NTP
National Toxicology Program

Survey of available testing methods for low dose toxicity, including new *in-vivo* and *in vitro* methods

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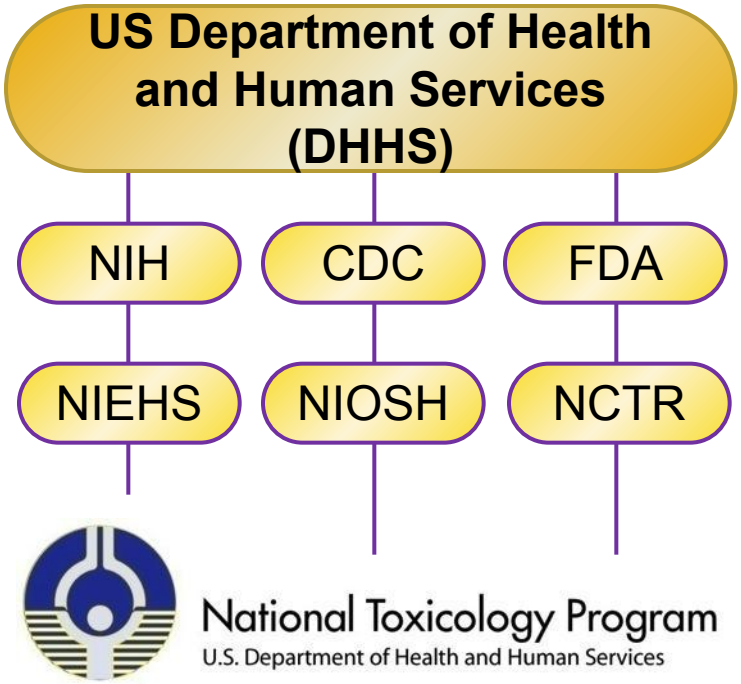
outline

- National Toxicology Program (NTP)
- Traditional toxicity assays
- Response to data poor toxicology emergency
- The low dose conundrum- BPA
- BPA alternatives- an approach

The US National Toxicology Program

- **Interagency program**
  - Established in 1978
  - Headquartered at NIEHS
- **Research on "nominations"**
  - Thousands of agents evaluated in comprehensive toxicology studies
  - Results communicated through technical reports, scientific publications, and the web
- **Analysis activities**
  - Report on Carcinogens
  - Office of Health Assessment & Translation
  - NTP Interagency Center for the Evaluation of Alternative Toxicological Methods



http://ntp.niehs.nih.gov

Diverse data for public health decisions

- Epidemiology
- Traditional animal and genetic toxicology studies
- Structure Activity Relationships (SAR)
- Tox 21 high throughput screening
- Alternative models (zebrafish, C. elegans)
- Toxicogenomics
- Read across

- Systematic review methods to evaluate and integrate findings

Standard NTP toxicology assays

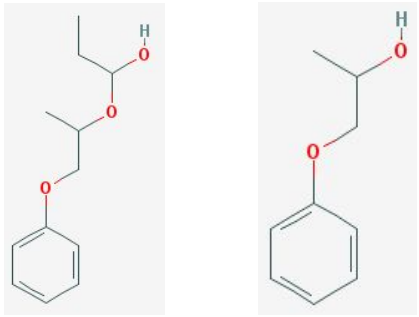
- Prechronic (14 and 90-day toxicology screens) rats, mice, both sexes
- Two-year rodent cancer studies
- Genetic toxicology (Salmonella mutation assay, blood and bone marrow micronucleus, pig-A assay, comet assay)
- Reproductive assessment by continuous breeding in rats
- Modified one-generation reproductive study
- Developmental assessments (follows FDA segment 2 guidelines)
- Immunotoxicity in mice (immune cell counts, functional responses, *in vivo* challenge assays, hypersensitivity assays)
- Absorption, Distribution, Metabolism, Excretion studies
- Toxicokinetic studies
- Toxicogenomic studies

January 9, 2014- A data poor emergency situation

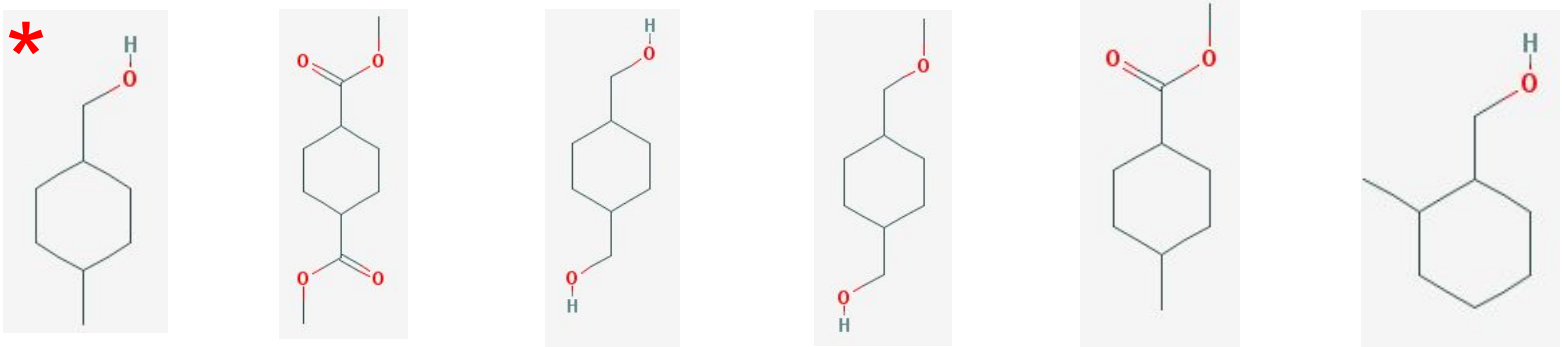
Charleston WV residents notice a “sweet smell” (like licorice) in the air.

Elk River, West Virginia-January 9, 2014

Phenyl Ethers PPH



Crude Methylcyclohexanemethanol (MCHM)



A liquid used to wash coal was spilled from a leaking tank into the Elk River approximately 1.5 miles upstream of the water intake facility serving 300,000 people.

Derivation of Drinking Water Advisory Level (DWAL)

Eastman Chemical releases results of toxicity studies.

CDC uses results from a 28-day repeat dose study to calculate a drinking water advisory level in water.

- Point of departure
  - **100 mg/kg/day**
- Safety factors
  - Limited database (10)
  - Rodent to human (10)
  - Sensitive individuals (10)
- Dose not anticipated to cause adverse effects
  - 0.1 mg/kg/day
- DWAL (10 kg child)
  - 1 ppm

Elk River chemical toxicology data

Uncertainties

- Few toxicology studies to support the MCHM DWAL
- No studies of MCHM in developing animals
- Very limited data on the minor components of the spill

Types of studies selected

Rapid predictive screens

- Structure Activity Relationships – commercial databases of known toxicology information for chemicals of similar structure
- High throughput screens (Tox21) – human cells for gene expression changes in pathways of toxicological concern
- C. elegans (roundworm) toxicity – expose nematodes for effects on reproduction, growth, and behavior
- Zebrafish embryo toxicity – expose embryos to monitor effects on structural and functional development
- Genetic toxicity – Ames test

Types of studies selected

Studies using rodents

- 5-Day toxicogenomic study – chemicals given to rats for 5 days, liver and kidney assayed for evidence of changes in the expression of genes known to be associated with responses to toxic chemicals
- Mouse dermal irritation and hypersensitivity studies – apply to mouse skin to assess potential to cause irritation and allergic responses
- Rat prenatal toxicity studies –determine effects on offspring of pregnant rats

5-Day toxicogenomics

Description

- Chemical orally administered to rats for 5 days- (0.1 to 500 mg/kg; 6 dose levels)
- Global gene expression measured (liver, kidney)
- Determine the most sensitive Molecular Biological Process (group of genes that function together to control a cellular process)
- Run Bench Mark Dose software
- Identify a biological “no effect level”, which typically occurs at a dose within a factor of 10 below that required for overt toxicity

Results in context of study goals

- *Strengthen the science base*
  - SAR predictions of developmental toxicity and irritancy confirmed
  - Rat prenatal toxicity study confirms prior NOEL (no observed effect level) of ~ 100 mg/kg/day (or ~ 1000 ppm in drinking water) for MCHM
  - 5-Day toxicogenomics studies show Molecular Biological Process activations at ~ 10 fold lower dose than phenotypic changes
  - Concentrations of MCHM and crude MCHM required to produce skin irritation and sensitization were much higher than expected
  - Low genotoxic potential minimizes concern for long-term health effects

Results in context of study goals

- *Determine if there are hazards for sensitive life stages*
  - Major components of spill did not affect C.elegans or zebrafish development
  - The fetus is more sensitive to toxicity than the pregnant adult rat (reduced fetal weights)
  - Toxicity occurred far above the drinking water advisory level that was derived by CDC
  - Subsequent State of WV birth weight survey was negative

Results in context of study goals

- *Screen minor components of the mixture to determine if any are more toxic than MCHM*
  - Minimal differences between the minor constituents and MCHM
  - One minor component (DMCHDC) was more toxic to developing zebrafish than MCHM, and was mutagenic

The collected findings supported the adequacy of the drinking water advisory level established at the time of the spill

Conceptual shift in environmental health science

OLD… chemicals act by overwhelming the body's defenses by brute force at very high doses

NEW… chemicals can act like hormones and drugs to disrupt the control of development and function at very low doses to which the average person is exposed

NEW… susceptibility to environmentally induced disease can vary widely, can persist long after exposure, and potentially across generations

NTP Bisphenol A studies

- Comprehensive GLP perinatal, 2-year, 7 days per week, 5-dose level gavage study in SD rats
- 2.5 to 25,000 µg/kg bw/day
- Control for litter effects, BPA in caging, water, feed, etc.
- Concurrent “positive” control
- Core protocol for interim (1 year) and 2-year animals
  - Vaginal cytology starting at 4 months to evaluate onset of aberrant cycles
  - Clinical chemistry, sperm analysis, organ weights, and target organ histopathology on interim sacrifice animals
  - At 2 years, complete necropsy with selected target organ histopathology
- Subset of animals for behavior testing
- All other animals for NIEHS-funded grantee studies; tissues from the same animals shared when feasible

Consortium members and areas of study

Name	Disease Focus	Endpoint	Aims Funded
Gail Prins	Prostate cancer	Prostate gene expression and cancer development *(PND 21; 6, 12, and 24 months)*	• Prostate gene expression • Prostate methylation • Renewal of stem cells • Assess PIN and cancer
Heather Patisaul	Learning and behavior	Brain transcriptomics *(Birth)* Behavior *(PND 21 and 90)*	• Brain gene expression • Behavioral assessment (*PND 21 and 90)*
Norbert Kaminski	Immune function	Spleen assessed *(PND 90 and 12 months)*	• Spleen T and B cells subpopulations • Response to stimulation • Estrogen receptor (ER) characterization • Gene expression
Kim Boekelheide	Testis function/sperm counts *(Continuous dosing only)*	Testis and epididymis (*PND 90 and 12 months)*	• Histological and morphological assessment of testis • Caudal sperm transcriptome • Caudal sperm methylome

Consortium members and areas of study

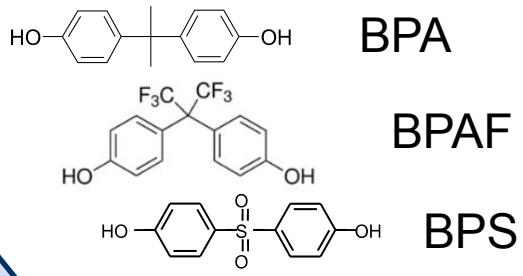
Name	Disease Focus	Endpoint	Aims Funded
Ana Soto	Breast cancer	Breast development and cancer *(PND 21 and 90; 6 months (whole mounts))*	• Breast morphology as precursor of cancer (*PND 21)* • Gene expression and DNA methylation (*PND 21)* • Assess pre-neoplastic lesions and neoplastic lesions (*PND 90 and 6 months)*
Shuk Mei Ho	Uterine cancer *Continuous dosing only*	Uterus histology and gene expression *(6, 12, and 24 months)*	• Histological identification of uterine hyperplasia/adenocarcinoma • Laser capture to assess methylome and transcriptome to identify early cancer genes
Nira Ben Jonathan	Obesity/adipose tissue	Adipose tissue disposition and weight gain *(PND 90; 6 and 12 months)*	• Fat depots and selected adipokines, gene expression • Serum hormones • Adipose cell number and size • BPA in fat tissues

Consortium members and areas of study

Name	Disease Focus	Endpoint	Aims Funded
Fred vom Saal	Male urogenital abnormalities	Urogenital system analysis *(Birth; 12 and 24 months)*	• 3D reconstruction of urogenital system • Examine animals for voiding and laser capture to assess gene expression in epithelium and stroma
Jodi Flaws	Ovarian function	Ovary *(Birth, PND 21 and 90, and 12 months)*	• Follicle number • Steroidogenic enzymes
Tom Zoeller	Thyroid and brain anatomy	Thyroid and brain development *(PND 15 and 21)*	• Changes in brain gene expression and histology due to BPA impact on thyroid hormones
Nestor Gonzalez-Cadavid	Penile function	Penile erection mechanism *(12 months)*	• Erection capability, transcriptomic profile, and stem cell analysis
Andrew Greenberg	Diabetes, blood glucose, and pancreas	Blood glucose and pancreas assessment *(12 months)*	• Assess blood glucose over time, beta cell mass, and insulin content

What is the biological activity of BPA analogues of emerging public health concern?

NTP assessment of BPA and its analogues



Literature Review

- Human epidemiology
- Animal
- *In vitro*

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Similarity Profiling

Biological Similarity

Structural Similarity

Laboratory Experiments

- *In vitro* (ER α/β, AR, transcriptomics, etc.)
- *In vivo* (Zebrafish, *C. Elegans*)

Long-term Studies

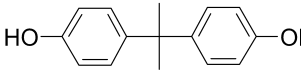
- Perinatal Repro and Developmental
- Adult oral exposure 90-day toxicity

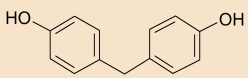
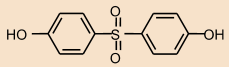
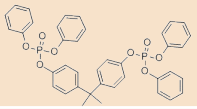
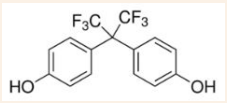
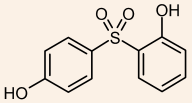
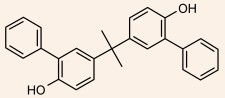
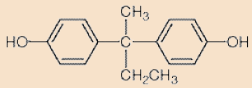
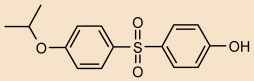
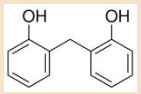
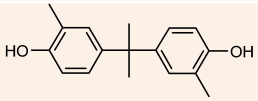
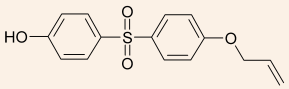
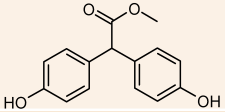
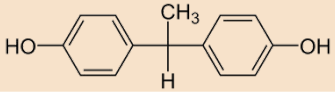
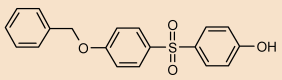
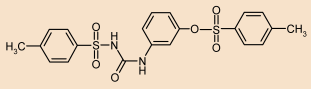
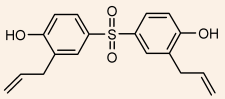
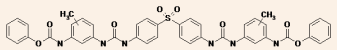
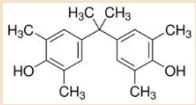
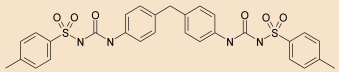
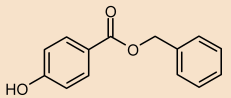
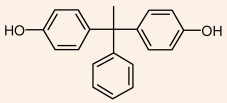
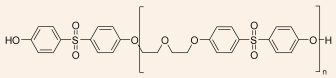
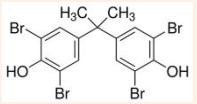
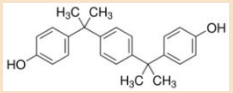
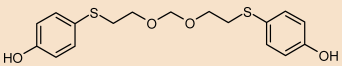
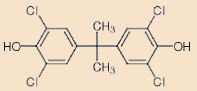
Short-term Studies

- ADME/TK
- Adult toxicity

Integrated Assessment of Bisphenols

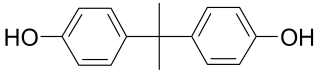
BPA analogues under study

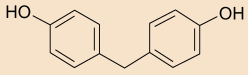
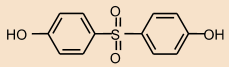
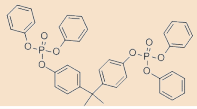
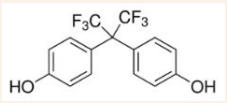
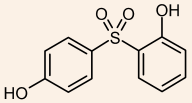
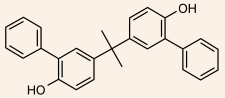
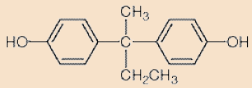
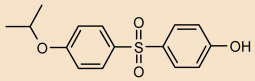
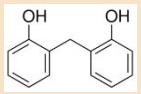
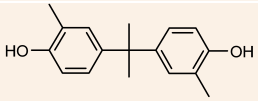
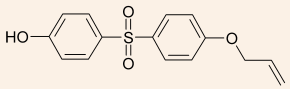
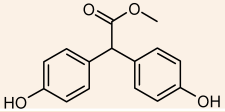
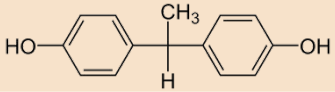
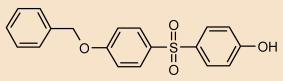
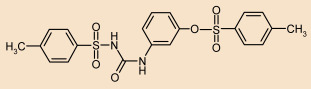
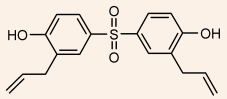
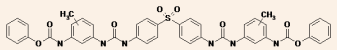
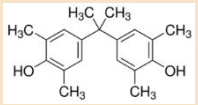
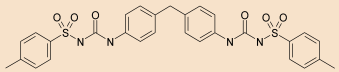
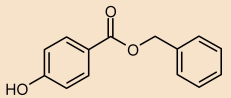
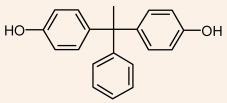
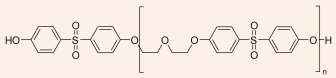
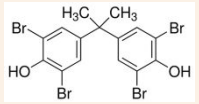
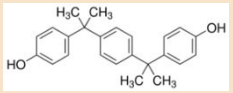
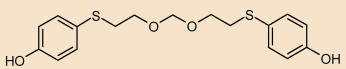
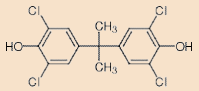

Bisphenol A

Structure	Chemical	Structure	Chemical	Structure	Chemical
	Bisphenol F (4,4)		Bisphenol S		BPA bis(diphenyl phosphate)
	Bisphenol AF		2,4-BPS		Bisphenol PH
	Bisphenol B		D8		Bisphenol F (2,2)
	Bisphenol C		BPS-MAE		MBHA
	Bisphenol E		BPS-MPE		Pergafast 201
	Bisphenol Z		TGSA		Urea Urethane Compound
	TMBPA		BTUM		PHBB
	Bisphenol AP		D-90		TBBPA
	Bisphenol P		DD-70		TCBPA

BPA analogues under study

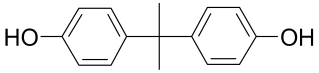
Red= known or potential use in thermal paper

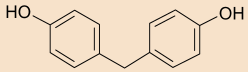
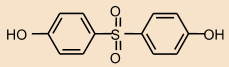
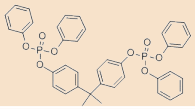
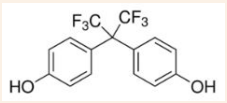
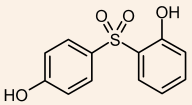
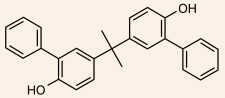
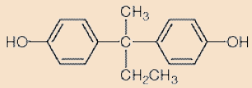
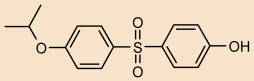
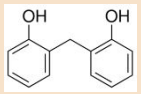
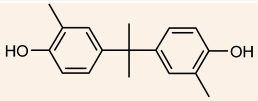
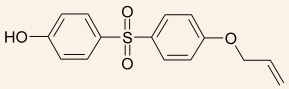
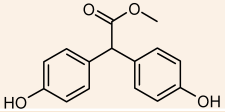
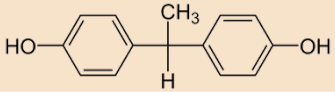
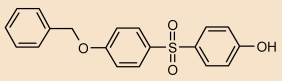
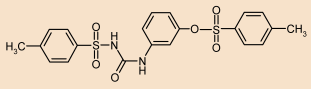
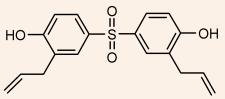
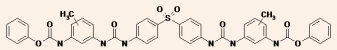
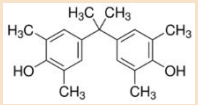
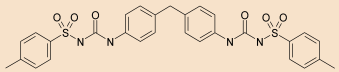
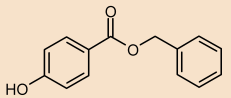
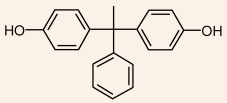
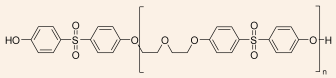
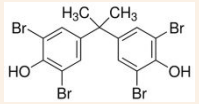
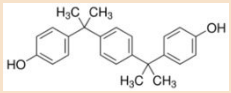
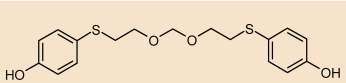
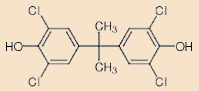
 Bisphenol A

Structure	Chemical	Structure	Chemical	Structure	Chemical
	Bisphenol F (4,4)		Bisphenol S		BPA bis(diphenyl phosphate)
	Bisphenol AF		2,4-BPS		Bisphenol PH
	Bisphenol B		D8		Bisphenol F (2,2)
	Bisphenol C		BPS-MAE		MBHA
	Bisphenol E		BPS-MPE		Pergafast 201
	Bisphenol Z		TGSA		Urea Urethane Compound
	TMBPA		BTUM		PHBB
	Bisphenol AP		D-90		TBBPA
	Bisphenol P		DD-70		TCBPA

BPA analogues under study

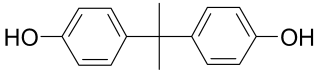
Blue = detected in environment or human samples

 Bisphenol A

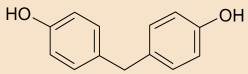
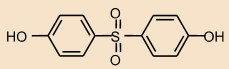
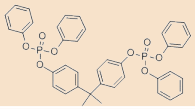
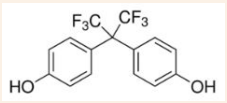
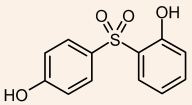
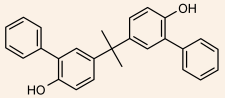
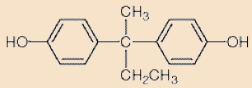
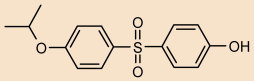
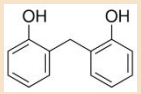
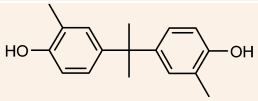
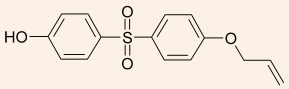
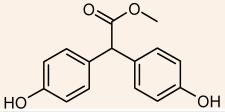
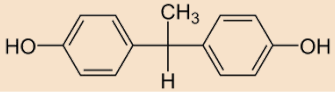
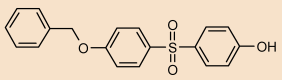
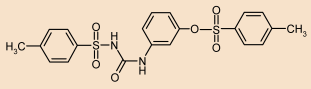
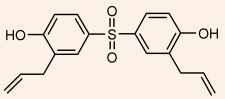
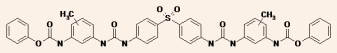
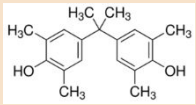
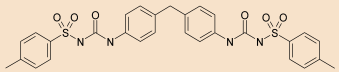
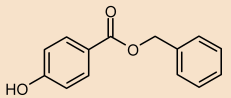
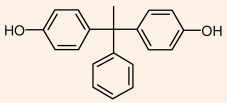
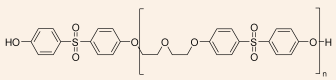
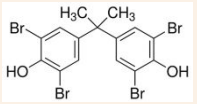
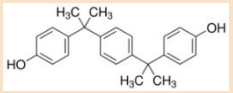
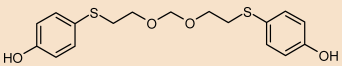
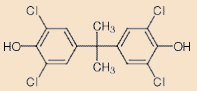
Structure	Chemical	Structure	Chemical	Structure	Chemical
	Bisphenol F (4,4)		Bisphenol S		BPA bis(diphenyl phosphate)
	Bisphenol AF		2,4-BPS		Bisphenol PH
	Bisphenol B		D8		Bisphenol F (2,2)
	Bisphenol C		BPS-MAE		MBHA
	Bisphenol E		BPS-MPE		Pergafast 201
	Bisphenol Z		TGSA		Urea Urethane Compound
	TMBPA		BTUM		PHBB
	Bisphenol AP		D-90		TBBPA
	Bisphenol P		DD-70		TCBPA

BPA analogues under study

Green= flame retardants; orange = plastic/resins/dental polymers



Bisphenol A

Structure	Chemical	Structure	Chemical	Structure	Chemical
	Bisphenol F (4,4)		Bisphenol S		BPA bis(diphenyl phosphate)
	Bisphenol AF		2,4-BPS		Bisphenol PH
	Bisphenol B		D8		Bisphenol F (2,2)
	Bisphenol C		BPS-MAE		MBHA
	Bisphenol E		BPS-MPE		Pergafast 201
	Bisphenol Z		TGSA		Urea Urethane Compound
	TMBPA		BTUM		PHBB
	Bisphenol AP		D-90		TBBPA
	Bisphenol P		DD-70		TCBPA

Systematic Review methodology

Databases Searched

- SciFinder
- Embase
- PubMed
- Scopus
- Toxline
- Web of Science

SR Tools Used

DistillerSR

DRAGON
An online tool for systematic review

HAWC
HEALTH ASSESSMENT WORKSPACE COLLABORATIVE
A content management system for human health assessments.

Gray Literature

- ECHA's REACH database
- HTS (Tox21/ToxCast data)

Data Streams

- Animal and *in vitro* data from literature searches
- Non-peer reviewed data obtained from ECHA's REACH database
- Hazard IDs developed for the US EPA DfE "Alternatives to BPA in Thermal Paper"
- High throughput screening data

Inventory of available literature

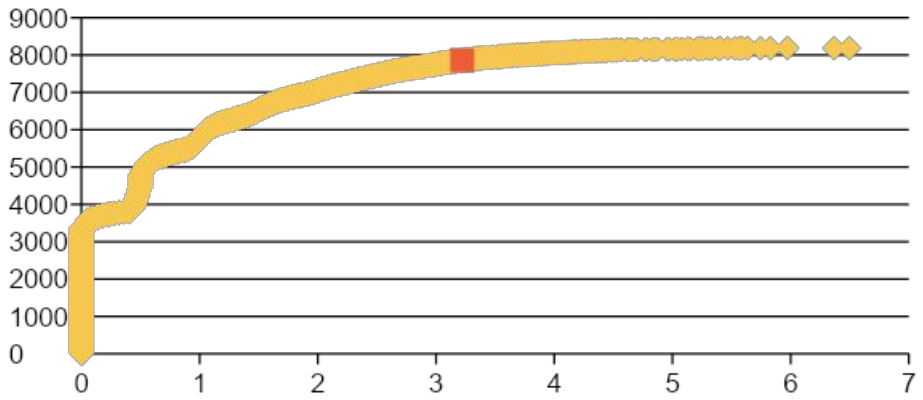
Chemical	Human	Animal	*In Vitro*
4,4-BPF	3	15	61
BPS	1	9	52
BPAF	0	10	41
BPB	0	9	35
BPC	0	5	22
BPE	0	3	23
BPZ	0	3	15
TMBPA	0	1	14
BPAP	0	2	9
BPP	0	0	6
2,2-BPF	0	2	1
BDP	0	1	2
BPPH	0	0	2
2,4-BPS	0	1	1
D-8	0	0	3
Pergafast 201	0	0	1

There were no records of human, animal, or mechanistic data for:

- BPS-MAE
- BPS-MPE
- BTUM
- D-90
- DD-70
- MBHA
- TGSA
- UU

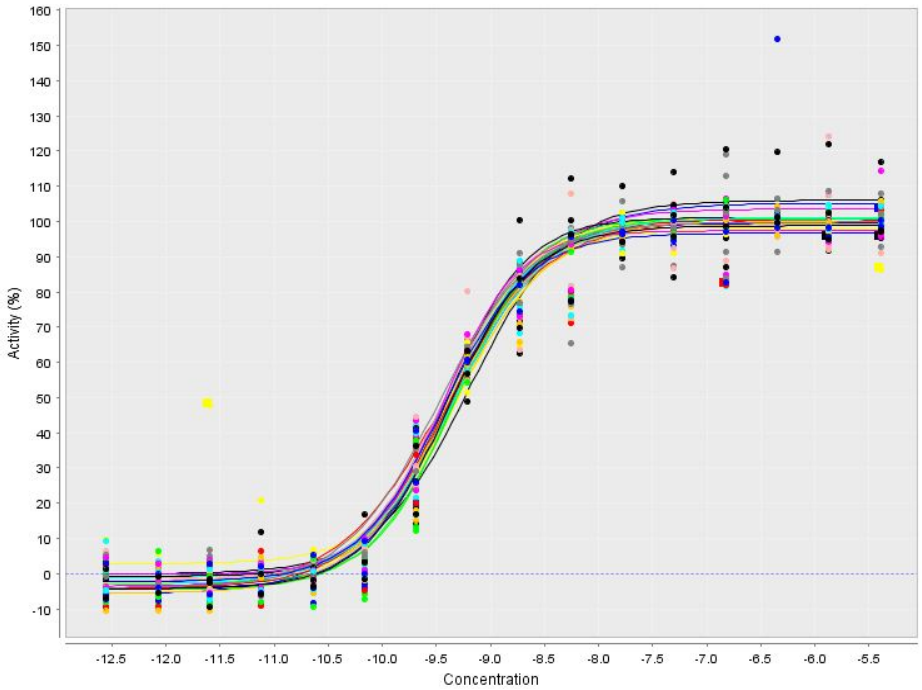
Tox21 high throughput screening capabilities

- Robotics
- Compound handling capabilities
- Informatics tools
- In the past 4 years, NCATS has been screening over 8,000 compounds against ~75 nuclear receptors and stress response pathways using cell-based assays
- Moving to transcriptomic assessments



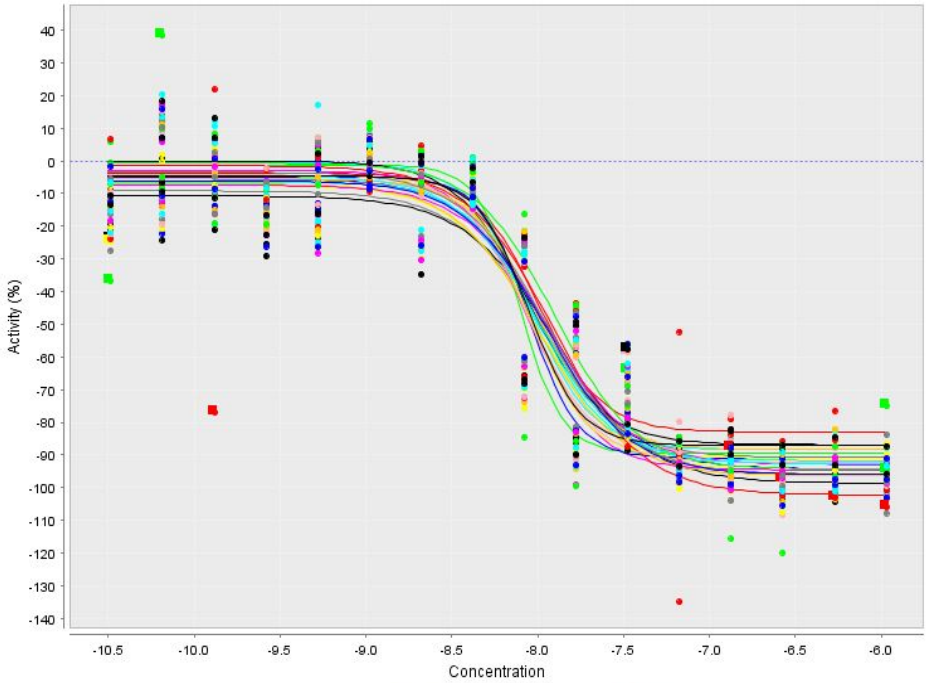
Estrogen Receptor alpha

β-estradiol (agonist) positive control dose response



ERα-bla	Online Validation Agonist (Mean ± SD)	Online Validation Antagonist (Mean ± SD)
EC50	0.40 ± 0.07 nM (n = 27)	0.01 ± 0.002 µM (n = 27)
S/B	3.68 ± 0.19	2.31 ± 0.08
CV (%)*	10.04 ± 1.02 (n = 18)	4.71 ± 1.05 (n = 18)
Z'	0.73 ± 0.05	0.68 ± 0.09

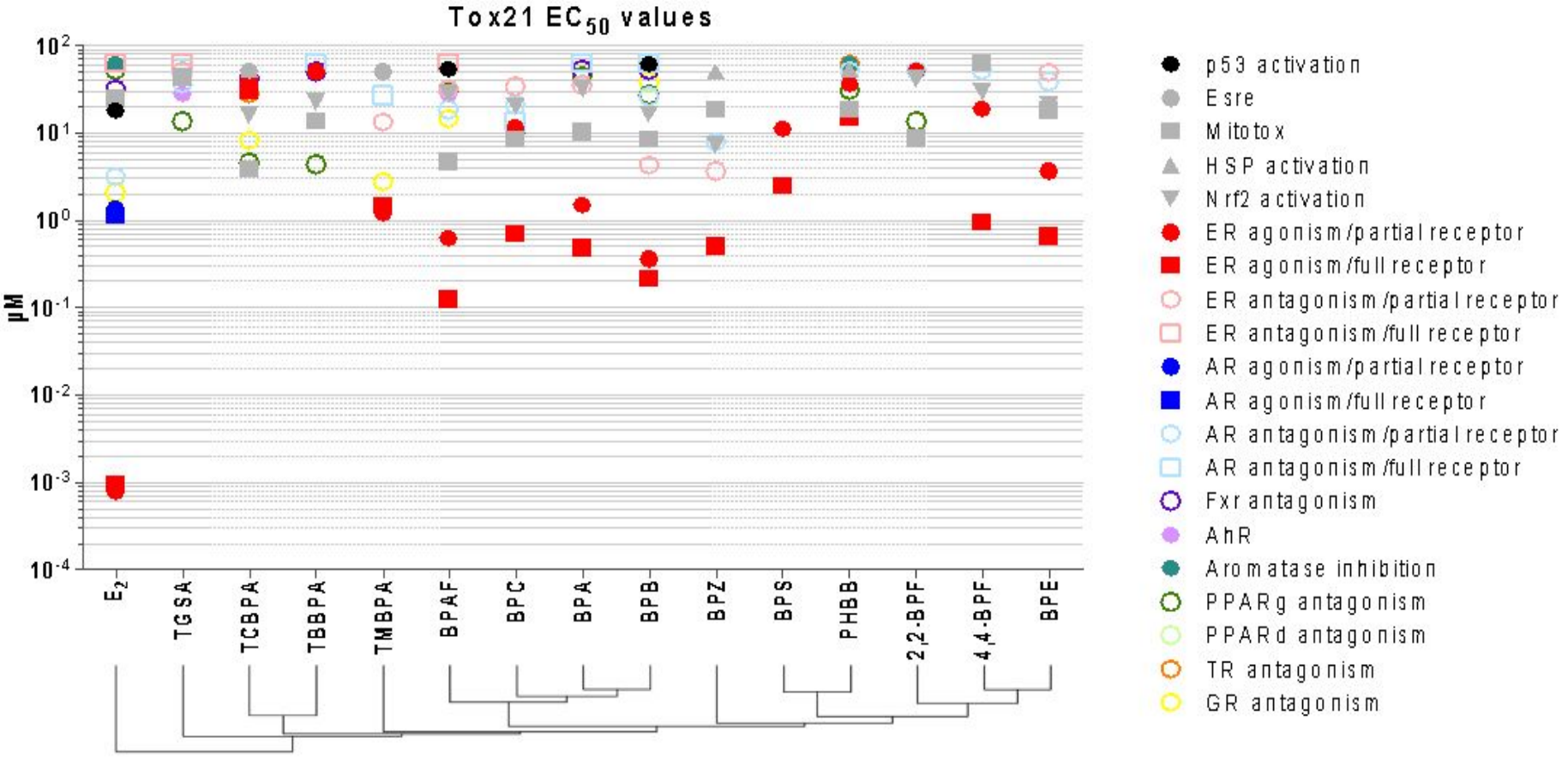
4-hydroxy tamoxifen (antagonist) positive control dose response



ERα-bla	Online Screening Agonist (Mean ± SD)	Online Screening Antagonist (Mean ± SD)	Online Screening Viability (Mean ± SD)
IC50	0.34 ± 0.42 nM (n = 461)	5.30 ± 1.88 nM (n = 464)	NA
S/B	4.65 ± 0.56	3.33 ± 0.82	132.86 ± 8.25
CV (%)**	3.57 ± 1.22 (n = 54)	3.81 ± 0.86 (n = 54)	9.76 ± 5.65 (n = 54)
Z'	0.53 ± 0.09	0.41 ± 0.10	0.75 ± 0.07

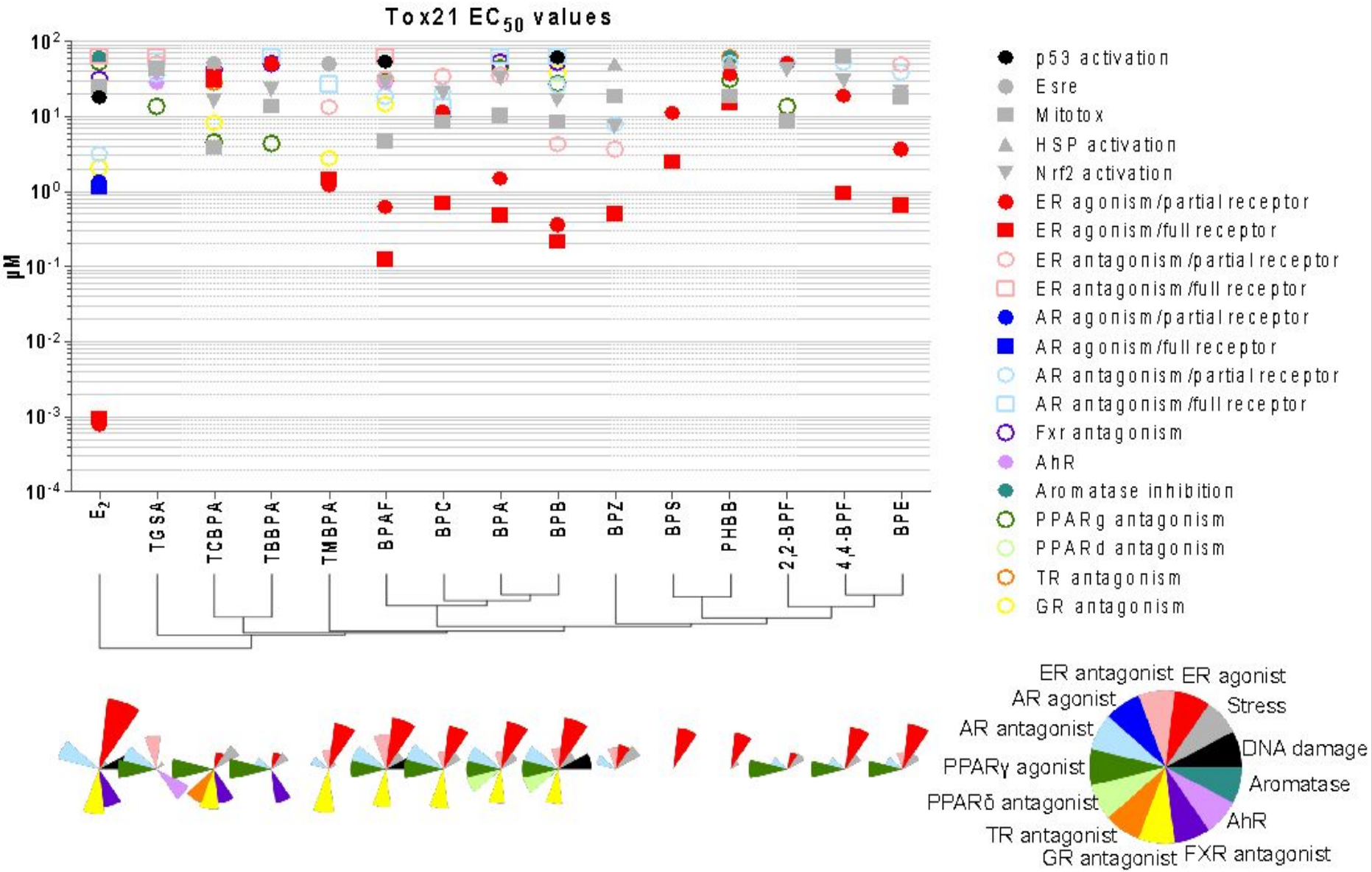
*CV values shown represent average of DMSO plates and low concentration plates
**CV values shown represent average of DMSO plates only

BPA Analogues in Tox21 Assays



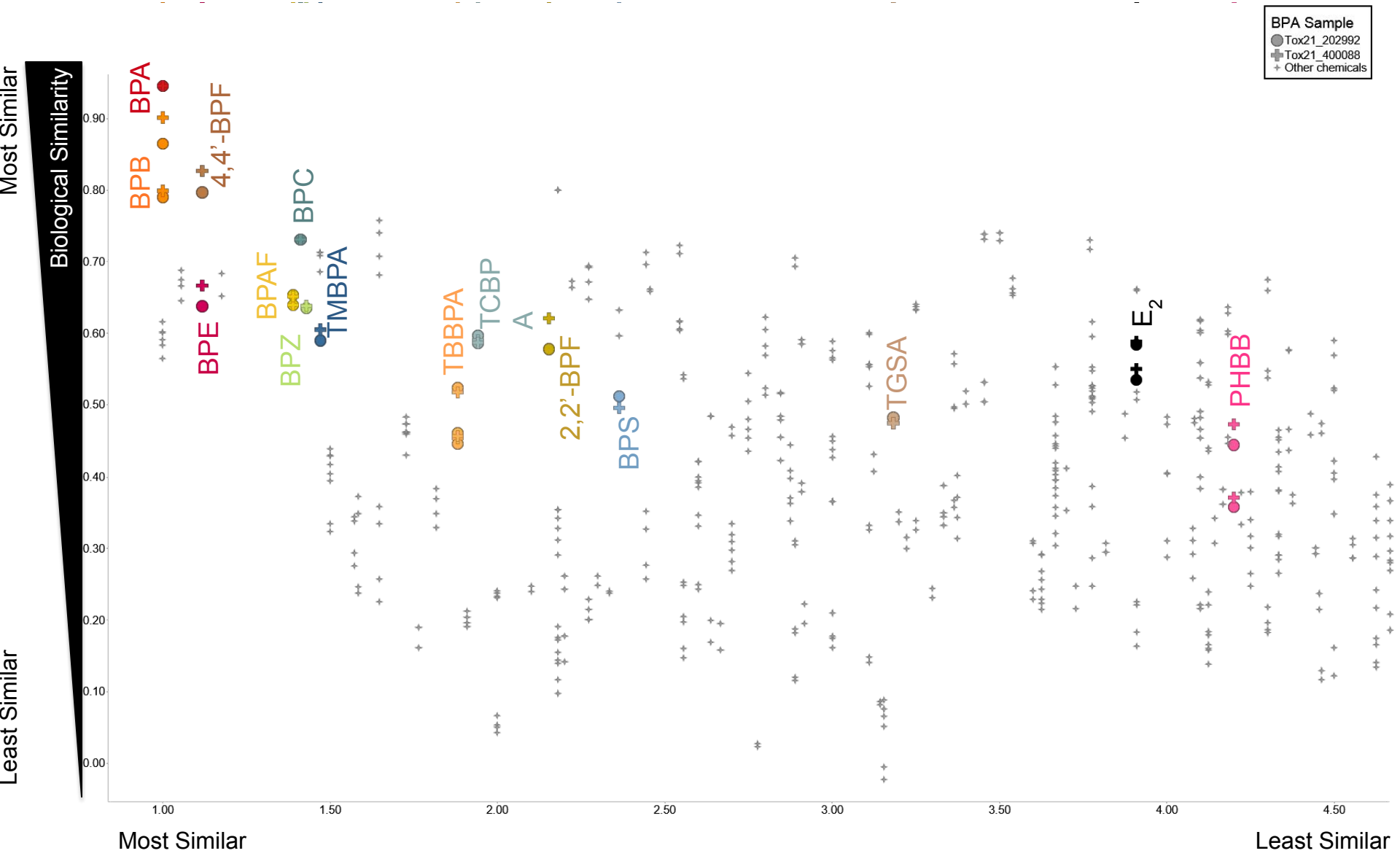
http://apps.sciome.com:3838/ActivityProfiling/

BPA analogues in Tox21 assays



http://apps.sciome.com:3838/ActivityProfiling/ http://comptox.unc.edu/toxpi.php

Similarity of BPA Analogues to BPA in Tox21



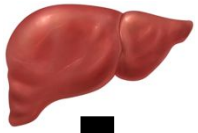
Conclusions

- Wide variety of approaches to assess potential toxicity
- Value in using a variety of screening assays in several species
- “Low dose” toxicity most often demonstrated in studies at molecular level
- Translation of low dose effects to traditional toxicity endpoints is under active investigation
- Methods measuring doses at which no measurable gene expression changes are observed in vivo or in vitro may hold promise for “agnostic” screening

Questions?

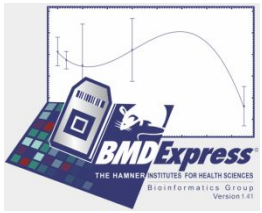
5-Day rat toxicogenomics

Molecular Biological Process BMD

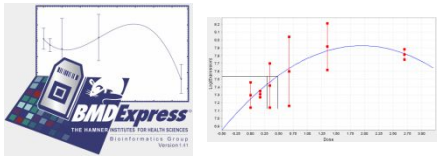


Liver RNA (6 dose levels, 4-5 animals/group)

RNA Expression Levels~20000 genes



Identify "Active" genes:
ANOVA(P<0.05) &
Fold change >|1.5|



Identify Gene-level BMDs:
"Active" genes are fit to 5 models. A BMD is derived from best fit model.

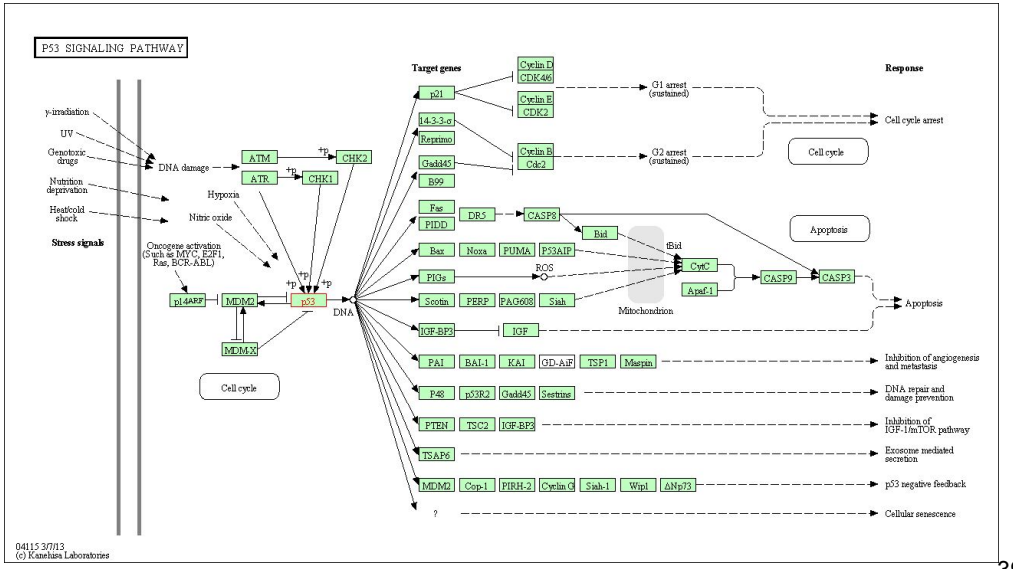
Genes sorted into Molecular Biological Processes (**MBP**)

Identify "Active" MBPs:
P<0.05 Fisher Exact test &
≥5% populated &
≥ 5 genes

Report Median MBP BMDs
(median BMD of gene in pathway or BP)

Molecular Biological Process

- A group of genes that function together control a cellular process (e.g. P53 signaling pathway, lipid metabolism, etc.)
  - Different types of Molecular Biological Processes
    - KEGG Pathways
    - GO Biological Processes

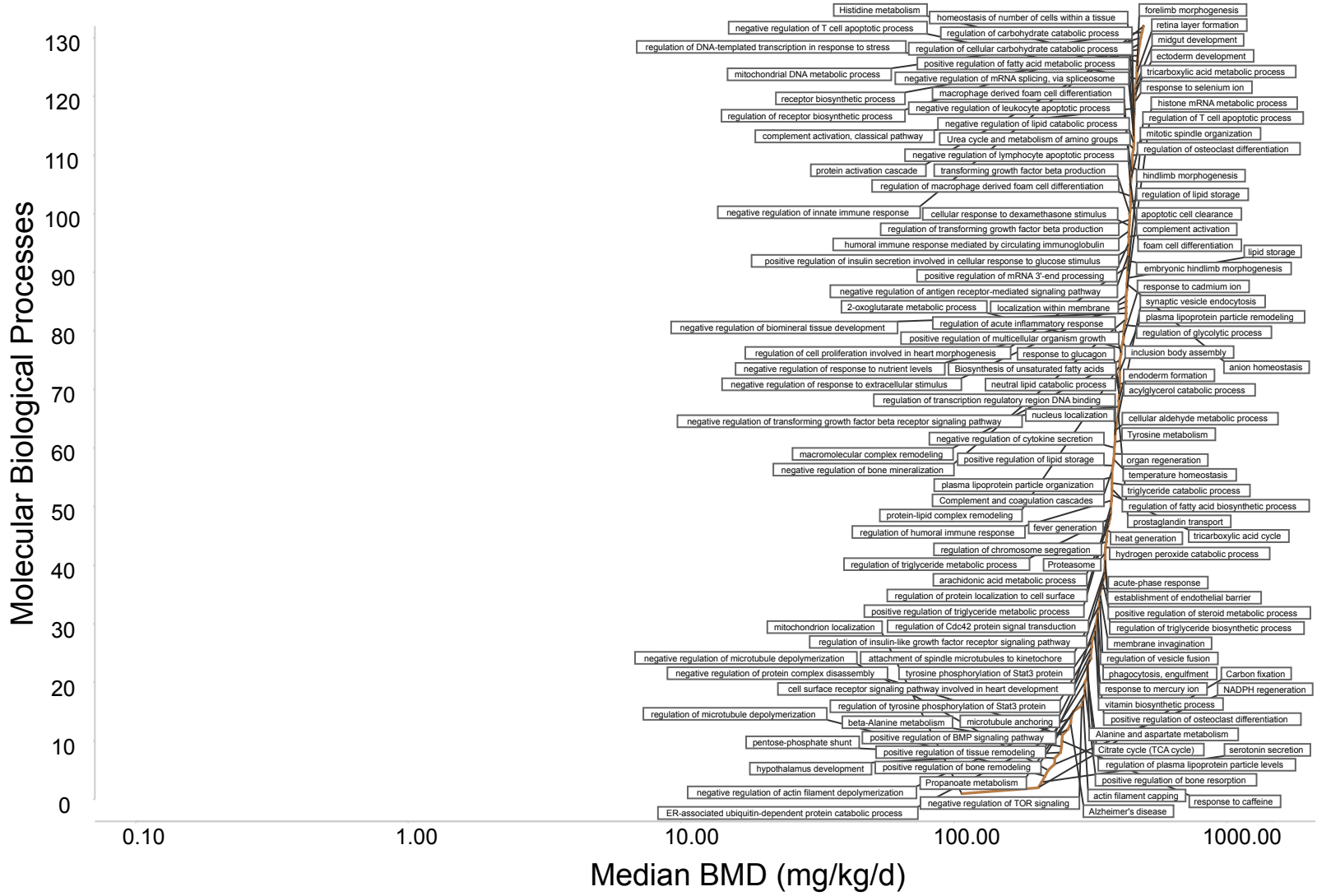


5-Day rat toxicogenomics

Findings

- Liver
  - MCHM
    - 22 Molecular Biological Processes active and had calculated BMD values
    - Minimum biological effect benchmark dose: 13 mg/kg/day- fatty acid metabolism
  - Crude MCMH mixture
    - 28 Molecular Biological Processes active
    - Minimum biological effect benchmark dose: 10 mg/kg/day- ribosome biogenesis

MCHM Molecular Biological Process Accumulation Plot



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