Abstract #: 3540
Poster #: P232

Considering Possible Mechanisms of Toxicity for Phthalates on California's Proposition 65 List Using BioSeek Data from ToxCast™

Nathalie Pham[1], Shoba Iyer[1], Melanie Marty[1], Martha Sandy[1], Gina Solomon[2], and Lauren Zeise[1]

[1]California Environmental Protection Agency's (CalEPA) Office of Environmental Health Hazard Assessment (OEHHA); [2]CalEPA

DISCLAIMER: The views expressed are those of the authors and do not necessarily represent the views and policies of OEHHA or CalEPA.

OEHHA
SCIENCE FOR A HEALTHY CALIFORNIA

Introduction

The US EPA's Toxicity Forecaster program (ToxCast™) is recognized as a rich source of information for the toxicity testing of environmental chemicals by batteries of high-throughput screening (HTS) assays. However, there still remains some degree of disconnect between the *in vitro* data from these HTS assays and the potential links to health and safety concerns in humans. To further elucidate the links between HTS and human health outcomes, we explore the relationships between the *in vitro* data from human cell lines (Bioseek (BSK)) for the chemical class *ortho*-phthalates and the observations from human-related studies as curated by the Comparative Toxicogenomics Database (CTD; http://ctdbase.org) in this study. The CTD identifies associations between human diseases to both chemicals and genes independently, as well as direct interactions between chemicals and genes/proteins. We mined these associations to identify potential links between *in vitro* assay data and *in vivo* toxicity for the *o*-phthalates. The *in vitro* associations identified in this analysis may prove useful for supplementing published evidence on phthalate mechanisms of toxicity, and may shed light on new suggested mechanisms not investigated previously.

Methods

CHEMICAL SELECTION (o-PHTHALATES)

The chemical class of *o*-phthalates was a candidate for study based on previous work (Pham et al., 2016) that explored the relationships between phthalates and their assay activity in US EPA's ToxCast program. The subset of phthalates was selected based on the overlapping chemical candidates from ToxCast and those listed in California's Proposition 65 as either causing reproductive toxicity (developmental, male, or female), or cancer (Title 27, California Code of Regulations, § 27001). Phthalates selected for this study are shown in Table 1.

ASSAY SELECTION (BIOSEEK PLATFORM—SELECTION OF ASSAYS WITH GENE TARGETS)

We selected the BioSeek (BSK, a division of DiscoveRx, Inc.) platform for assay exploration in this study. Selection was based on several criteria: 1) number of endpoints investigated (174 endpoints); 2) range of genes targeted in assays; and 3) translational capability to relevant *in vivo* endpoints (BSK data is captured through protein expression profiles in complex primary human cell culture models). BSK assays utilize eight cell-types. Each assay target includes a gain and a loss in signal endpoint.

The assay design for BSK incorporates enzyme-linked immunosorbent assay (ELISA) methodology in which protein-specific antibodies are used to tag and quantify levels of protein. Protein levels are detected by fluorescence. Changes in levels of protein expression in response to chemical agents are indicative of changes in signaling as they relate to a specific gene. The gene targets were identified from the assay profiles in the Interactive Chemical Safety for Sustainability (iCSS) ToxCast Dashboard (Table 2). The data from phases 1 and 2 of ToxCast Dashboard were retrieved on November 30, 2015 (http://www2.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data) and updated manually February 10, 2016.

PATHWAY SELECTION (CTD)- CURATED

We selected six hazard traits that could be linked to adverse pathways in the CTD: hepatotoxicity, cardiotoxicity, nephrotoxicity, reproductive toxicity, developmental toxicity, and cancer (Figure 1). Curated associations between gene targets identified in BSK and human disease/ adverse pathways in the CTD were extracted from the published scientific literature by CTD curators, or were derived from the Online Mendelian Inheritance in Man (OMIM) database using the mim2gene file from the NCBI Gene database. Gene-disease associations were retrieved from the CTD on January 20, 2016.

TOXPI VISUALIZATION

We used the ToxPi software to rank each phthalate for activity across the ToxCast BSK assays. ToxPi was developed at the Carolina Center for Computational Toxicology at the University of North Carolina at Chapel Hill (Reif et al., 2010). Each ToxPi slice represents a group of BSK assays categorized into a specific toxicity-associated pathway. Half-maximal response (AC_{50}) values for phthalates active in assays and values of 10^6 for phthalates inactive in assays were used as the input data. Each ToxPi slice length is proportional to the normalized potency of the assay value ($-\log_{10}(AC_{50}) + 6$) of the component assays included in that slice. The ToxPi slices are equally weighted and thus have the same width, regardless of the number of measured endpoints.

Table 1. Phthalates listed in California's Proposition 65 and also tested in BSK platform (ToxCast).

Chemical	Abbreviation	CAS-RN	Structure	Classification	Date Listed
Butyl benzyl phthalate	BBP	85-68-7		Developmental	12/2/05
Dibutyl phthalate	DBP	84-74-2		Developmental; Female and Male Reproductive	12/2/05
Di(2-ethylhexyl) phthalate	DEHP	117-81-7		Cancer; Developmental; Male Reproductive	10/24/03
Dihexyl phthalate	DHP	84-75-3		Female and Male Reproductive	12/2/05
Diisodecyl phthalate	DIDP	68515-49-1		Developmental	4/10/07

Table 2. Genes targeted in BSK assays.

BSK Assay Endpoint	Gene Target	Gene Name/ Function
CD38	CD38	Cluster of differentiation 38 molecule
CD40	CD40	Cluster of differentiation 40 molecule
CD69	CD69	Cluster of differentiation 69 molecule
Collagen III	Col3A1	Collagen alpha-1
EGFR	EGFR	Epidermal growth factor receptor
Eotaxin-3	CCL26	Chemokine ligand 26
E-selectin	SELE	CD62 antigen-like family member E
HLADR	HLADRA	Human Leukocyte Antigen - antigen D Related
ICAM-1	ICAM1	Intercellular Adhesion Molecule 1
IL-1a	IL-1a	Interleukin-1 alpha
IL6	IL6	interleukin 6
IL8	CXCL8	interleukin 8 (CXCL8)
IP10	CXCL10	interferon gamma-induced protein 10
LDLR	LDLR	Low density lipoprotein receptor
MCP1	CCL2	monocyte chemotactic protein 1; chemokine (C-C) ligand 2
MCSF	CSF1	Macrophage colony-stimulating factor
MIG	CXCL9	Monokine induced by gamma interferon
MMP1	MMP1	Matrix metalloproteinase-1
MMP-9	MMP9	Matrix metallopeptidase 9
PAI-1	SERPINE1	Plasminogen activator inhibitor-1
PGE2	PTGER2	Prostaglandin E2
P-selectin	SELP	P-selectin
SAA	SAA1	serum amyloid A1
TGF-B1	TGF-B1	transforming growth factor beta 1
Thrombomodulin	THBD	Thrombomodulin (TM)
TIMP-1	TIMP-1	a tissue inhibitor of metalloproteinase 1
TIMP-2	TIMP-2	a tissue inhibitor of metalloproteinase 2
Tissue Factor	F3	TF; coagulation factor III (thromboplastin)
TNFa	TNFa	tumor necrosis factor a
tPA	PLAT	tissue-type plasminogen activator (PLAT)
uPA	PLAU	Urokinase plasminogen activator
uPAR	PLAUR	Urokinase plasminogen activator receptor (PLAUR)
VCAM1	VCAM1	Vascular cell adhesion molecule 1
VEGFRII	KDR	Vascular endothelial growth factor receptor-2(KDR (type III receptor tyrosine kinase))

Figure 1. Adverse outcomes as listed by the CTD used for identifying possible toxicity pathways.

Developmental Toxicity
- Developmental delay
- Developmental disabilities
- Neural tube defects
- Motor skills disorder
- Neurologic manifestations
- Movement disorders
- Neonatal diseases and abnormalities
- Congenital abnormalities
- Birth defects
- Newborn diseases

Cardiotoxicity
- Cardiomyopathies
- Heart failure
- Myocardial ischemia
- Myocardial reperfusion injury
- Endomyocardial fibrosis
- Hypotension (hypertension)
- Coronary disease
- Heart diseases
- Cardiac arrhythmia
- Pericardial effusion
- Endocarditis
- Ventricular dysfunction

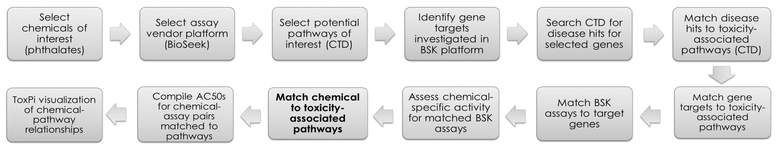
Reproductive Toxicity
- Endocrine system diseases
- Thyroid diseases
- Adrenocortical hyperfunction
- Sex chromosome aberrations
- Disorders of sex development
- Gonadal dysgenesis
- Urogenital abnormalities (male and female)
- Gonadal disorders

Hepatotoxicity
- Acute-or-chronic liver failure
- Drug-induced liver injury
- End stage liver disease
- Liver abscess
- Liver cirrhosis
- Liver diseases
- Liver failure
- Hepatitic insufficiency
- Hepatitic disease
- Liver dysfunction

Nephrotoxicity
- Nephritis
- Glomerulonephritis
- Nephrotic disease
- Nephrosis
- Nephrotic syndrome
- Nephropathy
- Renal failure
- Urologic diseases
- Hydronephrosis
- Renal dysplasia
- Renal tubular dysgenesis
- Nephrocalcinosis

Cancer
- Cancer (carcinomas, blastomas, adenomas)

Figure 2. Step-wise process for the determination of potential links between a chemical and a toxicity-associated pathway.



Results

Table 3. Chemical-specific hits for gene targets for each hazard trait.

A)

GENE TARGETS FOR DEHP (Total Hits: 15)

Assay Endpoint	Heptatotoxicity	Cardiotoxicity	Nephrotoxicity	Reproductive Toxicity	Developmental Toxicity	Cancer
CD38 (1)		X	X			
CD40 (2)						X
CD69 (1)				X		
Eotaxin-3 (1)						
HLADR (1)						
IL6 (1)	X	X	X	X		X
IL8 (1)	X		X			X
IP10 (2)	X			X		X
MCP1 (1)	X	X	X		X	X
MCSF (1)						
P-selectin (1)		X				
TNFa (1)	X	X	X			X
VCAM1 (1)		X				X

B)

GENE TARGETS FOR DBP (Total Hits: 17)

Assay Endpoint	Heptatotoxicity	Cardiotoxicity	Nephrotoxicity	Reproductive Toxicity	Developmental Toxicity	Cancer
CD38 (1)		X	X			
CD40 (2)						X
Collagen III (1)	X	X	X			
E-selectin (1)		X	X			
IL8 (1)	X		X			X
IP10 (1)	X			X		X
MCP1 (1)	X	X	X		X	X
MCSF (3)						
PGE2 (1)				X		
SAA (1)						
Thrombomodulin (1)		X				X
tPA (1)	X	X				
VCAM1 (2)		X				X

C)

GENE TARGETS FOR DHP (Total Hits: 23)

Assay Endpoint	Heptatotoxicity	Cardiotoxicity	Nephrotoxicity	Reproductive Toxicity	Developmental Toxicity	Cancer
CD38 (1)		X	X			
CD40 (2)						X
Collagen III (1)	X	X	X			
Eotaxin-3 (1)						
E-selectin (3)		X	X			
HLADR (1)						
IL8 (2)	X		X			X
MCP1 (4)	X	X	X		X	X
MCSF (2)						
P-selectin (1)		X				
Thrombomodulin (1)		X				X
VCAM1 (2)		X				X
VEGFRII (1)		X	X		X	X

D)

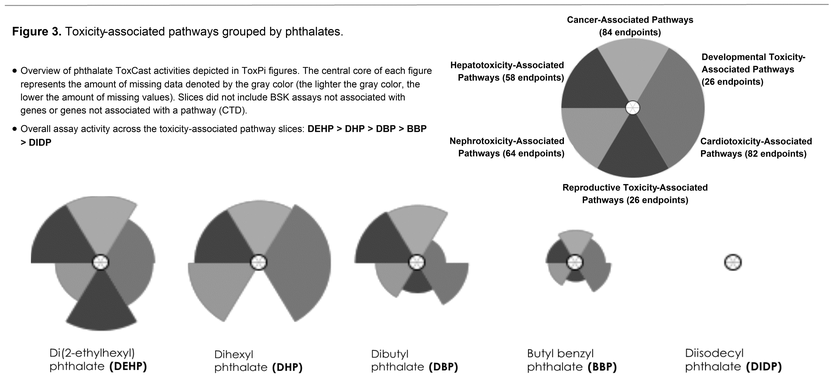
GENE TARGETS FOR BBP (Total Hits: 10)

Assay Endpoint	Heptatotoxicity	Cardiotoxicity	Nephrotoxicity	Reproductive Toxicity	Developmental Toxicity	Cancer
CD40 (1)						X
Collagen III (1)	X	X	X			
E-selectin (1)		X	X			
IL8 (1)	X		X			X
MCP1 (1)	X	X	X		X	X
MCSF (1)						
PGE2 (1)				X		
Thrombomodulin (1)		X				X
VCAM1 (2)		X				X

- **A)** Di (2-ethylhexyl) phthalate. **B)** Dihexyl phthalate. **C)** Dibutyl phthalate. **D)** Butyl benzyl phthalate. Diisodecyl phthalate has no active assays for gene hits. Specific gene targets and the total number of assay hits for each chemical is given. The parenthetical number next to each assay endpoint represents how many positive hits a chemical has for each gene regardless of cell type or signal direction.
- Gene hits with curated association to toxicity-associated pathways: **DHP > DBP > DEHP > BBP > DIDP**

Figure 3. Toxicity-associated pathways grouped by phthalates.

- Overview of phthalate ToxCast activities depicted in ToxPi figures. The central core of each figure represents the amount of missing data denoted by the gray color (the lighter the gray color, the lower the amount of missing values). Slices did not include BSK assays not associated with genes or genes not associated with a pathway (CTD).
- Overall assay activity across the toxicity-associated pathway slices: **DEHP > DHP > DBP > BBP > DIDP**



- The cardiotoxicity- and cancer-associated pathways had the most gene hits compared to all other hazard associated pathways. These two pathways also had the highest numbers of assay endpoints.
- Although DHP has the highest number of gene hits, DEHP has the most ToxCast assay activity across the ToxPi slices. This reflects the potency of DEHP for these *in vitro* assays.
- Association between a selected chemical and a potential toxicity pathway is influenced by both chemical activity (# of gene targets and hits) and chemical potency (AC_{50}).

Table 4. Curated literature studies (CTD) to support phthalate-gene relationships.

Dibutyl Phthalate (DBP)				
BSK Assay	**Gene**	**Interaction**	**Reference**	**Organism**
BSK_LPS_CD40_down	CD40	Results in increased expression of CD40 mRNA	Gaido, 2007	Mus musculus
BSK_hDFCGF_IP10_down	CXCL10	Results in increased expression of CXCL10 mRNA	Gaido, 2007	Mus musculus
BSK_BE3C_tPA_down	PLAT	Results in increased expression of PLAT mRNA	Johnson, 2011	Rattus norvegicus
BSK_CASM3C_Thrombomodulin_up	THBD	Results in decreased expression of THBD mRNA	Johnson, 2011	Rattus norvegicus
BSK_LPS_PGE2_down	*PTGER2*	*Results in decreased expression of PTGER2 mRNA*	*Ishido, 2005*	*Rattus norvegicus*
Di(2-ethylhexyl) Phthalate (DEHP)				
BSK Assay	**Gene**	**Interaction**	**Reference**	
BSK_LPS_PGE2_down	PTGER2	results in decreased expression of PTGER2 mRNA	Ishido, 2005	Rattus norvegicus
BSK_4H_Pselectin_down	SELPLG	results in increased expression of SELPLG mRNA	Ren, 2010	Mus musculus
BSK_LPS_TNFa_down	TNF	results in increased expression of TNF mRNA	Lee, 2011	Homo Sapiens
Butyl Benzyl Phthalate (BBP)				
NONE				
Dihexyl Phthalate (DHP)				
NONE				
Diisodecyl Phthalate (DIDP)				
NONE				

- DBP and DEHP both have several curated chemical-gene associations as captured by CTD that were supported by chemical activity in ToxCast assays
- BBP, DHP, and DIDP do not have any curated chemical-gene associations established by CTD.
- DBP has a curated chemical-gene association with PTGER2 (italicized). However, DBP was not active for the BSK assay for this endpoint.

Discussion and Conclusions

By analyzing the chemical−assay activity profiles in ToxCast and the chemical−gene associations from CTD, we found the following:

DEHP

- DEHP is listed under Proposition 65 for cancer, and developmental and reproductive toxicities, and DEHP showed medium to high assay activity in the ToxPi slices relevant to all three toxicities.
- DEHP had medium to high ToxCast assay activity relevant to hepatotoxicity, nephrotoxicity, and cardiotoxicity. It is important to note that the CTD has better coverage of these three pathways (i.e., these pathways have many associated genes and human diseases). In addition, literature studies showed a direct curated association between each of two genes (TNFa and SELPG) with DEHP (Table 4).

DHP

- Although listed in Proposition 65 as a reproductive toxicant and having the most gene hits in this study, DHP did not have any gene target hits associated with reproductive toxicity as captured by the CTD (Figure 3). This suggests DHP's main mechanism of action for reproductive toxicity may not operate through the genes screened in this study.
- DHP had high ToxCast assay activity for cancer and developmental toxicity suggesting other possible hazard traits to consider for this chemical.

DBP

- Listed in Proposition 65 for developmental and reproductive toxicity and showed low to medium ToxCast assay activity in the ToxPi slices.
- DBP had high ToxCast activity in pathways associated with cancer and nephrotoxicity which may shed light on alternative mechanisms of action for adverse effects by this chemical. Furthermore, direct chemical-gene curated associations have been established for DBP and the genes CD40, THBD, and CXCL10, all of which were associated with the cancer pathway and all of which were gene target hits by DBP (Table 3B, Table 4).

BBP

- Listed in Proposition 65 as a developmental toxicant, but showed low ToxCast assay activity in all the toxicity pathway categories.
- BBP activated the least number of BSK assays of all the phthalates—a little more than half of the number of assays by DHP. Low assay activity in ToxCast may also lead to a low number of gene-disease associations in CTD based on hits.

DIDP

- Listed in Proposition 65 as a developmental toxicant, but not active in any ToxCast BSK assays. This may be suggestive that the chemical structure of this compound is not compatible with any of the eight primary human cell systems in the assay platform.

In this study, we demonstrated the use of curated associations in the CTD to provide additional information on ToxCast chemicals. We derived a step-wise approach to potentially link candidate chemicals to adverse effects and/or toxicity endpoints via a specific set of *in vitro* assays and their associated genes. There are a few key things to note here. First, the activation of certain assays does not suggest a direct role of a particular gene in an adverse pathway. Furthermore, an absence of activation does not absolve a chemical from being linked to an adverse pathway. Rather, it is more likely that such adverse effects are associated with different mechanisms of action not currently captured in the BSK assays explored here. Further work is warranted to supplement such observations. Second, although the ToxPi slices are normalized and weighted equally, some hazard-associated pathways have far fewer endpoints incorporated in their slices than others. This discrepancy in endpoints may be potentially reduced with a different candidate list of genes. Here we also show data suggesting alternative routes for adverse effects, revealing other potential pathways to be considered for these chemicals. Lastly, we show that chemicals may have confirmed curated associations with a number of genes that are not currently linked to an adverse outcome or toxicity endpoint as captured in the CTD. Therefore ongoing expansion of the current CTD is necessary to address more gene associations and more possible adverse endpoints. Not only is it essential to develop an array of HTS assays to provide a wider range of biological coverage, but it is equally important to identify the right and appropriate batteries of assays for each specific adverse outcome or toxicity endpoint.

Future work will focus on investigating the gene associations of the phthalate metabolites using this step-wise process for comparison with the parents. Other work will also look into using this approach with additional ToxCast assay platforms such as Attagene (ATG) or OdysseyThera (OT). Expanding the current data in all the areas presented is needed to improve the accuracy of using ToxCast in vitro assay results to predict *in vivo* toxicities.

Acknowledgements

We thank the US EPA ToxCast team for ongoing discussion and guidance, particularly Drs. Keith Houck and Matt Martin for assistance with technical inquiries regarding the ToxCast assays, and Ms. Monica Linnenbrink for facilitating the logistics of these meetings. Special thanks to Dr. David Reif for helpful guidance on understanding the ToxPi GUI software.