Estimation of Human Equivalent Doses for Flavoring Compounds in ENDS Aerosols from *In Vivo* and *In Vitro* Points of Departure

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BACKGROUND AND PURPOSE

Electronic nicotine delivery systems (ENDS) products are not risk-free; however, evidence suggests reduced harm relative to combustible cigarettes. Thus, it is critical to assess individual constituents in ENDS aerosols as part of the product health risk assessment. Traditional risk assessment usually utilizes a point of departure (POD) derived from *in vivo* testing. The use of new approach methodologies (NAMs), such as *in vitro* and *in silico* methods, may offer novel approaches for chemical risk assessment, especially for chemicals with limited *in vivo* toxicity data.

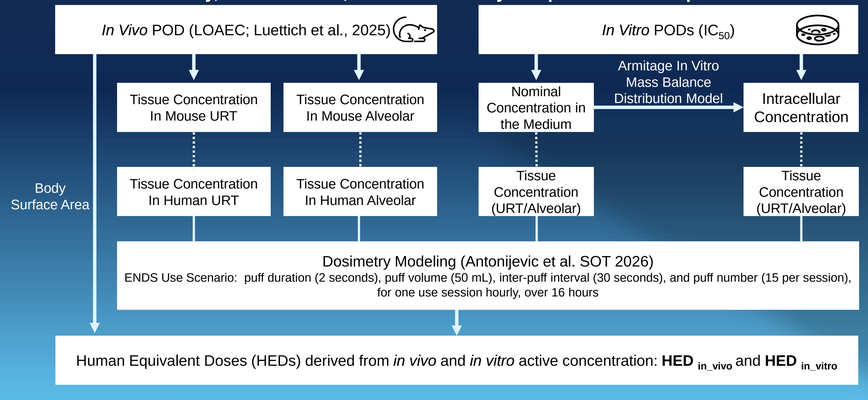
In this case study, we determined PODs based on an *in vivo* chronic inhalation study in the A/J mouse model (Luettich et al., 2025) in conjunction with the *in vitro* ToxTracker® assay for selected flavoring compounds present in the assessed ENDS aerosols. Subsequently, we calculated corresponding human equivalent doses (HEDs) using a physiologically based pharmacokinetic (PBPK) model that was specifically developed for ENDS aerosols (presented separately: Antonijevic et al. SOT 2026).

METHODS

PODs obtained from the *in vivo* study represent the estimated tissue concentrations of individual flavors in the upper respiratory tract (URT) and in the alveolar region (estimated using the mouse PBPK model), which were derived from the lowest-observed-adverse-effect concentration (LOAEC, total flavor load of 4.6% wt), simulating a 6-hours/day, 5-days/week exposure schedule as reported (for more details about the chronic inhalation study, see Luettich et al., 2025).

The PODs obtained from the ToxTracker assay included nominal half-maximal inhibitory concentration (IC_{50}) values for cytotoxicity (the nominal maximum tested concentration was utilized when a cytotoxicity IC_{50} was not determined), as well as the estimated intracellular concentration derived using the Armitage *in vitro* mass balance distribution model (Armitage et al., 2014).

These PODs were then used to estimate HEDs using the human PBPK model with puff topography and use scenario parameters consisting of the puff duration (2 seconds), puff volume (50 mL), inter-puff interval (30 seconds), and puff number (15 per session), for one use session hourly, over 16 hours, to mimic the daily use profile of ENDS products.



RESULTS

In Vitro PODs: Estimated Intracellular Concentrations vs. Nominal Medium Concentrations of Individual Flavors

- **When the *in vitro* mass balance distribution is considered (Fig. 1B), the estimated intracellular concentration is up to 215 times higher than the nominal medium concentration, except for isobutyraldehyde (CAS: 78-84-2, 69% of the nominal medium concentration) (Fig. 1A).**
- **Overall, for *in vitro*, estimated intracellular concentrations ≥ nominal medium concentrations**

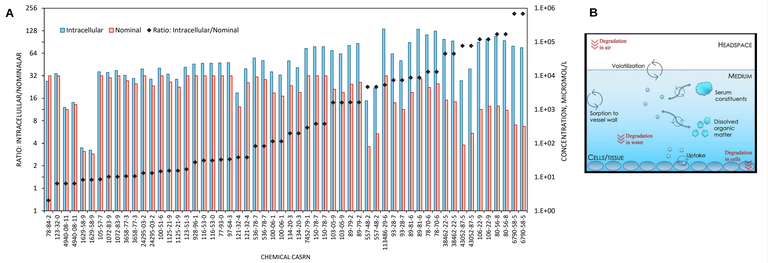


Figure 1. A. Nominal medium concentration and corresponding estimated intracellular concentration of individual flavor at IC_{50} (or Maximum Tested Concentration), µmol/L. All compounds were tested with and without S9 enzyme *in vitro*. For five compounds, the IC_{50} could not be determined within the tested range. Therefore, the maximum concentration (10 mM) was applied and a single value was reported. **B**. Schematic representation of the chemical biokinetics *in vitro* environment for the mass balance distribution model (Bloch et al., 2022).

In Vivo PODs: Estimated Tissue Concentrations of Individual Flavors in the URT vs. Alveolar Region

- **For *in vivo* PODs, alveolar and URT were selected as the target tissues based on the primary endpoints (lung tumorigenesis) and histopathological findings in the URT region of the chronic mouse inhalation study (Luettich et al., 2025).**
- **POD_{URT} > $POD_{Alveolar}$: URT (purple dots in Fig. 2) exhibited 2-5 orders of magnitude higher PODs than the alveolar region (green dots in Fig. 2).**
- **POD $_{in\_vitro}$ > POD $_{in\_vivo}$: Both the *in vitro* nominal medium concentrations and the estimated intracellular concentrations are higher than the estimated *in vivo* tissue concentrations in the mouse URT or alveolar region (Fig. 2).**

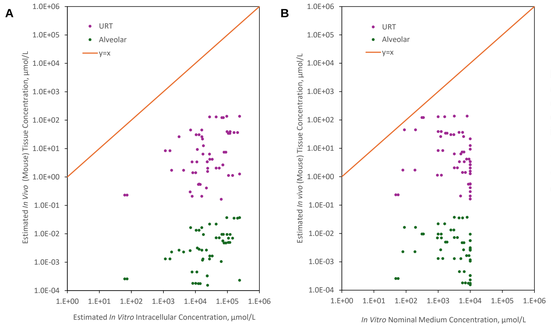


Figure 2 A) Comparison of estimated *in vitro* intracellular concentrations (A) or *in vitro* nominal medium concentrations (B) and the estimated in vivo tissue concentrations in mice.

The maximum tested concentration *in vitro* was 10 mmol/L (nominal concentration in the medium).

Human Equivalent Doses Derived from *In Vivo* PODs

- **Given the positive correlation between PODs and HEDs, the HEDs derived from *in vivo* PODs represents the conservative case because POD $_{in\_vitro}$ > POD $_{in\_vivo}$. HEDs derived from *in vivo* PODs are presented in Fig. 3.**
- **The HEDs converted from the mouse daily dose using body surface area scaling (blue zone in Fig. 3) were the most conservative, although this approach does not account for chemical-specific properties.**
- **HED_{URT} > $HED_{Alveolar}$: HED_{URT} is 1–300-fold greater than $HED_{Alveolar}$, which indicates that the alveolar region warrants closer evaluation in human risk assessment, although more symptoms were observed in the *in vivo* mouse study (Luettich et al., 2025).**

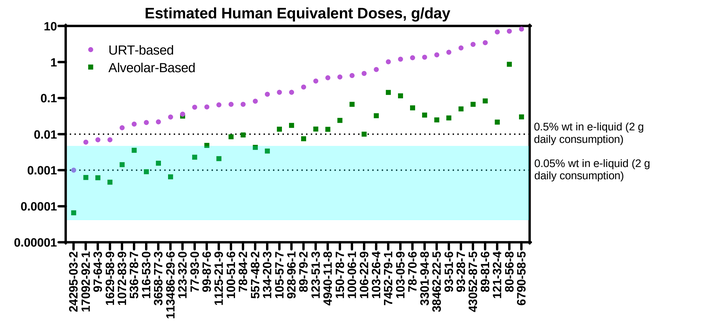


Figure 3. Estimated HEDs based upon tissue concentration in URT and alveolar, ranked by URT-specific results. The light blue zone represents the range of calculated HEDs derived from mouse LOAEC. Mouse daily dose (mg/kg/day) = LOAEC x minute ventilation x daily exposure duration x (5/7) / mouse body weight. Estimated human equivalent dose (mg/day) = Mouse Dose (mg/kg) x Human Body Weight / 12.3 (Nair and Jacob, 2016), assuming the human body weight of 60 kg.

CONCLUSIONS

- **This study explored multiple options for selecting PODs for estimating HEDs and demonstrated the potential utility of NAMs in risk assessment. The results showed that POD $_{in\_vitro}$ > POD $_{in\_vivo\_URT}$ > POD $_{in\_vivo\_Alveolar}$, consequently, HED $_{in\_vitro}$ > HED $_{in\_vivo\_URT}$ > HED $_{in\_vivo\_Alveolar}$. The body surface area scaling approach for the conversion from mice to humans provides most protection, while the tissue specific approach accounts for chemical specific properties and provides insights into site-specific toxicity in the mouse study, as well as their correlation in humans.**
- **The introduction of *in vitro* mass balance model refines the *in vitro-in vivo* extrapolation; however, appropriate validation and verification are needed for its application in quantitative risk assessment. As a proof of concept, the IC_{50} values from the cytotoxicity data of the ToxTracker assay were used. Since the assay is a genotoxicity assay, the genotoxicity endpoints would be more appropriate for the next step and may lead to lower *in vitro* PODs.**
- **Variability of puff topography and use scenario should also be considered to ensure that the risk assessment is protective of users.**

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