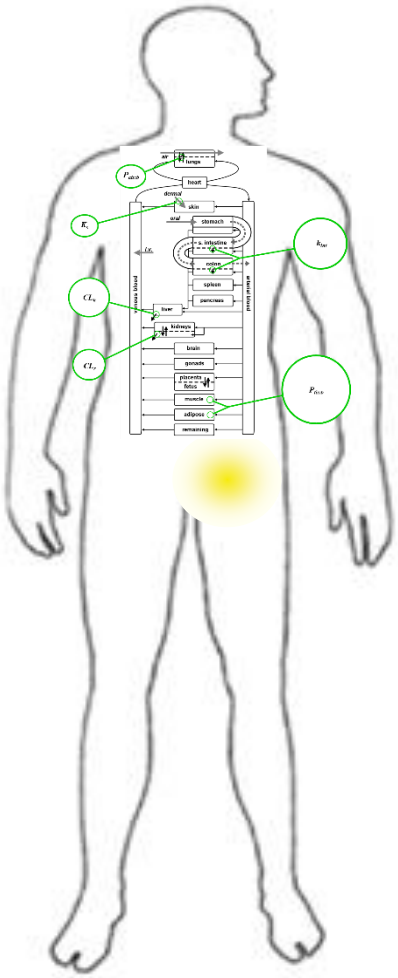


Risk assessment : extrapolation and interpretation of data

Predict toxicity:

"dynamics" + *kinetics*



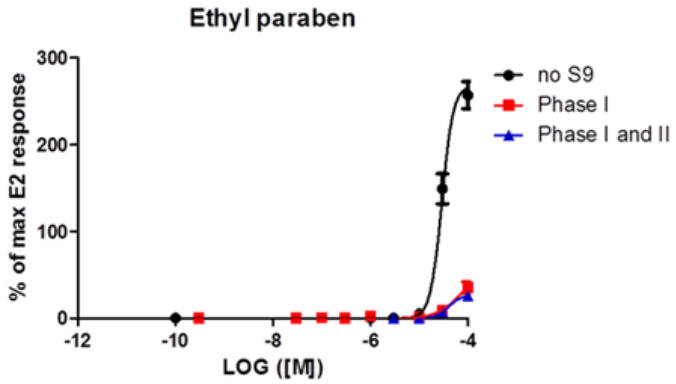


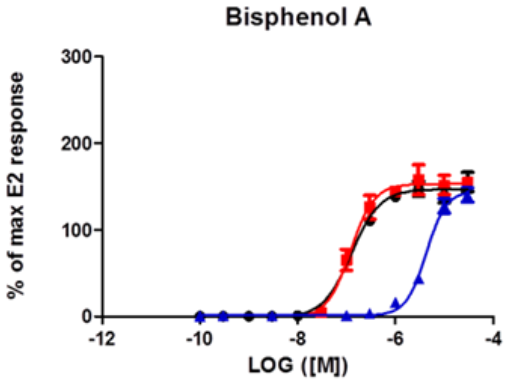
Input required:

- **fraction unbound (fu)**
- **hepatic clearance (CLh,int)**
- **intestinal permeability (Papp)**
- **logP, ionization**
- **Default assumptions: renal clearnce (CLr)**

TNO innovation for life

For mixtures: in vitro methods needed





activation/inactivation

Compound	ERα CALUX PC10 (M)		
	No S9	S9 Phase I	S9 Phase I+II
meso-hexestrol	2.4E-13	2.6E-13	**4.7E-12**
17α-ethinylestradiol	6.3E-13	1.0E-12	1.0E-12
17β-estradiol	2.2E-12	2.6E-12	**5.8E-12**
diethylstilbestrol	6.0E-12	**3.2E-11**	3.2E-11
norethynodrel	2.0E-10	4.0E-11	**1.6E-10**
17α-estradiol	2.5E-10	5.6E-10	**6.4E-10**
coumestrol	2.1E-09	2.1E-09	3.2E-09
tamoxifen	1.6E-08*	4.0E-09*	4.0E-09*
bisphenol A	2.0E-08	1.6E-08	**3.2E-06**
genistein	3.4E-08	3.7E-08	**1.4E-07**
4-tert-octylphenol	4.3E-08	**1.0E-06**	**NA (>1E-06)**
19-nortestosterone	6.1E-08	8.5E-09	1.4E-08
kepone	8.4E-08	6.3E-08	6.3E-08
4-cumylphenol	1.8E-07	**6.3E-07**	**6.9E-06**
butylbenzyl phthalate	5.0E-07	**2.5E-05**	**4.0E-05**
p,p'-methoxychlor	6.3E-07	2.0E-08	**2.5E-07**
testosterone	6.3E-07	2.5E-07	4.0E-07
kaempferol	7.6E-07	1.0E-06	**6.4E-06**
ethyl paraben	5.5E-06	**3.2E-05**	**3.8E-05**
corticosterone	NA (>1E-04)	NA (>1E-04)	NA (>1E-04)
linuron	NA (>1E-04)	3.2E-06	**1.0E-05**
spironolactone	NA (>1E-04)	1.0E-05	**NA (>1E-04)**
ketoconazole	NA (>1E-04)	4.0E-07	**1.3E-06**
reserpine	NA (>1E-04)	NA (>1E-04)	NA (>1E-04)
flutamide	NA (>1E-05)	3.2E-06	**NA (>1E-05)**
atrazine	NA (>3E-05)	1.3E-05	2.2E-05
vinclozolin	NA (>3E-05)	3.2E-06	**8.5E-06**

potency

Compound	no	S9 Phase I	S9 Phase I+II
meso-hexestrol	-12.6	-12.6	-11.3
17α-ethinylestradiol	-12.2	-12.0	-12.0
17β-estradiol	-11.7	-11.6	-11.2
diethylstilbestrol	-11.2	-10.5	-10.5
norethynodrel	-9.7	-10.4	-9.8
17α-estradiol	-9.6	-9.3	-9.2
coumestrol	-8.7	-8.7	-8.5
bisphenol A	-7.7	-7.8	-5.5
genistein	-7.5	-7.4	-6.9
4-tert-octylphenol	-7.4	-6.0	
19-nortestosterone	-7.2	-8.1	-7.9
kepone	-7.1	-7.2	-7.2
4-cumylphenol	-6.7	-6.2	-5.2
butylbenzyl phthalate	-6.3	-4.6	-4.4
p,p'-methoxychlor	-6.2	-7.7	-6.6
testosterone	-6.2	-6.6	-6.4
kaempferol	-6.1	-6.0	-5.2
ethyl paraben	-5.3	-4.5	-4.4
corticosterone			
linuron		-5.5	-5.0
spironolactone		-5.0	
ketoconazole		-6.4	-5.9
reserpine			
flutamide		-5.5	
atrazine		-4.9	-4.7
vinclozolin		-5.5	-5.1

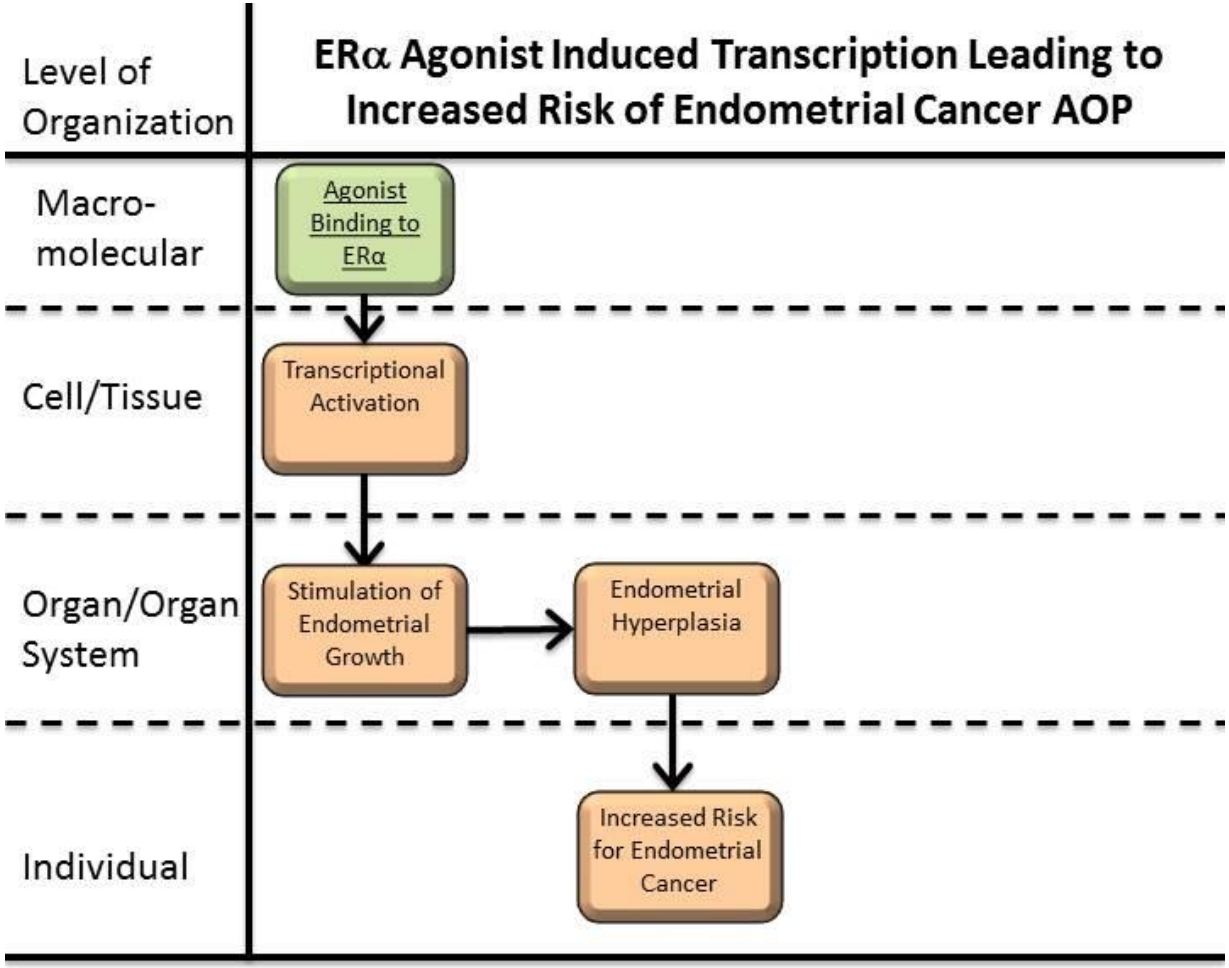
Van Vugt-Lussenburg et al. 2018, Reproductive Toxicology 75, 40-18

Use Adverse Outcome Pathways to link lower level tests to adversities

- Adverse Outcome Pathway (AOP): chain of linked **key events** at different levels of biological organisation that lead to an **adverse outcome**.
- Central elements to support chemical risk assessment based on mechanistic reasoning.

http://www.oecd.org/chemicalsafety/testing/adverse-outcome-pathways-molecular-screening-and-toxicogenomics.htm

AOP example: adverse effects by estrogens



OECD TG455

Molecular initiating event

↓

Key events

↓

Adverse Outcome

Becker et al., 2015 Regulatory Toxicology and Pharmacology, 72, 514-537

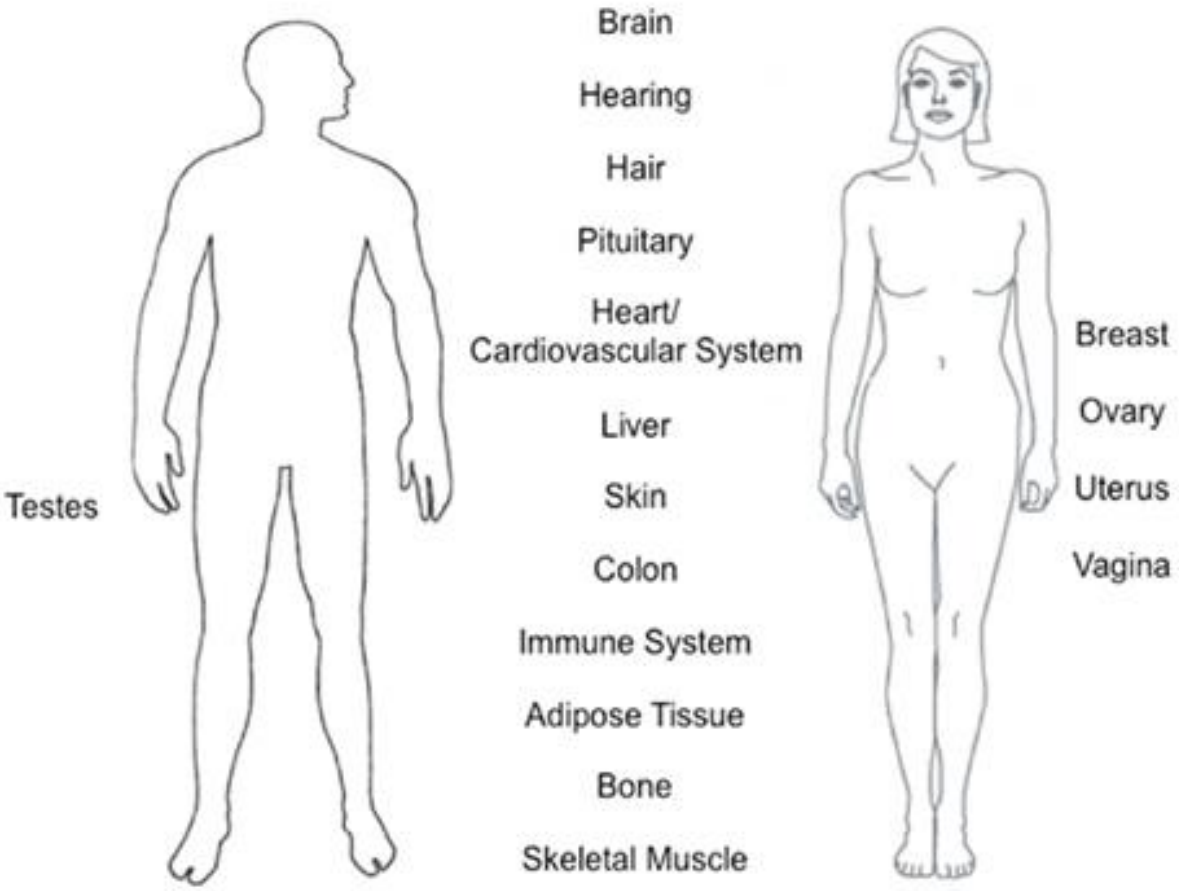
OECD; conceptual framework with many tests

OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

Note: Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

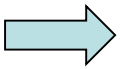
Level 1 Sorting & prioritization based upon existing information	•Physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability •Human & environmental exposure, e.g., production volume, release, use patterns •Hazard, e.g., available toxicological data	
Level 2 *In vitro* assays providing mechanistic data	•ER, AR, TR receptor binding affinity •Transcriptional activation •Aromatase & Steroidogenesis *in vitro* •Aryl hydrocarbon receptor recognition/binding	•High Through Put Prescreens •Thyroid function •Fish hepatocyte VTG assay •QSARs; Others (as appropriate)
Level 3 *In vivo* assays providing data about single endocrine Mechanisms and effects	•Uterotrophic Assay (estrogenic related) •Hershberger Assay (androgenic related) •Non-receptor mediated hormone function	•Fish VTG assay (estrogenic related) •Others (e.g. thyroid)
Level 4 *In vivo* assays providing data about multiple endocrine mechanisms and effects	•Enhanced OECD 407 (endpoints based on endocrine mechanisms) •Male and female pubertal assays •Adult intact male assay	•Fish gonadal histopathology assay •Frog metamorphosis assay
Level 5 *In vivo* assays providing data on effects from endocrine & other mechanisms	•1-generation assay (TG415 enhanced) •2-generation assay (TG416 enhanced) •Reproductive screening (TG421 enhanced) •Combined 28 day/reproduction screening test (TG 422 enhanced)	•Partial and full life cycle assays in fish, birds, amphibians & invertebrates (development & reproduction)

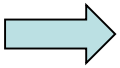
Many estrogen target tissues



- ***Dozens of AOPs needed for estrogens alone?***

Can a test for a Molecular Initiating event predict an adverse outcome?





OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals

Note: Document prepared by the Secretariat of the Test Guidelines Programme based on the agreement reached at the 6th Meeting of the EDTA Task Force

Level 1 Sorting & prioritization based upon existing information	•Physical & chemical properties, e.g., MW, reactivity, volatility, biodegradability •Human & environmental exposure, e.g., production volume, release, use patterns •Hazard, e.g., available toxicological data	
Level 2 *In vitro* assays providing mechanistic data	•ER, AR, TR receptor binding affinity •Transcriptional activation •Aromatase & Steroidogenesis *in vitro* •Aryl hydrocarbon receptor recognition/binding	•High Through Put Prescreens •Thyroid function •Fish hepatocyte VTG assay •QSARs; Others (as appropriate)
Level 3 *In vivo* assays providing data about single endocrine Mechanisms and effects	•Uterotrophic Assay (estrogenic related) •Hershberger Assay (androgenic related) •Non-receptor mediated hormone function	•Fish VTG assay (estrogenic related) •Others (e.g. thyroid)
Level 4 *In vivo* assays providing data about multiple endocrine mechanisms and effects	•Enhanced OECD 407 (endpoints based on endocrine mechanisms) •Male and female pubertal assays •Adult intact male assay	•Fish gonadal histopathology assay •Frog metamorphosis assay
Level 5 *In vivo* assays providing data on effects from endocrine & other mechanisms	•1-generation assay (TG415 enhanced) •2-generation assay (TG416 enhanced) •Reproductive screening (TG421 enhanced) •Combined 28 day/reproduction screening test (TG 422 enhanced)	•Partial and full life cycle assays in fish, birds, amphibians & invertebrates (development & reproduction)

What is needed for and EDC panel?

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- **Estrogen, Androgen, Thyroid, Steroidogenesis (EATS)**

EDC (EATS) panel: CALUX in combination with different assay types

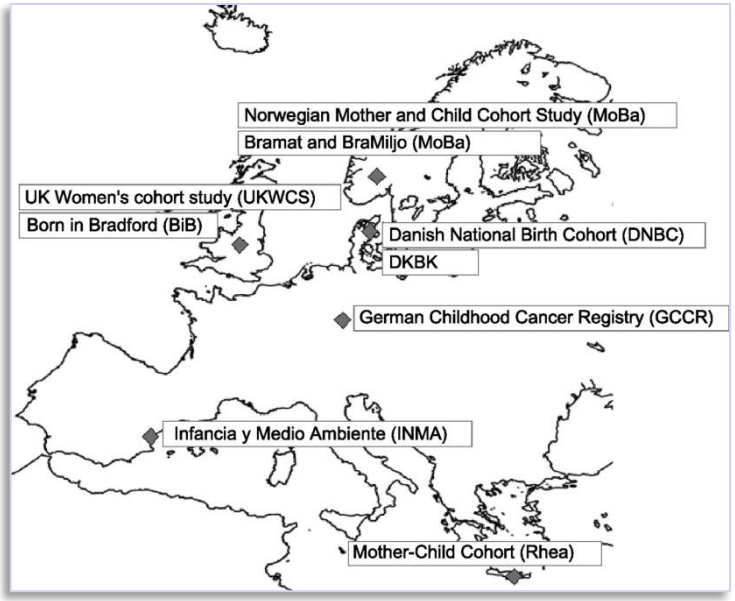
- **E: (anti)Estrogens: ERalpha CALUX (ER CALUX, ERbeta CALUX) (OECD TG455)**
- **A: (anti)Androgens: AR CALUX**
  (ECVAM validation and OECD TG guideline development)
- **T: Thyroid interference: TRbeta CALUX, TTR* and TPO assay (TG in preparation)**
- **S: H295R steroidogenesis, coupled to CALUX read-out (OECD 456)**

- **Phase 1 and 2 metabolic steps in EDC CALUX transactivation bioassays (TG in preparation)**
- **Others, if needed: PPARalpha, -beta, -gamma CALUX, PR CALUX, GR CALUX, etc.**

**Collet et al., submitted*

Specificity assays allows to measure exposure in humans

- **Pathway specific bioassays are valuable for human monitoring**
- **E.g. associations between DR-CALUX responses and:**



- **markers of childhood leukemia**
- **low birth weight**
- **shorter gestational time**
- **changes in AGD in young boys**
- **immune system functions later in life**

➢ **Derivation of thresholds /"trigger values" possible**

NewGeneris
Newborns and Genotoxic exposure risks

PROTECTED

Contents

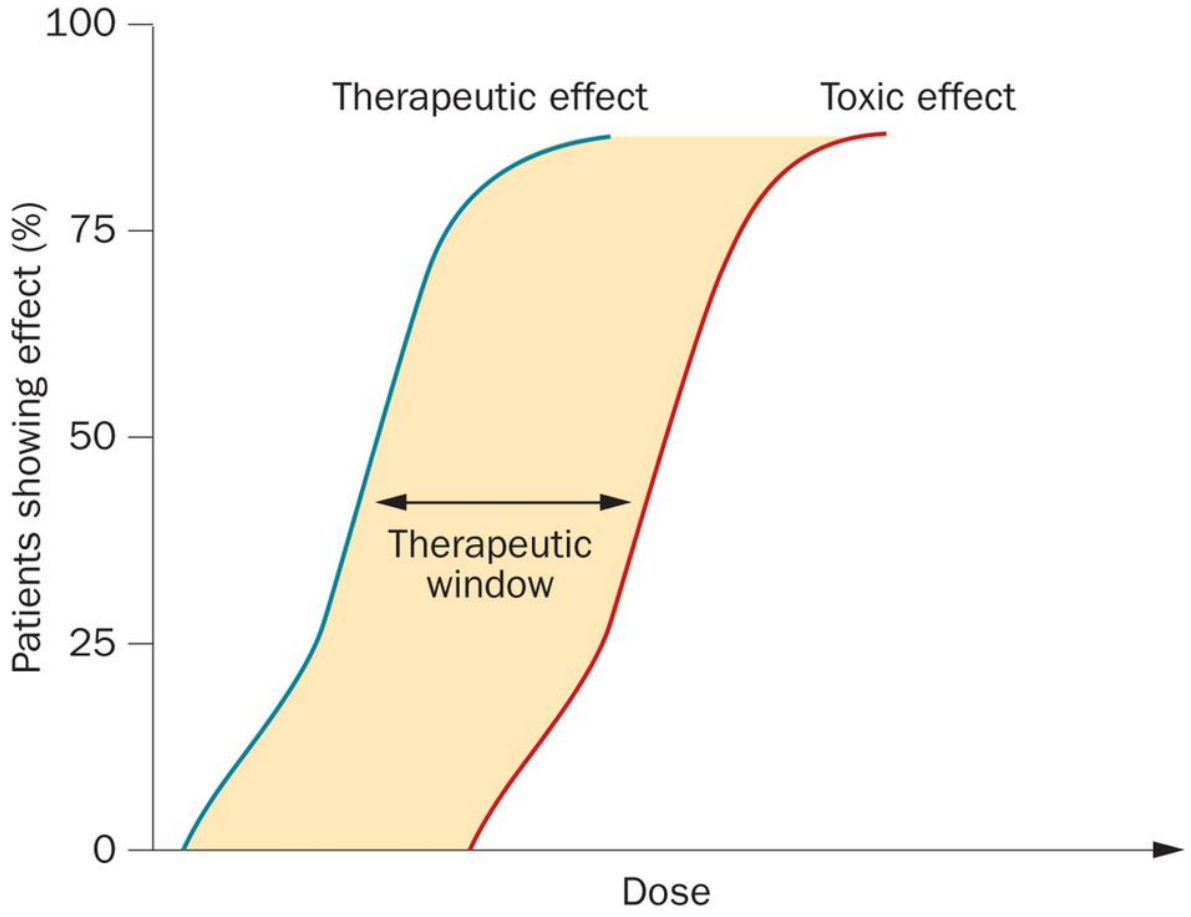
- Intro basic CALUX Quantitative High Throughput Assays
- Applications in safety assessment
- Specific requirements for different applications
- **Identification of nutraceuticals**

All chemicals are toxic, depending on the dose

Chemical	LD50 (mg/kg, orally to rat)
Sodium Chloride	3750
Aspirin	1750
Ethanol	1000
Morphine	500
Caffeine	200
Heroin	150
Lead	20
Cocaine	18
Parathion	13
Aflatoxin	10
Sodium cyanide	10
Nicotine	2
Strychnine	0.8
Sarin	0.4
Batrachotoxin	0.002
Tetanus toxin	0.000005
Botulinum toxin	0.00000003

*No principle difference between "**synthetic**" and "natural" chemicals*

Some chemicals are beneficial, but this depends on the dose

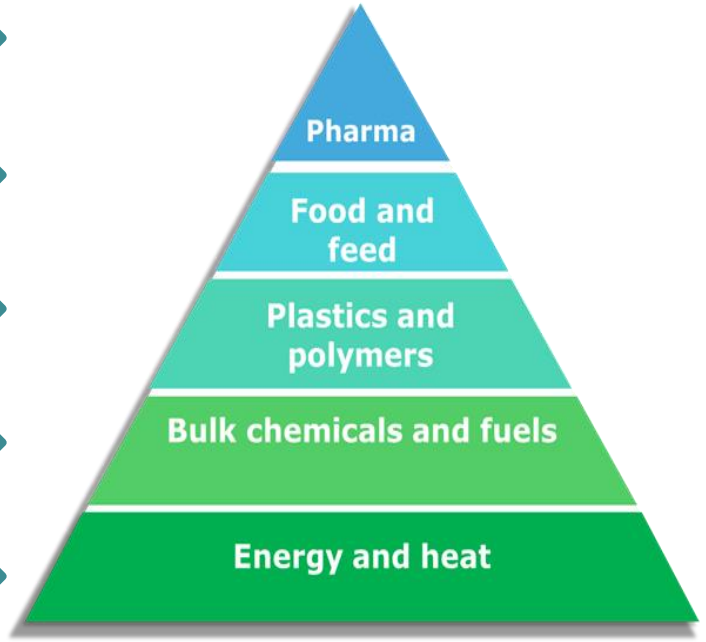


At low doses "toxic" chemicals can be beneficial: e.g. digoxin,aspirin, other secondary plant metabolites

Biomass valuation: screening for active ingredients

- Pharmaceuticals
- Ingredients, nutraceuticals
- Sugars, poly-saccharides, etc
- Lignin
- Residues

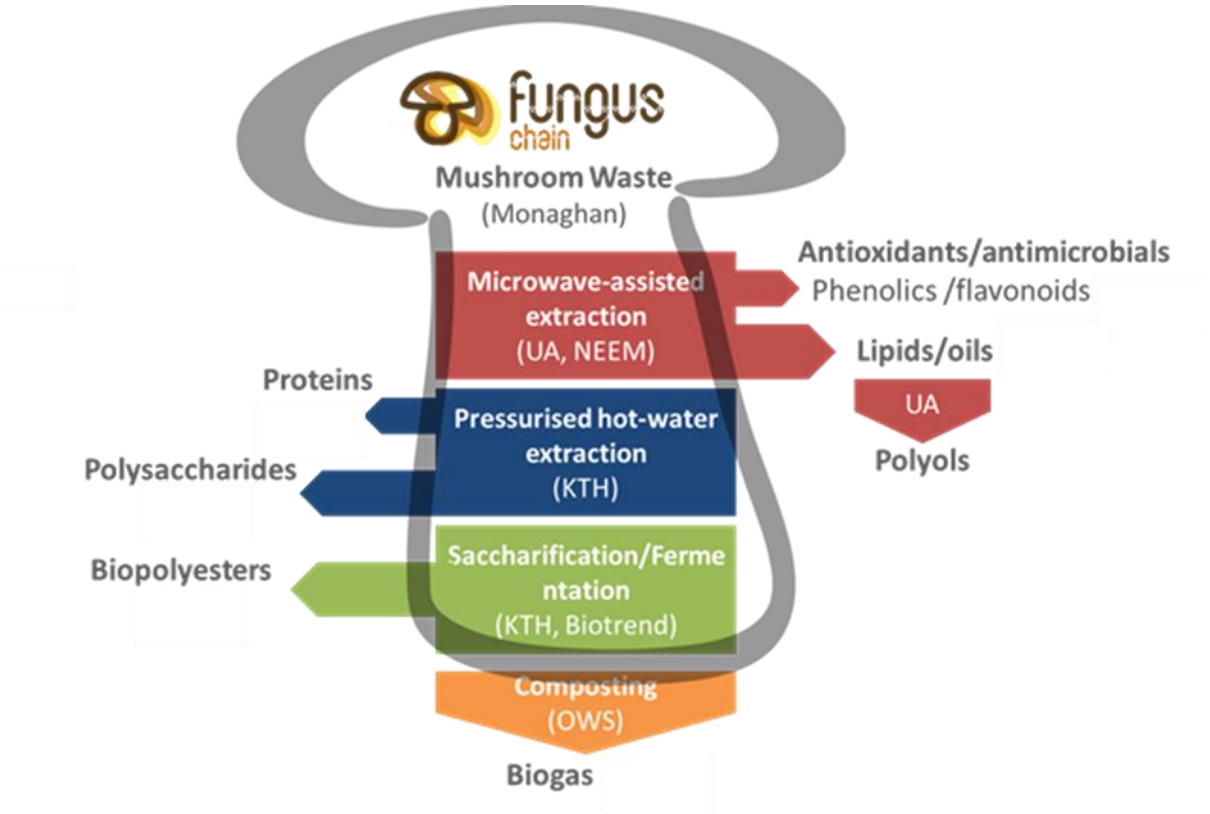
- Human drugs 1.5 trillion
- Veterinary drugs 22 billion



BE-Basic

Biomass valuation and safety assessment

One farm: 720 tons of waste per year

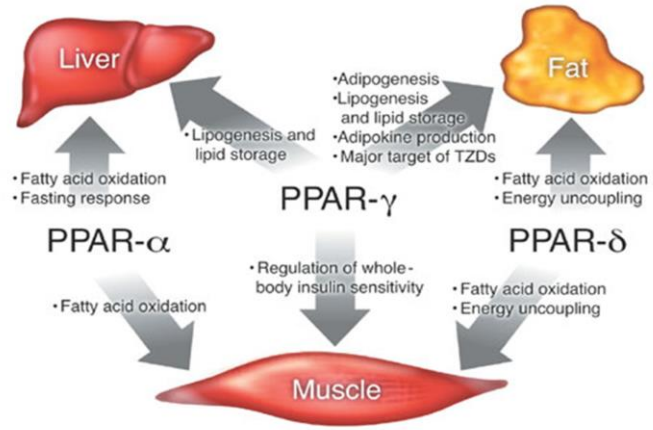


This project has received funding from the Bio Based Industries Joint Undertaking under the European Union's Horizon2020 research and innovation programme

Identification of modulators of fat metabolism

More than 1.4 billion adults were overweight in 2008, and more than half a billion obese

World Health Organization

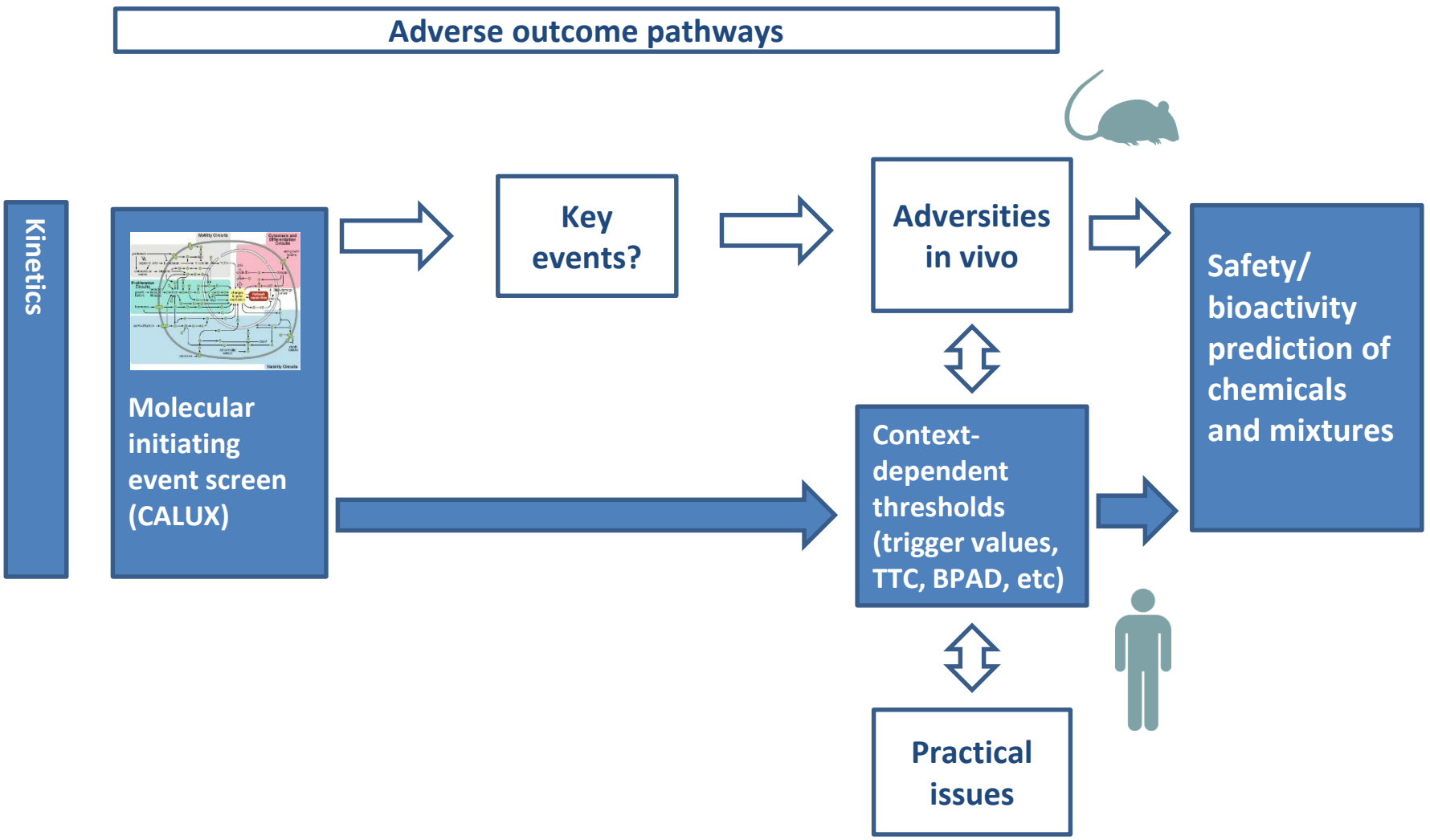


	PPARd (ng L-165,041 eq./g dry weight)	PPARg (ng Rosiglitazone eq./g dry weight)	PPARa (ng GW7647-eq./g dry weight)
Control	0	0	0
WBM	1696	53	0
Oyster	2565	2532	613
Shiitake	2377	841	334
LC	3326	2186	182
FMD12	2624	807	106
FMD16	2875	114	0
FPT31	3422	14	188
FBV334	490	718	0
DHC	2508	1304	0
HH	1266	2884	158
LS	0	578	328
NT	1071	440	0
CD	1220	2542	0
CX	0	1677	0
TTN	0	0	0

Low ---------->High
Equivalent units of PPAR activities

Ngoc et al., submitted

Generic scheme for bioanalysis



Conclusions

- **Specific CALUX high throughput panel of assays for EDCs**
- **Very good predictions of adversity (not the phenotype)**
- **Test batteries can be relatively simple when using specific assays**
- **Metabolic steps, pharmacokinetic modeling can improve predictions**
- **Linkage to regulations: OECD/ECVAM/ISO validation, incorporation in guidelines, AOP linkage**
- **Applicable for read-across, safe design/green chemistry**
- **Specially designed and very suitable for safety of complex mixtures**

Support & collaboration

High throughput toxicity screen	Human health	Water, environment	Biobased, nutraceuticals
RePrOTect	NewGeneris Newborns and Genotoxic exposure risks	METAEXPLORE	ePlantLIBRA
ZonMw	Netherlands Toxicogenomics CENTRE	TECHNEAU	ecogenomics
ChemScreen	PROTECTED	DEMEAU	BE-Basic
[:::] EUTOXRISK		AquaNES	ReSolve CREATING SAFE AND RENEWABLE SOLVENTS
			fungus chain