Development of a NAM-Based Classification Framework for Repeat Dose Chemical Toxicity: Insights from EPAA's NAM Designathon

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ABSTRACT / BACKGROUND

As part of EPAA's 'NAM Designathon 2023' (Worth et al. 2025) challenge for human toxicity we sought to identify a better classification system based on the NAMs framework of Ball et al. 2022.

This initiative focuses on categorizing chemicals into three levels of concern: low concern (without restriction), medium concern (requiring assessment to establish safe use levels), and high concern (restricted for some or all uses). The objective of this challenge was not to accurately classify, but to propose a hypothesis on how to categorise chemicals.

As the EPAA Designathon aims to encourage methods that do not use laboratory animals, the Staged Assessment group created a classification system following tiers 1-2 of the ECETOC framework (Ball et al., 2022) for hazard assessment and included an assessment of bioavailability to align with the Designathon classification scheme.

Methods: As part of the challenge, EPAA supplied a list of 150 chemicals, with equal distribution across three categories: low (L), medium (M), and high (H) concern. The specific category for each chemical and any related data were not disclosed.

We first searched for chemicals with adequate data sets in ToxCast (US EPA, 2024). We then evaluated conventional repeat dose toxicity data to determine their level of concern and selected and evaluated 12 chemicals (Table 4).

Assessment integrates evidence from:

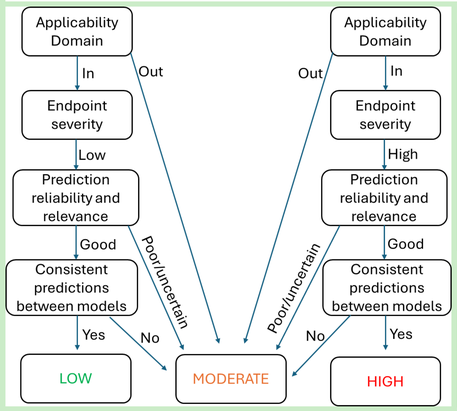
- *In silico* QSAR data, used to identify potential toxicity indicators and metabolites of concern. This information provided a preliminary assessment and helped review the adequacy of the *in vitro* database.
- *In vitro* PBPK modelling data on bioavailability, based on three PBK modelling tools (Table 1).
- *In vitro* data on bioactivity, based on dose-response (AC50 values).

Upon completing this assessment, the bioactivity and bioavailability categories for each selected chemical were placed in the EPAA Designathon matrix. A preliminary overall concern category was then determined, reviewed under the exclusion method (Framework Flowchart) and compared to existing *in vivo* conventional study data to evaluate the adequacy of the proposed chemical classification framework.

IN SILICO ASSESSMENT

For this analysis, several QSAR tools and models were run, including both expert rule and statistical based QSAR prediction methodologies.

- **Models** (Run on default settings): Derek & Meteor Nexus, OPERA, Leadscope Model Applier, ACD/Percepta, T.E.S.T., VEGA, QSAR Toolbox, TIMES Oasis and Dow in-house models.
- **Endpoints:** Carcinogenicity, mutagenicity, reproductive and developmental toxicity, endocrine activity, neurotoxicity, acute oral, some organ specific and general toxicity.



A high concern was assigned to chemicals that showed positive predictions across multiple severe endpoints, demonstrated consistency across different models, fell within the applicability domain, and were deemed relevant by experts. No low category was assigned based on *in silico* as a lack of alert is not the same as a negative one.

Figure 1: Decision Framework for in silico Output Level of Concern Assignment. Note: This is a rough guide, and expert knowledge sometimes overrode the framework.

BIOAVAILABILITY

Accumulation concern levels were evaluated with simulated 14-day plasma Cmax using a standard 0.1 mmol/kg dose with httk, PKSim and GastroPlus models.

Dose measurement were expressed in Molar/kg units over mg/kg to ensure consistency with activity assessment metrics.

Longer dosing periods of 28 days and 1 year did not have an observable effect on the Cmax for 800 chemicals from the ToxCast database.

Table 1: Summary of Bioavailability data from 3 models. High >500µM (Red); Mid 500- 50µM (Orange); Low <50µM (Green).

Consolidated model results (Cmax in µM for 0.1 mMol/Kg for 14 days)					
Substance	**Model inputs**	**httk**	**PK-Sim**	**GastroPlus**	**Overall**
Nitrobenzene	*in vitro*	44	3.7	5.1	**L**
Ouabain	*in silico*	13	0.013	18	**L**
1-chloro-4-nitrobenzene	*in silico*	194	21	11	**M**
Colchicine	*in vitro*	63	6.4	50	**M**
Phenol	*in vitro*	40	4.0	62	**M**
2,4,6-tri-tert-butylphenol	*in silico*	409	2.4	225	**M**
Carbaryl	*in vitro*	18	0.19	16	**L**
Chlorpropham	*in vitro*	36	0.9	25	**L**
Safrole	*in vitro*	232	40	117	**M**
Benzoic acid	*in silico*	1011	810	1097	**H**
4-nitrophenol	*in vitro*	86	8.4	125	**M**
Diethylphthalate	*in vitro*	29	1.9	23	**L**

BIOACTIVITY

Severity: Assays are categorized as high, medium or low. E.g. Estrogenic receptor assays are rated High; while PPAR binding is rated Low.

Potency: Dose-response curves are reviewed to ensure confidence in AC50 values. *In vitro* bioactivity data is primarily based on ToxCast, with the limitations it brings.

Potency Category	**<0.1 µM**	**0.1-10 µM**	**>10 µM**
	H	M	L

Figure 2: Potency categories determined by AC50

Chemical	**Colchicine**		Result:	H
	POT H	POT M	POT L	POT NO HIT
SEV H	**27**	**4**	**23**	**435**
SEV M	**18**	**5**	**3**	
SEV L	**74**	**56**	**7**	

Table 2: Original bioactivity matrix for Colchicine (High concern).

OVERALL ASSESSMENT

Bioavailability and Bioactivity outcomes are placed first into the EPAA Matrix and preliminary category is then reviewed using a weight of evidence approach.

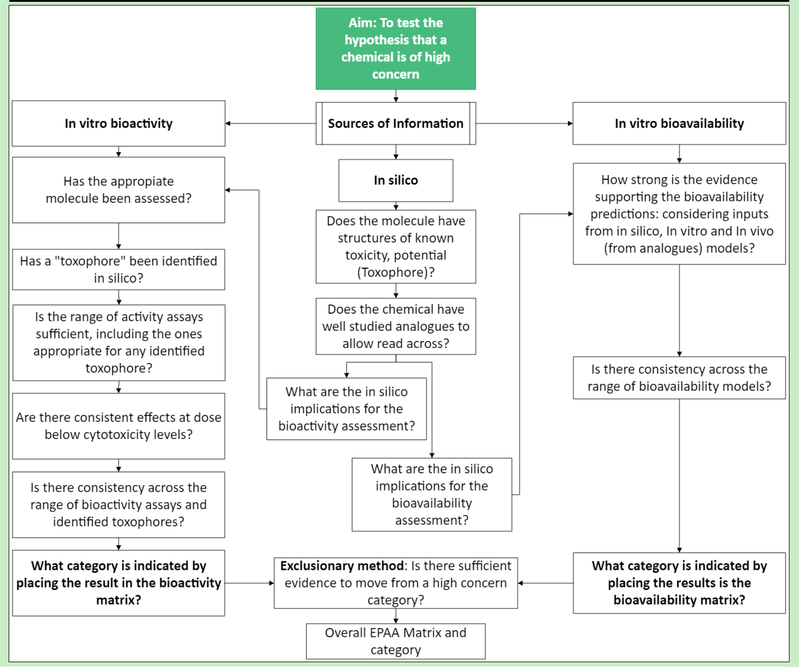
Chemical: **Safrole**	Activity H	Activity M	Activity L
Availability H			
Availability M			X
Availability L			

Table 3: Examples of the overall concern matrix for Safrole (Low concern).

Figure 3: Examples of one of the weight of evidence question for Safrole (Low concern)

Question	Answer	Conclusion
Is there sufficient evidence to move from High concern category?	Some indications of concern from *in silico*; No consistent indications from Bioactivity; Mid Bioavailability; Matrix indicates Low level of concern	**Low**

FRAMEWORK FLOWCHART



SUMMARY

Twelve chemicals have been assessed through the framework and compared with the conventional category (*in vivo*) derived from open literature review considering potency and severity in repeat dose studies (not using STOT RE criteria specifically).

The framework initially had a trend towards classifying chemicals in lower categories of concern. We have explored how adjusting the cut off values changed the distribution of chemicals.

Nevertheless, our evaluation shows that the EPAA Designathon matrix can be calibrated to meet protection standards but the level of confidence in exclusively NAM-based classification is heavily dependent on an adequate range of validated NAM procedures.

There are still areas to be explored, especially regarding the range of assays used, biological coverage of the assays, metabolites of concern, the definition of framework categorization, and how such a matrix would fit pragmatically into a tiered approach, including targeted *in vivo* studies.

Table 4: Initial outcome from the assessment framework using original categorisation criteria for in vitro bioactivity and in vitro bioavailability and category derived from conventional data.

Chemical	*In silico*	Bioactivity	Bioavailability	Overall NAM Category	Conventional Category (*In vivo*)
Nitrobenzene	**H**	**L**	**L**	**L**	**H**
Ouabain	**H**	**H***	**L**	**M**	**H**
1-chloro-4-nitrobenzene	**H**	**H***	**M**	**H**	**H**
Colchicine	**H**	**H**	**M**	**H**	**H**
Phenol	**H**	**M**	**M**	**M**	**M**
Tri Tertiary Phenol	**M**	**M**	**M**	**M**	**M**
Carbaryl	**H**	**M**	**L**	**L**	**M**
Chlorpropham	**M**	**M**	**L**	**L**	**M**
Safrole	**H**	**L**	**M**	**L**	**L**
Benzoic Acid	**M**	**M**	**H**	**M**	**L**
4-nitrophenol	**M**	**L**	**M**	**L**	**L**
Diethylphthalate	**M**	**M**	**L**	**L**	**L**

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