Perspectives on the Development, Evaluation, and Application of *in Silico* Approaches for Predicting Toxicity

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The views expressed in this presentation are those of the author and do not necessarily reflect the views or policies of the U.S. EPA
Conflict of Interest Statement

No conflict of interest declared.

Disclaimer:
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Outline

• Regulatory Drivers
• Computational *(in silico)* Toxicology [scope for today’s talk]
• Integrated Approaches to Testing and Assessment (IATA) – definitions and Adverse Outcome Pathway (AOP) informed
• Decision contexts and their impact on the approaches applied
• Risk-based prioritisation
  – Thresholds for Toxicological Concern (TTC)
• Read-across approaches
  – Generalised Read-across (GenRA)
  – Perfluorinated & polyfluorinated substances (PFAS)
• Summary remarks
• Acknowledgements
Regulatory and Non-Regulatory drivers

- Societal demands for safer and sustainable chemical products are stimulating changes in toxicity testing and assessment frameworks.

- Chemical safety assessments are expected to be conducted faster and with fewer animals, yet the number of chemicals that require assessment is also rising with the number of different regulatory programmes worldwide.

- In the EU, the use of alternatives to animal testing is promoted.

- Animal testing is prohibited in some sectors e.g. EU Cosmetics regulation.

- The European Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation lays out specific information requirements, based on tonnage level triggers. However, the regulation explicitly expresses the need to use non-testing approaches to reduce the extent of experimental testing in animals.
Regulatory and Non-Regulatory drivers

- REACH-like schemes also have been established in China, South Korea, and Turkey.

- In the US, the new Frank Lautenberg Chemical Safety for the 21st Century Act (LCSA) requires that a risk based prioritisation is conducted for all substances in commerce, ~40,000, many of which are lacking sufficient publicly available toxicity information.

- EPA Administrator signed memo 10/9/19 to “direct the agency to aggressively reduce animal testing, including reducing mammal study requests and funding 30% by 2025 and completely eliminating them by 2035”

- Risk based prioritisation is also an important aspect of regulatory frameworks in Canada (the Domestics Substance List), Australia and the EU.

- Non-testing approaches offer a means of facilitating the regulatory challenges in chemical safety assessment.
Computational (In Silico) Toxicology

- Databases/Dashboards of existing information
- Structure-Activity Relationships (SAR)
- Quantitative Structure-Activity Relationships (QSAR)
- Expert Systems
- Category formation (grouping) read-across
- Bioinformatics
- Chemoinformatics
- Biokinetics (PBPK)
Integrated Approaches to Testing and Assessment (IATA)

• “Integrated Testing Strategies (ITS) are …. approaches that integrate different types of data and information into the decision-making process. …”

• “A tiered approach to data gathering, testing, and assessment that integrates different types of data (including physicochemical and other chemical properties as well as in vitro and in vivo toxicity data). When combined with estimates of exposure in an appropriate manner, the IATA provides predictions of risk.”
General framework of an IATA

Problem formulation: Definition of the regulatory need (e.g., hazard identification, hazard characterisation, safety assessment etc.) and the information/parameters that are relevant to satisfy the need, including consideration of existing constraints and, if applicable, consideration of the level of certainty required.

Gather and evaluate existing information (in vivo, in vitro, in silico (e.g., QSAR), read across and chemical category data).

Make a weight of evidence assessment or apply predefined decision criteria (e.g., ITS, STS).

If available information does not provide sufficient evidence consider what additional information from non-testing, non-animal testing methods and, as a last resort, from animal methods would be needed to generate sufficient evidence.

Make a weight of evidence assessment or apply predefined decision criteria (i.e., ITS, STS).

Available information provides sound conclusive evidence for the specific regulatory need.
Typical Information within an IATA: IATA elements

- Historical information on the chemical of interest
- Non-standard *in vivo* tests
- Information from “similar” chemicals
- Predictions from other ‘non-testing’ approaches such as (Q)SAR
- *In chemico* tests
- *In vitro* tests
- Molecular biology, -omics
- Exposure, (bio-)kinetics
Mechanistic based and AOP-informed IATA

- As noted, there is a shift towards non-animal alternatives as a response to regulatory drivers.

- Integration of different non-animal approaches requires an organising framework to ensure that the different information sources are being interpreted in their appropriate context. This is particularly relevant for New Approach Methodologies (NAMs).

- AOPs serve to provide this organisational framework and hence play an important role in developing and applying IATA for different purposes as well as provide a roadmap for future QSAR development.

- AOPs provide the linkage from chemistry, through the Molecular Initiating Event (MIE) to Adverse Effect.

- Data from key events provides support to, and will enhance, read-across especially for regulatory acceptance as well as supports definition of domains for MIEs.
General workflow in Integrated Approaches to Testing and Assessment (IATA)

Problem formulation

- Multiple strategies e.g. in-house data, mining of relevant databases, literature search
- Expert Judgement

Generate additional information

- Generate additional information

Weight of Evidence assessment: Adequate information for decision-making?

- YES
- NO

Regulatory conclusion

- YES
- NO

From OECD
EPA CompTox Chemicals Dashboard

- A publicly accessible website delivering access:
  - ~875,000 chemicals with related property data
  - Experimental and predicted physicochemical property data
  - Integration to “biological assay data” for 1000s of chemicals
  - Information regarding consumer products containing chemicals
  - Links to other agency websites and public data resources
  - “Literature” searches for chemicals using public resources
  - “Batch searching” for thousands of chemicals
  - DOWNLOADABLE Open Data for reuse and repurposing

https://comptox.epa.gov/
CompTox Chemicals Dashboard: Landing Page
CompTox Chemicals Dashboard: Landing Page

• Different entry points depending on domain of interest
Bisphenol A
80-05-7 | DTXSID7020182
Search by DSDTox Substance Id.

Wikipedia

Bisphenol A (BPA) is an organic synthetic compound with the chemical formula (CH₃)₂C(OC₆H₄)₂CH₃ belonging to the group of diphenylmethane derivatives and bisphenols, with two hydroxyphenyl groups. It is a colorless solid that is insoluble in organic solvents, but poorly soluble in water. It has been in commercial use since 1937.

EPA is a starting material for the synthesis of plastics, primarily.

Read more

Intrinsic Properties

Structural Identifiers

Linked Substances

Presence in Lists

Record Information

Quality Control Notes
**Bisphenol A**  
80-05-7 | DTXSID7020182

**Executive Summary**

**Quantitative Risk Assessment Values**
- IRIS values available
- No PRRTV values
- EPA RSL values available
- Minimum RD: 0.056 mg/kg-day (chronic, IRIS, oral, 8)
- No RfC calculated
- VVME: POD not calculated

**Quantitative Hazard Values**
- Minimum oral PCO: 3.8 mg/kg/day (reproductive, HPV1S, oral, 6)
- No inhalation POD values
- Lowest Observed Bioactivity Equivalent Level: CYP1A1, CYP1A2, Tpo, ESR1, ESR1, NR113, PPPAR, NR112, Cyp2c11, MMP3, Esr1

**Cancer Information**
- No cancer slope factor
- No inhalation unit risk value
- Carcinogenicity data available: University of Maryland carcinogenicity warning
- No genotoxicity (findings reported)

**Reproductive Toxicology**
- 200 Reproductive toxicity PODs available

**REGIONAL SCREENING**

<table>
<thead>
<tr>
<th>Class</th>
<th>THQ</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>risk-based SSL (mg/kg)</td>
<td>THQ = 0</td>
<td>5.8</td>
</tr>
<tr>
<td>GIABS (unspecified)</td>
<td>THQ = 1</td>
<td>1</td>
</tr>
<tr>
<td>GIAGS (unspecified)</td>
<td>THQ = 0</td>
<td>1</td>
</tr>
<tr>
<td>AIBS (unspecified)</td>
<td>THQ = 0</td>
<td>0.1</td>
</tr>
<tr>
<td>RFD/o (mg/kg-day)</td>
<td>THQ = 0</td>
<td>0.05</td>
</tr>
<tr>
<td>screening level (residential Soil) (mg/kg)</td>
<td>THQ = 0</td>
<td>320</td>
</tr>
<tr>
<td>screening level (industrial Soils) (mg/kg)</td>
<td>THQ = 0</td>
<td>4100</td>
</tr>
</tbody>
</table>
Computational toxicology tools add value to most regulatory decisions

- Prioritisation
- Screening level hazard assessment
- Risk Assessment
- Exposure Assessment
Risk-Based prioritisation

- Could involve a combination of available experimental data and new approach methods (NAMs) such as HTTR, HTS
- One approach considered involved coupling Threshold of Toxicological Concern (TTC) with High Throughput Exposure (HTE) modelling to rank order substances for further evaluation
- TTC is a principle that refers to the establishment of a human exposure threshold value for (groups of) chemicals below which there would be no appreciable risk to human health
- Relies on past accumulated knowledge regarding the distribution of potencies of relevant classes of chemicals for which good toxicity data do exist

TTC is based on a predicted tumour risk of 1 in a million, derived through an analysis of genotoxic chemicals

TTC is based on frequency distributions (5th percentile) of NO(A)ELs of non-genotoxic chemicals
<table>
<thead>
<tr>
<th>Type of substance</th>
<th>μg/person/day (μg/kg-day for 60 kg adult)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alerts for potential genotoxic</td>
<td>Kroes: 0.15 (0.0025 μg/kg-day)</td>
</tr>
<tr>
<td>carcinogenicity</td>
<td>ICH: 1.5 (0.025 μg/kg-day)</td>
</tr>
<tr>
<td>Acetylcholinesterase inhibitors</td>
<td>18 (0.3 μg/kg-day)</td>
</tr>
<tr>
<td>(AChEI)</td>
<td></td>
</tr>
<tr>
<td>Organophosphate/carbamate</td>
<td></td>
</tr>
<tr>
<td>Cramer Class III</td>
<td>90 (1.5 μg/kg-day)</td>
</tr>
<tr>
<td>Cramer Class II</td>
<td>540 (9.0 μg/kg-day)</td>
</tr>
<tr>
<td>Cramer Class I</td>
<td>1800 (30 μg/kg-day)</td>
</tr>
</tbody>
</table>
Cumulative Distributions of Cramer Structural Class NOELs

- Decision tree of 33 questions
Predicted HT exposures

• Wambaugh and colleagues (2014) developed a rapid heuristic high throughput exposure (HTE) model that enables prediction of potential human exposure to thousands of substances for which little or no empirical exposure data are available.

• The HTE model was calibrated by comparison to NHANES urinary data that reflects total exposure (all routes/sources)
Integrating TTC with predicted HT exposures

- Compared the conservative Cramer Class III TTC value of 1.5 μg/kg-day to the previously calculated median and upper 95% credible interval (UCI) of total daily median exposure rates for 7968 chemicals.

  only 273 (fewer than 5%) were found to have UCI daily exposures estimates that exceeded the Cramer Class III TTC value of 1.5 μg/kg-day.

Initial evaluation showed the approach of using the ratio of exposure to TTC (HTE: TTC) appeared promising for risk-based prioritisation.
• Refined the approach using the Kroes et al. structure-based workflow for TTC

• None of the substances categorised as Cramer Class I or Cramer Class II exceeded their respective TTC values.
• No more than 2% of substances categorised as Cramer Class III or acetylcholinesterase inhibitors exceeded their respective TTC values.
• Majority of chemicals with genotoxicity structural alerts did exceed the relevant TTC – recommendations were proposed for next steps

Patlewicz et al., 2018
Risk-Based prioritisation

- Investigate relevance of existing TTC values for substances of interest to EPA
- Extracted data from EPA’s ToxValDB, which aggregates in vivo testing data from over 40 sources including US federal and state agencies, as well as international agencies such as the European Chemicals Agency and the World Health Organisation
- Objectives were:
  - Reproduce the TTC values developed by Munro et al (1996)
  - Follow the Kroes et al (2004) workflow to assign substances present in ToxVal to their respective Cramer classes and use the associated repeat dose toxicity data to derive new TTC values
  - Evaluate whether the TTC values from ToxVal and Munro are statistically equivalent
  - Derive confidence intervals for the new TTC values
  - Compare and contrast the chemistry of the two data sets to rationalise any (dis)similarities in TTC values
Risk-Based prioritisation

Follow the Kroes et al (2004) workflow to assign substances present in ToxVal to their respective Cramer classes and use the associated repeat dose toxicity data to derive new TTC values.

Evaluate whether the TTC values from ToxVal and Munro are statistically equivalent & derive confidence intervals for the new TTC values.
Risk-Based prioritisation

- Bootstrap sampling used to quantify the uncertainty around the 5th percentiles values for both ToxVal and Munro data sets
- Differences were observed for substances assigned as Cramer Class III

- Presence of OP/carbamates in the Munro Cramer class III set largely explained the difference in 5th percentile values

Nelms et al, submitted
Definitions: Chemical grouping approaches

“Analogue approach” refers to grouping based on a very limited number of chemicals (e.g. target substance + source substance)

“Category approach” is used when grouping is based on a more extensive range of analogues (e.g. 3 or more members)

A chemical category is a group of chemicals whose physico-chemical and human health and/or environmental toxicological and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (or other similarity characteristics).
Definitions: Read-across

- **Read-across** describes the method of filling a data gap whereby a chemical with existing data values is used to make a prediction for a ‘similar’ chemical.

- A **target chemical** is a chemical which has a data gap that needs to be filled i.e. the subject of the read-across.

- A **source analogue** is a chemical that has been identified as an appropriate chemical for use in a read-across based on similarity to the target chemical and existence of relevant data.

<table>
<thead>
<tr>
<th>Property</th>
<th>Source chemical</th>
<th>Target chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute toxicity?</td>
<td>![Chemical structure]</td>
<td>![Chemical structure]</td>
</tr>
<tr>
<td>Known to be harmful</td>
<td>Predicted to be harmful</td>
<td></td>
</tr>
</tbody>
</table>

- Reliable data

- Missing data
Navigating through the minefield of read-across frameworks: A commentary perspective

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Where do other NAM fit?
How should we transition to data-driven approaches?
What about characterising the uncertainty of the predictions made?
Navigating through the minefield of read-across tools: A review of in silico tools for grouping

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ABSTRACT

Read-across is a popular data gap filling technique used within analogue and category approaches for regulatory purposes. In recent years there have been many efforts focused on the challenges involved in read-across development, its scientific justification and documentation. Tools have also been developed to facilitate read-across development and application. Here, we describe a number of publicly available read-across tools in the context of the category/analogue workflow and review their respective capabilities, strengths and weaknesses. No single tool addresses all aspects of the workflow. We highlight how the different tools complement each other and some of the opportunities for their further development to address the continued evolution of read-across.

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## Selected read-across tools

<table>
<thead>
<tr>
<th>Tool</th>
<th>AIM</th>
<th>ToxMatch</th>
<th>AMBIT</th>
<th>OECD Toolbox</th>
<th>CBRA</th>
<th>ToxRead</th>
<th>GenRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analogue identification</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Analogue Evaluation</td>
<td>NA</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>NA</td>
</tr>
<tr>
<td>Data gap analysis</td>
<td>NA</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Data gap filling</td>
<td>NA</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Uncertainty assessment</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>X</td>
<td>NA</td>
<td>NA</td>
<td>X</td>
</tr>
<tr>
<td>Availability</td>
<td>Free</td>
<td>Free</td>
<td>Free</td>
<td>Free</td>
<td>Free</td>
<td>Free</td>
<td>Free</td>
</tr>
</tbody>
</table>

- AIM: Analogical Identification Model
- ToxMatch: ToxMatch
- AMBIT: Automated Meta-Analysis of Biological Information Technology
- OECD Toolbox: OECD Toolbox
- CBRA: Chemical Biological Risk Assessment
- ToxRead: ToxRead
- GenRA: Genetic Risk Assessment

### Notes:
- X: Available
- NA: Not Available
- Data matrix can be exported
- Data matrix viewable
- For Ames & BCF
GenRA (Generalised Read-Across)

• Predicting toxicity as a similarity-weighted activity of nearest neighbours based on chemistry and bioactivity descriptors (Shah et al, 2016)

• Generalised version of the Chemical-Biological Read-Across (CBRA) developed by Low et al (2013)

• Goal: To establish an objective performance baseline for read-across and quantify the uncertainty in the predictions made

\[ y_i^{\beta, \alpha} = \frac{\sum_j^k S_{ij}^{\alpha} x_j^{\beta}}{\sum_j^k S_{ij}^{\alpha}} \]

Jaccard similarity:

\[ s_{ij} = \frac{\sum_l (x_{il} \land x_{jl})}{\sum_l (x_{il} \lor x_{jl})} \]

\[ x_j^{\beta} = \text{activity of } c_j \text{ in } \beta \]

\[ s_{ij}^\alpha = \text{Jaccard similarity between } x_i^\alpha, x_j^\alpha \]

\[ k = \text{up to } k \text{ nearest neighbours} \]
GenRA v1.0 - Approach

I. Data
1,778 Chemicals
3,239 Structure descriptors (chm)
820 Bioactivity hitcall (bio)
ToxCast

574 toxicity effects (tox)
ToxRefDB

II. Define Local neighbourhoods
Use K-means analysis to group chemicals by similarity
Use cluster stability analysis
~ 100 local neighbourhoods

III. GenRA
Use GenRA to predict toxicity effects in local neighbourhoods
Evaluate impact of structural and/or bioactivity descriptors on prediction
Quantify uncertainty
Read-across workflow in GenRA v1.0

**Decision Context**
Screening level assessment of hazard based on toxicity effects from ToxRefDB v1

**Analogue identification**
Similarity context is based on structural characteristics

**Data gap analysis**
for target and source analogues

**Uncertainty assessment**
Assess prediction and uncertainty using AUC and p value metrics

**Read-across**
Similarity weighted average - many to one read-across

**Analogue evaluation**
Evaluate consistency and concordance of experimental data of source analogues across and between endpoints
GenRA tool in reality

- Integrated into the EPA CompTox Chemicals dashboard
GenRA tool in practice

- Structured as a workflow
GenRA tool in practice

Step Two: Data Gap Analysis & Generate Data Matrix

Data gap analysis
GenRA tool in practice

Step Three: Run GenRA Prediction

Run GenRA

Target

Source analogues

Run Read-Across

Download: Filetype
GenRA tool in practice

Short Communication

Generalized Read-Across (GenRA): A workflow implemented into the EPA CompTox Chemicals Dashboard

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1Oak Ridge Institute for Science and Education (ORISE), Oak Ridge, TN, USA; 2National Center for Computational Toxicology (NCCT), Office of Research and Development, US Environmental Protection Agency, Research Triangle Park (RTP), NC, USA

Abstract

Generalized Read-Across (GenRA) is a data driven approach which makes read-across predictions on the basis of a similarity weighted activity of source analogues (nearest neighbors). GenRA has been described in more detail in the literature (Shah et al., 2018; Helman et al., 2018). Here we present its implementation within the EPA’s CompTox Chemicals Dashboard to provide public access to a GenRA module structured as a read-across workflow. GenRA assists researchers in identifying source analogues, evaluating their validity and making predictions of in vivo toxicity effects for a target substance. Predictions are presented as binary outcomes reflecting presence or absence of toxicity together with quantitative measures of uncertainty. The approach allows users to identify analogues in different ways, quickly assess the availability of relevant in vivo data for those analogues and visualize these in a data matrix to evaluate the consistency and concordance of the available experimental data for those analogues before making a GenRA prediction. Predictions can be exported into a tab-separated value (TSV) or Excel file for additional review and analysis (e.g., doses of analogues associated with production of toxic effects). GenRA offers a new capability of making reproducible read-across predictions in an easy-to-use interface.
GenRA - Next Steps

• Ongoing research:

• Summarising and aggregating the toxicity effect predictions to guide end users - what effect predictions are we most confident about (digesting & interpreting the predictions more efficiently)

• Consideration of other information to define and refine the analogue selection & evaluation - e.g. physicochemical similarity, metabolic similarity, reactivity similarity, bioactivity similarity (transcriptomics similarity)...
  - EPA New Chemical Categories
  - Quantifying the impact of physicochemical similarity on read-across performance (Helman et al., 2018)
GenRA – Next Steps

- Dose response information to refine scope of prediction beyond binary outcomes
  - Transitioning from qualitative to quantitative predictions – how to apply and interpret GenRA in screening level hazard assessment
  - Starting with quantitative data – e.g. acute rat oral toxicity (Helman et al. (2019), ToxRefDB v2 (Helman et al. (2019))
Case study: Acute toxicity

• Transitioning GenRA to make quantitative predictions

• Investigated extending GenRA using the acute oral rat systemic toxicity data collected as part of the ICCVAM Acute toxicity workgroup

• NICEATM-NCCT effort to collate a large dataset of acute oral toxicity to evaluate the performance of existing predictive models and investigate the feasibility of developing new models
Acute toxicity: Dataset creation

<table>
<thead>
<tr>
<th>Database Resource</th>
<th>Rows of Data (number of LD50 values)</th>
<th>Unique CAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECHA (ChemProp)</td>
<td>5533</td>
<td>2136</td>
</tr>
<tr>
<td>JRC AcutoxBASE</td>
<td>637</td>
<td>138</td>
</tr>
<tr>
<td>NLM HSDB</td>
<td>4082</td>
<td>2238</td>
</tr>
<tr>
<td>OECD (eChemPortal)</td>
<td>10206</td>
<td>2314</td>
</tr>
<tr>
<td>PAI (NICEATM)</td>
<td>364</td>
<td>293</td>
</tr>
<tr>
<td>TEST (NLM ChemIDplus)</td>
<td>13689</td>
<td>13545</td>
</tr>
</tbody>
</table>

Rat oral LD50s:
- 16,297 chemicals total
- 34,508 LD50 values

Require unique LD50 values with mg/kg units

15,688 chemicals total
21,200 LD50 values

Preprocessing for modelling

11,992 chemicals
16,173 LD50 values

Karmaus et al., 2018; Kleinstreuer et al., 2018
Exploratory Data Analysis

- Found DSSTox matches for 7011 substances
- Extracted MW values
GenRA approach: Overall ‘global’ performance

- Search for a maximum of 10 nearest neighbours on entire dataset
- Use a min similarity threshold of 0.5

Linear regression used to fit predicted and observed LD50 values
- \( R^2 = 0.61 \)
- \( \text{RMSE} = 0.58 \)
- A few outliers, but not too extreme
- Residuals clustered around zero with no obvious patterns
Coverage vs Similarity vs Performance

- **Coverage vs Similarity**

  ![Coverage vs Similarity](image1)

  Based on the grid searches performed, $k = 10$, $s = 0.5$ were reasonable parameters to tradeoff coverage vs prediction accuracy.

  ![R2 for up to k source analogues](image2)

  ![Coverage for exactly k neighbors](image3)
Monte Carlo Cross Validation

- Estimate confidence in $R^2$
- 75-25 train-test splits
- $R^2$ values range from 0.46 to 0.62
- GenRA performs robustly on this acute tox data set

Helman et al. (2019)
Evaluating ‘local’ performance

Clustered chemicals into 100 groups on the basis of ToxPrint fingerprints

Explored performance on the basis of individual clusters to gauge what sorts of chemicals resulted in significantly improved performance (R2) relative to the overall ‘global’ performance reported using 10 nearest neighbours and a similarity of 0.5

Average R2 values improved (R2>0.61) for 19 out of the 100 clusters, some up to 0.91

Carbamate containing substances
• Are there pairs of substances that are very similar structurally with very high LD50 differences, so called activity cliffs

The number of chemical pairs that fell within the activity cliff quadrant was very low relative to the total number of chemical pairs captured.

This suggests that the chemical fingerprints were able to capture sufficient information to make robust predictions of acute oral toxicity.
EPA Using New Approach Methods to Help Fill Information Gaps for PFAS

- ~1,223 PFAS currently in TSCA inventory for use in US
- ~ 602 of those currently active
- + unknown number of degradation and manufacturing byproducts

EPA 2019 PFAS Action Plan recognised need for approach to grouping approaches

Research Area 1: What are the human health and ecological effects of exposure to PFAS?

- Using computational toxicology approaches to fill in gaps. For the many PFAS for which published peer-reviewed data are not currently available, the EPA plans to use new approaches such as high throughput and computational approaches to explore different chemical categories of PFAS, to inform hazard effects characterization, and to promote prioritization of chemicals for further testing. These data will be useful for filling gaps in understanding the toxicity of those PFAS with little to no available data. In the near term, the EPA intends to complete assays for a representative set of 150 PFAS chemicals, load the data into the ComTox Chemicals Dashboard for access, and provide peer-reviewed guidance for stakeholders on the use and application of the information. In the long term, the EPA will continue research on methods for using these data to support risk assessments using New Approach Methods (NAMs) such as read-across and transcriptomics, and to make inferences about the toxicity of PFAS mixtures which commonly occur in real world exposures. The EPA plans to collaborate with NIEHS and universities to lead the science in this area and work with universities, industry, and other government agencies to develop the technology and chemical standards needed to conduct this research.
Assembled a PFAS Chemical Library for Research and Methods Development

- Attempted to procure ~3,000 based on chemical diversity, Agency priorities, and other considerations

- Obtained 480 total unique chemicals
  - 430/480 soluble in DMSO (90%)
  - 54/75 soluble in water (72%)
    (incl. only 3 DMSO insolubles)

- Issues with sample stability and volatility

- Categories assigned based on three approaches
  - Buck et al., 2011 categories
  - Markush categories
  - OECD categories
  - Manual assignment

Kathy Coutros, Chris Grulke, and Ann Richard
Selecting a Subset of PFAS for Tiered Toxicity and Toxicokinetic Testing

Goals:

- Generate data to support development and refinement of categories and read-across evaluation
- Incorporate substances of interest to Agency
- Characterise mechanistic and toxicokinetic properties of the broader PFAS landscape

Selected 150 PFAS in two phases representing 83 different categories

- 9 categories with > 3 members
- Lots of singletons
# In Vitro Toxicity and Toxicokinetic Testing

## Toxicological Response

<table>
<thead>
<tr>
<th>Toxicological Response</th>
<th>Assay</th>
<th>Assay Endpoints</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatotoxicity</td>
<td>3D HepaRG assay</td>
<td>Cell death and transcriptomics</td>
<td>Measure cell death and changes in important biological pathways</td>
</tr>
<tr>
<td>Developmental Toxicity</td>
<td>Zebrafish embryo assay</td>
<td>Fertilization, lethality, and structural defects</td>
<td>Assess potential teratogenicity</td>
</tr>
<tr>
<td>Immunotoxicity</td>
<td>Bioseek Diversity Plus</td>
<td>Protein biomarkers across multiple primary cell types</td>
<td>Measure potential disease and immune responses</td>
</tr>
<tr>
<td>Mitochondrial Toxicity</td>
<td>Mitochondrial membrane potential and respiration (HepaRG)</td>
<td>Mitochondrial membrane potential and oxygen consumption</td>
<td>Measure mitochondrial health and function</td>
</tr>
<tr>
<td>Developmental Neurotoxicity</td>
<td>Microelectrode array assay (rat primary neurons)</td>
<td>Neuronal electrical activity</td>
<td>Impacts on neuron function</td>
</tr>
<tr>
<td>Endocrine Disruption</td>
<td>ACEA real-time cell proliferation assay (T47D)</td>
<td>Cell proliferation</td>
<td>Measure ER activity</td>
</tr>
<tr>
<td>General Toxicity</td>
<td>Attagene cis- and trans-Factorial assay (HepG2)</td>
<td>Nuclear receptor and transcription factor activation</td>
<td>Activation of key receptors and transcription factors involved in hepatotoxicity</td>
</tr>
<tr>
<td></td>
<td>High-throughput transcriptomic assay (multiple cell types)</td>
<td>Cellular mRNA</td>
<td>Measures changes in important biological pathways</td>
</tr>
<tr>
<td></td>
<td>High-throughput phenotypic profiling (multiple cell types)</td>
<td>Nuclear, endoplasmic reticulum, nucleoli, golgi, plasma membrane, cytoskeleton, and mitochondria morphology</td>
<td>Changes in cellular organelles and general morphology</td>
</tr>
</tbody>
</table>

## Toxicokinetic Parameter

<table>
<thead>
<tr>
<th>Toxicokinetic Parameter</th>
<th>Assay</th>
<th>Assay Endpoints</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrinsic hepatic clearance</td>
<td>Hepatocyte stability assay (primary human hepatocytes)</td>
<td>Time course metabolism of parent chemical</td>
<td>Measure metabolic breakdown by the liver</td>
</tr>
<tr>
<td>Plasma protein binding</td>
<td>Ultracentrifugation assay</td>
<td>Fraction of chemical not bound to plasma protein</td>
<td>Measure amount of free chemical in the blood</td>
</tr>
</tbody>
</table>

*Assays being performed by NTP and EPA*
Current work in progress

- How do the structural categories inform read-across? How are the categories enriched by the bioactivity (tiered toxicity and toxicokinetic) data being generated?
**Attagene cis- and trans- Factorial Assay**

- **CIS Assay**
  - 47 Endogenous Transcription Factors
    - Xenobiotic pathways
    - Cell growth/differentiation
    - Endocrine pathways
    - Stress response

- **TRANS Assay**
  - 24 human nuclear receptors
  - GAL-4 formats (NR ligand-binding domains)

- HepG2 cells
  - Concentration-response testing
  - 24-hour exposure
Preliminary Category-Based Analysis of the Attagene Transcription Factor Assay

Estrogen Receptor Activity

NRF2 Activity

PPARα Receptor Activity

*7 categories with STD > 0.6

Keith Houck and Grace Patlewicz
High-Throughput Phenotypic Profiling (aka ‘Cellular Pathology’)
Preliminary Category-Based Analysis of the Phenotypic Profiling Assay

MCF7 Cells

U2OS Cells

Pairwise Euclidean Distance

Structural_Category

Joshua Harrill, Johanna Nyffeler, and Grace Patlewicz
Current PFAS Grouping Approaches Use Different Levels of Aggregation

<table>
<thead>
<tr>
<th>Chemical Categories/Group</th>
<th>Level of Structural Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
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<tr>
<td>E</td>
<td></td>
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<tr>
<td>F</td>
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<td>G</td>
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<tr>
<td>H</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Available source *in vivo* tox study
Incorporating Mechanistic and Toxicokinetic Data to Inform PFAS Category Aggregation

Chemical Categories/Group

A  B  C1  C2  D  E  F  G  H  I  J  ...

* Needed in vivo tox study  * Available source in vivo tox study
Challenges with the analysis to date...

- Initially structural category assignments were largely expert driven.
- This was pragmatic based on what resources were available at the time, however it is difficult to assign membership reproducibly and objectively with a manual naming convention.
- Moreover this does not facilitate profiling of other PFAS inventories/libraries of interest e.g. OECD.
PFAS “Categories”: Per & Poly-fluorinated alkyl substances

• “Expert”-assigned PFAS categories – manual, subjective
  – Buck et al. (DuPont), based on chemical & series informed by synthetic pathways (e.g., fluorotelomers)
  – data-gathering, occurrence reports, ecotox
  – OECD PFAS listing (>4500 chemicals) – manually assigned groupings

<table>
<thead>
<tr>
<th>Class</th>
<th>Category_Name1</th>
<th>Category_Name2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>Fluorotelomer alcohols</td>
<td>Fluorotelomer (linear) n:2 alcohols</td>
</tr>
<tr>
<td>Sulfonic Acid</td>
<td>Perfluoroalkyl sulfonic acids</td>
<td>Perfluoroalkyl (linear C4-C10) sulfonic acids</td>
</tr>
<tr>
<td></td>
<td>Perfluoroalkyl ethers</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fluorotelomer phosphates</td>
<td></td>
</tr>
</tbody>
</table>
“Expert-assigned” OECD PFAS Categories, e.g.

- 4730 PFAS in list
- 173 expert-assigned categories under 8 general headings (bold)
- Broad “catch-all” terms (in red)
- Structural elements, but NOT structure-based
- Requires expert to assign new chemicals to categories

<table>
<thead>
<tr>
<th>Category</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>perfluoroalkyl carbonyl compounds</strong></td>
<td>CnF2n+1_C(O)_R</td>
</tr>
<tr>
<td>perfluoroalkyl carbonyl halides</td>
<td>R = F/Cl/Br/I</td>
</tr>
<tr>
<td>perfluoroalkyl carboxylic acids (PFCAs), their salts and esters</td>
<td>R = OH, ONa, OCH₃, etc.</td>
</tr>
<tr>
<td><strong>other perfluoroalkyl carbonyl-based nonpolymers</strong></td>
<td>to be refined</td>
</tr>
<tr>
<td>perfluoroalkyl carbonyl amides / amid ethanol and other alcohols</td>
<td>R = NH₂, NH(OH), etc.</td>
</tr>
<tr>
<td>perfluoroalkyl carbonyl (meth)acrylate</td>
<td>R = R’_OC(O)CH=CH₂</td>
</tr>
<tr>
<td>perfluoroalkyl carbonyl (meth)acrylate polymers</td>
<td></td>
</tr>
<tr>
<td>1-H perfluoroalkyl carboxylic acids</td>
<td>H(CF₂)nCOOH</td>
</tr>
<tr>
<td><strong>perfluoroalkane sulfonyl compounds</strong></td>
<td>CnF2n+1_S(O)(O)_R</td>
</tr>
<tr>
<td>perfluoroalkane sulfonyl halides</td>
<td>R = F/Cl/Br/I</td>
</tr>
<tr>
<td>perfluoroalkane sulfonic acids (PFSAs), their salts and esters</td>
<td>R = OH, ONa, OCH₃, etc.</td>
</tr>
<tr>
<td><strong>per- and polyfluoroalkyl ether-based compounds</strong></td>
<td>CnF2n+1_O_CmF₂m+1_R</td>
</tr>
<tr>
<td>per- and polyfluoroalkyl ether sulfonic acids (PFESAs), their salts and esters, as well as derivatives</td>
<td>CnF2n+1_O_CmF₂m+1_SO₃H</td>
</tr>
<tr>
<td><strong>fluorotelomer-related compounds</strong></td>
<td></td>
</tr>
<tr>
<td>perfluoroalkyl iodides (PFAIs)</td>
<td>CnF2n+1_I</td>
</tr>
<tr>
<td>n:2 <strong>fluorotelomer-based</strong> non-polymers</td>
<td>CnF2n+1_C2H4_R, to be refined</td>
</tr>
</tbody>
</table>
Translating Expert Categories to Markush

Expert category

- Fluorotelomer acrylates
- Fluorotelomer alcohols
- Polyfluorinated alcohols
- Fluorotelomer sulfonates
- N-alkyl perfluoroalkyl sulfonamidoacetic acids
- N-alkyl perfluoroalkyl sulfonamidoethanols
- Perfluoroalkyl aldehydes
- Perfluoroalkyl amides
- Perfluoroalkyl carboxylates
- Perfluoroalkyl acyl fluorides
- Perfluoro vinyl esters
- Perfluoroalkyl ketones
- Semi-fluorinated alkenes
- Perfluoroalkyl vinyl ethers
- Perfluoroalkyl alkyl ethers
- Fluorotelomer amines
- Perfluoroalkyl sulfonamides
- Semi-fluorinated alkanes
- Perfluoroalkyl sulfonates
- Perfluoroalkyl sulfamido amines
- Polyfluoroalkyl carboxylates
- Perfluoroalkyl ethers
- Fluorotelomer phosphates
Example of Markush representation

- Perfluoralkyl sulfonates
  - DTXSID: DTXSID0892979
  - CASRN: NOCAS_892979

- Perfluorobutanesulfonic acid
  - DTXSID: DTXSID0503030
  - CASRN: 375-73-5

- Perfluoropentanesulfonic acid
  - DTXSID: DTXSID031664
  - CASRN: 1753-23-1

- Perfluorodecanesulfonic acid
  - DTXSID: DTXSID04148
  - CASRN: 335-77-3

- Perfluoroheptanesulfonic acid
  - DTXSID: DTXSID05920
  - CASRN: 375-82-8

- Perfluorobutanesulfonic acid
  - DTXSID: DTXSID062600
  - CASRN: 2706-91-4

- Perfluorooctanesulfonic acid
  - DTXSID: DTXSID071356
  - CASRN: 68255-12-1

- Perfluoropropanesulfonic acid
  - DTXSID: DTXSID0876531
  - CASRN: 423-41-8

- Perfluorodecanesulfonic acid (PFOS)
  - DTXSID: DTXSID0873011
  - CASRN: 79780-39-5
Exploiting fixed fingerprints to facilitate objective structural categories

- For the ~150 set, have aimed to harmonise the 3 schemes using fixed ToxPrints
- Defined rules on membership based on specific features
- Extendable to incorporate other information i.e. bioactivity
Take home messages

• Computational toxicology approaches impact many aspects of regulatory contexts
• Outlined how computational approaches fit within an IATA
• Illustrated how we have explored coupling TTC & HTE for a risk-based prioritisation application
• Discussed read-across approaches, tools & their frameworks
• Proposed a harmonised framework for read-across approaches
Take home messages

• Outlined GenRA, how it was developed and how it is aligned with this framework – public tool

• Initial GenRA (baseline) considers structural similarity but current work has evaluated the quantitative impact of physicochemical similarity (as it relates to bioavailability) and transitioning to dose predictions e.g. acute toxicity LD50

• Highlighted the research efforts of using chemical structural groupings to underpin selection of representative PFAS for toxicity and toxicokinetic testing using NAMs
Acknowledgements

• Many but in particular...
  • Imran Shah
  • George Helman
  • Tony Williams
  • Richard Judson
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  • Jason Lambert

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• Joshua Harrill
• Johanna Nyffeler
• Rusty Thomas