Establishing confidence in new approach methodologies (NAMs): Use of NAMs to refine and strengthen SAR read-across for branched-alkyl carboxylic acids

Corie Ellison, PhD
Procter & Gamble
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Thanks to my P&G Colleagues…

- Shengde Wu
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Research article

Structure-activity relationship read-across and transcriptomics for branched carboxylic acids

Shengde Wu, Corie Ellison, Jorge Naciff, Michael Karb, Cindy Obringer, Gang Yan, Yuqing Shan, Alex Smith, Xiaohong Wang, George P. Daston*

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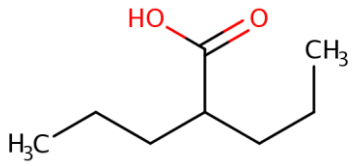
Thanks to the EPIC webinar organizers

Case Study Outline

- Aim of the case study
- NAMs to address Toxicodynamic
  - Transcriptomics
  - Molecular Docking
- NAMs to address Toxicokinetics
  - In vitro ADME assays
  - PBPK modelling
- Summary
  - Case study conclusions
  - Establishing confidence in the implemented NAM approach

Case Study Problem Statement

Valproic acid (VPA)

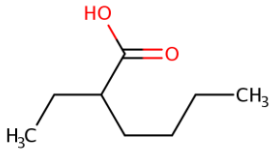


Widely used pharmaceutical

A <u>human teratogen</u>, causing spina bifida in about 1% of babies exposed prenatally

Causes neural tube defects and other abnormalities in animal models

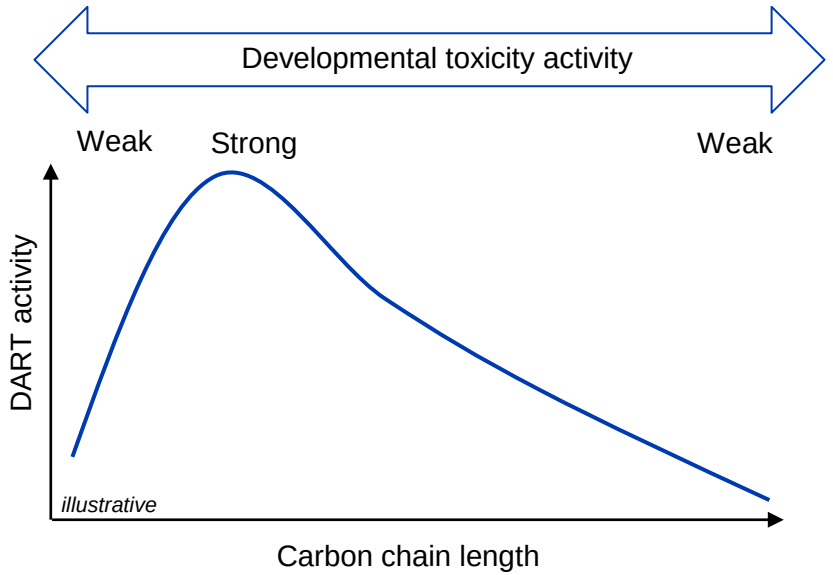
Activity trend for other branched carboxylic acids



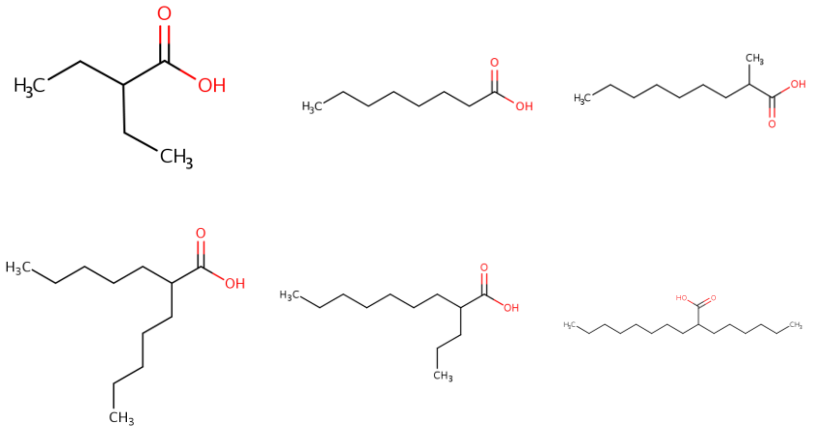
2-Ethylhexanoic acid (EHA)

Isomer of VPA

<u>Developmentally toxic</u> in rodent models



Additional branched carboxylic acids



Used in the production of surfactants, lubricants, plastics and other products

Much less data on the developmental toxicity potential

<u>Do they have the potential to have VPA-like effects on development</u>?

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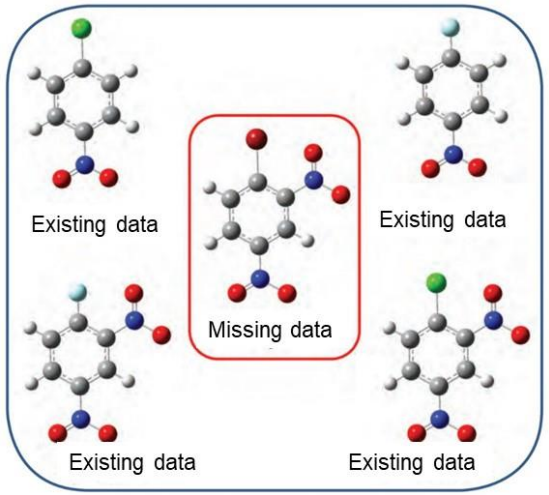
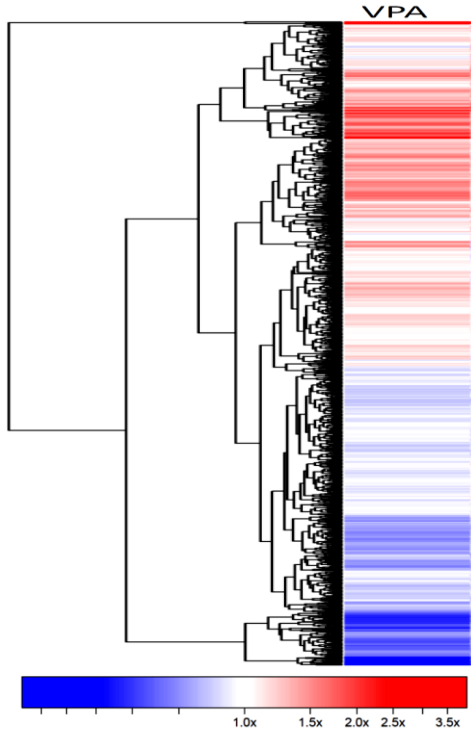
Hypothesis Driven Selection of NAMs

New Approach Methodologies (NAMs) refer to any technologies, methodologies, approaches, or combinations thereof that can be used to provide information on chemical hazard and potential human exposure that can avoid or significantly reduce the use of testing on animals (US EPA 2022)

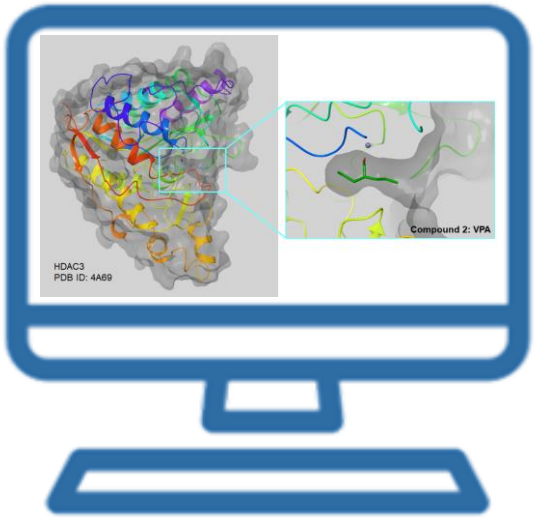
TOXICOLOGICAL STRUCTURE ACTIVITY RELATIONSHIP (SAR) READ ACROSS



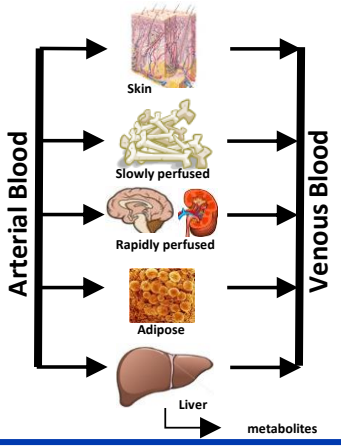
TRANSCRIPTOMICS



IN SILICO MOLECULAR DOCKING



IN VITRO & IN SILICO TOXICOKINETICS



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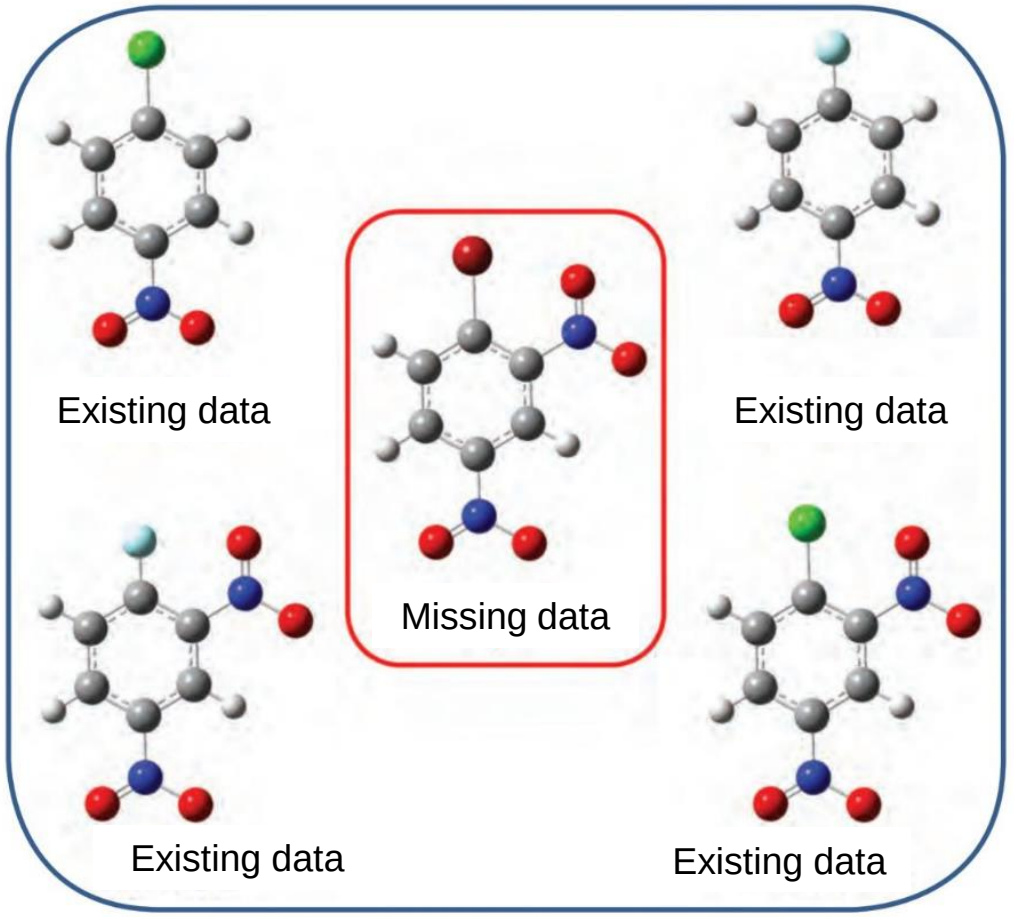
Predicting Toxicity

Toxicological Structure Activity Relationship (SAR) read across



https://pubs.rsc.org/en/content/ebook/978-1-84973-384-7

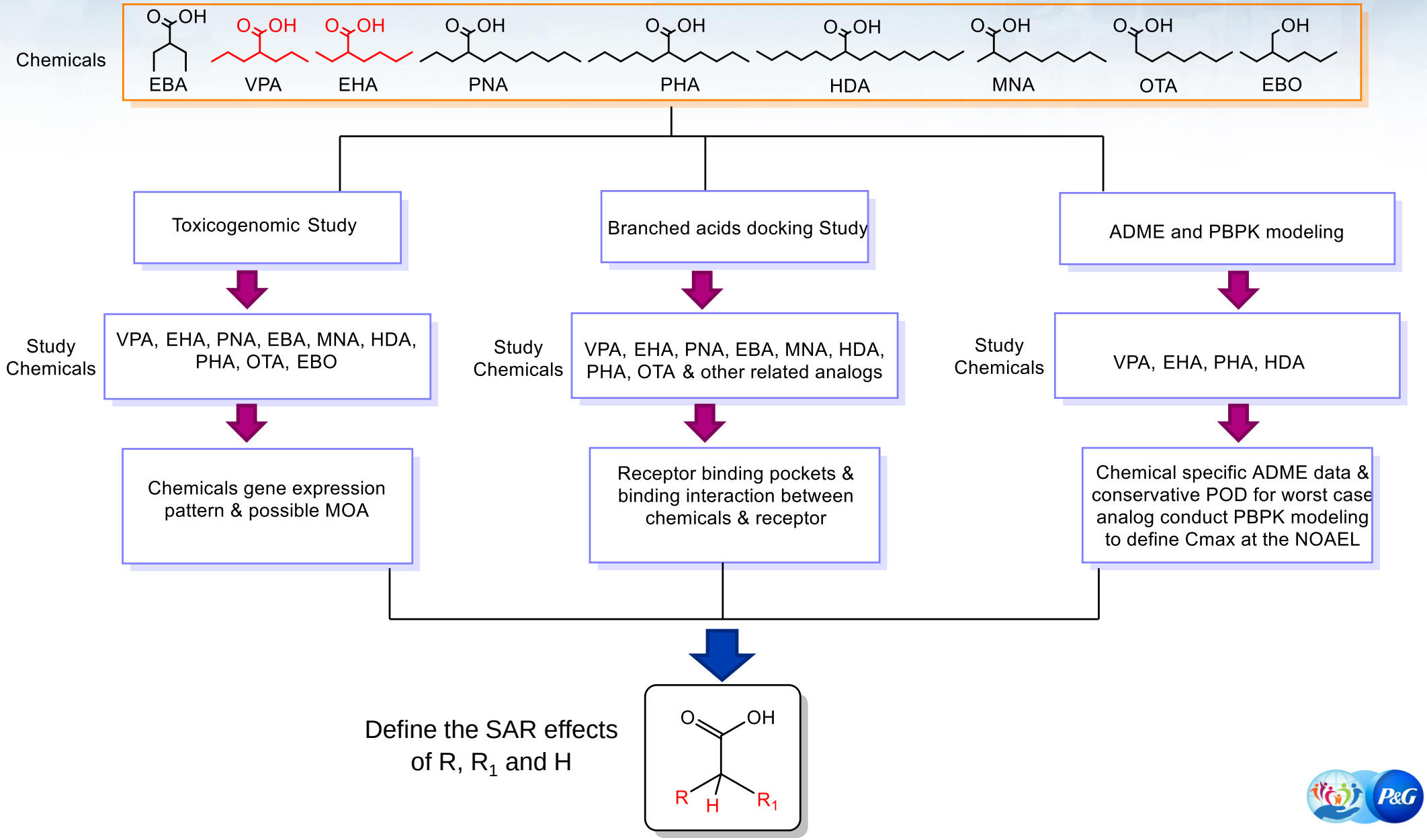
Read-across is an old concept that fits within the scope of NAMs

The basic premise for read-across is that compounds with similar chemical structure will have the same biological activity

Read-across requires: (1) identifying and rating the quality of analogs; (2) demonstrating that close structural analogs have similar activity

Use read across and other NAMs to inform on developmental toxicity potential of branched carboxylic acids in case study

NAMs Used to Support/Refine Toxicological SAR Read Across



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Transcriptomics

Study Design

4 cell types

- MCF-7 (breast epithelial adenocarcinoma),
- A549 (lung epithelial carcinoma),
- HepG2 (hepatocellular carcinoma)
- iCell (cardiomyocytes from induced pluripotent stem cells)

3 doses (up to 1,000 uM)

6 hr of exposure

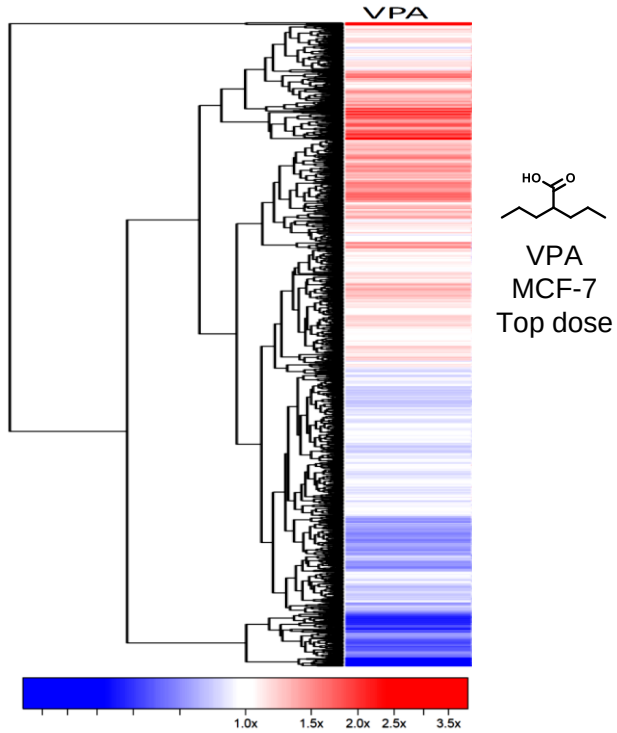
N = 3 or 4

L1000 platform to detect gene expression

The gene expression data uploaded to the Gene Expression Omnibus

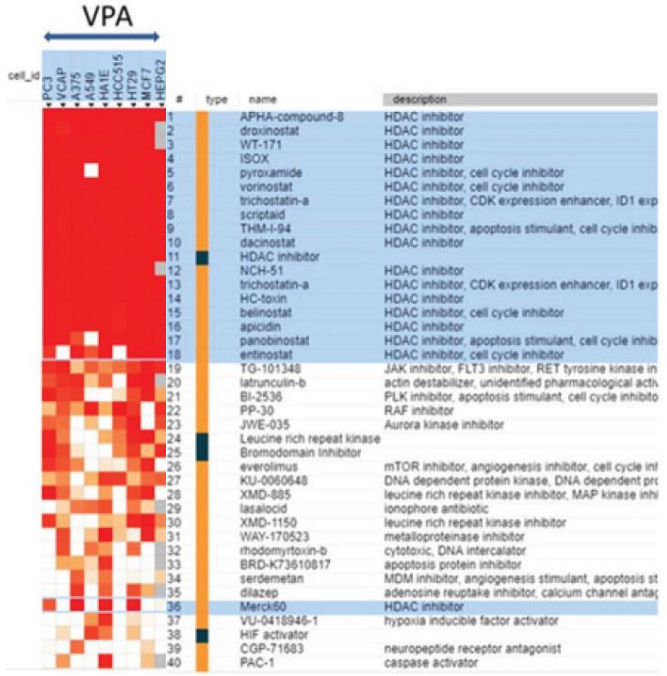
Transcriptional Signature

Determined for each chemical, dose & cell type combination



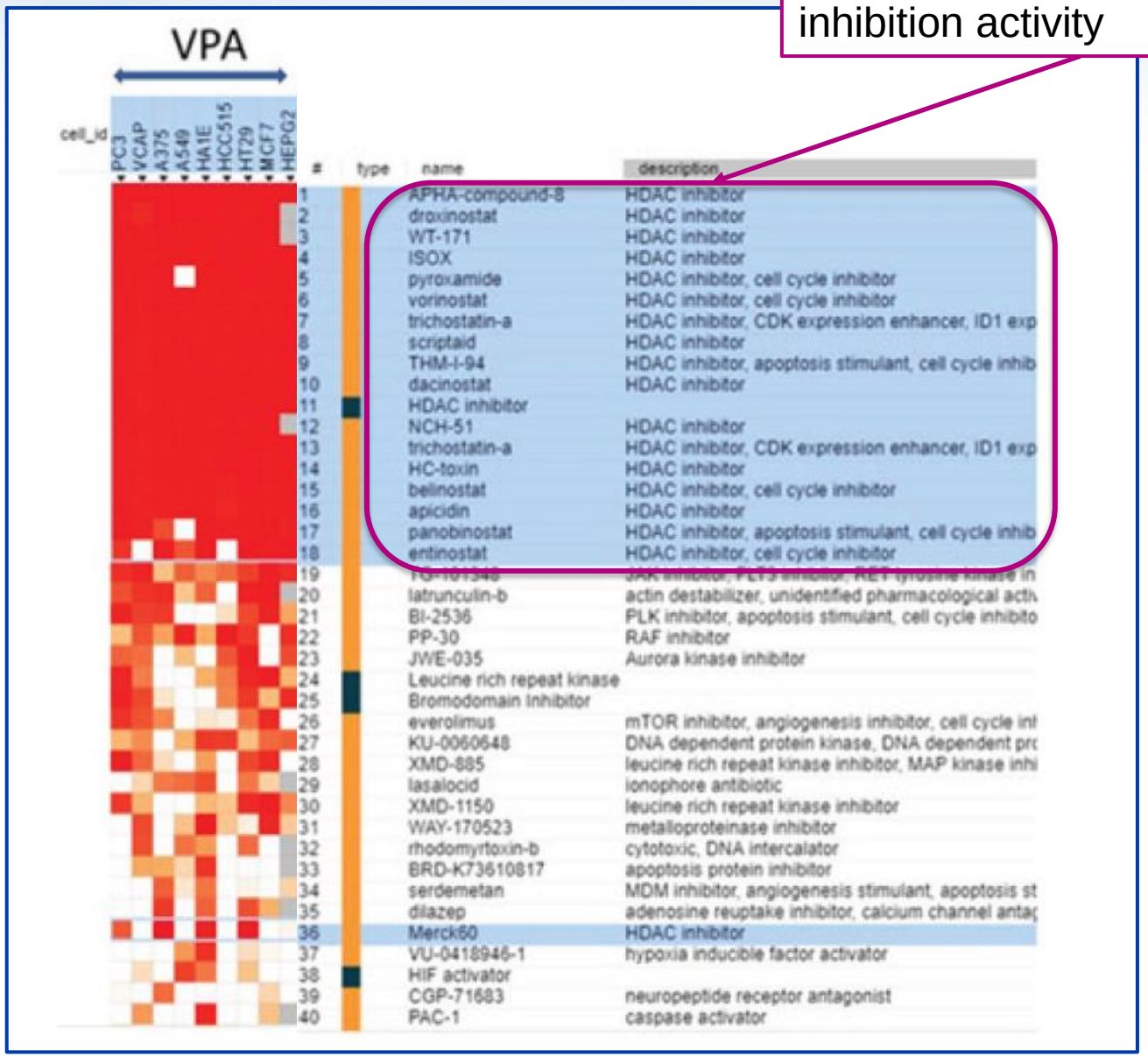
CMap Analysis

Used to determine degree of similarity of transcriptional signature as a measure of comparable biological activity



Connectivity Mapping of VPA to Entries in Database

Histone deacetylase (HDAC) inhibition activity

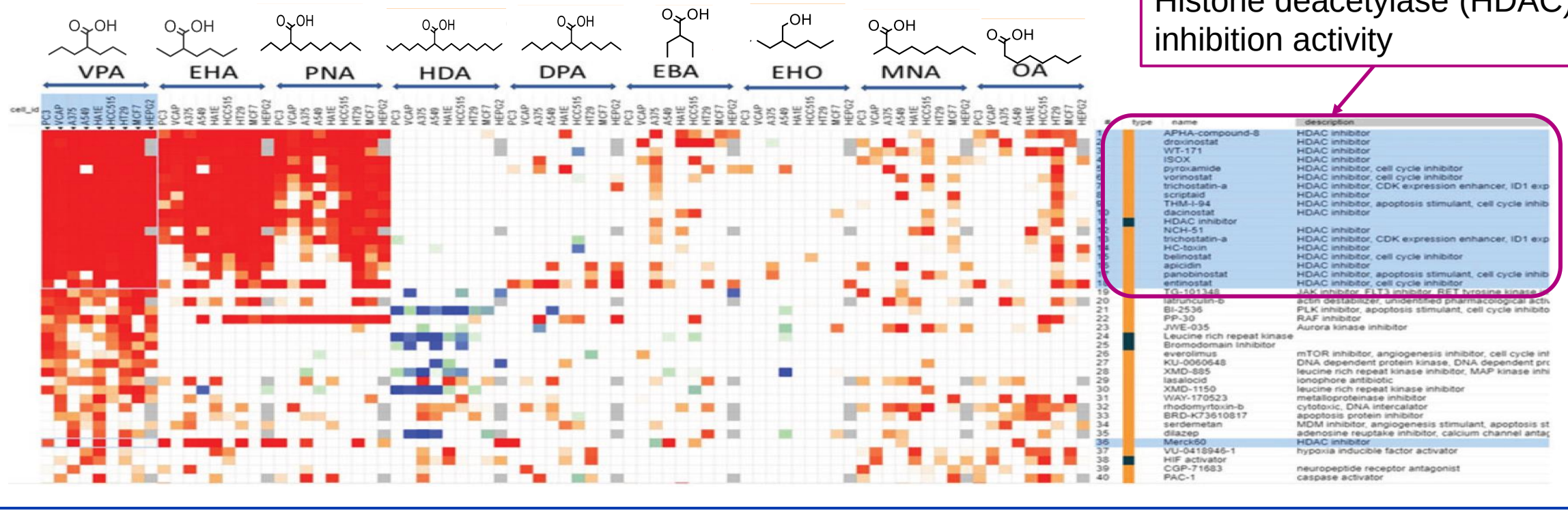


Transcriptional signature for VPA was evaluated for connections in a large CMap database (https://clue.io/touchstone).

Approach is useful to identify chemicals with similar mode of action

CMap results indicate that VPA connects with histone deacetylase (HDAC) inhibitors

Connectivity Mapping of Branched Carboxylic Acids to Entries in Database



C-MAP results indicate:

- VPA, EHA, PNA have highly correlated transcriptional signatures
- VPA, EHA, PNA have similar transcriptional signatures as HDAC inhibitors
- PHA, MNA, HDA, EHO, EBA, OA have different gene expression pattern as HDAC inhibitors and VPA, EHA, PNA

HDAC inhibition may be associated with valproate-like effects on development for these acids (VPA, EHA; possibly PNA)

MOA of HDAC Inhibitors to Cause Teratogenic Potential

- Many HDAC inhibitors can induce specific malformations in rodents
- Common MoA is histone hyperacetylation and the consequent alteration of gene expression pattern.

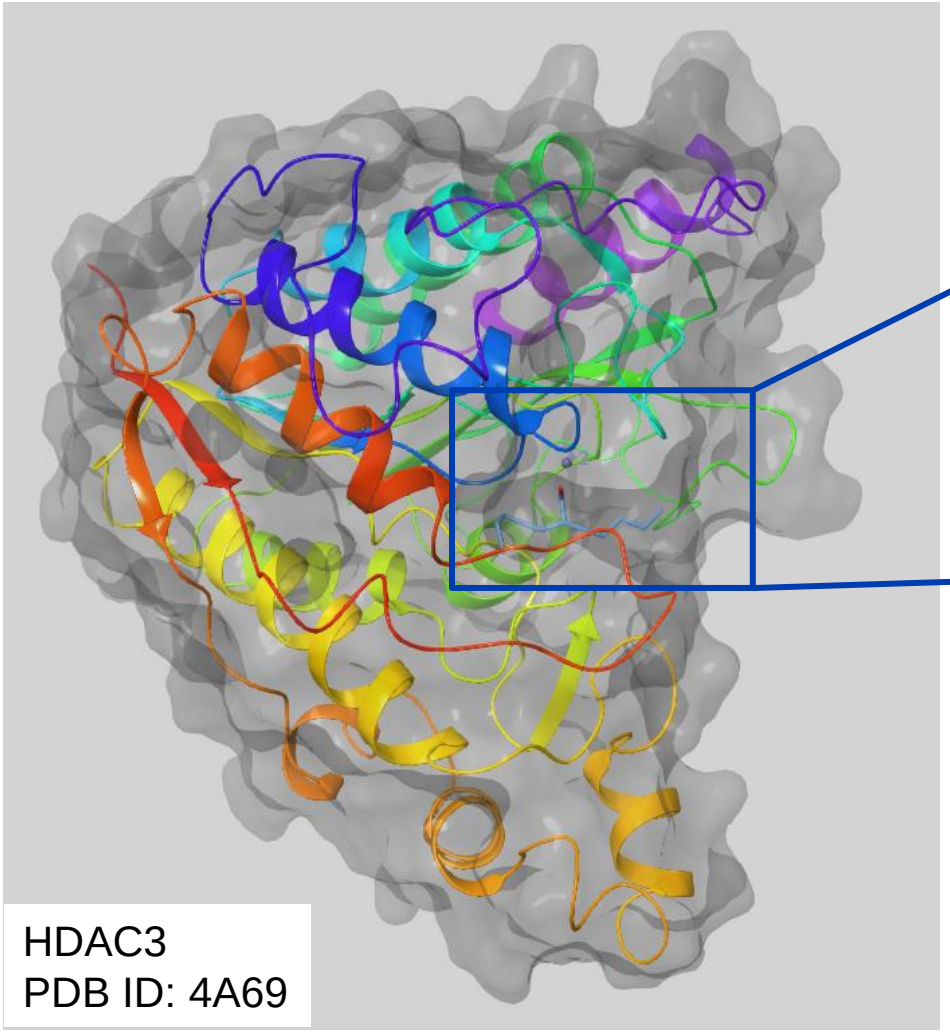
HDAC knockout mouse phenotype related teratogenic activity

Class I HDAC1 HDAC3	Class I HDAC2 HDAC3	Class II HDAC	Class I HDAC1
Embryolethal at very early stages of development, probably due to cell proliferation and general growth arrest	Postnatal lethality respectively due to cardiac or skeletal defects	Lethality or altered phenotype in growth response of specific tissues (cartilage and heart muscle)	Knockdown or mutant zebrafish embryos are characterized by developmental defects at the level of the heart, neuroepithelial derivatives, craniofacial cartilages and pectoral fins

Can we support the transcriptomic results with molecular docking information?

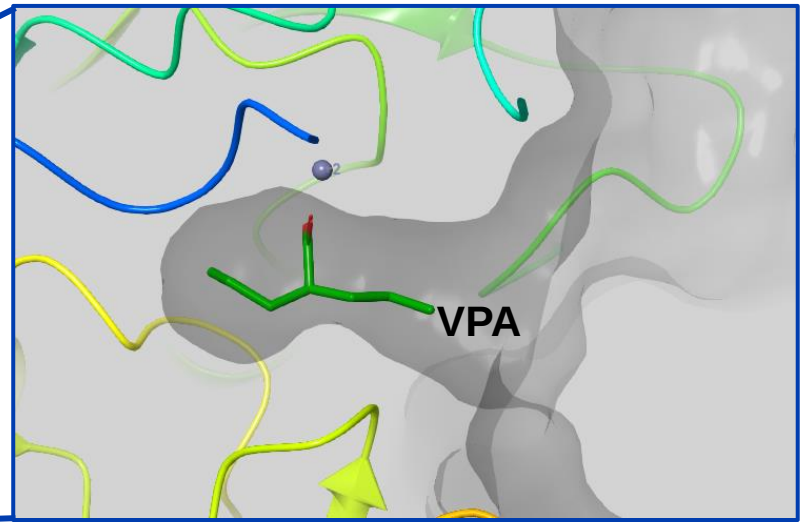
Molecular Docking with Histone Deacetylase

Structure of histone deacetylase 3 (HDAC3)



HDAC3
PDB ID: 4A69

Close up view of HDAC3 binding pocket

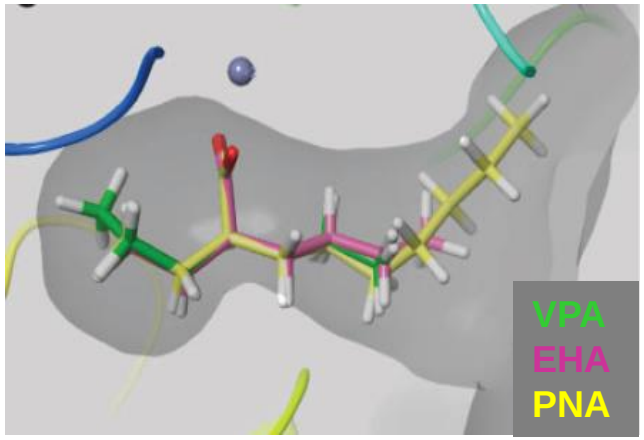


HDAC receptor contains two asymmetric binding pockets:

(1) small pocket which only fits a shorter alkyl chain
(2) larger pocket which can tolerate longer alkyl chains

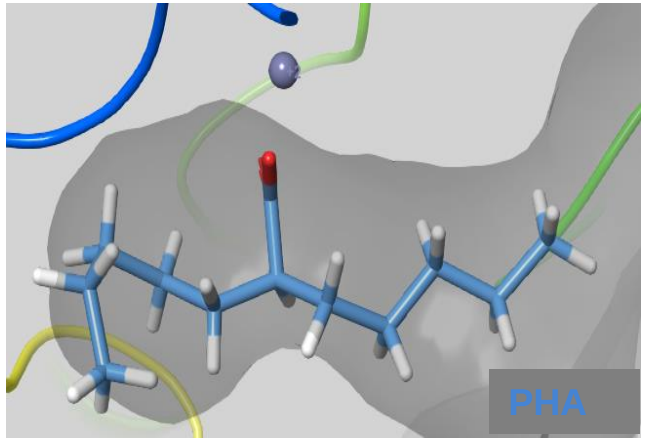
VPA is a good ligand for HDAC3

Molecular Docking Results



VPA, EHA, PNA

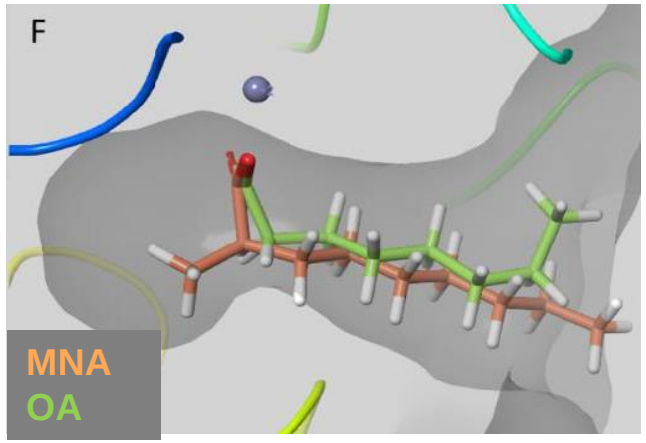
Good fit into HDAC binding pocket



PHA, HDA

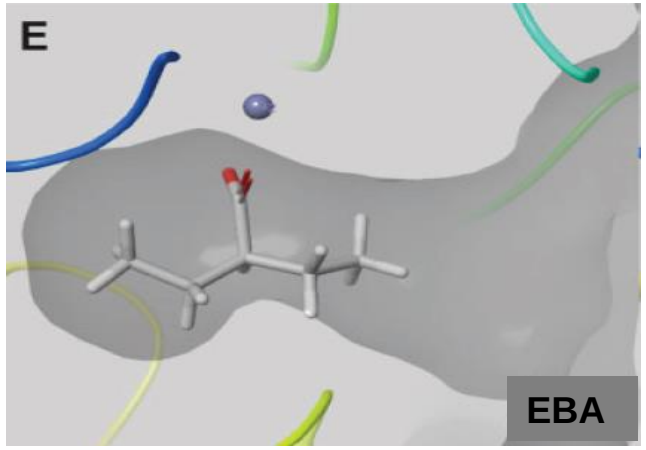
Poor fit into HDAC binding pocket

Too bulky



MNA, OA, EBA

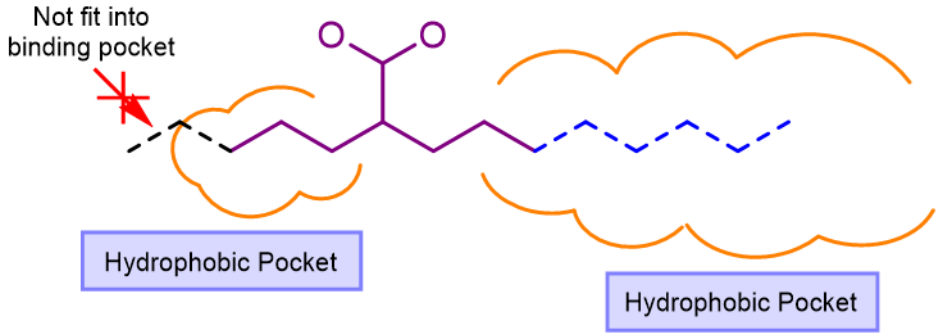
Poor fit into HDAC binding pocket



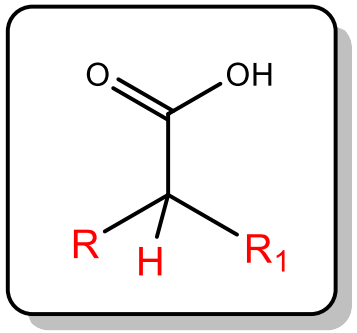
At least one R group too small

Similar results obtained for HDAC1, HDAC2 and HDAC3

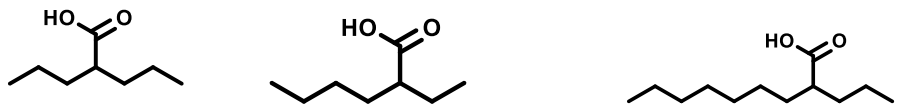
SAR Results Based on Transcriptomics and Docking



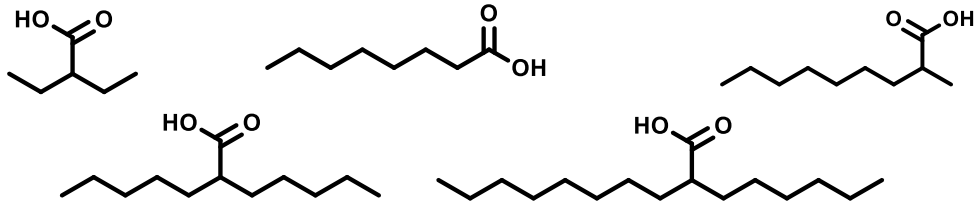
Depending on the size of branched alkyl chains they can serve as an ‘anchor’ within a hydrophobic pocket in the HDAC receptor



Teratogenic if:
C1 < R < C5 and C2 < R1 < C8



These acids shared similar gene expression pattern and showed HDAC inhibition potential. They have similar MOA and could be considered as suitable analogs for developmental potential



These chemicals shared different gene expression pattern to VPA, EHA and PNA and did not show HDAC inhibition potential. They could be considered as very weak or non-developmental potential

What About Toxicokinetics

Toxicodynamics

Differences seen with transcription profiles between the branched carboxylic acids

Differences seen with HDAC inhibition potential between the branched carboxylic acids

VPA, EHA, PNA

PHA, MNA, HDA, EHO, EBA, OTA

Toxicokinetics

Are there differences in the Absorption, Distribution, Metabolism, Excretion (ADME) or toxicokinetics (TK) between the branched carboxylic acids

Do similar administered doses result in similar internal exposures

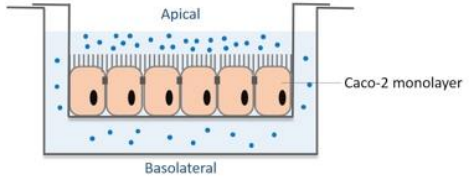
Explore these questions further with a subset of branched carboxylic acids:

VPA, EHA, PHA and HDA

Toxicokinetic Based NAMs

In Vitro Toolbox

Caco-2 Permeability

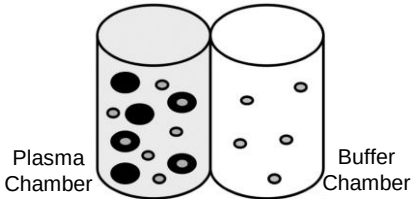


10 uM test chemical concentration

5 timepoint sampling

Monitor test chemical permeability from donor to receiver chamber

Plasma Protein Binding



Female rat plasma

10 and 100 uM test concentration

Determine fraction unbound at equilibrium

Hepatocyte Metabolism

Cryopreserved primary female rat hepatocytes in suspension

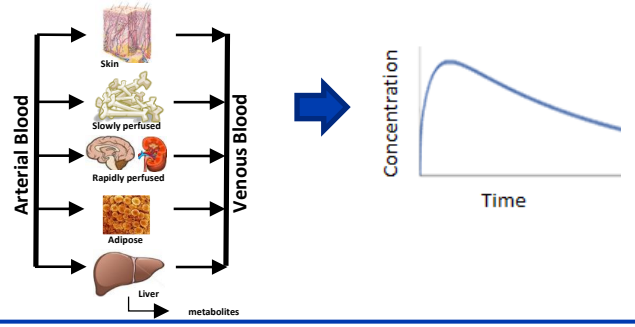
10 uM test concentration

8 timepoint sampling

Monitor parent depletion

In silico Models - *Integrate Data and Predict Outcomes*

Chemical properties + *Physiology* = *Physiologically Based Kinetic Models*

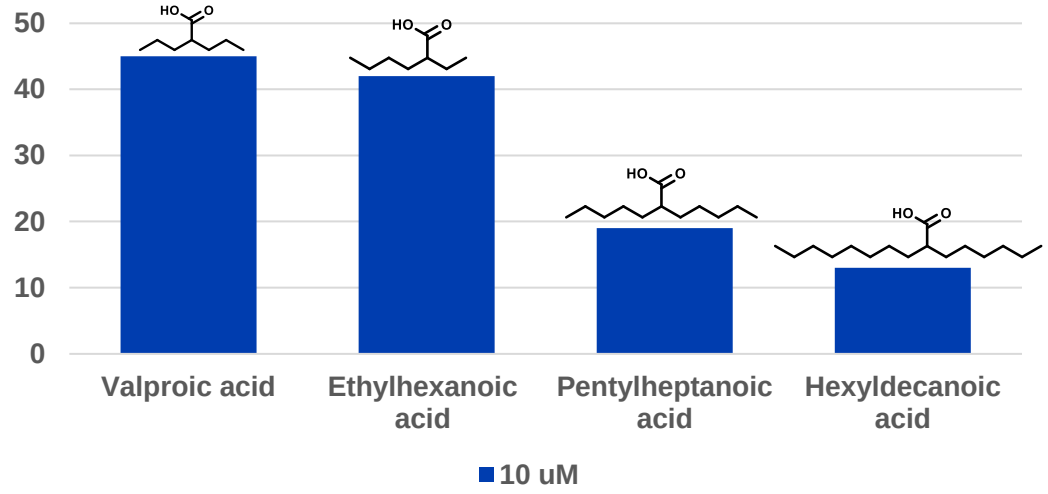


In Vitro ADME Data for Four Branched Carboxylic Acids

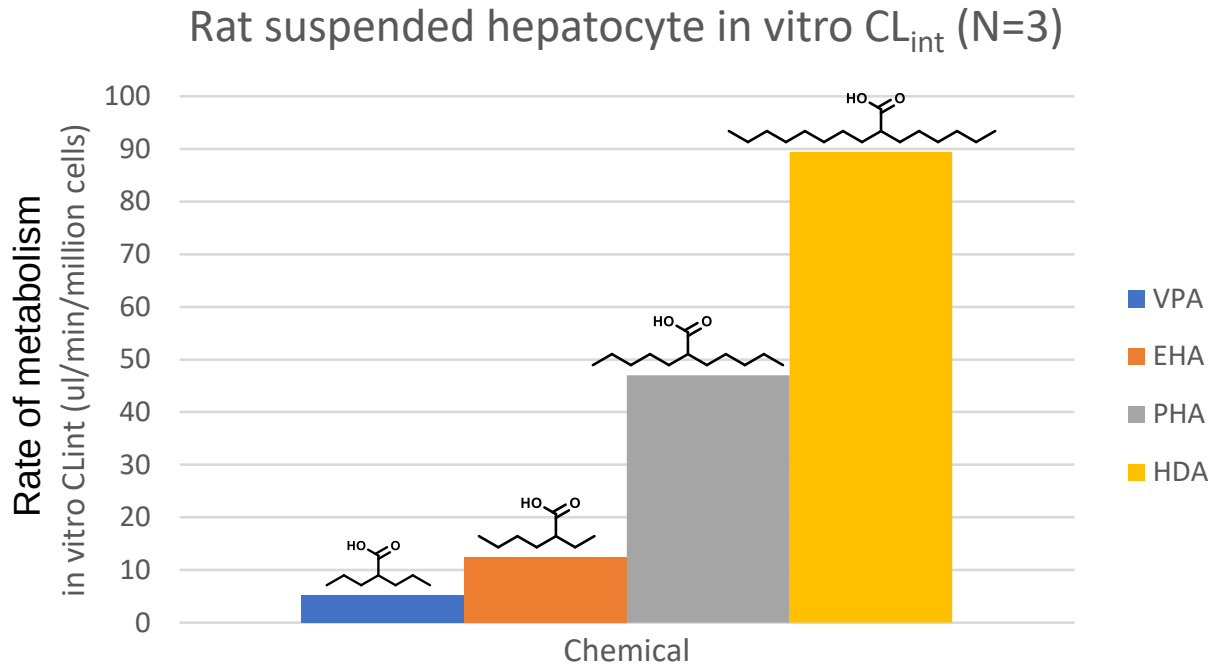
CACO-2 PERMEABILITY

Chemical	Permeability
Valproic Acid	High
2-Ethylhexanoic acid	High
2-Pentylheptanoic acid	High
2-Hexyldecanoic acid	Not quantifiable

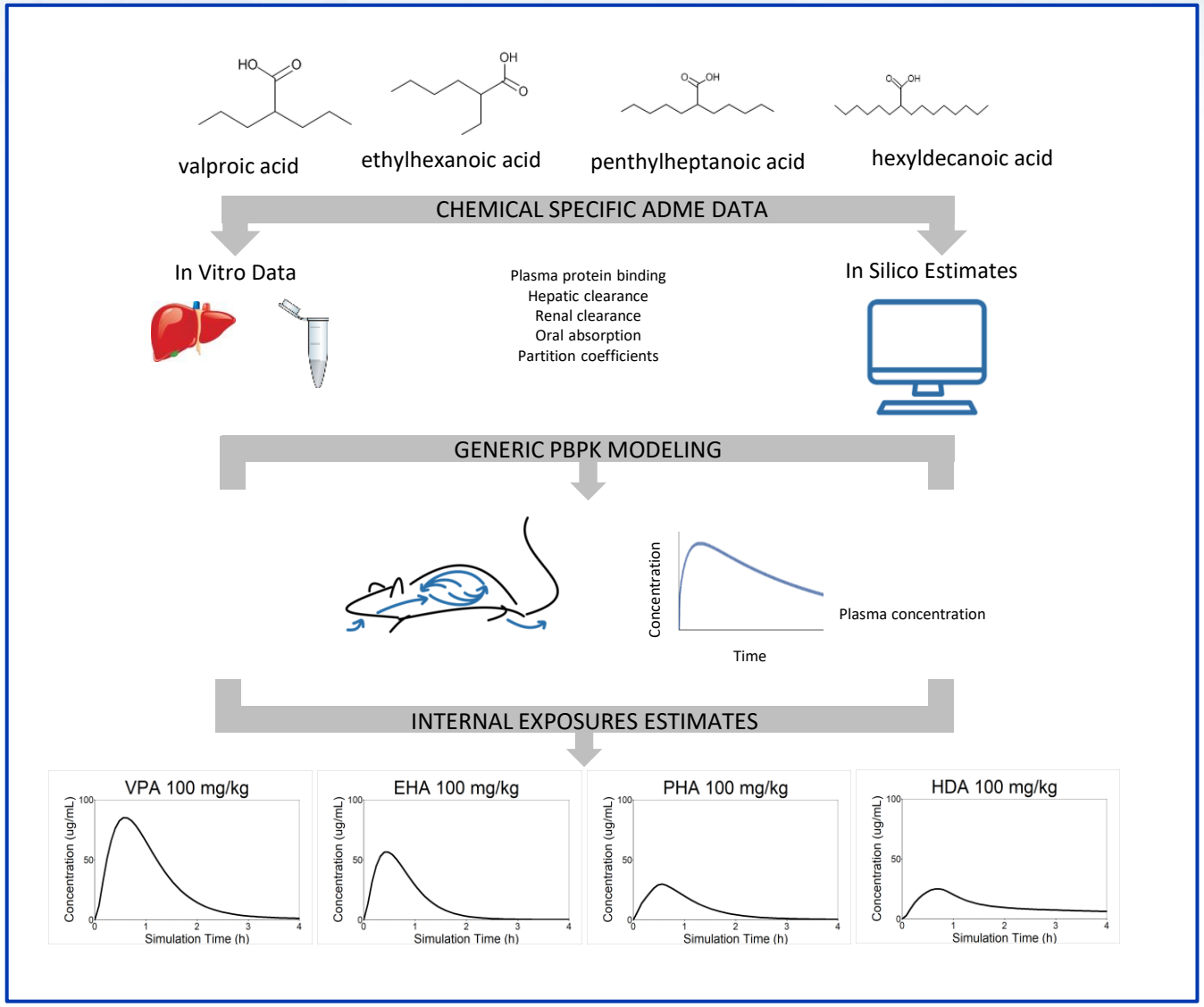
PLASMA PROTEIN BINDING



RAT HEPATOCYTE METABOLISM



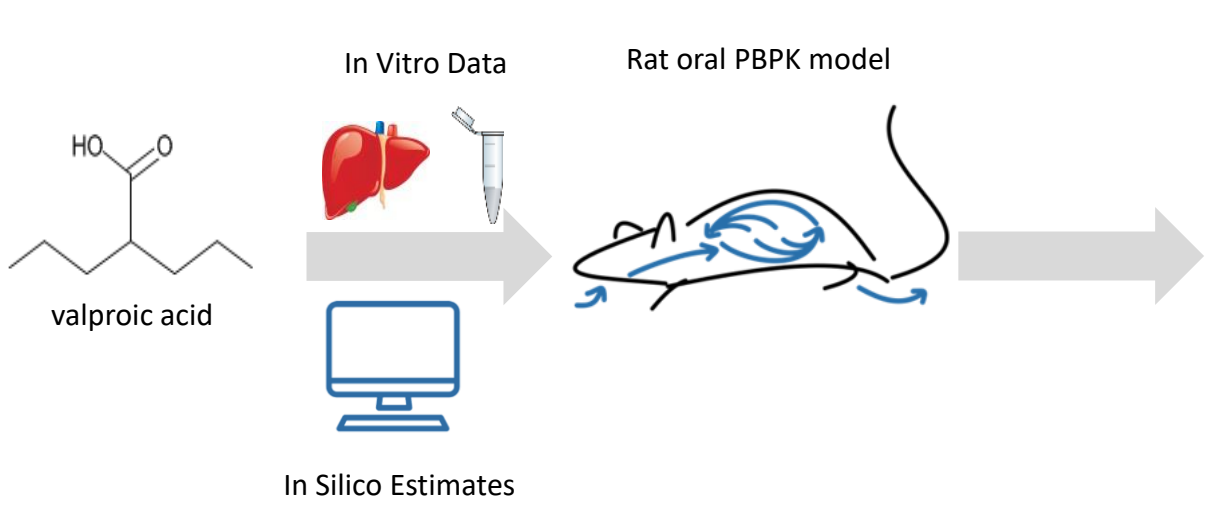
PBPK Modeling Approach

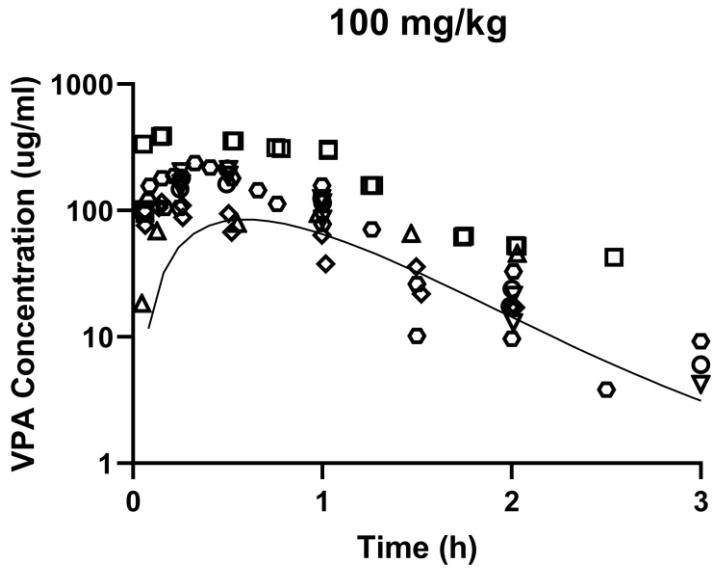


- Use ADME data and generic PBPK model to characterize TK for VPA, EHA, PHA and HDA.
  - How do the TK profiles compare?
  - What is the internal exposure when given the same oral dose?
  - What are oral equivalent doses?
- TK profiles can be used to evaluate TK (dis)similarity between the chemicals

PBPK Model Evaluation

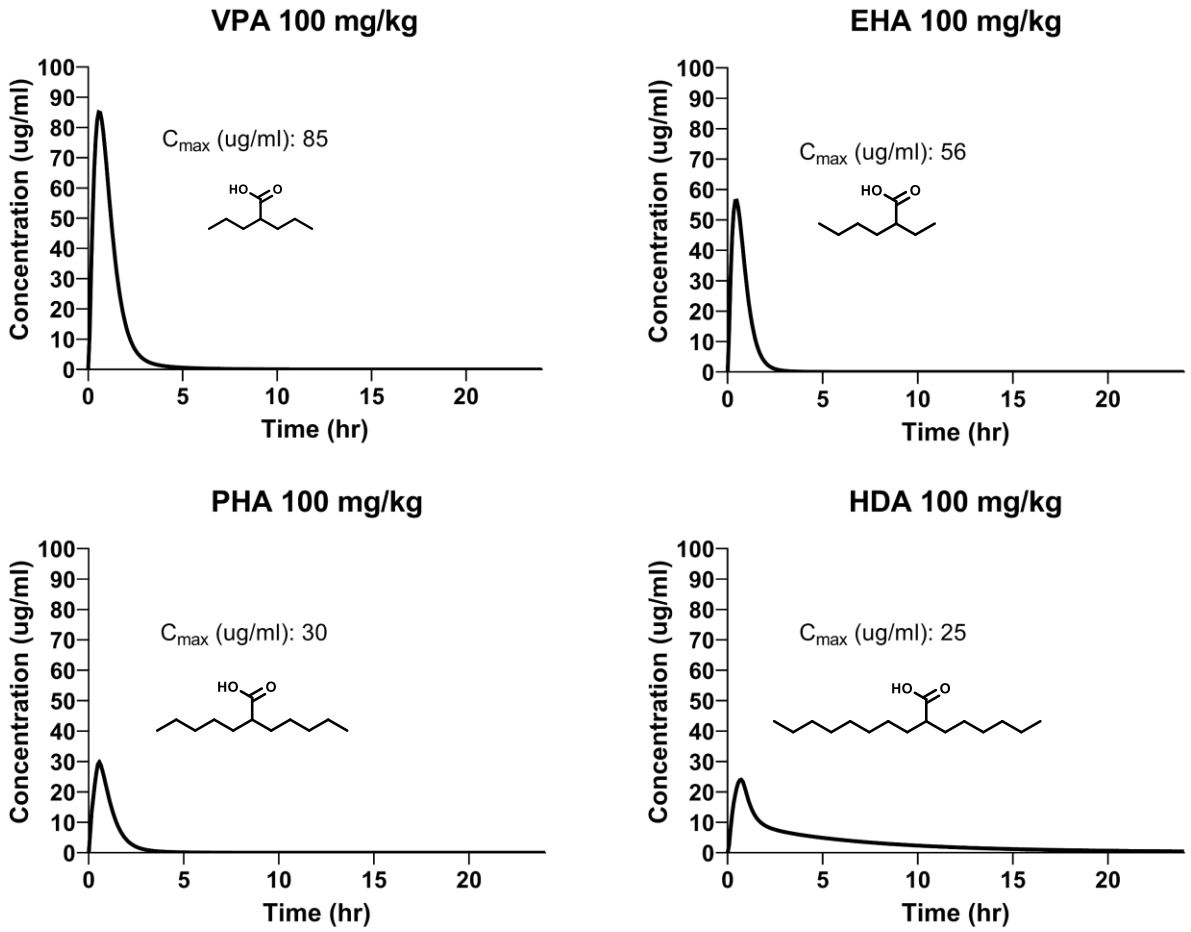
	Physiology	In vitro parameters	In silico parameters	PBPK simulation	In vivo data
VPA	Rat	oral permeability, plasma protein binding, hepatic metabolism	LogP, water solubility, pKa, blood:plasma ratio, tissue partition coefficients	existing simulations, relevant tox exposures	11 PK data sets
EHA					1 PK data set
PHA				relevant tox exposures	0 PK data sets
HDA					





Internal Exposure Estimates for a Common Oral Gavage Dose to Rats

The PBPK model for 4 different branched carboxylic acids was used to estimate the internal exposure following a 100 mg/kg oral gavage dose



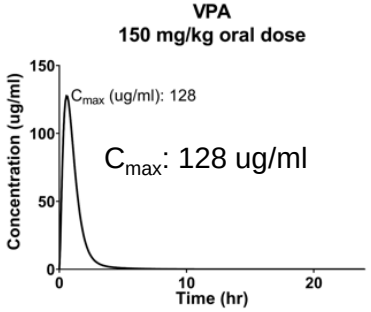
For a common dose, longer chain carboxylic acids result in lower internal exposures, due to faster hepatic metabolism

Thus, compared to shorter chain branched carboxylic acids, the longer chain carboxylic acids must be given a higher oral dose to results in equimolar internal exposures

Oral Equivalent Doses Based on Cmax

VPA rat developmental NOAEL = 150 mg/kg (Nau 1986)

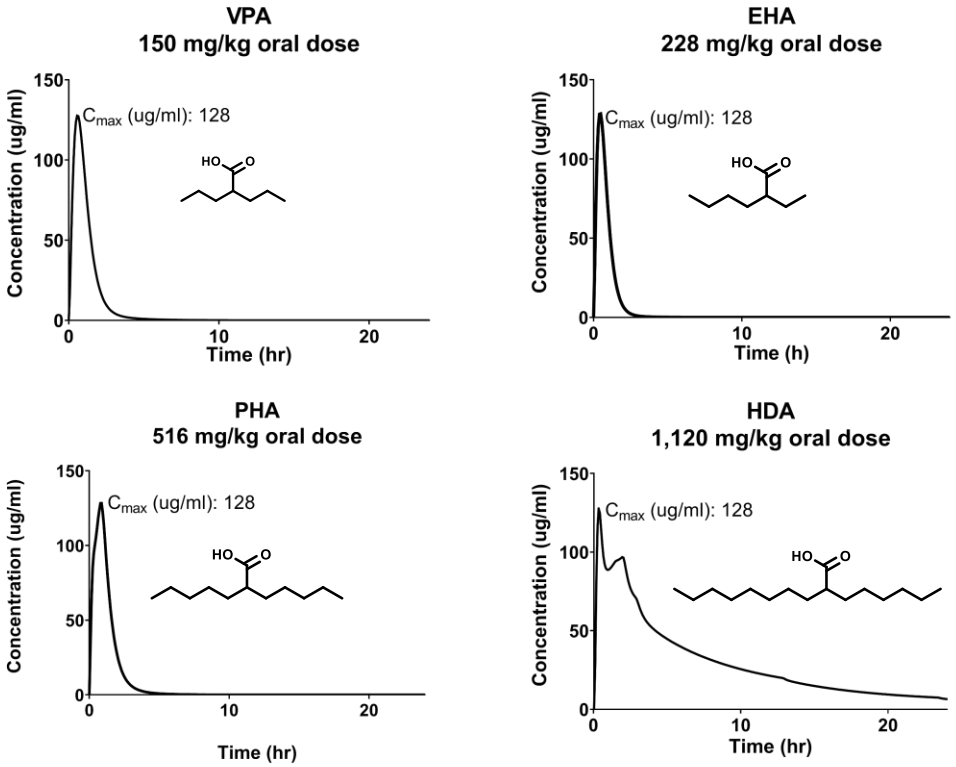
Simulate 150 mg/kg in VPA PBPK model



C_{max}: 128 ug/ml

For other branched carboxylic acids, determine what oral dose results in a plasma Cmax of 128 ug/ml

PBPK model simulations that result in a plasma Cmax of 128 ug/ml

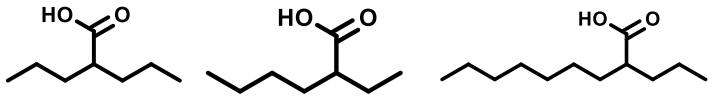


When targeting a plasma Cmax of 128 ug/ml (equivalent to VPA NOAEL), longer chain branched carboxylic acids have a higher oral equivalent dose compared to the shorter chain branched carboxylic acids

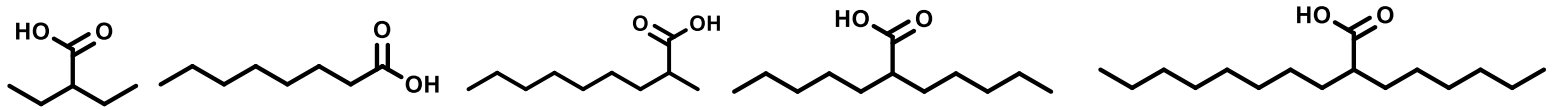
Summary

- **Toxicokinetics**:
  - As the branched chain length increases, the rate of hepatic metabolism increases: for a common oral dose, the longer chain chemicals result in lower internal exposures than VPA
  - PBPK modeling indicates that the longer chain branched carboxylic acids would require approximately 3-7x higher oral doses to results in a similar internal exposure as VPA

- **Toxicodynamics**:
  - SAR evaluation indicated that alkyl substituents of the carboxylic acid have a significant impact on developmental effects
  - As the branched chain length increases, the developmental effects of the chemicals appears to decrease
  - VPA, EHA, PNA have similar gene expression patterns as well as HDAC inhibition potential and could be considered as suitable analogs for developmental potential
  - EBA, OA, MNA, PHA, HDA have different gene expression pattern to VPA, EHA and PNA and did not show HDAC inhibition potential. They could be considered as very weak or non-developmental potential



VPA, EHA, PNA



EBA, OA, MNA, PHA, HDA

Establishing Confidence in the Current NAM Approach

The approach was hypothesis driven

Multiple independent lines of evidence (e.g., NAMs) converging to a common answer

- Transcriptomics, molecular docking, in vitro metabolism, PBPK modeling were able to separate the branched carboxylic acids into different groups

The chemistry and biology align across multiple chemicals

- Structure activity relationships could be identified
- Mechanistic understanding was achievable

Guidance exists on how to conduct a read-across assessment

- European Chemicals Agency (ECHA)'s read-across assessment framework (RAAF)
- Scholarly publications (e.g., Blackburn et al., 2011; Wu et al., 2010)

Methods exist to estimate confidence in read across assessment

- Methods to estimate confidence in the assessment (e.g., Blackburn and Stuard, 2014)

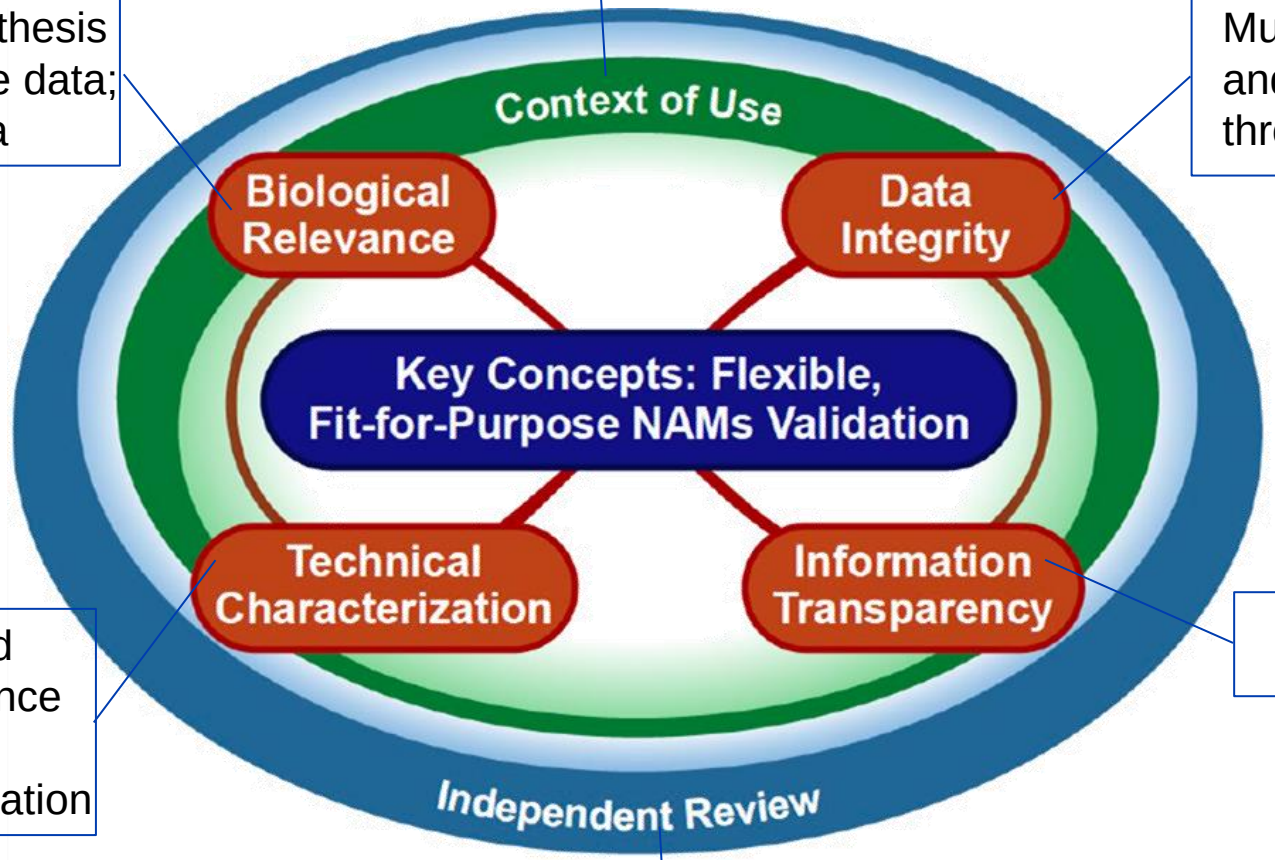
Mapping NAM Approach to ICCVAM 2023 Report

Figure 1 - Key concepts to consider during development and implementation of flexible, fit-for-purpose NAMS validation strategies

Determined via the case study problem statement and hypothesis driven approach

Mechanistic understanding; hypothesis driven approach; use of reference data; comparison to legacy animal data

Multiple independent checks of raw and processed data conducted throughout the process



State of the art approaches used with appropriate controls, reference chemicals, analytical methods, statistical models and documentation

All NAM data published

Approach reviewed and published in ToxSci

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Thank you for your attention and time