

# Application of Structure-Activity Relationship (SAR) and Cluster Analysis to ToxCast™

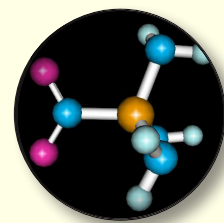
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## Introduction

The U.S. Environmental Protection Agency (EPA) is developing ToxCast™ as a cost-effective and efficient platform to rapidly prioritize the screening and testing of large numbers of chemicals. ToxCast™ will provide development of new tools, such as *in vitro* assays that may predict the potential for adverse outcomes in whole animals, and predictive metabolism and biotransformation models to identify relevant endpoints for toxicological pathways. Once gathered, analysis of these data for likely *in vivo* toxic effects will require innovative approaches.

Cluster analysis has successfully been applied to organize and evaluate sets of structurally similar compounds for regulatory decision making. U.S., EU, Canadian, and Japanese regulatory authorities have accepted cluster analysis methodology for High Production Volume (HPV) chemicals, and existing substance registration reevaluation programs to organize the experimental and structure-activity relationship (SAR) toxicology databases of structurally similar chemicals. Structurally related compounds generally have similar and/or predictable chemical and biological profiles (toxicology and metabolism). Evaluation of a clustered data set, rather than single compounds, provides the means to proceed with regulatory decision-making for the related compounds as a unit, when data from group members are sufficient for risk assessment. When data are insufficient, a targeted data development plan can be generated for group members as a unit, rather than for each compound.



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This same cluster analysis methodology can also be applied to ToxCast during the Phase I (proof of concept involving 300 chemicals and 400 different High Throughput Screening (HTS) assays) and beyond into Phase II (expansion of predictive signatures to 1,000 chemicals and continued proof of concept) and Phase III (the concept is validated and applied to the categorization of more than 1,000 chemicals). In Phase I, and beyond, initial sets of structurally similar compounds can be grouped according to functional elements and other structural characteristics that would affect their likely metabolic pathway.

The overall objective in the application of cluster analysis is to identify a lead compound(s) to represent the cluster, analyze the cluster for data gaps, and then to define and minimize the whole animal testing needed to characterize the cluster. Structurally related compounds are grouped (clustered) based on modeled SAR predictions from programs like DEREK and TOPKAT™, *in vivo/in vitro* screening results from databases like ToxCast™ and ACToR, and physicochemical properties from EPI Suite™. Once grouped into 'clusters' by metabolic and structural similarity, the physicochemical data and results from experimental and modeled toxicology can be compiled and evaluated for completeness. Cluster analysis makes it possible to 'Read-Across' the available data and use it to define group characteristics such as mechanism of action and target organ toxicity, and to predict reactivity of structural elements that are relevant and predictive of the toxicity pathways for all members of the cluster.

## Assembling Data Sets for Cluster Analysis and Read Across

Structurally similar compounds with common functionality can be clustered (grouped) and evaluated based on common physicochemical and biological properties—mode/mechanism of action, common metabolic pathways, results from *in vivo/in vitro* assays, and if available, experimental testing data. EPA's Strategic Plan for Evaluating the Toxicity of Chemicals, www.epa.gov/osa/spc/toxicity testing (March 2009) defines mode of action as the sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in an adverse health effect. Mechanism of action implies a more detailed understanding and description of events, often at the molecular level, than is meant by mode of action.

Although clustering can provide a foundation to organize data sets of structurally similar compounds, proposed clusters also need to be challenged to determine whether either the predictive SARs and/or experimental toxicology data can be bridged across all members of the cluster to fill data gaps, or whether it is necessary to generate additional experimental toxicology data on one (or a few) of the cluster compounds. The lead compound(s) chosen to represent the set would typically be predicted to be the most active based on physicochemical properties, metabolism, and/or existing toxicology data.

The following factors need to be considered for each compound in the cluster, in order to evaluate the soundness of the proposed clustering and the validity to "Read Across" the data for members of the cluster.

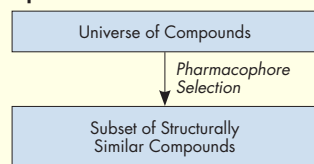
- Consistency in the trends of *in vitro* and *in vivo* screening, and existing acute and repeat dose toxicology data. Outliers and inconsistencies in expected trends need to be explainable. If not, the compound(s) should be eliminated from the proposed cluster.
- Quantitative SAR (QSAR) modeling data can reliably predict a similar endpoint in another chemical. If not reliable, the modeling data should not be used.
- The cluster represents a range of predictable physicochemical properties (e.g., Log  $K_{ow}$  increases with increasing carbon chain length).
- The cluster compounds have a common mode/mechanism of action (U.S. EPA 2009), and common metabolites.

To date, there are no algorithms or system databases to integrate toxicology, metabolism, and relevant physicochemical properties necessary to conduct cluster analysis. It is necessary to rely primarily on expert judgment to objectively determine whether the data:

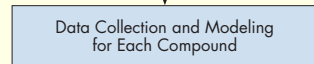
- Are sufficient to fully read across (can be bridged)—to proceed to an evaluation of human health and/or environmental risk assessment for the group of compounds that compose the cluster
- Have data gaps that warrant additional targeted *in vivo* and/or *in vitro* screening or whole animal testing for one or more additional compound.

### Conceptual Cluster Analysis and Read Across Framework

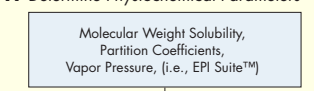
#### 1. Develop Cluster



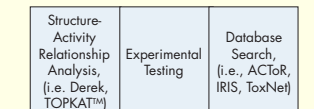
#### 2. Organize Cluster



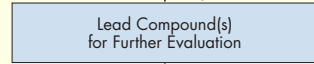
#### A Determine Physicochemical Parameters



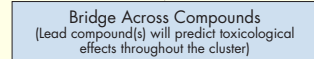
#### B Compile Toxicology



#### 3. Cluster Analysis



#### 4. Read-Across Cluster



## Cluster Analysis Tools

### Physicochemical Analysis: Estimation Programs Interface, EPI Suite™

EPI Suite™ is a Windows-based suite of physical/chemical property and environmental fate estimation programs developed by the EPA's Office of Pollution Prevention Toxics and Syracuse Research Corporation (SRC), (www.epa.gov/oppt/exposure/pubs/episuite.htm). EPI Suite™ is a screening-level tool to be used when experimental physicochemical data are not available. Other tools for chemical assessment and design are available on other EPA websites, www.epa.gov/oppt and http://www.epa.gov/epahome/Data.html.

### SAR and QSAR Databases

SAR and QSAR tools, methods, and databases can be used to correlate the biological activity of a chemical and its structure, and make predictions for uncharacterized compounds based on similarity of structure (shape, size, chemical arrangement, and distribution of functional groups). SARs and QSARs are based on expert systems that are generally applicable to a wide range of toxicology endpoints. SAR predictions are expressed qualitatively (e.g., similarly toxic, less toxic, more toxic), where as QSAR expert systems can provide quantitative responses and outputs based on regression equations.

EPA has used SAR and QSAR data from commercially available expert system software such as Derek and TOPKAT™. Other SAR options in use include MultiCASE, Oncologic, and ToxTree. The capabilities of Derek and TOPKAT™ are summarized below.

Derek for Windows (v.11, 2009). SAR software available from by Lhasa, Ltd. (www.lhasalimited.org/index.php?cat=2&sub\_cat=64), is an SAR expert knowledge-based system that predicts whether a chemical is toxic in humans, other mammals, and bacteria. Molecular structures are entered into the program, either via a chemical editor program or by importing Molfiles, .skc, or SDFiles. The program applies SARs and other expert knowledge rules to derive a reasoned conclusion about the potential toxicity of the query chemical. Results are supported by evidence in the form of literary citations, examples, and comments. The application provides a high throughput screen for more than 30 endpoints, including carcinogenicity, chromosome damage, genotoxicity, hepatotoxicity, HERG channel inhibition, irritation (eye, GI tract, respiratory tract, skin), mutagenicity, reproductive toxicity, respiratory and skin sensitization, thyroid effects, and cholinesterase inhibition.

Derek provides SAR predictions based on a gradient of qualitative terms such as, certain (proof that the proposition is true), probable (at least one strong argument that the proposition is true), plausible (weight of evidence supports the proposition), equivocal (there is evidence for and against), improbable (there is at least one strong argument that the proposition is false), and impossible (proposition is false).

TOPKAT™ is QSAR software available from Accelrys (accelrys.com/products/discovery-studio/toxicology) and uses cross-validated models based on experimental data of highly consistent protocols (including data published by NTP and FDA studies). DS TOPKAT™ uses patented Optimum Prediction Space (OPS) technology to assure that the compounds under investigation are well represented in the models. TOPKAT™ has the capabilities to address acute and chronic quantitative toxicology endpoints such as weight-of-evidence rodent carcinogenicity, Ames mutagenicity, rat oral LD50, rat inhalation LC50, rat chronic LOAEL, rat MTD and environmental/physicochemical properties.

TOPKAT™ results are expressed as a numeric term indicating probability of the assessed endpoint. Interpretation of that numeric term is based on the reported number of compounds within the database that are structurally similar, and the success of the TOPKAT™ program in predicting the outcome (positive or negative for the assessed endpoint).



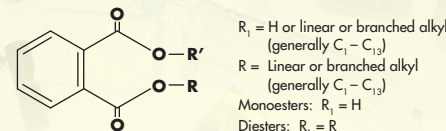
## Phthalate Ester Example Cluster Analysis Read Across

An example cluster analysis was developed for the phthalate compounds included in the HPV Program. Phthalates comprise a class of economically important HPV chemicals that provide durability and flexibility to polyvinyl (PVC) plastics, and are used in industrial applications, household products such as detergents, and in many personal care products such as soap, shampoo, hair sprays, and nail polish. Low molecular weight phthalates are also used as solvents for other chemicals. The toxicology of these compounds has been extensively studied because of their widespread use and high potential for human exposures.

The estimated physicochemical information, along with reading across the available toxicology data, can be used to estimate toxicological trends and properties for the other compounds in the phthalate example cluster. Physicochemical data (Table 1) are integrated with toxicology data as shown in Tables 2 and 3. Table 2 summarizes HPV toxicology and Derek SAR data. Table 3 lists phthalate *in vitro* estrogen assay test results from the ToxCast database.

### Chemical Identity

Following is the generic structure of economically important phthalate esters. The carbon side chain may vary to include either linear and branched monoesters or linear and branched diesters of the same carbon chain group. The aromatic ring is generally unsubstituted.



### Physicochemical Data Analysis

Physicochemical data were generated using EPI Suite™ for the example phthalates. Data were developed by the model for some parameters, while the remainder (Table 1) are based on experimental study data. All of the phthalates in this proposed cluster are 1,2-benzenedicarboxylic acids with ester groups for which the majority have a linear carbon chain length of C1–C9 but with one compound, di(2-ethylhexyl), is a C8 branched carbon chain.

Physicochemical properties are reported for water solubility and lipophilicity because these should affect dermal and systemic absorption. Vapor pressure is reported because it is a potential indicator of whether inhalation absorption is feasible. As demonstrated by the data summarized in Table 1, the characteristics of the side chain esters affect the physicochemical properties of the compound. Overall, it can be concluded that with increasing ester carbon chain length there is an increase in log  $K_{ow}$  with decreased water solubility. Moreover, the phthalate monoesters are more polar than diesters. The phthalate esters are, as a class (C1–C8 examples indicated below) nonvolatile.

**Table 1.** Phthalate Ester EPI Suite™ 3.2 Physicochemical Calculations

CAS RN	MW	Water Sol WKOW mg/L or	Log $K_{ow}$	Vapor pressure mm Hg @25°C
CASRN 84-74-2: Dibutyl Phthalate <sup>1</sup> R <sup>1</sup> , R <sup>2</sup> = nC <sub>4</sub> H <sub>9</sub>	278.4	11.2 (exp) <sup>2</sup>	4.61 (exp) <sup>2</sup>	2.01 E-05
CAS 131-11-3: Dimethyl Phthalate <sup>1</sup>	194.2	2,014	1.6 (exp) <sup>2</sup>	3.08E-03
CAS 4376-18-5: Methyl Hydrogen Phthalate <sup>1</sup> R <sup>1</sup> =H, R <sup>2</sup> = CH <sub>3</sub>	180.2	5,958	1.13 (exp) <sup>2</sup>	0.00024
CAS 131-70-4: Monobutyl Phthalate <sup>1</sup> R <sup>1</sup> =H, R <sup>2</sup> = nC <sub>4</sub> H <sub>9</sub>	222.2	450.81	2.84	3.87 E-005
CAS 117-81-7: Diethylhexylphthalate <sup>1</sup> R <sup>1</sup> , R <sup>2</sup> = branched C <sub>8</sub> H <sub>17</sub>	390.57	0.00113	7.6 (exp) <sup>2</sup>	1.42E-07 (exp) <sup>2</sup>
CAS 84-75-3: Dihexyl Phthalate <sup>1</sup> R <sup>1</sup> , R <sup>2</sup> = C <sub>6</sub> H <sub>13</sub>	334.5	0.0115	6.82 (exp) <sup>2</sup>	1.4E-05(exp) <sup>2</sup>
CAS RN 117-84-0: Dioctyl Phthalate <sup>1</sup> R <sup>1</sup> , R <sup>2</sup> = C <sub>8</sub> H <sub>17</sub>	390.6	0.000423	8.10 (exp) <sup>2</sup>	1.00E-07 (exp) <sup>2</sup>

<sup>1</sup> Phthalates listed in ToxCast database

<sup>2</sup> (exp) indicates that EPI Suite™ provided experimental data.

### Phthalate Mammalian Metabolism

Phthalate esters may be metabolized by aromatic ring oxidation, at the 4, 5 position of the benzene ring, or by partial ester hydrolysis to generate monoesters; metabolites are excreted in the urine. Human metabolism studies indicate that the oxidized aromatic ring metabolites predominate for the longer carbon chain phthalate esters, such as di(2-ethylhexyl), diisononyl and di(2-propylhexyl). In the case of the shorter chain phthalates, such as dipropyl and dibutyl, the simple monoesters are the major metabolites. Overall, these data indicate that the phthalate toxicology for shorter chain and longer chain monoesters should be considered as two distinct subclusters for toxicology evaluation and testing.

Reference: Wittassek M. and Angerer, J., 2008. Phthalates: metabolism and exposure. International Journal of Andrology, 31:131–138

### Phthalate HPV Toxicology

In February 2007, the American Chemistry Council (ACC) Phthalate Esters Panel submitted to EPA HPV robust summaries and test plans for a cluster of 19 phthalate esters ranging in ester carbon chain length C1–C13 and with linear and branched examples. These data are available the EPA website at http://www.epa.gov/chemrtk/pubs/summaries/viewsrch.htm. The ACC Phthalate Esters Panel separated the 19 phthalates into 3 separate subcategories based on MW (ester carbon chain length but not carbon chain branching), physicochemical and biological properties. These phthalate ester subcategories were described as Low MW (C1–C2); Transitional (C4–C8); and High MW (C9–C13).

The HPV robust summary toxicology data that are summarized in Table 2 for illustrative purposes. Because the phthalates represent a class of highly studied compounds (which is typically not the case), Read Across data gaps were not identified.

The acute toxicity data indicate that the phthalate mono and diesters, are generally as a class, not acutely toxic by either the oral or dermal routes of exposure, with the exception of CAS 84-75-3 (dihexyl phthalate) and are also not likely to act on DNA to cause mutagenicity or genotoxicity. However, compounds of medium linear carbon chain length C6–C8, as demonstrated by CAS 84-75-3 and CAS 117-81-7 are more toxic than the C1–C4 phthalates based on both acute and repeat dose testing. Branched ester congeners, as demonstrated by CAS 117-81-7 and CAS 68515-47-9, are less toxic in both acute and repeat dose testing than the linear esters. Overall, the concept of two subclusters, as indicated by the mammalian metabolism, appears to be substantiated.

HPV data indicate reproductive effects were observed for both linear, CAS 131-11-3 (C1), and branched phthalates. These findings were

**Table 2.** Phthalate Ester Experimental Toxicology Data

CAS RN	Acute <sup>1</sup> Oral LD50 (mg/kg)	Acute dermal LD50	Mutagenicity/ Genotoxicity	Repeat Dose <sup>2</sup> NO(A)EL mg/kg/day	Reproductive NO(A)EL mg/kg/day	Derek SAR endpoints (at least plausible)
CASRN 84-74-2: Dibutyl Phthalate	ND	ND	(-) Ames (+) lymphoma, +S9 (-) human leukocytes	125 (rat) [IRIS database, 1 year, not GLP]		-Teratogenicity -Testicular atrophy
CAS 131-11-3: Dimethyl Phthalate	6,900 (rat)	N/D	(-) Ames (-) chromosomal aberration	Not stated (high)	840 (maternal), >3,570 (teratogenicity)	-Teratogenicity -Testicular atrophy
CAS 4376-18-5: Methyl Hydrogen Phthalate	ND	ND	N/D	N/D	ND	-Teratogenicity
CAS 131-70-4: Monobutyl Phthalate	ND	ND	N/D	N/D	ND	No alert
CAS 117-81-7: Diethylhexylphthalate	6,860-34,000 (rat) 9,800-49,000 (mouse) 33,222-33,320 (rabbit) 26,000 (guinea pig)	4000 (rat) 4,000 (mouse)	(-) Ames (-) UDS (-) lymphoma (-) HGPRT	1,093 (rat) 28 day oral 1 (rat) 8 week inhalation (OECD 412)	95 (maternal mouse), 48 (F1 mouse) two generation oral 0.3 (maternal and fetal rat), teratogenicity OECD 414; inhalation	-Teratogenicity -Testicular atrophy
CAS 84-75-3: Dihexyl Phthalate	30 (rat)	ND	(-) Ames	N/D	430 LOAEL (mouse), testicular atrophy, teratogenicity	-Teratogenicity -Testicular atrophy
CAS RN 117-84-0: Dioctyl Phthalate	53,700 (rat) 13,000 (mouse)	ND	(-) Ames	39 oral OECD 408, 90 day	7.5 (maternal, F1, F2 mouse) 9,780 (maternal, fetal mouse) oral	-Teratogenicity -Testicular atrophy
CAS 68515-47-9: Diisotridecyl Phthalate	>10,000 (rat)	>3,160 (rabbit)	(-) Ames	N/D	ND	ND

<sup>1</sup> 28 day/90 day subchronic study data.

<sup>2</sup> Derek endpoints 'teratogenicity' and 'testicular atrophy' are triggered by the general category 'phthalate mono- or di-ester', and are listed as 'plausible', meaning that a review of the literature by Lhasa Ltd. scientists indicates a positive weight of evidence.

**Table 3.** ToxCast *In Vitro/In Vivo* Assay and Enzyme Metabolism Data

CAS RN	Estrogen receptor binding NCGC datasheet ER $\alpha$ , Novascreen datasheet ER $\beta$ and hER IC50, $\mu$ M				ADME data Novascreen datasheet IC50, $\mu$ M		
	ER $\alpha$ Agonist	ER $\alpha$ Antagonist	ER $\beta$	hER	hCYP1A2	hCYP2C19	rCYP2A2
CASRN 84-74-2: Dibutyl Phthalate	inactive	inactive	inactive	inactive	11	12.6	inactive
CAS 131-11-3: Dimethyl Phthalate	inactive	inactive	inactive	inactive	inactive	inactive	inactive
CAS 4376-18-5: Methyl Hydrogen Phthalate	inactive	inactive	inactive	inactive	inactive	inactive	inactive
CAS 131-70-4: Monobutyl Phthalate	inactive	inactive	inactive	inactive	inactive	inactive	16.6
CAS 117-81-7: Diethylhexylphthalate	inactive	inactive	inactive	inactive	inactive	inactive	inactive

## Conclusions

- Cluster Analysis has the potential to tremendously decrease the time, cost, and number of animals needed for toxicology testing.
- During Phase I, use of cluster analysis (whole animal toxicology data, metabolism physicochemical modeling and (Q)SAR tools) in combination with ToxCast can identify, through Read Across, both data gaps for additional testing and understanding of the mechanisms and pathways of toxicity.
- The predictive capability of cluster analysis should be improved in the future (Phase II proof of concept expansion and Phase III categorization) with the development of improved and updated (Q) SAR tools.

also reflected in the Derek SAR and reported qualitatively as "plausible" teratogenicity because of testicular atrophy endpoints. Monobutyl phthalate does not trigger the same Derek prediction as dibutyl phthalate, indicating that there may be differences in mono and diester phthalate esters, but the length of the ester side chains does not change the overall Derek SAR profile.

### Phthalate ToxCast Toxicology Data

ToxCast HTP data are summarized in Table 3 (including estrogen receptor *in vivo* and *in vitro* toxicology and enzyme metabolism data). These selected data illustrate the use of ToxCast data to search for mechanism of action based on possible reproductive effects caused by estrogenic effects.

Table 3 demonstrates that the available phthalates were inactive in four estrogen receptor assays. Given the literature report (i.e. dihexyl phthalate) for testicular atrophy, additional ToxCast *in vivo/in vitro* assay results for androgen effects (receptors and other HTP reproductive/developmental assays should also be investigated to determine mechanistic/toxicity pathways and to provide clarification for structural characteristics (i.e., mono versus diesters, carbon chain length and branching) that influence the pathways of toxicity.

Table 3 also provides ADME data that could be used to predict the metabolites generated *in vivo*, including whether a species difference exists that would affect magnitude of effect, or whether interspecies safety factors are relevant. These data indicate that the number of ester side chains (mono or diesters) is relevant to the predicted effect because dibutyl phthalate is metabolized by two human CYP enzymes, while monobutyl phthalate is metabolized by neither. Monobutyl phthalate is, however, metabolized by the rodent form of CYP2A2, indicating a possible species difference.

