**External Validation of STopTox as an Alternative to Animal Testing for Toxicological Assessments**

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**Competing Interests**

AT and ENM are co-founders of Predictive, LLC, which develops novel alternative methodologies and software for toxicity prediction. All the other authors declare no conflicts.

**Data Collection Protocol**

**Integrated Chemical Environment (ICE) Overview:**

1. ICE contains highly curated and verified datasets for various specific toxicity endpoints.
2. The datasets can be downloaded by accessing the link:
   1. (<https://ice.ntp.niehs.nih.gov/DATASETDESCRIPTION>, accessed June 2024)
3. Datasets of interest can be directly downloaded as xlsx files from their “datasets tab.” All original and complete endpoint-specific datasets from ICE are available in the folder: “ICE Datasets.”
   1. File descriptions:
      1. Oral Toxicity – 9110 chemicals for 4 endpoints
      2. Acute Dermal Toxicity – 275 chemicals for 3 endpoints
      3. Acute Inhalation Toxicity – 1781 chemicals for 3 endpoints.
      4. Eye Irritation Toxicity – 454 chemicals for 7 endpoints
      5. Skin irritation/Corrosion – 564 chemicals for 16 endpoints
      6. Skin Sensitization – 1956 chemicals for 26 endpoints.
4. All datasets were filtered to include only compounds tested *in vivo* using the same OECD Test Guidelines the original STopTox was designed to predict. Specifically,
   1. Eye Irritation/Corrosion
      1. OECD TG 405
   2. Skin Irritation/Corrosion
      1. OECD TG 404
   3. Skin Sensitization
      1. OECD TG 429
   4. Acute Oral
      1. OECD TG 401, 420, 423, 425
   5. Acute Dermal
      1. OECD TG 402
   6. Acute Inhalation
      1. OECD TG 403

**Data Collection Protocol:**

The search was executed independently for the endpoints available in the SToptox web platform. The endpoints of interest were three acute systemic (acute oral, acute dermal, and acute inhalation toxicity) and three topical (eye irritation/corrosion, skin irritation/corrosion, and skin sensitization).

1. First, we navigated to ICE’s “Search” tool.
2. The scope of the search used was “Intersection.”
3. For “Chemical Input,” all 26 available chemical quick lists were selected.
4. For “Datasets,” all *in vivo* assays were included for each endpoint. Using skin sensitization as an example:
   1. Navigate to the sensitization tab and filter for “Murine Local Lymph Node Assay (LLNA).”
5. Select “Run.”
   1. In this example, 886 records were found for 322 unique substances.
6. The file was exported, and the CASRN of the chemicals in ICE were cross-referenced with those in STopTox’s training set.
   1. Duplicate chemicals or compounds missing SMILES were removed.
   2. The remaining compounds were analyzed, and their hazard category predictions were performed using the STopTox web platform (<https://stoptox.mml.unc.edu/>, accessed February 2025). The predictions were then compared to the original *in vivo* outcome for external validation.
7. Steps 1-6 were repeated for all endpoints available in the SToptox Web platform.

***Endpoint-specific “dataset” filters used:***

1. Eye Irritation/Corrosion: “Rabbit Draize Skin Irritation/Corrosion Test”
2. Skin Irritation/Corrosion: “Rabbit Draize Skin Irritation/Corrosion Test”
3. Skin Sensitization: “Murine Local Lymph Node Assay (LLNA)”
4. Acute Oral: “Rat Acute Oral Toxicity”
5. Acute Dermal: “Rat Acute Dermal Toxicity”
6. Acute Inhalation: “Rat Acute Inhalation Toxicity”

***Model Benchmarking:***

1. OECD QSAR Toolbox (<https://qsartoolbox.org/>, accessed February 2025, Version 4.7.1) was downloaded.
2. SMILES lists were created using the same compounds that were outside the STopTox training set, available in the GitHub.
3. The SMILES list for each endpoint was loaded into the standalone platform and predicted using the QSAR Toolbox models.
4. Models information:
   1. Eye Irritation:
      1. Test organisms (species): Rabbit
      2. Endpoint: Primary Irritation Index
      3. Type of method: in Vivo
      4. Prediction approach: QSAR
      5. SAR/QSAR: Severe Skin Irritation in Rabbit - Danish QSAR DB CASE Ultra model"
   2. Skin Irritation/Corrosion:
      1. Test organisms (species): Rabbit
      2. Endpoint: Primary Irritation Index
      3. Type of method: in Vivo
      4. Prediction approach: QSAR
      5. SAR/QSAR: Severe Skin Irritation in Rabbit - Danish QSAR DB battery model
   3. Skin Sensitization
      1. Endpoint: EC3 <OR> S M W N <OR> Skin sensitisation
      2. Type of method: in Vivo
      3. Assay: GPMT <OR> LLNA
      4. Prediction approach:
      5. SAR/QSAR: Skin sensitization for DASS
   4. Acute oral:
      1. Test organisms (species): Rat
      2. Endpoint: LD50
      3. Duration: 48 h
      4. Prediction approach: QSAR
      5. SAR/QSAR: Acute toxicity in Rat, Oral - Danish QSAR DB ACDLabs model
5. Predictions were compared to the original calls assigned and evaluated using CCR, SE, SP, PPV, and NPV formulas.