Post-Meeting Peer Review Summary Report

External Peer Review of a Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolite Clearance (U.S. EPA, 2020)

December 17, 2020

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I. BACKGROUND

The U.S. EPA CPHEA is currently evaluating a Request for Reconsideration (RFR), specifically to consider use of a PBPK model in a potential IRIS reassessment of chloroprene, CAS No. 126-99-8. The 2020 report by Ramboll entitled "Incorporation of In Vitro Metabolism Data in a Physiologically Based Pharmacokinetic (PBPK) Model for Chloroprene," describes new analyses and corresponding revision of a PBPK model for chloroprene, specifically using metabolic parameters derived from in vitro studies. Initial quality assurance evaluation by EPA of the previously published versions of the model (Yang et al., 2012) (for dosimetry in mice, rats and humans) identified issues which the additional data and analyses described in the report seek to address. These unpublished results have not been subjected to a formal peer review process. Such a peer review process is important in establishing the appropriateness, validity, and applicability of the revised PBPK model, in particular considering that no *in vivo* PK data are available to validate or calibrate model predictions in humans. Further, the model predicts the rate of metabolism of chloroprene to presumed toxic metabolites, but not the tissue concentrations of these metabolites.

Typically, metabolism and clearance of chemical entities in humans is assumed to be slower than in smaller mammals, with scaling by $BW^{0.75}$ used to predict the relative clearance in the absence of specific data. However in vitro data have been previously reported by the oxidative metabolite (1-chloroethenyl)oxirane (1-CEO) (Himmelstein et al., 2004). Further, while the report suggests that the toxic metabolite(s) may be completely consumed in the metabolizing tissues (liver and lung), this is contradicted by the induction of tumors in distal sites, in particular mammary tissue, which suggests that clearance by blood perfusion is a factor. Therefore a supplemental analysis (U.S. EPA, 2020) has also been developed to extrapolate the in vitro clearance of 1-CEO by the observed pathways to *in vivo*, to make the various rates comparable to each other and to clearance by blood perfusion, and to ultimately obtain relative total clearance rates in human and rodent liver and lung, and systemic distribution rates, that can be used to evaluate relative risk and whole-body dosimetry.

For this peer review, nine (9) experts with experience and expertise in one or more of the following areas: physiologically based pharmacokinetic (PBPK) modeling, statistics, mass transport fluid dynamics and molecular diffusion, and metabolic rates *in vitro* were selected as peer reviewers to answer 19 charge questions and to evaluate and provide written comments on a report on physiologically based pharmacokinetic (PBPK) modeling for chloroprene (Ramboll, 2020) and a supplemental analysis of metabolite clearance (U.S. EPA, 2020).

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II. CHARGE TO REVIEWERS

Estimation of Mass Transfer Resistance in the *In Vitro* Metabolism Experiments

A model of the *in vitro* incubation system was used to estimate the metabolic parameters from the *in vitro* data. This model is based on certain assumptions and physical parameters, such as the volume of the *in vitro* incubation vials and volumes of air and liquid media in the vials.

The model of the *in vitro* system initially used for the analysis of the *in vitro* experiments to estimate the corresponding metabolic parameters (Yang et al., 2012; Himmelstein et al., 2004) assumed that the chloroprene in the air and liquid (incubation medium) phases was at equilibrium at all times after the start of the experiment; i.e., concentration in the medium was set equal to the concentration in the air times the equilibrium partition coefficient (CM = CA*P). At EPA's suggestion, the model was changed to explicitly describe separate air and liquid media compartments, with a mass-transfer coefficient (Kgl) limiting the rate of distribution between them, as described by Kreuzer et al. (1991) and others.

1. Please evaluate the validity and uncertainties of these two approaches to estimation of the kinetics in the vitro system and therefore in the estimation of metabolic parameters:

a) treating the air and liquid phases as always being at equilibrium (original model); or

b) treating the air and liquid phases as distinct compartments with the rate of transfer limited and determined by a mass-transfer constant (Kgl).

Experiments were conducted to determine the Kgl for the *in vitro* system, however the value of Kgl obtained from those experiments is not consistent with some of the observed metabolic data (Ramboll (2020) Supplemental Material B), and Kgl would need to be at least 8 times higher to obtain results consistent with those data and to obtain a Km consistent with metabolic parameters reported for other VOCs. This inconsistency may exist because the experiments conducted to estimate Kgl used an incubator mixing speed of 60 rpm while the experiments of Himmelstein et al. (2004) and Yang et al. (2012) used 500 rpm. Also, the experiments to measure Kgl were performed without microsomal protein and the report hypothesizes that the presence of microsomal protein (1–3 mg/mL) in the metabolic experiments could increase mass transfer. It is noted that the mean value of the partition coefficient, P, estimated from the Kgl data in the absence of microsomal protein was 0.48 (Ramboll (2020) Supplemental Material B) while that reported by Himmelstein et al. (2001) for chloroprene equilibration with media containing heat-inactive protein was 0.69, 44% higher. To be clear, simulations of the metabolically active experiments used to estimate the metabolic parameters used P = 0.69, so have accounted for the difference in the equilibrium partition coefficient, but are still not consistent with the highest activity data when using the value of Kgl obtained from the 60 rpm data.

2. Please comment on the likelihood that either the presence of microsomal protein (1–3 mg/mL) or that the higher mixing speed used in the metabolic experiments (500 rpm) vs. the mass transfer experiments (60 rpm) would increase the rate of chloroprene mass transfer between the air and liquid phases in the in vitro system by a factor of 8 or greater, relative to the rate observed in the mass-transfer experiments.

An analysis provided in Supplemental Material B of Ramboll (2020) demonstrates that estimates of the metabolic parameter Km depend strongly on the value of Kgl. Two approaches were used to estimate the value of Kgl:

a) the measured Kgl was increased by (500/60), the ratio of mixing speeds in the metabolic experiments vs. Kgl experiments, yielding Kgl = 0.2 L/h; and

b) a Bayesian analysis used to estimate Kgl from the metabolic data yielded a mean Kgl = 0.22 L/h.

3. Given the two-compartment in vitro model structure, please comment on the two approaches for estimating Kgl and whether the value obtained is sufficiently reliable to support valid estimates of metabolic parameters and assess the uncertainties in those estimates.

Estimation of Metabolic Parameters from *In Vitro* Metabolism Experiments

The following questions address the robustness of the available metabolic data for application in the model. The questions are written with the assumption that the choice of Kgl is appropriate. Using this value of Kgl while evaluating the remaining analysis of *in vitro* metabolic data as described in Supplemental Material B of Ramboll (2020) results in parameter values listed in Table S-3 of Supplemental Material A of Ramboll (2020). For the chloroprene *in vitro* experiments, the human liver microsome samples were obtained from a pool of 15 donors while the human lung microsomes were obtained from a pool of 5 individuals (Himmelstein et al., 2004). For the 7-ethoxycoumarin *in vitro* experiments used to estimate the relative lung:liver metabolic activity, represented by the parameter A1, tissue samples were not pooled; activity was measured in liver microsomes obtained from 10 donors while the human lung activity was measured using microsomes from 12 donors (Lorenz et al., 1984). Other information on the specific microsomal samples, preparation methods and *in vitro* experiments are in Lorenz et al. (1984), Himmelstein et al. (2004) and Yang et al. (2012).

4. Please comment on the pool sizes for the human microsomes used to estimate chloroprene metabolic rates in vitro, and the number of tissue samples (donors) evaluated for 7-ethoxycoumarin activity, for the estimation of average metabolic activity for human adults.

5. Discuss the appropriateness of the data used and the statistical modeling approach with regard to representing average (or mean) adult human, mouse, and rat metabolic parameters. In particular, please comment on whether a sufficient number of microsomal samples (incubations) were analyzed to represent the average values and to characterize metabolic variation across species, sexes, and tissues.

6. Considering the experimental and computational methods, please comment on the potential order of magnitude and direction of bias of the quantitative uncertainties in the estimated in vitro metabolic rates that may be related to these factors, collectively.

Additional discussion on the estimation of lung metabolic parameters in rats and humans is provided in Supplemental Material C of Ramboll (2020) in a section entitled "IVIVE for first order metabolic clearance in rat and human lung." However, the metabolic rate parameter values for the human lung were ultimately selected as described in the main report in a subsection

entitled "Estimation of chloroprene metabolism in the human lung" because the *in vitro* chloroprene experiments with human lung microsomes showed minimal metabolism.

7. Please comment on the use of the relative 7-ethoxycoumarin activity in human lung vs. liver tissue to predict the average rate of chloroprene oxidative metabolism in the human lung.

8. Please comment on the possible use of a parallel approach, based on the relative activity of 7-ethoxycoumarin or another marker CYP2E1 substrate, to estimate the rate of metabolism in the rat lung and the human kidney.

IVIVE Calculations for Chloroprene

IVIVE extrapolation is summarized in the *Model Parameters* section of the Ramboll (2020) report, with details on scaling factors in Supplemental Material C of Ramboll (2020) and results in Table S-4 of Supplemental Material A. (Calculations are provided in an Excel workbook, Supplemental Material D of Ramboll (2020). The U.S. EPA performed a quality-assurance evaluation of the workbook to assure the calculations are as described in the report text and tables.) Wood et al. (2017) evaluated the ability of IVIVE to predict clearance for oral dosing of a number of pharmaceutical compounds with data in rats and humans and reported a systematic bias towards under-prediction with increasing clearance. However, the Wood et al. (2017) results may not be relevant to chloroprene because of differences in the route of exposure, chemical properties, metabolizing enzymes, and rate-determining processes for the set of compounds analyzed. In particular, Wood et al. (2017) evaluated IVIVE for oral dosing of drugs, but not for the inhalation of volatile compounds like chloroprene. While, IVIVE for oral exposure to drugs may be more difficult and is subject to additional sources of uncertainty compared to inhalation of volatile compounds due to variability in intestinal absorption and metabolism (Yoon et al., 2012; Liao et al., 2007), analysis of Wood et al. (2017) specifically focuses on predictions of hepatic *clearance* of drugs, for which metabolism in the liver is a significant component. Thus, the analysis of Wood et al. (2017) may be considered relevant to chloroprene since it addresses the ability to predict metabolic clearance via IVIVE, not oral absorption. The U.S. EPA is not aware of a systematic evaluation of IVIVE accuracy like that of Wood et al. (2017) but focused on volatile organic (chlorinated) compounds like chloroprene for the inhalation route.

9. Please evaluate the choices of extrapolation factors and formulas used for the IVIVE calculations. Please discuss the soundness of the metabolic parameters in Table S-4 as estimates for average adult female and male mice and rats, and average adult humans (combined sexes).

PBPK Model Structure, Physiological Parameters, and Partition Coefficients

10. Please discuss the appropriateness of the PBPK model structure presented by* Ramboll (2020) *for estimation inhalation dosimetry in an EPA Toxicological Review of chloroprene. Please consider in particular the model structure for the kidney, liver, and lung; i.e., tissues in which chloroprene metabolism is predicted by the model.

Arterial blood concentrations in B6C3F1 mice after inhalation exposures to chloroprene are shown in Figure 3 of Ramboll (2020). In particular, it is noted that when chloroprene exposure was increased 2.5-fold from 13 to 32 ppm, the mean arterial concentration increased less than 1.5-fold. Further, the mean arterial concentrations from 90 ppm exposure, which is seven (7) times higher than 13 ppm, are only about 4 times higher than those measured at 13 ppm. These data might indicate that some process not included in the PBPK model may have reduced chloroprene uptake or somehow increased metabolic efficiency at 90 and 32 ppm relative to 13 ppm. A factor to be considered is the high variability with large standard deviations for many of the data points, as illustrated in Figure 3 of Ramboll (2020). The PBPK model structure implies that blood levels should increase in proportion to exposure as long as blood concentrations remain below the level of metabolic saturation and should increase at a faster rate above saturation, unless there is some other exposure-related change in model parameters. However, the plethysmography data evaluated do not show a clear or significant dose-response Ramboll (2020). Figure 7 of Ramboll (2020) presents the extent of agreement of the model predictions with the blood concentrations in mice following inhalation exposure. It is noted that the inhalation PK data are from a single exposure (animals were not previously exposed to chloroprene) and the non-proportionality is evident by the 3-hour time-point.

11. Given these data, please evaluate the likelihood that changes in respiration rate or metabolic induction might be factors in the observed PK relationship between exposure and internal dose. Please comment on any other physiological or biochemical mechanisms that might be explanatory factors in the apparent discrepancy or whether experimental variability in the data may explain these differences.

In the *Model Parameters* section of the Ramboll (2020) report, the authors describe the apparent discrepancy between the rate constant for cardiac output (QCC) from Brown et al. (1997) and other data. The sensitivity of the predicted blood concentration to unscaled cardiac output is shown in Figures 5 and 6 of the report.

12. Please comment on the analysis presented here and the proposed choice of QCC for the mouse.

13. Given the specific considerations above, please comment on the appropriateness of the values selected for the physiological parameters in Table S-1 and partition coefficients in Table S-2, for prediction of chloroprene dosimetry.

<u>Overall PBPK Model Soundness and Applicability</u>

Model-predicted doses in model tissue compartments corresponding to tissues in which neoplasm were observed in the rat and mouse bioassay, with corresponding cancer incidence for 80 ppm chloroprene inhalation exposure, are provided in the EPA background document. In potential application to human health risk assessment, the relative risk of tumors in human liver and lung will depend on the relative rate of metabolism predicted in those tissues, compared to the mouse or rat (as well as the relative rate of clearance). Estimation of risks for tissues other than liver and lung could depend on the relative estimates of chloroprene venous blood or tissue concentration. An evaluation of the model's applicability and degree of uncertainty should

consider both the absolute model predictions (i.e., does the model accurately predict the absolute rates of metabolism and blood/tissue concentrations in each species?) and also the ability to predict the relative rate of metabolism or relative concentration in human vs. rodent tissues, though some inaccuracy in the absolute values may exist. See "Background for the Peer Review" document for additional context.

Demonstration of the PBPK model's ability to predict *in vivo* PK data is shown by the level of agreement between model predictions and chloroprene venous blood concentrations in Figure 7 of Ramboll (2020). For reference, where there are data, and as a rule of thumb, EPA often seeks dosimetric estimates from a model that are within a factor of two of empirical results. The results of the sensitivity analysis shown in Figure 8 for arterial concentrations indicate that these data and specific predictions are not sensitive to the estimated metabolic parameters: a relatively large range in the estimated metabolic parameters (such as the apparent difference between male and female mouse parameters) would yield similar predictions of blood concentrations. However, as demonstrated in Figure 9, the estimation of lung dose metrics is sensitive to the estimated metabolic parameters.

14. Please comment on the capacity of the PBPK model to provide sound estimates of chloroprene inhalation dosimetry in mice, rats, and humans. In particular, please comment on the reliability of model predictions of the rate of chloroprene metabolism in liver and lung for use in animal-to-human extrapolation. Please also comment on the reliability and uncertainty of model predictions of chloroprene concentrations in blood and other tissues from inhalation exposures. Please provide your scientific judgement about the potential order of magnitude of quantitative uncertainty in these estimates.

Proposed Uncertainty Analysis of *In Vitro* Metabolic Data and PBPK Model Predictions

The U.S. EPA seeks input on initial analyses that it has conducted, its proposed approach to evaluate quantitative uncertainty of the metabolic parameters estimated from *in vitro* data, and its proposed approach to incorporate the metabolic parameter uncertainty into an estimate of uncertainty in the PBPK model predictions U.S. EPA (2020).

15. Please comment on the analysis and statistical assumptions for control data from* Yang et al. (2012) *as an approach for evaluating the underlying experiments, data, and distribution of RLOSS for use in subsequent uncertainty analyses of the metabolic data.

16. Considering the preliminary results for RLOSS provided, please provide any specific suggestions you may have for how the analyses methods might be improved.

A similar analysis was conducted using data from five control incubations obtained by Himmelstein et al. (2004). Comparison of the results for RLOSS based on Yang et al. (2012) control data vs. Himmelstein et al. (2004) control data indicates that the value of RLOSS may have been lower in the Himmelstein et al. (2004) study. The two sets of experimental *in vitro* studies were conducted in the same laboratory by the same principle investigator (Matthew Himmelstein), but given the period of time between the two studies, the applicability of non-concurrent control data is a source of uncertainty.

17. Please comment and provide any specific suggestions you have on the possible use of either:

a. separate distributions of RLOSS obtained from the* Yang et al. (2012) *vs.* Himmelstein et al. (2004) *studies when analyzing the uncertainty for the different metabolic parameters obtained with data from the respective studies; or

b. combining the control incubation data and analysis to obtain a distribution applicable to all metabolic data.

U.S. EPA (2020) describes intended methods for evaluating the uncertainty in the metabolic parameters obtained from the *in vitro* data, given the distribution in RLOSS already obtained. The analysis is particularly focused on the human liver and lung data, which were obtained with pooled microsomes from 15 individuals for liver microsomes and 5 individuals for lung microsomes.

18. Please evaluate the planned analysis as an appropriate statistical approach for evaluating the uncertainty in the metabolic parameters for the pooled tissue samples. Note any additional quantitative factors whose uncertainty you believe would not be addressed by this approach. Please provide any specific suggestions you have on how the analysis should be modified.

U.S. EPA (2020) describes intended methods for evaluating the uncertainty in the PBPK model predictions for the rate of metabolism in liver, lung, and kidney, and in predictions of chloroprene venous blood concentrations. Since the analysis is focused on estimation of population average doses, uncertainty in human physiological parameters would be quantified as uncertainty in the *mean* values for a healthy adult, rather than overall population variance. For model predictions based on the parameter A1 (lung:liver metabolic ratio obtained from data for 7-ethoxycoumarin) and a similar parameter for the kidney (A2), uncertainty in A1 or A2 based upon variance in tissue-specific values reported for the corresponding *in vitro* studies will be included.

19. Please comment on whether the planned analysis for PBPK-predicted dose metrics as outlined by* U.S. EPA (2020) *is an appropriate approach for evaluating quantitative uncertainty in the estimated internal doses. Please provide any specific suggestions you have on how the analysis could be improved.

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III. GENERAL IMPRESSIONS

Leslie Z. Benet, Ph.D.

In reading my report one should be aware that my expertise related to this issue is as one trying to use IVIVE to predict the pharmacokinetics and dynamics of drugs. I have some experience with environmental toxicology issues having served as the PI of the UCSF NIEHS Superfund Program Project Grant: "Health Effects of Toxic Substances" 1987-1995 and did during that time have some interactions with Drs. Andersen and Clewell. I come to this evaluation of chloroprene with strong opinions about the validity of IVIVE and PBPK in general. In the last 6 years, we have published 7 peer reviewed papers investigating the reasons why IVIVE predictions for drugs were so poor. Our most recent (published September 10, 2020, AAPS J **22**:120) paper "Investigating the Theoretical Basis for *In Vitro-In Vivo* Extrapolation (IVIVE) in Predicting Drug Metabolic Clearance and Proposing Future Experimental Pathways" finds that the 60-80% of drugs exhibiting poor IVIVE predictability violate the theoretical principles upon which IVIVE is premised. We attribute this to two major differences between Chemistry and Pharmacokinetics with respect to metabolism. In Chemistry, intrinsic clearance (the ratio of V_{max}/K_m at low substrate concentrations) is determined in vitro in a fixed volume that is drug independent, while in vivo intrinsic clearance is a function of volume of distribution of drug in the liver, which will be drug dependent and in most cases a different volume of distribution than that for the in vivo metabolic enzymes. Secondly, in Chemistry, V_{max} was originally and continues to be defined in terms of a concentration change, while in Pharmacokinetics V_{max} has been defined as an amount change that results in the ratio V_{max}/K_m as a clearance parameter, rather than as a rate constant. But pharmacokineticists have not derived the Michaelis-Menten relationship in terms of amounts. Rather they use the chemistry concentration derivation and then just change the V_{max} units, without any theoretical justification, because it is more convenient. This error is only stated in our recent paper, but the derivation differences are the subject of a further to be published manuscript. So I come to the chloroprene evaluation wary of the validity of the IVIV extrapolations.

I am also very wary of PBPK modeling from a mechanistic point of view. I agree that PBPK models are highly useful, with predictions that can be trusted, especially when sensitivity analyses are employed. However, the objective of PBPK models is to fit the data, ignoring and often hiding basic scientific principles and including "fudge" factors. I am highly suspect of supposed mechanistic findings resulting from PBPK model fitting and have detailed in a number of recent publications various mechanistic errors inherent in the basic drug metabolism PBPK models, which doesn't necessarily make them less useful in a particular situation. There are certainly a number of assumptions in the chloroprene PBPK models of Himmelstein, Yang and Clewell (Ramboll) that are hard to accept in terms of basic scientific principles.

Yet, in spite of my concerns with IVIVE and PBPK modeling, overall I find the Ramboll analysis to be persuasive and potentially useful. Since I don't believe that PBPK modeling can be trusted to strengthen the scientific basis for the model or accurately reduce uncertainty, and I have detailed why IVIVE methodology doesn't recognize basic theoretical flaws, I have no Tier 1 or Tier 2 suggestions. If PBPK models have to be scientifically valid, we will have no PBPK useful models. PBPK models based on IVIVE and other data need to predict relevant outcome

measures, in this case with respect to chloroprene potential toxicity. I believe the Ramboll analysis does that.

Jeffrey J. Heys, Ph.D., P.E.

The report by Ramboll (2020) presents a refinement of an existing PBPK model of Chloroprene metabolism. The model is based on the experimental measurements and PBPK model of Himmelstein et al. (2004), which was later refined by Yang et al. (2012) and Clewell et al. (2019). My overall impression of the report is that I think there is potential in the approach being taken to better understand chloroprene metabolism and, ultimately, cancer risk. The historical approach, which is extrapolating in vivo animal data to humans, has a major limitation – animals are different from humans. In some cases, those differences might have a significant impact on predicted cancer risk. The Ramboll report provides an argument that chloroprene metabolism is an example of a system where simple extrapolation from an animal model to predict cancer risk may result in an inaccurate prediction because of metabolic differences.

Even though I see potential in the approach presented in the report, my second impression of the report is that it is simply inadequate at this time. The quality and comprehensiveness of the data presented in the report is limited. Almost 20 years have passed since most of the data were collected, and there are still gaps where data is needed to better understand metabolism in the human lung, mass transfer, and more. The model itself has gaps, the report includes examples where the data contradicts model assumptions, and large uncertainty exists with some parameters. The report does not include key equations used in the analysis (e.g., the equation for mass transport between the air and fluid) and some parameters are not defined (e.g., Kgl). In summary, the report represents a step in a potentially important direction, but more steps are needed.

Jochem Louisse, Ph.D.

The approach to characterize human hazards based on animal toxicity data with help of PBPK modelling to take interspecies differences in toxicokinetics into account is a step forward in a more science-based risk assessment of chemicals. The data presented in the documents that are used for PBPK model development are in general clear, well presented and accurate. For the assessment of the quality of the in vitro kinetic data, it would have been of help to (also) present the data obtained with the microsomal incubations in an excel-file, thereby also providing a clear overview of the number of measurements, information on number of independent experiments, etc. This information has been presented a bit scattered in the documents provided. Also, the importance of the metabolism data related to the kidney is not clear to me, as it does not play a significant role in chloroprene clearance and only the lung is indicated as being the relevant organ for toxicity. Also, no data on metabolism with kidney microsomes is presented in the main report, whereas kinetic constants for the kidney have been added to the Supplemental Table S-3 and S-4.

The PBPK models have been developed by renowned scientists, with ample experience in development and application of PBPK models for volatile chemicals, and with knowledge on their kinetics. The mouse PBPK model was evaluated by comparison of PBPK model-predicted

blood concentrations upon inhalation exposure to chloroprene blood concentrations in mice in vivo. That evaluation shows that the model accurately predicts chloroprene blood concentrations in mice upon inhalation exposure, providing confidence that chloroprene blood concentrations are adequately predicted also by the human PBPK model. It can, however, not be concluded from this evaluation that the amount of oxidized chloroprene in the lung (the dose metric of interest for the interspecies comparison) has been adequately predicted by the PBPK model(s), as the chloroprene blood concentrations are hardly dependent on chloroprene oxidation in the lung, i.e. chloroprene oxidation in the lung plays a very limited role in the determination of chloroprene blood concentrations.

Since kinetic parameters have been determined based on a substrate depletion approach and since depletion is limited in the incubations with lung microsomes (as well as with kidney microsomes), there may be more uncertainty in the kinetic parameters for lung metabolism (and kidney metabolism). No chloroprene depletion was found in in vitro incubations with human lung microsomes and therefore the liver Km was used for the lung and the Vmax was estimated based on reported differences in 7-ethoxycoumarine O-deethylation in human liver microsomes vs human lung microsomes. I am of the opinion that that approach is not adequate to estimate parameter values for chloroprene oxidation in the lung, resulting in too much uncertainty in the PBPK model-based prediction of chloroprene oxidation in the human lung. Since the prediction of the amount of oxidized chloroprene/day/g lung is very sensitive to parameters for lung metabolism, I think it is important to have more certainty in these parameter values.

Altogether, I am of the opinion that the presented PBPK model should not be used as such to extrapolate the mouse data to a human effect dose for application in the risk assessment. In vitro kinetic data on toxic metabolite formation of chloroprene (epoxide metabolite), and kinetic data on detoxification (epoxide hydrolysis) in the mouse vs human lung should be obtained and applied in the PBPK model to increase confidence in the prediction of kinetic-dependent species differences (mouse vs human) in chloroprene toxicity.

Annie Lumen, Ph.D.

The report by Ramboll is well written and the overall description about what was done and why it was done a particular way was transparent and clear. Assumptions, methodological details, and choice of approaches for IVIVE scaling and PBPK modeling were clearly stated. The model developers are experts in PBPK modeling with prior experience in inhalation modeling as supported by publications in the peer-reviewed literature. The conclusions based on their specific assumptions and approaches were reasonable.

EPA's supplementary document on alternate uncertainty analysis was also clear and the details of the proposed analysis, though beyond my statistical knowledge, was well laid out and its technical transparency appreciated. The analyses appear to be comprehensive and aimed to address plausible uncertainties associated with the invitro parameter estimations and the overall PBPK model predictions.

Please note that I'm a PBPK modeler and do not have sufficient expertise in metabolic assays or statistical approaches. I've indicated this where applicable in the response to charge questions and addressed the ones that I had specific inputs on.

Kenneth M. Portier, Ph.D.

The PBPK model for chloroprene described in the Ramboll (2020) report along with the supplemental materials and the EPA supplemental analysis (US EPA 2020) represent a massive amount of work by careful laboratory and computational scientists. The results of this modeling exercise are well presented, displaying a logical approach to model structure, parameter estimation methodology and model analysis plan that at the same time seems quite reasonable in the underlying assumptions. As with all mathematical models that attempt to describe quite complex biochemical and toxicological processes in rodents and humans, there are issues with this model that limit its current full utilization as a tool for incorporation of in vitro test findings to predictions of in vivo processes, all in support of ongoing chemical risk assessments. While not fully ready for use today, it is clear the with some additional and moderate efforts, this model, or one derived from it, will play a strong supporting role in future chemical risk assessments.

Kan Shao, Ph.D.

The document under review discusses a physiologically based pharmacokinetic (PBPK) model developed solely on in vitro studies for chloroprene in the mouse, rat, and human which will be used to support an inhalation risk assessment for lung cancer. Previous PBPK models for methylene chloride and vinyl chloride were used as the base for developing a PBPK model for chloroprene and selecting an appropriate dose metric. A few improvements and/or adjustments have been applied in the new PBPK model to enhance its performance and reliability, such as using pharmacokinetic data obtained from an in vivo study to validate the PBPK model, estimating lung metabolism parameters by employing liver metabolism parameters with the ratio of liver and lung activity.

Overall, the Ramboll (2020) report has a mixed quality. On the one hand, almost the entire document (especially the first three sections: Introduction, Materials and Methods, Results) is disorganized: (1) sub-sections were simply put together without logical connection and presentation; (2) the lack of detailed information and explanation makes the document difficult to follow and understand; (3) some same/similar information has presented multiple times throughout the entire document (including the supplemental materials) which is unnecessary. Therefore, to help reviewers to follow the report smoothly, a flowchart describing the key steps in the entire study and how they are connected with each other is highly recommended. On the other hand, the report did a good job to provide detailed justification (mainly in Section IV Discussion) for a number of important assumptions, selections, and implementations (although some of the methods can be improved) , such as the selection of dose metric, the assumption to treat Km for metabolism in human lung and liver the same, etc. The Uncertainty Analysis report prepared by the US EPA was well written and organized in a clear and easy-to-follow format with detailed explanation and justification for most of the key issues.

The technical quality (from a quantitative analytic perspective) of the two reports is hindered by a few issues, including how to justify the necessity and validity of introducing a new parameter "Kgl" to quantify the air and liquid mass-transfer, the lack of detailed analytic results prevent better evaluating the statistical approaches' ability to characterize uncertainty and variability, etc. Detailed comments, explanations, and suggestions are provided after the corresponding charge questions. Consequently, better addressing and more clearly explaining these issues will certainly significantly improve the quality of the reports.

Jordan Ned Smith, Ph.D.

Ramboll (2020) developed a physiologically based pharmacokinetic model (PBPK) for chloroprene. Based on the success of previous PBPK models for risk assessment of methylene chloride and vinyl chloride, Ramboll (2020) proposed this model to support chloroprene risk assessment. After reviewing the model, I agree that it has the potential to be useful for human risk assessment of chloroprene. The model offers many strengths over conventional risk assessment techniques. However, several aspects of the model could be improved, which would increase confidence in the ability of the model to provide accurate predictions of human dose metrics relevant for risk assessment.

Using the chloroprene PBPK model for risk assessment offers many strengths over conventional techniques. Previous risk assessment efforts with similar compounds and proposed dose metrics have demonstrated a PBPK model's utility for integrating pharmacokinetic knowledge and measurements (e.g. metabolism, partition coefficients, etc.) across a variety of sources and physiology values (e.g. ventilation rates, body weights, etc.) to quantitatively predict dose metrics in humans. This model was constructed by experts knowledgeable in the development and application of PBPK models. The model uses a conventional and well-accepted structure, and the most important parameters (metabolism parameters, partition coefficients, and a handful of physiological parameters) are either measured in vitro or are well-established reference values. Measured parameter values provide confidence over those predicted by algorithms or extrapolated from animal models. The model accurately simulates concentrations of chloroprene in blood of mice exposed to 13 ppm chloroprene by nose only inhalation reasonably well but overpredicts higher exposures (32-90 ppm) by ~2-fold. The model can be used to integrate uncertainty of sensitive parameters and translate that uncertainty to selected dose metrics of interest for risk assessment. EPA efforts to integrate various sources of uncertainty into this model offers a quantitative approach for assessing overall uncertainty, an important consideration in risk assessment.

Several areas of improvement that would increase confidence in the ability of the chloroprene model to provide accurate predictions of human dose metrics relevant for risk assessment. Mouse to human extrapolation of lung metabolism is based 7-ethoxycoumarin activity assuming that CYP2E1 is the primary enzyme responsible for metabolizing chloroprene. This assumption needs experimental evidence to support it, including identification of enzymes involved with chloroprene metabolism at relevant concentrations and assessing the potential of those enzymes to be induced. Additional experiments to measure Kgl would aid in obtaining accurate in vitro metabolism parameters. It may be prudent to evaluate the potential of chloroprene plasma protein binding as an additional process affecting chloroprene metabolism and distribution. In a perfect

world, a better dose metric would be some derivative/variant of the actual ultimate toxicant: the epoxide metabolite, which would be subjected to various distribution processes and metabolic detoxification pathways not considered in the current model (e.g. epoxide hydrolases). As such, developing a parallel PBPK model of the epoxide metabolite and utilizing the respective dose metrics would capture the net result of all these other pharmacokinetic processes and provide a better dose metric for risk assessment.

Overall addressing some or all areas of improvement would help capitalize on the many strengths of this model and support risk assessment of chloroprene.

Raymond S.H. Yang, Ph.D.

The researchers involved in all the relevant studies to this Project, starting from the early 2000 until the present days, have all been reputable scientists from good laboratories and institutions in the fields of Toxicology and Risk Assessment. Their publications all appeared in top-notch peer-reviewed toxicology journals. The quality of their work was good and the studies were well planned and executed. There is no reason to question the accuracy of the information presented. The clarity of presentation is good, and the conclusions, given the stated purposes and with the exceptions of the issues discussed below, are, in general, scientifically reasonable.

In doing this review, I am coming from the general direction of "Are the assumptions and approaches used in the PBPK modeling and related IVIVE in the Ramboll/Denka application/report reasonable?" and "How well did this application of PBPK modeling and the related IVIVE improve the existing cancer risk assessment of chloroprene?" It was wise for the EPA to encourage the discussion to be focusing on PBPK modeling and IVIVE during the Meeting because of the limitation of time. However, since the "bottomline" of this project was the Ramboll/Denka request for the EPA to "relax" the present risk estimate of chloroprene by 137X, it was impossible to ignore the application of these techniques (PBPK Modeling and IVIVE) to the risk assessment of chloroprene. Thus, in my discussion, some aspects of risk assessment of chloroprene were included. My feeling is that if I were a risk assessor on this Project, given the reactive nature of chloroprene and the potency of its carcinogenicity demonstrated in the animal studies, I would rather err on the side of caution. Further, since some of my comments during the Meeting had generated somewhat intense debates, I decided to adopt the following practice: During and following the meetings, I had absorbed and digested the relevant discussions and I had done further reading and thinking; I placed my further thoughts under a "Post-meeting Thoughts" section following my pre-Meeting comments in the appropriate Charge Questions.

My overall opinion is that it is **NOT PRUDENT** for the EPA to grant the requested 137X relaxation of the risk estimate in the IRIS risk assessment, at this time, to Ramboll/Denka based on the science presented for this Review, as well as on my own evaluation of some of the related state-of-the-science relevant to this Project. My general impression was that Ramboll/Denka had dismissed or ignored some of the available science and chose a simplistic approach of relying on a previously successful example (Revision of Methylene Chloride Risk Assessment) by the same lead scientists. In doing so for this highly reactive chemical, chloroprene, the Ramboll/Denka petition left many holes in their scientific arguments. Thus, while PBPK Modeling is a very

useful tool for risk assessment, the Ramboll/Denka application is not scientifically strong enough, at this time, to support their petition.

The key elements for the basis of my decision were given under my comments in Charge Question 14, and the details were given under the related comments in different Charge Questions. I also made many key recommendations to both Ramboll and the EPA colleagues under the various Charge Questions.

Yiliang Zhu, Ph.D.

Ramboll Report

Overall the updated PBPK model for chloroprene is sound in structure and appears sufficiently flexible for predicting tissue specific dosimetry in liver, lung, and venous blood. Using PBPK models for extrapolations between species and between organs is scientifically appealing for evidence integration. Challenges remains to calibrate the model for species, tissues and to quantify uncertainty and variability.

The Ramboll Report (2020) provides a number of aspects of model updating. The presentation of the report can benefit from a re-organization and from including more specific details that are crucial for comprehending the process and for making a critical appraisal. The follow are some examples, along with recommendations.

Although main sections of the report are labelled use numerals, subsections are labeled using different fonts. Using numerals (e.g. section 2.1, section 2.1.1 and section 2.1.1.1 etc.) will make the hierarchical structure more visible and navigation easier.

The report followed a standard format of journal papers, with a method section and result section. This created a separation between steps for a single study. For example, the experiment on the mass transfer and that of female mouse experiment for venous blood concentration addressed different aspects of the PBPK models. With proper transition from one section to another, a separate section for each experiment might offer a more cohesive presentation.

Lack of necessary and adequate details appears common in the main report. At the center of the section of "Re-estimation of in vitro metabolism parameters" (pp 9-10) is the estimation of mass transfer coefficient Kgl. Yet the main document only summarizes the results, does not provide details on the kinetic model, the experiment, or the methods used for estimation. One has to frequently go back and forth between the report and Supplemental Material B (Supp B). But the kinetic model for mass transfer between air and liquid was not present in Supp B either. A MCMC analysis was conducted to re-estimate Km, Vmax and Kgl for the male mouse data (Himmelstein et al 2004) as well as all other in vitro data. Specifics are lacking in both cases (Supplementary Material B). RLOSS and KF are mentioned in the last paragraph of that section, without any introduction or preparation. These concepts and notations are in EPA's Supplement: Uncertainty Analysis.

All Supplemental Materials should have page numbers. Figures should have proper legends. The use of MCMC was central to all the estimation and uncertainty analysis in both the main report and EPA's Supplement. However, the description of MCMC analyses is notably lacking critical details, making a critical appraisal difficult.

The term "95% confidence interval" in association with a posterior distribution is improper. Consider replace it with either a mid-95% inter-percentile range, or 95% credibility interval on the basis of the posterior distribution derived from MCMC analysis.

EPA Supplement

The Supplement is well written and the analysis plan is well laid out. It is clear that EPA is seeking input on important aspects of implementing MCMC analysis. There are some key assumptions that EPA has made in the interim that may lead EPA to implement MCMC in a way that is less conventional, particularly that one MCMC for each incubation sample. MCMC based on joint likelihood of all incubation samples would be more statistically sound.

IV. RESPONSE TO CHARGE QUESTIONS

Estimation of Mass Transfer Resistance in the *In Vitro* Metabolism Experiments

A model of the *in vitro* incubation system was used to estimate the metabolic parameters from the *in vitro* data. This model is based on certain assumptions and physical parameters, such as the volume of the *in vitro* incubation vials and volumes of air and liquid media in the vials.

The model of the *in vitro* system initially used for the analysis of the *in vitro* experiments to estimate the corresponding metabolic parameters (Yang et al., 2012; Himmelstein et al., 2004) assumed that the chloroprene in the air and liquid (incubation medium) phases was at equilibrium at all times after the start of the experiment; i.e., concentration in the medium was set equal to the concentration in the air times the equilibrium partition coefficient (CM = CA*P). At EPA's suggestion, the model was changed to explicitly describe separate air and liquid media compartments, with a mass-transfer coefficient (Kgl) limiting the rate of distribution between them, as described by Kreuzer et al. (1991) and others.

Question 1.
Please evaluate the validity and uncertainties of these two approaches to estimation of the kinetics in the vitro system and therefore in the estimation of metabolic parameters:
Question 1.a. treating the air and liquid phases as always being at equilibrium (original model); or

Leslie Z. Benet, Ph.D.

It seems very reasonable and scientifically sound to suggest that the air and liquid phases are distinct compartments and the rate of transfer would be limited by a mass transfer constant. Thus, experiments were run to determine the Kgl for the in vitro systems. However, the experimental outcome value of 0.024 L/h is unreasonable and inconsistent with observed metabolic data. If this value had been consistent with the metabolic data no further analysis would have been undertaken. Thus, in a typical PBPK approach, one then considers other experimental conditions that could explain the discrepancy and result in the expected outcome.

Jeffrey J. Heys, Ph.D., P.E.

The original model (Himmelstein, Carpenter et al. 2004) assumed that mass transport between the air and liquid phases was 'instantaneous.' This assumption was support by measurements of chloroprene concentration in the air (or headspace) of the vial containing phosphate buffer at a 1-to-10 (liquid-to-air) ratio (Figure 1A in Himmelstein, Carpenter et al. 2004). The starting concentrations in the headspace were from 0.02 to 22 nmol chloroprene/ml, and, while it is difficult to make a precise, quantitative determination, it does appear that the headspace concentration decreases slightly (maybe 10-20%) over the 1 hour that measurements were taken. This small decrease indicates that the mass transfer is not necessarily 'instantaneous' and there is the potential for some mass transfer limitations. In the Ramboll (2020) report, when the air and liquid phases are treated as distinct compartments, equilibrium is achieved after 2-5 minutes. Considering that the metabolic kinetics of interest have characteristic timescales of 10-20

minutes, in many cases, an assumption of instantaneous mass transport does not seem appropriate in my opinion. It is important to note that the measurements (Himmelstein, Carpenter et al. 2004) were taken with an agitation rate of 500 rpm according to the Ramboll report and (Clewell, Campbell et al. 2019), which is much higher than the Ramboll report's equilibrium experiment. At the higher agitation rate, mass transfer could be much higher and potentially sufficiently close to instantaneous to be neglected.

It is difficult to quantify the uncertainty associated with mass transfer limitations, but if non-negligible mass transfer limitations are present and a model is developed that assumes instantaneous equilibrium, then the model concentration in the liquid phase could be elevated relative to the actual physical system for at least the first few minutes that are simulated. The error associated with assuming instantaneous mass transfer would depend on the reaction rates of interest. If downstream reactions require hours to achieve equilibrium, the error associated with assuming instantaneous mass transfer is likely to be negligible. If downstream reactions require minutes to achieve equilibrium, the errors would be non-negligible.

Jochem Louisse, Ph.D.

I have no expertise in mass transfer of volatile chemicals on air:liquid interfaces, so I cannot provide scientifically substantiated input on this point.

Considering that model fits and estimated Vmax values of both approaches (1) assuming always equilibrium (data from Yang et al. (2012)) and (2) taking a limited transfer into account (new analysis)) are highly similar and that estimated Km values only differ at max two-fold, I doubt whether one should estimate kinetic parameter values applying a Kgl that is also estimated, but that has not been substantiated by the experimental data.

Since the impact of protein concentration on Kgl is not known and given that (to my understanding) different protein concentrations have been applied in the incubations, the use of one Kgl for incubations with different protein concentrations may not be adequate.

Annie Lumen, Ph.D.

I've not conducted such assays to speak from experience about the validity of treating the air and liquid phases to be always at equilibrium for these class of chemicals.

If this has not been confirmed experimentally it might be good to evaluate by making this assumption that the two phases are in equilibrium if and by how much the metabolic parameters would be under-estimated by (**Tier 2**). Especially, since under-estimation of metabolic capacities could contribute to lesser health-conservative dose metrics.

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and experience and I have no comments to add.

Kan Shao, Ph.D.

I comment on this question only from a perspective of quantitative analysis. Using this simplified assumption may ignore the potential uncertainty and variability in the rate of air:liquid transfer, which should be reasonably characterized. A sensitivity analysis is suggested to justify the validity of the assumption (**Tier 2**).

Jordan Ned Smith, Ph.D.

Data in Figure B-1 indicates that equilibrium between the two phases is not instantaneous, as equilibrium occurs in ~6 min. Within the first 6 min, the concentration ranges from 0 to the equilibrated value (~50 µg/L). Since metabolism occurs during this first 6 min. and metabolic loss is measured within an hour (longer measurements may indicate that mass transfer is not rate limiting) and depends on substrate concentration, mass transfer probably needs to be considered to accurately estimate metabolism parameters. High correlation between Kgl and Km offers further evidence that Kgl is impactful for metabolism parameter estimation. Overall, data in Figure B-1 provides evidence that a limiting mass transfer process is present and should be accounted for in the *in vitro* metabolism assays.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

No response provided

Question 1.b. treating the air and liquid phases as distinct compartments with the rate of transfer limited and determined by a mass-transfer constant (Kgl).

Leslie Z. Benet, Ph.D.

No response provided

Jeffrey J. Heys, Ph.D., P.E.

The treatment of the air headspace and liquid phases as distinct compartments that are connected by a mass transfer constant was based on (Schlosser, Bond et al. 1993), which was based on (Csanady, Guengerich et al. 1992). The Schlosser et al. paper includes an analysis of the partition and mass transfer of benzene from the headspace into buffer. Approximately 10 minutes are required to achieve equilibrium for benzene, which suggests that liquid-phase kinetics that occur on a similar (or faster) time scale will be inhibited by lower concentration associated with mass transport limitations.

Benzene and chloroprene are different molecules, but they share a number of similar physical properties that are relevant in mass transport. For example, according to the EPA online tool for site assessment (https://archive.epa.gov/ceampubl/learn2model/part-two/onsite/JnE_lite.html), chloroprene and benzene have very similar diffusivity values in air (approximately $9 \times 10^{-2}\ cm^2/s$) and water (approximately $1 \times 10^{-5}\ cm^2/s$). Mass transport in the air of the headspace should be rapid and a negligible source of mass transfer resistance. Mass transport in the aqueous buffer will occur via a combination of convection and diffusion. Further, mass transport in the liquid buffer will be strongly dominated by convection (I would estimate the Péclet number, which is the ratio of convective to diffusive transport, to be on the order of 10^5) with the exception of a very thin film at the air-water interface. At the air-water interface, solubility (and diffusion) will govern transport and it is here that benzene and chloroprene differ. The Henry's law constant for Chloroprene (0.61) is more than twice that of benzene (0.28). Similarly, measurement of solubility in water are significantly higher for chloroprene than benzene by a factor of approximately 5. These physical properties suggest that mass transfer is likely to be more important and limiting in benzene-water systems than chloroprene-water systems. The chloroprene equilibrium data in Ramboll (2020), Figure B-1, supports this claim as chloroprene equilibrium between the air and water/buffer phases requires approximately 2 to 5 minutes (interestingly, the solubility of chloroprene is approximately 5 times higher than benzene and equilibrium is achieved in $1/5^{th}$ the time of the benzene system). The chloroprene equilibrium data between air and water phases (Figure B-1 in Ramboll, 2020) implies that kinetics on the time scale of minutes (i.e., less than an hour) will be impacted by mass transfer limitations and these limitations should be considered. Of course, as noted above, the equilibrium data in Ramboll, 2020 may not be relevant to fitting the in vitro data because the in vitro experiments on the mouse liver used a much higher mixing rate with potentially higher mass transfer.

Tier 2 Suggested Recommendation: Retain the current two compartment model with separate air and liquid phases. If the experiments recommended in response to question 2 demonstrate that mass transfer is nearly instantaneous, then the model can be modified to use a single compartment and an equilibrium assumption.

Jochem Louisse, Ph.D.

As indicated in my answer to question 1a, I have no expertise in mass transfer of volatile chemicals on air:liquid interfaces, so I cannot provide scientifically substantiated input on this point.

Annie Lumen, Ph.D.

Given the volatility of Chloroprene and based on mass-action kinetics this approach seems reasonable. This is also supported by Figure B-1 in the supplementary material B as other panel members had pointed out in the public meeting.

However, since the experimental estimations to determine Kgl required further assumptions about mixing speeds and non-specific binding and subsequent MCMC analysis showed high correlation to Km, a key metabolic parameter, a rigorous uncertainty analysis (than to select one

representative value) on the Kgl parameter and its propagated impact on metabolic parameter estimations might be necessary. This is my recommendation (**Tier 1**). Specifically, to evaluate the degree of under-estimation of metabolic parameters from the current estimates, in other words what is the maximum dose-metric value will the lowest possible value of Kgl (0.11 L/hr as stated in Pg. 6 of Supplementary Materials B or whatever the appropriate equivalent is) would yield in each tissue (**Tier 1**).

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and experience and I have no comments to add.

Kan Shao, Ph.D.

Because Chloroprene is highly volatile, it is important to quantify the mass-transfer coefficient to estimate the amount of Chloroprene entered into the liquid phase. However, the estimated value from the new experimental study was too low to support the observed rates of metabolism in the media. Consequently, a MCMC simulation analysis was applied utilizing the data in Himmelstein et al (2004) to estimate Kgl, Vmax and Km simultaneously. Results show that the resulting Kgl value from the MCMC analysis was consistent with the expected value. A few issues should be addressed to better evaluate if introducing an additional "Kgl" parameter is worthwhile together with the quantifications of uncertainty and variability:

(1) Comparing the estimated results (including confidence intervals) of Vmax and Km before and after introducing the "Kgl" parameter, so that the impact of introduction "Kgl" can be evaluated (**Tier 1**).
(2) Investigating the impact of the specified prior distribution, i.e., log-uniform distribution, on the "Kgl" estimation. The estimated confidence interval shown on Figure B-5 is very narrow, I am wondering if this is related to the specified uninformative prior for these parameters. Additionally, it seems to me that the resulting posterior distribution of "Kgl" is not closely related to the specified lower bound "0.11" used in the prior distribution for "Kgl". Therefore, it is worth to investigate if the "Kgl" estimate is sensitive to its specified prior distribution (**Tier 1**).

Jordan Ned Smith, Ph.D.

There appears to be evidence of a limiting mass transfer process in the *in vitro* metabolism assays (See my comments from 1.a). As such, it seems appropriate to include a term for the mass transfer process in order to obtain accurate metabolic parameters from the *in vitro* experiments.

Raymond S.H. Yang, Ph.D.

Given the discussions during the meeting, I would like to make the following two recommendations (**Tier 1**):

1. Dr. Clewell and Team at Ramboll provide more detailed descriptions, to be included in this Report, of the incubation system as well as explaining how 500 rpm stirring was achieved in such a system.

2. Dr. Schlosser and Team at the EPA provide written description of how he and the EPA colleagues examining the kinetic behavior of the above system and reached their conclusion that the high speed agitation at 500 rpm had not denatured the microsomal enzymes.

I would also like to note that in the two publications (Cottrell et al., 2001; Munter et al., 2003) on in vitro metabolism of chloroprene, there didn't seem to be any concern about the mixing speed of the enzyme assay system.

References quoted for this section:

Cottrell L, Golding BT, Munter T, Watson WP (2001) In vitro metabolism of chloroprene: Species differences, epoxide stereochemistry and a de-chlorination pathway. Chem. Res. Toxicol. 14:1552-1562.

Munter T, Cottrell L, Golding BT, Watson WP (2003) Detoxication pathways involving glutathione and epoxide hydrolase in the in vitro metabolism of chloroprene. Chem. Res. Toxicol. 16:1287-1297.

Yiliang Zhu, Ph.D.

No response provided

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Experiments were conducted to determine the Kgl for the *in vitro* system, however the value of Kgl obtained from those experiments is not consistent with some of the observed metabolic data (Ramboll (2020) Supplemental Material B), and Kgl would need to be at least 8 times higher to obtain results consistent with those data and to obtain a Km consistent with metabolic parameters reported for other VOCs. This inconsistency may exist because the experiments conducted to estimate Kgl used an incubator mixing speed of 60 rpm while the experiments of Himmelstein et al. (2004) and Yang et al. (2012) used 500 rpm. Also, the experiments to measure Kgl were performed without microsomal protein and the report hypothesizes that the presence of microsomal protein (1–3 mg/mL) in the metabolic experiments could increase mass transfer. It is noted that the mean value of the partition coefficient, P, estimated from the Kgl data in the absence of microsomal protein was 0.48 (Ramboll (2020) Supplemental Material B) while that reported by Himmelstein et al. (2001) for chloroprene equilibration with media containing heat-inactive protein was 0.69, 44% higher. To be clear, simulations of the metabolically active experiments used to estimate the metabolic parameters used P = 0.69, so have accounted for the difference in the equilibrium partition coefficient, but are still not consistent with the highest activity data when using the value of Kgl obtained from the 60 rpm data.

Question 2.
Please comment on the likelihood that either the presence of microsomal protein (1–3 mg/mL) or that the higher mixing speed used in the metabolic experiments (500 rpm) vs. the mass transfer experiments (60 rpm) would increase the rate of chloroprene mass transfer between the air and liquid phases in the in vitro system by a factor of 8 or greater, relative to the rate observed in the mass-transfer experiments.

Leslie Z. Benet, Ph.D.

There is precedent in drug kinetics for the addition of protein to increase hepatic uptake of drugs, so I could envision a similar phenomenon occurring at the air liquid interface. Also the correction of the different mixing speeds to increase the Kgl nicely results in a reasonable value in terms of observed metabolic data. Ramboll then carried out a new MCMC analysis of Himmelstein male mouse metabolism data that there was collinearity between K_m and Kgl "indicating that these two parameters are not completely independent." I would not agree with the statement on page 10 of our Instructions that "An analysis provided in Supplemental Material B of Ramboll (2020) demonstrates that estimates of the metabolic parameter Km depend strongly on the value of Kgl." The statement in Ramboll quoted above is more accurate. But, the 0.2 L/h value estimated and the Bayesian analysis estimate of 0.22 are consistent with the metabolic data.

Jeffrey J. Heys, Ph.D., P.E.

I will begin my comments by addressing the question of whether or not the mixing speed differences are likely to increase the rate of chloroprene mass transfer between the air and liquid phases. There is an extensive body of research that has looked at the impact of mixing speed on mass transfer. Of potential interest here are the numerous studies that examine the impact of mixing speed on the rate of mass transfer of oxygen in bioreactors. A common finding in these studies is that the mass transfer rate is roughly proportional to the mixing speed to the 2/3 power, e.g., $k_L A \propto (rpm)^{0.67}$ (Vantriet 1979, Versteeg, Blauwhoff et al. 1987, Galaction, Cascaval et al. 2004, Karimi, Golbabaei et al. 2013). Unfortunately, test results are unlikely to have application to the question posed here. In these systems, the gas phase is typically bubbled up through the liquid of the bioreactor. The objective of the mixing system is primarily to shear and break up bubbles to increase the surface area available for mass transport. In other words, the goal is to make large bubbles smaller. My understanding of the chloroprene mass transfer system described in the question above is that the mixing rate does not significantly increase the surface area for mass transfer. If my understanding is incorrect, most of my statements below are going to be incorrect.

For a single molecule of chloroprene to be transported from the air space in the vial to an enzyme in the liquid phase (where it can be reacted to a different molecule) requires several transport steps:

1. Transport through the air to the air-liquid interface (likely to be fast based on the diffusivity of chloroprene in air).
2. Transport from the gas to the liquid phase (governed by solubility)
3. Transport through the liquid film at the interface (governed by liquid phase diffusivity according to film theory or penetration theory, e.g. chapter 3 in Seader and Henley (2006))
4. Transport through the liquid to either a specific or non-specific binding site (likely to be fast based on the large Péclet number, which I would estimate to be on the order of 10^5, suggesting that convective transport is much greater than diffusive transport).

Based on a rough, order-of-magnitude assessment, it is my opinion that steps 2 and/or 3 are the rate limiting step(s). Step 2 is not significantly impacted by the mixing rate. Step 3 will be impacted by the concentration gradient across the liquid film, which could be impacted by mixing rate, but it is unlikely to be a large or linear impact.

Assuming the mass transport rate is limited by step 2 or 3, it is important to consider the concentration difference of chloroprene in the liquid phase between the liquid at the air-liquid interface and the bulk liquid. The size of this concentration gradient will determine the mass transport rate across the thin liquid layer at the liquid-air interface. If 90% of the chloroprene in the bulk liquid were to bind specifically or non-specifically to protein, lipophilic components or other components of the microsomes, it would increase mass transport in the limiting steps (especially step 3 above) by a factor of 5-10 compared to the measurements of pure buffer without microsomes. Considering that the chloroprene concentration of the liquid is less than $50\ \mu g/L$ (Figure B-1 in Ramboll, 2020) and the liquid has $1-3\ g/L$ of microsome, it does not seem impossible to me that some non-specific binding in lipophilic components is present. I believe the absence of microsomes in the air-liquid equilibrium experiments are a potential explanation for some of the higher rates of mass transport apparently observed in the metabolism studies.

Tier 2 Suggested Recommendation: In Csanady, Guengerich et al. (1992), they state that equilibrium experiments employed "heat-inactivated microsomes or phosphate buffer." I think that conducting air-liquid equilibrium studies with heat-inactivated microsomes would provide some insight into the magnitude of non-specific binding (it would not provide insight into specific binding).

Tier 2 Suggested Recommendation (alternative if the previous, Tier 2 Suggested Recommendation is not feasible): If the first recommendation is not feasible, it also seems relatively straightforward to conduct equilibrium experiments identical to those shown in supplement B at various mixing rates up to at least 500 rpms to determine the impact of mixing on mass transport.

Jochem Louisse, Ph.D.

I have no expertise in mass transfer of volatile chemicals on air:liquid interfaces, so I cannot provide scientifically substantiated input on this point.

I would think that the rate of chloroprene mass transfer would increase with higher mixing speed (larger surface area of transfer) and with the presence of microsomal proteins in the buffer. Proteins in the buffer may function as a sink and increase the transfer to the buffer. However, I do not know whether this could lead to an increased rate of chloroprene mass transfer between the air and liquid phases by a factor 8 or greater.

Annie Lumen, Ph.D.

Although logically it seems plausible that both presence of microsomes and increase in mixing speed could affect mass transfer, I don't have the expertise in experimental set-up or mass-

transfer to comment on whether a factor of 8 increase in the rate for Chloroprene transfer can be attributed to these explanations and them alone. I will defer this question to other members on the panel.

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and experience and I have no comments to add.

Kan Shao, Ph.D.

I have no expertise in this area and thus am unable to comment.

Jordan Ned Smith, Ph.D.

I find it plausible that both hypotheses (protein binding and mixing speed) could result in faster mass transfer of chloroprene from gas to liquid phases. As a moderately lipophilic compound (log Kow: 2.2), chloroprene could bind to lipophilic components of microsomes (compared to buffer), effectively increasing the apparent solubility, concentration gradient, and rate of mass transfer from gas to liquid. Additionally, if chloroprene exhibits protein binding properties, a similar increase in solubility and rate of mass transfer could be observed. Our group has observed significant impacts of protein binding on diffusion transport of highly bound chemicals across a monolayer of cells (Smith et al. 2017; Carver et al. 2018). Increasing the mixing speed could increase the size of the boundary condition between the gas and liquid phases or increase the surface area available for mass transfer, both increasing the rate of transport.

Tier 2 Suggestion: I recommend that these hypotheses are tested using the *in vitro* metabolism experiment. Phase transfer experiments with inactivated microsomes at various concentrations could be used to measure Kgl at different RPMs. Properly designed experiments could address these hypotheses specifically and definitively.

Smith JN, Carver ZA, Weber TJ, Timchalk C. 2017. Predicting transport of 3,5,6-trichloro-2-pyridinol (TCPy) into saliva using a combination experimental and computational approach. *Toxicological Sciences*. 157(2): 438-450. DOI: 10.1093/toxsci/kfx055.

Carver ZA, Han AA, Timchalk C, Weber TJ, Tyrrell KJ, Sontag RL, Luders T, Chrisler WB, Weitz KK, Smith JN. 2018. Evaluation of Non-invasive Biomonitoring of 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Saliva. *Toxicology*. 410: 171-181. DOI: 10.1016/j.tox.2018.08.003.

Raymond S.H. Yang, Ph.D.

Since there seemed to be a lot of discussions on Bayesian analyses, I would like to add the following comments: If there are so many uncertainties of the microsomal enzyme incubation system [e.g., the lipid content of the microsome, mixing rates, unable to repeat experiments (DuPont had closed their labs), lack of data,…etc.], then Kgl becomes a “fudge factor.” If so, is

it meaningful to go through Bayesian analyses using such sophisticated computational technique as Markov Chain Monte Carlo (MCMC) simulations?

Yiliang Zhu, Ph.D.

No response provided

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An analysis provided in Supplemental Material B of Ramboll (2020) demonstrates that estimates of the metabolic parameter Km depend strongly on the value of Kgl. Two approaches were used to estimate the value of Kgl:

a) the measured Kgl was increased by (500/60), the ratio of mixing speeds in the metabolic experiments vs. Kgl experiments, yielding Kgl = 0.2 L/h; and

b) a Bayesian analysis used to estimate Kgl from the metabolic data yielded a mean Kgl = 0.22 L/h.

Question 3.
Given the two-compartment in vitro model structure, please comment on the two approaches for estimating Kgl and whether the value obtained is sufficiently reliable to support valid estimates of metabolic parameters and assess the uncertainties in those estimates.

Leslie Z. Benet, Ph.D.

This parameter is to be used in a PBPK analysis. But this is not the prediction, this is an in vitro parameter to be used in making the prediction. It is consistent with the metabolic data and I believe sufficiently reliable.

Jeffrey J. Heys, Ph.D., P.E.

As stated in comments to the previous question, I do not think that increasing Kgl linearly with mixing speeds is reliable. This correction assumes, I think, that mass transport in the bulk liquid is the rate limiting step and since convection dominates transport in the bulk liquid, increased convection increases the transport rate. I think this explanation is incorrect because mass transport in the bulk liquid is unlikely to be the rate limiting step. There is one important counterargument that should be considered. I believe mass transport is limited by solubility of chloroprene in the liquid (low solubility results in small concentration gradients to drive transport) and by a thin film in the liquid phase at the air-liquid interface. The thickness of the film is likely to decrease with increased mixing speed. Therefore, increased mixing rate is likely to increase the mass transport rate – it is unlikely to be linear, however, because solubility, which is not affected by mixing, will also limit mass transport by limiting the concentration gradients in the liquid film.

I have little experience with Bayesian analysis so I will not comment on the reliability of that estimate. However, it is true that Kgl will strongly depend on Km because Km will influence the concentration gradients in the liquid phase, which will influence mass transport.

Jochem Louisse, Ph.D.

I have no expertise in mass transfer of volatile chemicals on air:liquid interfaces, nor in Bayesian analyses, so I cannot provide scientifically substantiated input on this point.

To my opinion, only if the model fit to the data of the new model (with a Kgl) would be significantly better than with the original model (no Kgl, assuming always equilibrium between water and air), one should use the new model to estimate Km and Vmax. I do not know how to statistically evaluate this, but I think it is not adequate to use a model in which 3 parameters are estimated (Kgl, Km, Vmax) that does not provide a significant better fit than a model in which 2 parameters are estimated (Km, Vmax).

If data become available using the appropriate conditions regarding agitation rate (500 rpm) and microsomal protein concentrations to determine a Kgl, this value could be applied in the two-compartment model to estimate Km and Vmax of chloroprene oxidation. I am of the opinion, however, that it is more worth the effort to invest time and resources for experimental work in order to assess toxic metabolite (epoxide) formation and epoxide detoxification in in vitro incubations with tissue fractions (especially of lung, see my comments to later questions) than in obtaining an experimentally derived Kgl.

Annie Lumen, Ph.D.

The two approaches by themselves have their weaknesses; the experimental determinations were too low and required inferential calculations and the second method shows Kgl is not independently identifiable. However, their concordance when taken together provides low to moderate confidence in the reliability of the Kgl estimate. As such, please consider my recommendation stated in Q1b to evaluate the uncertainties in the Kgl estimate and the magnitude of its influence on metabolic parameter estimations (**Tier 1**).

I wanted to note that for the second approach, I was a little confused when the Kgl value of 0.22 L/h estimated for male mouse liver was stated to be used in the re-analysis of metabolism data for all tissues (Pg. 8 of Supplementary Materials B) but elsewhere in the same document Kgl was said to be fixed at 0.45 L/hr in the MCMC analysis (Figure legends for Figures B-6, B-7,B-8 in Pgs. 11,12,13). During the meeting, it was clarified that 0.45 L/hr was not used in the final metabolic parameter estimations. The implications of this as it relates to Figure B-6, B-7, B-8 and estimated parameters needs to be verified (**Tier 2**).

Furthermore, in the meeting I got some clarification on the logic behind the following steps described in Supplementary Material B "The flux of chloroprene between air and media (Kgl) was estimated by fixing the Km in the male mouse liver microsomal study to 1.0 μmol/L and estimating both Vmax and Kgl. Initial testing of the model showed that the male mouse liver had the strongest data upon which to base the Kgl (i.e. steepest slope as low start concentrations). In the estimation of Kgl, the broad distributions reported above for metabolic parameters were retained. The geometric mean of Kgl was retained as a fixed value for the analysis of all the in vitro studies including the male mouse liver which was re-analyzed to estimate Vmax and Km after the Kgl was fixed.".

Since Kgl and Km were correlated, to start somewhere, the Km for the male mouse liver was fixed to 1.0 μmol/L. I do not have the statistical expertise to evaluate the implications of this assumption/fixing of initial value from a statistical point of view. The authors of the report have cited the range of Km values for similar compounds as supportive reasoning for the choice of this value fixing for Chloroprene. I recommend that since this overall process is to estimate the respective metabolic parameters in each tissues/species including Km perhaps it would be useful to at least understand whether and by how much would this initial choice of fixed Km value impact the final metabolic parameter estimations – perhaps a range of values around the 1.0 μmol/L (below and above) be evaluated to see if the initialization of that value carries any considerable impact (**Tier 1**).

Kenneth M. Portier, Ph.D.

Adjusting the Kgl estimate by (500/60), the ratio of mixing speeds in the metabolic experiments vs. Kgl experiments, assumes that the mass-transfer coefficient is proportional to agitation rate. The justification for this assumption is reported to be based on a personal communication.

Recommendation (Tier 2): Perform a literature search to better justify that the mass-transfer coefficient for volatile compounds is likely to be proportional to mixing speeds.

The fact that the Kgl estimate computed using an agitation rate adjustment (0.2 L/h) and the Kgl estimate computed through the Bayesian analysis (0.22 L/h) are similar may only be a fluke. Further analysis may be needed.

The Bayesian analysis consisted of applying Monte Carlo Markov Chain methodology to estimate simultaneously the Kgl, Vmax and Km parameters from metabolism data for the male mouse. A high degree of collinearity was observed between the Km and Kgl estimates in the estimated posterior distribution.

The report indicates that "uninformative priors" were applied for the three parameters. Because data-informed bounds are placed on the some of the prior parameters to avoid assigning probability density to implausible values, these priors should be labeled as "weakly non-informative priors." The Supplemental B report notes that "There is no evidence that the posterior distributions from this analysis were clipped by the use of these lower bounds on the priors." While this is a supportive observation, the sensitivity of the posterior distributions to bounds on prior distributions should be examined, or, if this has already been done, results described more clearly in the report. Furthermore, the lower bounds placed on the prior distributions of Kgl (0.11 L/hr) and Km (0.5 mmol/L) are quite low and below values indicated in the literature review. I agree that there is little evidence that the posterior distributions are impacted by these bounds. Simulations have shown that bounds and indeed the assumed form of the prior distributions can have an impact on the form of the posterior distributions and the associated credible intervals. That impact should be examined and if possible quantified.

Recommendation (Tier 2): Perform a sensitivity analysis on the impact of placing bounds on the range of prior distributions as well as modifying the form of the prior distribution.

The current assumptions are given in Table B-2. Table B-1 suggests that an informed upper bound for ln(km) is closer to -7 than to 5. What is the impact of assuming a priori that Km ~ Log-Uniform (-10, -7) instead of Log-Uniform (-10,5)? What is the impact of assuming a priori that Kgl ~ Log-Uniform (-4,0) instead of Log-Uniform (-3,0)? It is plausible that the lower bound for Kgl is below exp(-3)=0.05 .

I assume above that Log() indicates the natural log. Figure B-3 is confusing since it suggests that Km values range from $0.01=10^{-2}$ to $0.25=10^{-0.6}$ whereas Table B-1 suggests a range from 10^{-5} to $10^{-4.1}$.

Recommendation (Tier 1): Resolve the confusion between the bounds on km provided in Table B-1 and the suggested limits in Figure B-3.

The collinearity between the Kgl and Km estimates is mentioned in Supplement B and illustrated by Figure B-3. But the "confidence" (better labeled as "credible") ellipse plots for the posterior chains show in Figure B-9 and B-10 and in the spreadsheets in Supplement D show that Vmax and Km estimates are also collinear when Kgl is fixed at 0.22 L/hr. It is not clear how the estimated credible ellipse changes if a slightly different value of Kgl is assumed.

In the re-analysis, the a priori distribution assumed for the standard deviation is a Lognormal with mean 1 and standard deviation 1 with the distribution truncated at 0.1 and 100. Lambert et al. 2005 discusses 6 additional ways the a priori distribution for the standard deviation can be specified and illustrates how this assumption can significantly impact MCMC findings and interpretations. The study by Lampert et al. could be used to design a study of the sensitivity of model findings to how the prior distribution of the standard deviation is specified.

Recommendation (Tier 2): Following the approach by Lampert et al (2005), perform a sensitivity analysis to determine how specification of the prior distribution of the standard deviation impacts the estimates of Vmax, Km and Kgl in the re-analysis.

Cited: Lambert PC. Sutton AJ. Burton PR. Abrams KR. and Jones DR. 2005. How vague is vague? A simulation study of the impact of the use of vague prior distributions in MCMC using WinBUGS. Statist. Med. 24:2401-2428.

Kan Shao, Ph.D.

I am generally not very comfortable with how these two approaches were presented. According to the description provided in the document under review, Approach a) is basically a hypothesis proposed by one of the key authors in the team (i.e., Dr. Schlosser), then a Bayesian model was employed to estimate the "Kgl" value based on the data from another study which resulted in a value similar to the hypothesized value in a). In my opinion, this logic chain is disconnected, i.e., using one statistical analysis to justify a biological experimental concept is relatively weak. Therefore, my suggestions to the Ramboll/EPA team are either (1) conducting a lab experiment to verify the "Kgl" value (**Tier 2**) or (2) focusing on Approach b) to perform a more detailed analysis (as suggested in my response to Charge Question 1) to understand the possible value range of "Kgl" with uncertainty and sensitivity (**Tier 1**).

Jordan Ned Smith, Ph.D.

Both approaches estimate approximately the same parameter value. I am not sure if assuming that Kgl is proportional to mixing speeds adequately justifies the ratio without further theoretical basis (e.g. changes in boundary condition, surface area, etc.). The Bayesian approach is outside of my expertise for comment.

Tier 2 Suggestion: As recommended in question 2, measuring Kgl with an experiment specifically designed to assess mixing speed and microsome concentration with inactivated microsomes would be a preferred approach.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

Computationally and numerically, the first approach seemed to have yielded a fudge factor estimate. Even under the physical-chemical plausibility of the effect of mixing rate and microsomal protein, a thorough uncertainty analysis is needed to give confidence to such estimates.

The Bayes approach to estimation is acceptable, the results of the estimates of Kgl, Vmax, and Km obtained in this report are not without concerns, however.

It appears that MCMC was applied in three situations: estimation of mass transfer coefficient on the basis of chloroprene concentrations in the aqueous phase; a re-analysis of male mouse liver (Himmelstein et al 2004), and then re-analysis of all in vitro data (Himmelstein et al 2004; Yang et al 2012). For the first two MCMC analyses little was presented in the report or Supplementary Material B (Supp B) about the underlying kinetics models or MCMC implementation, a significant concern of transparency and reproducibility. The absence of specific details also hinders a critical appraisal of the analyses. Supp B contains more description about MCMC implementation for the re-analysis of all in vitro data, but the level of details remained inadequate and insufficient.

Figure B-1 presents the results for the first case, mass transfer coefficient Klg. The plot is color-coded, but there are no legends. Figure B-1 does not necessarily clarify the experiment with respect to sample size. In the description of the experiment (Supp B p 2), step 1 states a total of 12 vials for each series of tests and step 7 indicates that starting the replication R-15, 5-second contact samples would be replaced by 600-second contact samples; there are at least 9 data points at 600-second contacting point seen in Figure B-1. The “best estimate” of Kgl was 0.024 (sd=0.0054) with a partition coefficient of 0.48 (SD=0.02). “Best estimate” was not defined however. Because this Kgl estimate was too small to be consistent with the metabolic data, Ramboll conducted a re-analysis of male mouse liver data (Himmelstein et al 2004) using MCMC to jointly estimate Kgl, Vmax, and Km and to compare with mixing rate adjusted Kgl estimate. This is the second analysis.

The results for the second case were summarized in Figures B-4 (marginal posterior density plots) and B-5 (model predicted concentration over time). Ramboll took posterior mean as the point estimate for Kgl, Vmax, and Km. Traditionally the posterior mode is preferred over mean because the region surrounding the mode has the greatest probabilities. When a posterior distribution is highly skewed, using the mean as a point estimate is particularly unwanted. From Figure B-4 we can see the best estimate of Km (0.62) and Kgl (0.22) on ln-scale corresponds to -1.5 and -0.48, respectively, is well into the right trail area of the posterior density. Furthermore, within the context of a Bayes posterior distribution, it is more customary to report either the 2.5%-97.5% range (mid-95%-percentile range) or the 95% highest posterior density area. Reporting posterior mean plus minus 2 SD as a confidence interval is not an accepted practice.

Ramboll subsequently conducted the third MCMC, a more formal Bayes estimation of a two-compartment model for all the in vitro data. Its description was inconsistent and confusing. In the section "Re-estimation of In Vitro Metabolism Parameters" of Supp B, it stated first: "[T]he estimated value of Kgl (0.22L/H) was used in a re-analysis of the metabolism data for all tissues, then continued on p9: "[T]he flux of chloroprene between air and media (Kgl) was estimated by fixing the Km in the male mouse liver microsomal study to 1.0 μmol/L and estimating both Vmax and Kgl", and then again: "[T]he geometric mean of Kgl was retained as a fixed value for the analysis of all the *in vitro* studies including the male mouse liver which was re-analyzed to estimate Vmax and Km after the Kgl was fixed."

The items listed below constitute a tier-one recommendation for this as well as other MCMC Bayes analyses.

Tier 1 recommendation:

- Given that the three kinetic parameters Vmax, Km, and Kgl are biologically and statistically dependent, the MCMC analysis must sample data from the joint posterior distribution. This requires specification of the likelihood for the parameters, a prior for each parameter, the joint posterior, and MCMC implementation strategies.
- Supp B failed to describe the model log(u) in the likelihood (Supp B, Eq 1) and the joint posterior distribution. The first step to implement MCMC is to specify the likelihood function where log(u) must be explicit with respect to u=(Vmax, Km, and Kgl), and the kinetic model underlying log(u) should be also specified. A log-normal likelihood is reasonable. Re-parameterization of the kinetic parameters may be useful or even necessary to utilize the fact that a normal likelihood in conjunction with appropriate prior (e.g. non-informative) implies normal posterior for the kinetic parameters.
- A non-informative prior for each kinetic parameter can be specified if an informative prior is not plausible. However, use of the log-normal distribution as a prior for SD is highly unusual, justifications are needed. Common priors for SD include uniform and inverse gamma (ref: Gelman A. Prior distributions for variance parameters in hierarchical models. Bayesian Analysis (2006) 1, Number 3, pp. 515–533).
- MCMC can be implemented most effectively in an iterative fashion as illustrated below:
  a) draw posterior k samples from P(σ |data);
  b) for fixed σ (e.g. the kth sample), draw k samples from the posterior distribution p(Kgl | σ ;data)

c) for fixed σ and Kgl, draw k samples from posterior p(Km | Kgl, σ; data)
d) for fixed σ, Kgl, and Km, draw k samples from posterior p(Vmax | Km, Kgl, σ; data)
e) iterate between steps a)-d)

- The iterative approach above ensures a multivariate posterior distribution resembling what Figure B-3 depicts. Consider presentation of the MCMC results in a joint fashion when feasible.
- Describe the MCMC sampling process and report results in greater details to ensure transparency and reproducibility.
- Describe convergence criteria adopted, including graphic tools such as trace plot.

Additional Clarifications Requested from EPA

After review of the draft post-meeting comment report, EPA had some additional clarifying questions regarding Dr. Zhu's comments on the supplemental EPA uncertainty analysis.

EPA's additional clarifications and Dr. Zhu's responses are listed below:

In response to Charge Question 3, Dr. Yiliang Zhu stated:
"MCMC is most effectively implemented in an iterative fashion as illustrated below:
f) draw posterior k samples from P(σ|data);
g) for fixed σ(e.g. the kth sample), draw k samples from the posterior distribution p(Kgl |σ;data)
h) for fixed σ and Kgl, draw k samples from posterior p(Km | Kgl, σ; data)
i) for fixed σ, Kgl, and Km, draw k samples from posterior p(Vmax | Km, Kgl, σ; data)
j) iterate between steps a)-d)"
A hierarchical Bayesian parameter estimation approach was suggested by Dr. Zhu and others during the panel discussion.

EPA: Could Dr. Zhu provide further explanation (i.e., an explicit statement of the proposed likelihood function and prior distributions) or a reference describing a comparable approach?

Dr. Zhu's response: The normal likelihood function can be either applied directly to the original observation or after taking log-transformed of observation (concentration). Ramboll has alluded to the use of log-transformation (equation #1, Supp. B), which makes sense.

The mean ("mu" or "log(mu)") of the normal distribution is a function of the solution to the differential equations, hence a function of all the kinetic parameters. Reparametrization of these parameters may be necessary to make the mean (or log (mu)) linear in the parameters so that the posterior is more normal-looking.

Closed-form solutions to PBPK is not always available for non-linear kinetics. Numerical approximation might be necessary.

For (reparameterized) kinetic parameters, noinformative prior or normal prior (in conjunction with normal likelihood) are common and work well.

For σ, common prior include non-informative or inverse gamma. (Gelman . Prior distributions for variance parameters in hierarchical models. Bayesian Analysis (2006) 1. No. 3 515-533).

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in general smaller (i.e. 5-10). In my opinion, to assess the relevance of a certain batch of pooled microsomes, one should have information on the conversion of model substrates for that batch (also to allow comparison of results obtained with other batches). Such data on model compounds (for various biotransformation enzymes) are often provided by suppliers of microsomes for the batches they sell. As a control, one should test such (a) model substrate(s) in house to show adequate activity under the applied conditions in house when performing studies to estimate kinetic constants for biotransformation of the chemical of interest. Information on activity of model substrates in the batches used by Himmelstein et al. (2004) and Yang et al. (2012) have not been provided and may not be available for this evaluation. As such, one cannot judge whether the batches that have been used are representative for the metabolic conversion of an average human. It must be noted that a batch obtained from a relatively small pool of donors may provide the same 'average' metabolic conversion rates for certain substrates as a batch obtained from a relatively large pool of donors. See as an example metabolic conversion data reported from a 15-donor pool (https://www.xenotech.com/wp-content/uploads/2020/03/H0604_1010191.pdf) compared to a 200-donor pool (https://www.xenotech.com/wp-content/uploads/2020/03/H2600_1910096.pdf).

Given that no or very limited chloroprene was oxidized by human lung microsomes, one would like to be reassured that the lung microsomes showed activity (e.g. showing activity for a model substrate). To my opinion, such controls are required if these data are to be used in a regulatory setting.

The number of tissue samples (donors) evaluated for 7-ethoxycoumarin conversion (O-deethylation as determined with a fluorometric assay) amounts to 15 and 12, for liver and lung, respectively. All 15 liver samples and 9 of the 12 lung samples showed 7-ethoxycoumarin conversion. As indicated before, there is no general consensus on what number of donors would be required for an adequate representation of the average human.

Recommendation (Tier 2): Assess whether information on metabolic conversion of model substrates are available for the microsomal badges that have been used for the in vitro kinetic studies. These data should then be compared with metabolic conversion data of well-characterized batches of human microsomes (e.g. available by suppliers of these materials). This may provide some insight into whether the microsomal badges that have been used in the original studies can be considered representative for the 'average' human.

Annie Lumen, Ph.D.

As indicated earlier, I don't have experience conducting such assays or have sufficient exposure to the experimental aspect of the field to make an informative comment on appropriateness of the pool sizes or number of tissue samples.

Kenneth M. Portier, Ph.D.

The accuracy of the average liver, lung, and kidney metabolic activity parameters (Vmax, Km and KF) depends on the degree to which the individuals incorporated into the "pool" represent the human population. The precision of the average metabolic activity parameters depends on the inherent variability in metabolic activity among individuals as well as the sample size.

Accuracy:

Himmelstein et al. (2004) and Yang et al. (2012) used pooled samples. These articles do not provide demographic information on microsome donors although Yang et al. (2012) may have provided these demographics in their Supplement A to which I did not have access. Without this information the accuracy or conversely the potential for bias in the average parameter estimates cannot be assessed.

Lorenz et al. (1984) provides demographic information on the individual liver and lung microsome donors. Needle biopsies were taken of the liver from 15 adults aged 22 to 67 of both sexes and needle biopsies were taken of the lung from a different 12 adults aged 32 to 81. It is not clear which of 15 adults were the 10 donors of liver microsomes. The demographic information suggests that the average metabolic activity measured in the liver microsomes and the human lung microsomes are fairly representative of activity in human adults. No information is available for potentially susceptible subpopulations such as children, the elderly, or other demographic subpopulations such as Native Americans and Alaska Natives.

Recommendation (Tier 3): Summarize the donor demographics from the (2012) Supplement A in the model report. Discuss how limited demographics in the sample pool can potentially bias the final parameter estimates.

Precision:

Without some understanding of interhuman variability in microsome activity, the precision of the estimates obtained by Himmelstein et al. (2004) and Yang et al. (2012) is not estimable.

Lorenz et al. (1984) does provide sample sizes and estimated standard deviations along with observed minimum and maximum values for the three enzymes analyzed. With this, the adequacy of the number of donors (samples) can be assessed, provided we first specify the precision required for the underlying parameter.

As an example of how such a sample size calculation might be accomplished, I offer the following:

A sample size determination can be accomplished based on a test of the coefficient of variation, CV, defined as $\eta = \sigma/\mu$ where μ is the true population mean for the metabolic activity being measured and σ is the standard deviation in the population. This CV test is described in Banik et al. (2012) and is referred to in Exponent (2017). The test hypothesis is

H_0: CV =CV_0 versus H_a: CV > CV_0

The test statistic used is from Miller (1991) and depends on an assumption of normality and the asymptotic distribution of the sample CV. Let CVe be the estimated CV. Then the test statistic M defined as $(CVe - CV_0)/S_{CVe}$ has a standard normal distribution where S_{CVe} is defined as the square root of $\{(CVe^4 + 0.5CVe^2)/n\}$. Note that the Banik et al. (2012) paper also describes a test statistic by Sharma and Krishna (1994) that is more robust to the assumption of normality and which can more easily be solved directly for *n*.

This test has the benefit of not having to specify a variance term but to rely only on the estimated value of the CV and the target difference, d_{cv}, between the observed and expected CV. The Miller (1991) test statistics can be solved for *n* similar to what is done for determining sample size when the precision target is based on specification of a target relative error. In this case, with type I error α specified and type II error β (equivalently power=1-β) specified and prespecified CV target of d_{cv}, the sample size is:

$$n = (Z_{1-\alpha/2} + Z_{1-\beta})^2 [CVe^4 + 0.5\ CVe^2]/\ d_{cv}^2$$

For example, assume a type 1 error of 0.05 and that the true population CV is 33% which is approximately the observed CV for the liver enzymes in Lorenz et al. (1984). The sample sizes for specified power and target differences between the true and observed CV are given in Table 1. So, with an *n* of 15, there is a low chance that a difference in CV of 20% or greater might be missed, but a high chance that a CV difference of 5% were missed. If the true CV is closer to 50% (also shown in Table 1) which is approximately the CV of measured lung enzyme activity in Lorenz et al. (1984), there is a high chance that a CV difference of <15% would be missed but low chance that a difference >35% would be missed.

The bottom line of this analysis is that 10 to 12 samples is not likely to produce a very precise estimate of the average liver and lung metabolic activity. A more reasonable sample size in this scenario is closer to 35-40.

The parameter A1 referenced in the preference to this question is derived as the ratio (X/Y = Lung/Liver) of the specific activities of 7-ethoxycoumarin in liver and lung by Lorenz et al. (1984). The sample mean and standard deviation for Lung is given as $\mu_x = 0.0006$, $\sigma_x = 0.0003$, and the sample mean and standard deviation for Liver is given as $\mu_y = 0.418$ and $\sigma_y = 0.157$ all measures in nano-mole product/min/mg protein. The estimated mean for R=X/Y is given as $\mu_x / \mu_y = 0.0006/0.418 = 0.001435$. An estimate of the variance of R can be derived using the Delta method or by a Taylor's series expansion (see http://www.stat.cmu.edu/~hseltman/files/ratio.pdf) as

$$Var(R) = (\mu^2_x / \mu^2_y) [\sigma^2_x / \mu^2_x + \sigma^2_y / \mu^2_y] = (2.0592x10^{-6}) [0.25 + 0.141] = 7.81x10^{-6}$$
$$SD(R) = 2.793x10^{-3} = 0.002793$$

It is unlikely that the distribution of R is anywhere near a Normal (Gaussian) distribution. Still, given these values, it is unlikely that R is close to 1.

Recommendation (Tier 2): Provide an estimate of the standard deviation of A1, compute an approximate confidence interval and use this to discuss the likelihood that A1 is close to 1.

Table 1 Estimated sample sizes for assumed CV of 33% and 50% for combinations of target differences and test power.

Type I Error	0.05
Z_alpha	1.645
Assumed CV	0.33

			Target Difference						
Power	**Type II Error**	**Z_beta**	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**	**35%**
0.9	0.1	1.2816	228	57	26	15	10	7	5
0.8	0.2	0.8416	164	41	19	11	7	5	4
0.7	0.3	0.5244	125	32	14	8	5	4	3
0.6	0.4	0.2533	96	24	11	6	4	3	2
0.5	0.5	0.0000	72	18	8	5	3	2	2
0.4	0.6	-0.2533	52	13	6	4	3	2	2
0.3	0.7	-0.5244	34	9	4	3	2	1	1
0.2	0.8	-0.8416	18	5	2	2	1	1	1
0.1	0.9	-1.2816	4	1	1	1	1	1	1

Type I Error	0.05
Z_alpha	1.645
Assumed CV	0.5

			Target Difference							
Power	**Type II Error**	**Z_beta**	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**	**35%**	**50%**
0.9	0.1	1.2816	643	161	72	41	26	18	14	7
0.8	0.2	0.8416	464	116	52	29	19	13	10	5
0.7	0.3	0.5244	353	89	40	23	15	10	8	4
0.6	0.4	0.2533	271	68	31	17	11	8	6	3
0.5	0.5	0.0000	203	51	23	13	9	6	5	3
0.4	0.6	-0.2533	146	37	17	10	6	5	3	2
0.3	0.7	-0.5244	95	24	11	6	4	3	2	1
0.2	0.8	-0.8416	49	13	6	4	2	2	1	1
0.1	0.9	-1.2816	10	3	2	1	1	1	1	1

References

Banik S, Kibria BMG, Sharma D. 2012. Testing the Population Coefficient of Variation. J. of Modern Applied Statistical Methods. 11(2): 325-335.

Exponent (2017). Sample size calculation or intraspecies variability in OP in vitro inhibition study. Memo from R. Reiss (Exponent) to N. Zinn (U.S. EPA).

Kitz R. and Wilson, IB. 1962. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. J. Biol. Chem., 237: 3245-3249.

Miller EG. 1991. Asymptotic test statistics for coefficient of variation. Communications in Statistics-Theory & Methods. 20: 3351-3363.

Kan Shao, Ph.D.

As mentioned earlier, additional analysis results should be presented to better evaluate if introducing the "Kgl" parameter is appropriate, i.e., how the estimates of Vmax and Km changed before and after including "Kgl" (**Tier 1**).

Regarding the pool sizes for the human microsomes, the estimate results presented in Supplemental Materials A and B demonstrate that the pool sizes are reasonably sufficient to generate adequate parameter estimates with confidence intervals. However, sensitivity analysis on the prior distribution is highly recommended which will be very useful to determine whether the relatively small confidence intervals are resulted from narrow priors or sufficient sample sizes (**Tier 2**).

Jordan Ned Smith, Ph.D.

The question of how many individuals is adequate within a pool to represent the average human is frequently asked in metabolism and modeling studies. Obviously, more is better than less; however, in my experience, there is little scientific consensus as to how big of pool adequately describes an average human. Himmelstein et al. (2004A) reports 10-15 and 5 individuals for liver and lung measurements, respectively. Yang et al. (2012) does not report number of individuals. Lorenz et al. (1984) reports 15 and 12 individuals for liver and lung, respectively. Unless substantial phenotypic differences exist for the enzymes responsible for chloroprene metabolism, these numbers are probably adequate.

Perhaps a better descriptor would be a measure of relevant substrate marker activities from the pooled samples compared to known population distributions. Vendors frequently provide substrate marker activities with purchased samples as a way to compare activities of various enzymes in samples. These measurements would offer evidence from an enzyme activity perspective rather than a number of individuals within the pool perspective and could be used to quantitatively compare how similar/dissimilar samples used to estimate metabolic parameters are to known populations. Unfortunately, substrate marker activities were not reported in any of the references for comparison.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

Briefly, precision and accuracy are two common metrics of the performance of the estimator of these kinetic parameters. The corresponding statistical translation of these two metrics is standard error and bias, respectively. Whereas it is feasible to evaluate precision at the given pool size, it is not plausible, in the absence of adequate knowledge of variability in adult human

metabolic activity, to assess if the tissue donor represents the entire adult human population. Uncertainty analysis is warranted to inform potential bias and variability across human adults.

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Question 5.
Discuss the appropriateness of the data used and the statistical modeling approach with regard to representing average (or mean) adult human, mouse, and rat metabolic parameters. In particular, please comment on whether a sufficient number of microsomal samples (incubations) were analyzed to represent the average values and to characterize metabolic variation across species, sexes, and tissues.

Leslie Z. Benet, Ph.D.

I am not concerned with the validity of metabolic data across species, sexes and tissues. The strange assumptions of the models utilized, as discussed subsequently, are much more relevant than any concern with the values utilized.

During the discussion of this Charge Question and correspondence between committee members on the night of October 5, a reference to species differences based on ontogeny was identified. I made the following comment.
I'm not sure an ontogeny paper is the best way to look at this question. Here is an alternate paper supplied by my co-author Dr. Sodhi. She summarizes the results as follows and I bolded 2E1:

Ohtsuki et al., 2012. Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. Drug Metab Dispos. 2012;40(1):83-92.
•Table 3: Correlation between protein and mRNA expression levels
•Isoform specific correlations:
High correlation: CYP3A4, 2B6 and 2C8
Medium correlation: CYP2C19, 2D6, 3A5/7
Low correlation: CYP1A2, 2C9, 2A6, **2E1**, 4A11 and UGTs
•Table 4: Correlation of enzyme activity to either (A) protein or (B) mRNA expression levels
Correlations between activity and protein levels are better (or equivalent to) the correlation between activity and mRNA expression
The authors point out that CYP2B6 activity has slightly better correlation with mRNA expression (r^2 = 0.904) than with protein levels (r^0.849) but these are pretty similar.
•Figure 4: Correlation of enzyme activity to protein and mRNA levels of CYP3A4
In particular for low activity CYP3A4, enzyme activity correlates better with protein expression (closed circles and solid lines) than with mRNA expression (open squares dotted lines)
Table 6: Correlation of transporter protein to mRNA (no activity measurements were possible) terrible correlations between transporter activity and mRNA, aside from OATP1B1 (r^2 = 0.727)

Jeffrey J. Heys, Ph.D., P.E.

This is not my area of expertise so I will not comment directly on whether a sufficient number of microsomal samples were analyzed. I will, however, express a concern that in Himmelstein et al. (2004), it states, "Human donor demographic information … is available not reported here for the sake of brevity." Clearly, a diverse demographic cohort is important for this study, but I cannot confirm if this cohort is sufficiently diverse.

Jochem Louisse, Ph.D.

I have no expertise in statistical analyses, so I cannot provide insight from the statistical point of view.

My comments regarding pool sizes are given above (comments to question 4). When looking to the data presented in the Himmelstein et al. (2004) and Yang et al. (2012) papers, many data points have been obtained for liver microsomes and mouse lung microsomes, whereas in general fewer data points were obtained for the other conditions.

It is not clear to me whether all data have been obtained from one experiment or whether independent studies have been performed. It would have been of help for the analysis to present an overview of the data points that have been obtained in the Himmelstein et al. (2004) and Yang et al. (2012) papers, e.g. in a Table like this :

		Himmelstein et al. (2004), experiment 1	Himmelstein et al. (2004), experiment 2	Yang et al. (2012), experiment 1	Yang et al. (2012), experiment 2
Female mouse	Liver	... concentrations ... technical replicates per concentration ... time points per replicate			
	Lung				
	Kidney				
Human	Liver				
	Lung				
	Kidney				

I think it is important to have at least data of two independent experiments per type of microsome (so for a certain tissue/species/sex) in order to assess the robustness of the method (the experimental setup).

I think it is adequate to use 3-5 vials per condition within a single experiment. Since I am not a statistician, I cannot scientifically substantiate this.

Regarding concentrations, it is important to have some concentrations (in the liquid phase) below the Km and above the Km in order to allow an adequate estimation of Km and Vmax.

I think one should be cautious to determine kinetic constants of substrate depletion data when substrate depletion is minimal. From that perspective, I would be cautious with determination of kinetic constants for incubations other than with the liver microsomes and with the male mouse lung and male mouse kidney microsomes. If substrate depletion is minimal, metabolic parameters may better be obtained by using a metabolite formation approach.

Recommendation (Tier 1): Make a clear overview of the kinetic data in a Table (see example above) that has been used to derive the kinetic constants for chloroprene conversion. This allows a better assessment as to whether the data, with regard to number of replicates, independent experiments, etc., can be considered as being adequate to provide robust data.

Recommendation (Tier 2): Perform in vitro studies with well-characterized batches of microsomes for which data on metabolic conversion of model substrates are available (provided by vendor). Include one or more of these substrates in the studies (reference chemicals) to assess whether the system works (quality control). I am actually of the opinion that such quality controls should always be included if one generates data to be used in a regulatory setting. From that perspective, this can also be seen as a Tier 1 recommendation.

Annie Lumen, Ph.D.

With regards to the data, my comment is same as in response to Q4. With regards to statistical analysis, please see my comment in response to Q3 pertaining to the logic in the choice of Km and Kgl fixing vs. metabolic parameter estimation.

Kenneth M. Portier, Ph.D.

As pointed out in my comments to question 4, the appropriateness of the data depends on the representativeness of the individuals included in the sample. From Himmelstein et al. (2004) pools of liver microsomes and cytosol were purchased for male B6C3F1 mice, Fischer F344 rats, Wistar rats, and Golden Syrian hamsters, all from Charles River Laboratories of Raleigh, NC. No indication is provided as to the ages of these animals or the number individuals that are represented in the pool.

The human data are from pooled liver microsomes from 15 individuals, lung microsomes from a pool of 5 individuals, and lung cytosol was from a single male. The demographics of these individuals is apparently known to the researcher, but details are not provided in the research paper. Still, one would have to know something about the variability of liver and lung microsome activity from individual-to-individual and whether it varies by age, sex, health status, etc. to be able to address the adequacy of the pool. For humans, the paper by Lorenz et al. (1984) does provide an indication that activity does vary significantly as a fraction of the mean activity and samples of size 15 are sufficient to estimate the true mean to within ±10% for the liver microsomal activity and sample size of 5 is sufficient to estimate the true mean to within ±22%

for the lung microsomal activity. One sample is clearly inadequate to represent lung cytosol unless lung cytosol characteristics do not vary at all from individual-to-individual.

For rats, a pool of 5 individuals is sufficient to estimate the true mean to within ±5% for liver microsomal activity and ±14% for lung microsomal activity. For mice, the values are ±3% and ±7% respectively for liver and lung. It is quite likely that the rat and mouse pools contain adequate samples to provide quite accurate estimates of average microsomal activity.

The 3-5 incubation vials do not measure population variability but address measurement uncertainty – this is pointed out in several of the documents. As a result, the tight fit of the curves displayed in Figure B-5 is not unexpected. It would be expected that in a well-performed laboratory study that measurement uncertainty would be quite small.

In conclusion, I would say that metabolic variation across species, sexes, and tissues is only moderately well characterized in the available documentation. For humans, little evidence is provided that representative individuals have been included in the pools analyzed.

Kan Shao, Ph.D.

From a statistical modeling perspective, my general feeling is that the statistical modeling approach (mainly the Bayesian MCMC simulation method described in Supplemental Material B) was appropriate, and the performance of MCMC simulation (based on a few indicators, such as the posterior sample trace plot and the PSRF values) was adequate to be based on for making inferences. On the other hand, I also would like to point out that the performance of MCMC simulation and consequently the estimation results are closely related to the modeling implementation: (1) using a single level MCMC analysis instead of a hierarchical structure essentially treated the samples from various incubation vials equally and increased the sample size, which may reduce the uncertainty/variability in the posterior sample and facilitate the convergence of posterior sample; (2) the selected prior distributions for the parameters may also have important impact on the resulting estimates. So, my suggestion is to employ additional sensitivity analysis to investigate how the various settings in the MCMC method may impact the results (**Tier 2**).

Jordan Ned Smith, Ph.D.

Statisticians can provide the best commentary on whether the samples used by Ramboll are adequate for the statistical methods applied.

See my comment above regarding the size of the human pool. Due to inbreeding, pool size requirements for rodents are less rigorous than those for humans, as long as the animal model is consistent with those used in the in vivo studies. In my experience, the number of measurements and technical replicates made here to accurately measure the sample are probably adequate. Again, statisticians can offer a more objective evaluation. These measures do not provide information on inter-individual population variability of metabolism, since measurements are made in pooled samples to represent an average mouse or human. Measurements made in tissue

samples from individual mice or humans would provide a better measure of population variability.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

See comments to Q3 and Q4.

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Question 6.
Considering the experimental and computational methods, please comment on the potential order of magnitude and direction of bias of the quantitative uncertainties in the estimated in vitro metabolic rates that may be related to these factors, collectively.

Leslie Z. Benet, Ph.D.

I feel that the assumptions in the Ramboll re-estimation of the in vitro metabolic parameters are reasonably justified except in the most important case. The only differences between the Ramboll analysis and the Yang et al. values in Table S-3 that are relevant to the analysis are the Km liver values in mice and rats. In female mice the Ramboll model Km value is 47% of the Yang value. In male mice the decrease is 55%. In male rats the decrease is 38%, while in female rats there is a 32 % increase. In all of the decreases, the Yang value falls well outside the 95% confidence interval of the Ramboll value. But this Km difference is important since the Ramboll model utilized these values to predict lung Km. Perhaps I am not seeing it, but I do not find any specific comments about these in vitro liver Km differences between the two models, their justification and most importantly their significance in the final model prediction.

Jeffrey J. Heys, Ph.D., P.E.

No comment

Jochem Louisse, Ph.D.

If substrate concentrations applied are both below and above the estimated Km values, and if the decrease of the parent chemical is sufficient (difficult to judge based on the figures presenting the data on a log scale), I think the data are appropriate to reliably determine Km and Vmax values of the reactions. This seems to be valid for the incubations with liver microsomes, but is a bit more difficult to judge for the incubations with mouse female lung microsomes (human lung microsomes not considered, as these data are not used). It must be noted, however, that the fits presented to the female mouse liver data do not seem to be optimal and linear fits to these (log-transformed) data may be more appropriate.

Recommendation (Tier 1): to facilitate assessment of the kinetic data, it would be of help to not only present the data points in graphs but to include these in accompanying tables, allowing better assessment by the reviewers.

Something that is not taken into consideration in the analysis is that kinetic parameters obtained for chemical conversion using in vitro incubations can be largely dependent on the microsomal protein concentration applied. To obtain robust and reliable in vitro kinetic data one must choose a microsomal protein concentration for which a 2-fold increase and a 2-fold decrease in protein concentration provides a 2-fold faster and 2-fold slower conversion, respectively (i.e. to be in the linear range regarding protein concentration). This should be optimized for each chemical and for each type of microsome and is important to obtain high quality in vitro kinetic data that can be used for IVIVE.

The Himmelstein et al. (2004) paper states '*The protein concentration (0.25–3 mg/ml) and duration of incubation (0–60 min) were also optimized during preliminary experiments.*', suggesting that this has been covered. However, no data are available to evaluate this. The Yang et al. (2012) paper does not indicate that conditions regarding protein concentrations have been optimized.

Recommendation (Tier 1): Assess whether in vitro kinetic data of optimization studies are available and include these in the table presenting all in vitro kinetic data. This allows reviewers to assess whether kinetic studies have indeed be performed at optimal conditions.

Annie Lumen, Ph.D.

Below are some areas of uncertainties that stood out in my view based on which I have provided a rough estimate of the magnitude of uncertainties:

- Uncertainty in the data to be sufficiently informative for those low metabolism tissues (including background loss issue)

- Overall uncertainty in IVIVE assumptions, methods, and calculations

- Pool sizes might not be representative of a true population average of a polymorphic metabolic enzyme

- Lack of clarity in the enzyme- (CYP2E1 or CYP2F across species) or metabolic pathway- (1-CEO/2-CEO vs. others) specific IVIVE across species

- Evaluating the worst-case scenario using Kgl value of 0.11L/h instead of 0.22 L/h given the high correlation to Km (**Tier 1**).

- An average value of A1 was chosen to be used from Lorenz et al. 1984. Given that this value is critical for the estimation of the proportional Vmax values in the human lung tissue and that we don't have any data in human lung to verify this estimation, I thought it would be helpful to consider the range values the parameter A1 can take and evaluate its

impact as a part of supplemental uncertainty analysis if seen fit (**Tier 1**). For example, from Table 2 of Lorenz et al. 1984 a range of value for A1 can be derived. Of which the highest bound of A1 value is 0.0083 (0.0013/0.156) which is approximately 6-fold higher than the average value currently proposed to be used (**Tier 1**).

- Based on the description in Pg. 17 of Ramboll report I calculated the metabolic clearance in the lung to be 0.24 L/h/g of protein (0.052*0.00143*1000/ 0.316) but the report indicates an estimate of metabolic clearance of 0.16 L/h/g of protein. I doubt this glitch is real and could be something that I've missed but I thought I'll bring it up since it caught my eye and I'm fine with being proved wrong (**Tier 1**).

Based on these factors I estimate uncertainties associated with IVIVE approaches to be approximately two orders of magnitude in the direction of under-estimating metabolic capacities and therefore the tissue-specific dose-metric.

Kenneth M. Portier, Ph.D.

The question asks about order of magnitude and potential bias in estimation of in vitro metabolic rates and not the extent to which the in vitro estimates are or are not biased estimates of in vivo metabolic rates (the topic discussed in question 5). To answer this question, one really needs to know something about the factors that affect variability in microsome functioning, both among cells of the target tissue and among samples from individuals of the same species.

Since laboratory rats and mice are inbred strains, one would expect that there would be little bias in the estimation of the mean metabolic rates from the pooling of samples from a moderate number of individuals.

For humans, the bias is unknown and unknowable without additional information on how microsome performance differs within and among individuals.

The sensitivity analysis discussed in my comments to Question 2 might be able to answer a part of this question; the part regarding biases introduced by limits on a priori distributions for the metabolism parameters in the MCMC re-estimation exercise.

Kan Shao, Ph.D.

The comparison results presented in Table S-3 in the Supplemental Material A confuse me. First of all, without showing the confidence interval estimates for these parameters, it is very difficult to judge the magnitude of uncertainties in the estimated results obtained using Yang et al (2012) approach (**Tier 1**). Given the differences between the Yang et al (2012) approach and the Bayesian method employed in the present study, my feeling is that the uncertainties in these estimated factors of the current analysis were underestimated. In contrast to the Bayesian hierarchical model structure and the way to model Km of lung and liver in human in Yang et al (2012) study, the single-level modeling and assumption that Km of lung and liver in human are the same will certainly reduce the interval of parameter estimates (i.e., reduce uncertainty) and make posterior sample easier to converge. Consequently, it is likely that the uncertainty

quantified in the present analysis may be underestimated. Additionally, it is not clear why the estimated value of Km_liver and the estimated value of Km_lung of the present analysis presented in Table S-3 in the Supplemental Material A are different given they were assumed to be the same (**Tier 2**).

Jordan Ned Smith, Ph.D.

Several factors contribute to potential bias and uncertainty in the in vitro measurements. Metabolism rates measured with tissues from animals probably do not contribute much bias since they are from the same animal models used in the in vivo assays. Since further details (e.g. age, sex, evaluation of enzyme activities compared to population, etc.) on the human samples are not available, it is not clear if those metabolism estimates are biased. A targeted assay to measure Kgl directly would reduce uncertainty regarding that parameter and resulting metabolism parameters. A sensitivity analysis of the model used to parameterize metabolism parameters would provide quantitative evidence to which parameters are most sensitive for describing the observed metabolism data.

Tier 2: Suggestion: I suggest a sensitivity analysis should be conducted to better identify sensitive parameters.

Raymond S.H. Yang, Ph.D.

To comment on this Charge Question, I need to bring in a close analog, 1,3-butadiene, for comparison.

There is only one chlorine atom difference between chloroprene and 1,3-butadiene; in fact, chloroprene is 2-chloro-1,3-butadiene. Thus, these two chemicals are very close analogs, and, therefore, sharing close physico-chemical properties, and therefore most likely, sharing close pharmacokinetic, metabolic, and toxicological properties.

Csanady et al. (1992) had studied the biotransformation of 1,3-butadiene and its key metabolite, butadiene monoepoxide by hepatic and pulmonary tissues from humans, rats, and mice. These investigators were able to obtain kinetic constants for the oxidation of 1,3-butadiene or butadiene monoepoxide in the liver and lung of humans, mice, and rats [e.g., Vmax for lung microsomes for human, 0.15±0.04 (unit: nmol/mg protein/min), for mice, 2.31±0.26, for rat, 0.16±0.01; Table 1, p. 1147]. Similarly, Kohn and Melnick (2001) presented, among others, lung Vmax values for 1,3-butadiene and epoxybutene (i.e., 1,2-epoxybut-3-ene or butadiene monoepoxide), and revealed a very complex metabolic scheme for mice and rats (Fig. 3, p.296).

Given the above, the questions to ask are:

1) If other scientists (two groups as shown above) were able to obtain such kinetic constants in lung for 1,3-butadiene, and even for the reactive metabolite, butadiene monoepoxide, in 1992 or 2001, why were the industrial scientists at DuPont having problems deriving kinetic constants for lungs in humans and mice for chloroprene?

2) Assuming it is impossible to derive lung kinetic constants for chloroprene because of technical problems, and if a surrogate must be used to derive a ratio of lung metabolic activities between human and animals, wouldn't 1,3-butadiene and butadiene monoepoxide (available at Aldrich Chemical Co. in 1992) be far better surrogates than 7-ethoxycoumarin for the present Project?

The present Inhalation Unit Risks (IURs) are 5.0×10^{-4} per $\mu g/m^3$ for chloroprene and 3×10^{-5} per $\mu g/m^3$ for 1,3-butadiene (IRIS Summaries accessed 9/25/2020 online). I don't know how exactly EPA regulates chemical emissions in the field for a given location such as St. John, LA; I assume that they would consider the local weather condition including wind direction...etc., among other things. However, given the above information, EPA should be regulating chloroprene 17X more stringent than 1,3-butadiene, if both chemicals were emitted at St. John. On page 4 (last line) of Louisiana Environmental Action Network submission (Public submission on Aug 21, 2020). Denka was challenging the NATA/IRIS Chloroprene air emission concentrations of 0.2 $\mu g/m^3$; thus, extrapolate linearly (i.e., 1/5 of IUR exposure), the interpretation would be EPA, in this case, would accept a risk of 1/10,000 extra cancer at St. John, LA which is still fairly "relaxed" when de Minimus value is at 1/1,000,000. It is nevertheless within the bounds of federal regulatory practices as surveyed by Travis et al. (1987).

According to Louisiana Environmental Action Network submission, Denka had repeatedly violated the 0.2 $\mu g/m^3$ limit, often as high as 103 to 467X over the limit in the St. John air (page 5, 2nd paragraph). This is not good for such a reactive chemical!
It is noteworthy that both chloroprene and 1,3-butadiene caused multi-sites tumors away from the inhalation route of entry (i.e., lungs) with similar lowest effective doses for carcinogenicity in mice at 12.8 ppm (NTP, 1998; IRIS Summary) and 6.25 ppm (NTP, 1993; IRIS Summary), respectively. These similarities in toxicological responses suggest: (i) chloroprene, like 1,3-butadiene, does circulating in the body; and (ii) the toxicodynamics of both compounds are similar in vivo.

As a toxicologist and a risk assessor, I would have a very difficult time to deviate the IUR for chloroprene too far from the IUR for 1,3-butadiene (see also my discussion under Methylene Chloride below in Charge Question 14).

References quoted for this section:

Csanady GA, Guengerich FP, Bond JA (1992) Comparison of the biotransformation of 1,3-butadiene and its metabolite, butadiene monoepoxide, by hepatic and pulmonary tissues from humans, rats and mice. Carcinogenesis 13:1143-1153.
Kohn MC, Melnick RL (2001) Physiological modeling of butadiene disposition in mice and rats. Chem. Biol. Interactions 135-136:285-301.
NTP (1993) Toxicology and carcinogenesis studies of 1,3-butadiene (CAS No. 106-99-0) in B6C3F1 mice (inhalation studies). NTP TR 434.
NTP (1998) Toxicology and carcinogenesis studies of chloroprene (CAS No. 126-99-8) in F344 rats and B6C3F1 mice (inhalation studies). NTP TR 467.
Travis CC, Richter SA, Crouch EAC, Wilson R, Klema ED (1987) Cancer risk management. Environ Sci Technol 21:415-420.

Post-Meeting Thoughts:

During the Virtual Meeting, the Ramboll colleagues indicated their strong belief that chloroprene is a completely different chemical from 1,3-butadiene in that chloroprene is highly reactive and it "falls apart" in the body and that's the reason Dr. Himmelstein was unable to obtain human metabolic rate constants such as Vmax and K_M for PBPK modeling. Further, they strongly object the suggestion of using 1,3-butadiene as a surrogate instead of 7-ethoxycoumarin. A letter-to-the-editor and related appendix were provided by Dr. Clewell.

I read all the documents provided by Dr. Clewell; further, through Dr. Clewell's reference list, I obtained and read, respectively, the Munter et al. (2003) and through further searching the literature, an earlier paper from the same group, Cottrell et al. (2001). I also appreciate Dr. Andersen's discussion on the assay systems (email attachment dated October 13, 2020). After much reading and thinking, I am afraid that I am not convinced by Ramboll colleagues' arguments. My thoughts and reasonings are as follows:

1) Table 1 in Cottrell et al. (2001) and Figure 9 and Table 1 in Munter et al. (2003) indicated the existence of quantitative data of enzymatic velocity vs. substrate (i.e., chloroprene) in rats, mice, and humans typical of enzyme kinetic studies. Double reciprocal plots of such data would derive Vmax and K_M for all species studied. I wasn't aware of the existence of these two papers (Cottrell et al., 2001 and Munter et al., 2003) but Ramboll scientists knew them. Why didn't they even mention these studies in their submitted Report? Why didn't they think about using the information therein (or using data from 1,3-butadiene) rather than resorting to use 7-ethoxycoumarin as a surrogate? (**Tier 1** recommendation: Ramboll should use the kinetic information in Cottrell et al. (2001) and Munter et al. (2003) in their chloroprene PBPK modeling. For instance, capturing as many data points as needed, digitally, from the curves in Figure 9 by software such as Getdata-Graph-Digitizer. After obtaining Vmax and Km from double reciprocal plots for chloroprene for this reaction, PBPK model simulations could be done without the issue of using surrogates. Since Cottrell et al. (2001) and Munter et al. (2003) also did comparisons of microsomal enzyme assays with or without epoxide hydrolase (EH) inhibitor, quantitative information on the detoxication of EH was buried in there somewhere to be uncovered. Therefore, I believe that the Ramboll PBPK modeling work might be improved greatly without doing further experiments by simply using the info in these two papers.)
2) The Cottrell et al. (2001) and Munter et al. (2003) are excellent studies including the following efforts: (i) synthesis of chloroprene and its monoepoxide for their experiments (because of unavailability of these chemicals); (ii) elucidation, through enzymatic studies, separation and isolation of target metabolites, and instrumental analyses (HPLC, LC/MS, GC/MS, NMR), comprehensive metabolic pathways of chloroprene; (iii) discussions on species differences, stereochemistry, and a dechlorination pathway.
3) These investigators conducted and published the work Dr. Himmelstein was not able to do at about the same time in early 2000s.
4) The enzymatic incubation was carried out in gastight vials (line 7 under "Metabolism of Chloroprene to **3a,b** and **4a,b**", page 1289, Munter et al., 2003). The incubation time of 30 min at 37˚ C, and the successful subsequent extraction, isolation, and analyses indicated that

the parent compound and the metabolites were stable enough to undergo these experimental procedures.

5) From the careful description of the experimental details and the "Caution" sections for safety (1st time I've ever seen in my career) at the beginning of "Materials and Methods" and "Experimental Procedures" in the Cottrell et al. (2001) and Munter et al. (2003) papers, respectively, these investigators were meticulously aware of the fact that chloroprene and its metabolites were reactive and volatile.
6) The following direct quote from the "Conclusions" of the Cottrell et al. (2001) had reinforced my points stated above in my pre-meeting comments: "…The overall trends in the stereochemistry of the P450 catalyzed epoxidation were similar to those described for the structurally related dienes, butadiene, and isoprene. The fundamental difference between chloroprene and the other dienes is that the chlorine atom leads to the formation of chloro-aldehydes and ketones in addition to epoxides…" Thus, Ramboll scientists should effectively use the 1,3-butadiene pharmacokinetic and metabolism data as additional supporting information in the building of a robust and complete PBPK model for chloroprene. (This last sentence should be considered as a **Tier 1** recommendation).
7) Another important note is that the Cottrell et al. (2001) and Munter et al. (2003) studies are microsomal enzyme studies. They did not investigate the role of glutathione S-transferase (GST) which is a cytosolic enzyme in chloroprene metabolism. GST, being a high capacity low affinity enzyme (Andersen et al., 1987), would serve as an important detoxifying enzyme for chloroprene epoxides as indicated by similar data in 1,3-butadiene metabolism studies (Csanady et al., 1992; Kohn and Melnick, 2000; 2001). (**Tier 1** recommendation: Ramboll scientists should incorporate kinetic information of reactive metabolites detoxication into their PBPK modeling.)
8) The Cottrell et al. (2001) and Munter et al. (2003) studies also reported the GSH detoxication of reactive metabolites of chloroprene as a body's chemical defense system as outlined in Scheme 2 in Cottrell et al. (2001) and Scheme 1 in Munter et al. (2003). These chemical detoxication processes, as other detoxifying enzymatic processes, would certainly impact on the "dose metric" of the Ramboll/Denka PBPK modeling approach. Ramboll scientists should incorporate such detoxication processes into their PBPK modeling. (This last sentence is **Tier 1** recommendation).

Mice are certainly more efficient in converting chloroprene to its monoepoxide. However, such enzymatic efficiency and capability does not stop at this particular biochemical reaction. In Kohn and Melnick (2001), evidence was presented that mice were more than twice as efficient to detoxify the monoepoxide of 1,3-butadiene than the rats which would similarly reduce the internal dose metric of chloroprene that Ramboll/Denka report used as the key argument for the revision of the IRIS risk assessment for chloroprene. Furthermore, using the methylene chloride risk assessment as an example as Ramboll/Denka did, GST is a low affinity but high capacity enzyme whereas Cytochrome P450 2E1 is a high affinity but low capacity enzyme for methylene chloride (Andersen et al. 1987). The same would hold true for chloroprene. This, again, would argue against the accumulation of the monoepoxide (i.e., the dose metric used by Ramboll/Denka).

The above discussions illustrate that the overall toxicokinetics of chloroprene, in vivo, are much more complicated than what the Ramboll Report suggested. Given the cancer clusters reported

near the DuPont/Denka plants, as well as the repeated violations of these plants in their emissions of chloroprene, in one of the Public comments (Louisiana Environmental Action Network, 2020, the EPA would be prudent to examine these issues carefully before making a final decision on Ramboll/Denka's latest request.

References quoted for this section:

Csanady GA, Guengerich FP, Bond JA (1992) Comparison of the biotransformation of 1,3-butadiene and its metabolite, butadiene monoepoxide, by hepatic and pulmonary tissues from humans, rats and mice. Carcinogenesis 13:1143-1153.

Kohn MC, Melnick RL (2000) The Privileged Access Model of 1,3-Butadiene Disposition. Environ. Health Perspect. 108(Supple. 5):911-917.

Kohn MC, Melnick RL (2001) Physiological modeling of butadiene disposition in mice and rats. Chem. Biol. Interactions 135-136:285-301.

Cottrell L, Golding BT, Munter T, Watson WP (2001) In vitro metabolism of chloroprene: Species differences, epoxide stereochemistry and a de-chlorination pathway. Chem. Res. Toxicol. 14:1552-1562.

Munter T, Cottrell L, Golding BT, Watson WP (2003) Detoxication pathways involving glutathione and epoxide hydrolase in the in vitro metabolism of chloroprene. Chem. Res. Toxicol. 16:1287-1297.

Yiliang Zhu, Ph.D.

Bias and uncertainties are determined by a number of factors, including primarily 1) correct-specification of the kinetic models, 2) design of the experiment to solicit data necessary for tease apart biologically dependent kinetic parameters, and 3) statistical estimation methods. Note that statistical methods would not be able to correct for any bias introduced in the kinetic models.

It is unclear whether or not, or how RLOSS was incorporated into the underlying PK model, which guides the MCMC re-estimation of the in vitro metabolic parameters Vmax, Km, and Kgl. Supplement B describes the re-estimation process, but does not provide a description of the PK model. When MCMC failed for female mouse kidney and human lung, RLOSS was then introduced into the analysis. Omission of RLOSS or bias in the estimate of RLOSS would be a source of significant bias for the metabolic parameters, with the order of magnitude comparable to RLOSS under a first order kinetic.

In contrast, the EPA supplement (EPA 2020) describes a two-compartment PK model that incorporates RLOSS, so does the MCMC (EPA, 2020). It is unclear if the same model was used in Ramboll (2020). Synchronization and consistency between the two documents with respect to this model is necessary.

Experimental design can also be crucial for reliable estimation of kinetic parameters. When no data is available near metabolism saturation, it is difficult to get reliable estimate of Vmax and Km separately because the two become non-identifiable. Spreading measurements of tissue concentration over time is also crucial for estimation reliability.
Finally, statistical methods can also introduce bias. Concerns about the MCMC method for re-estimation of all in vitro data have been commented on. See comments to Q3.

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Additional discussion on the estimation of lung metabolic parameters in rats and humans is provided in: "IVIVE for first order metabolic clearance in rat and human lung." However, the metabolic rate parameter values for the human lung were ultimately selected as described in the main report in a subsection entitled "Estimation of chloroprene metabolism in the human lung" because the *in vitro* chloroprene experiments with human lung microsomes showed minimal metabolism.

Question 7.
Please comment on the use of the relative 7-ethoxycoumarin activity in human lung vs. liver tissue to predict the average rate of chloroprene oxidative metabolism in the human lung.

Leslie Z. Benet, Ph.D.

I think this is a useful and reasonable approach.

Jeffrey J. Heys, Ph.D., P.E.

The metabolic clearance rate in the human lung is arguably the most important part of the model. In the "IVIVE for first order metabolic clearance in rat and human lung" section of Supplement C, the report describes how for the rat and human lung only a first-order rate (instead of a Michaelis-Menten rate law) was used for fitting the experimental data. This approach, however, is described as potentially leading to an overestimation of lung metabolism and risk estimate at higher exposures.

The human lung rate parameters (K_m and V_{max}) were, instead based on the value in the liver (K_m) and a mapping of a value in the liver (V_{max}) to the lung using A1. The A1 value was obtained using the specific activity of 7-ethoxycoumarin, a very different molecule from chloroprene with a higher molecular weight and lower solubility. I am reluctant to support this approach and would recommend at least a few alternatives be considered.

Tier 3 Recommendation: The best alternative, in my opinion, is to design experiments on human lung cells that allow K_m and V_{max} to be estimated. This may require a longer experiment, it might require higher concentrations, but these are two of the most important parameters in the model, so it is likely to be valuable. Recommendation (alternative recommendation if the previous recommendation is not feasible, and there are two options in this alternative): One option, which was dismissed in the Ramboll report, is to simply use a linear rate model instead of a Michaelis-Menten (or saturation) rate law. Another option is to continue to use a parameter (A1) for mapping the liver data to the lung, but to determine A1 using a molecule that is more similar to chloroprene if possible.

Jochem Louisse, Ph.D.

To my opinion, the approach used to estimate chloroprene activity in the human lung, using a scaling factor applied to the human liver data (based on the ratio of activity of 7-ethoxycoumarin

O-deethylation obtained in human lung vs liver microsomes) should not be used to estimate metabolic parameter values for chloroprene oxidation in the human lung.

One first concern is that the data on 7-ethoxycoumarin O-deethylation (Lorenz et al., 1984) cannot be evaluated on their quality. Experimental setup can largely affect the outcomes of in vitro kinetic studies and optimization is required to obtain adequate and reliable kinetic data on chemical conversion. One would like to determine kinetic constants in vitro using conditions in which activity is linear with regard to microsomal protein concentration and time. Since the Lorentz et al. (1984) paper only presents activity values in a table, it cannot be assessed whether the underlying data are adequate to determine the reported activities.

Another issue with the approach used is that this would only be a possible valid approach if 7-ethoxycoumarin O-deethylation and chloroprene would be converted by exactly the same enzymes. It is suggested in the documents that this would be the case (CYP2E1-mediated conversion), but 7-ethoxycoumarin O-deethylation has also been reported to be mediated via other enzymes, amongst others CYP1, CYP2B, CYP2D6 and CYP3A4 (see, for example, Table 3 of Oesch et al. (2019); www.doi.org/10.1007/s00204-019-02602-7). Also, chloroprene oxidation is not necessarily only mediated via CYP2E1. In the documents it is indicated that also CYP2F1 plays a role, but possibly also other metabolic enzymes may play a role. In light of these uncertainties, I am of the opinion that scaling metabolism in the human lung using a factor based on reported differences in 7-ethoxycoumarin O-deethylation in human liver vs human lung, is not adequate, and introduces large uncertainties in the PBPK model-based prediction of chloroprene oxidation in the human lung.

Since different (CYP) enzymes may play a role in the conversion of chloroprene in the human liver compared to the human lung, I am also of the opinion that using the Km for human liver as Km for the human lung may not be adequate. If data are available that indicate that chloroprene is indeed converted only (or mainly) by CYP2E1, applying the Km of the liver also for the lung may be acceptable.

Although the obtained scaling ratio based on the 7-ethoxycoumarin O-deethylation data is close to the reported lung/liver ratio of CYP2E1 mRNA, this may just be a fluke, especially since it has been reported that CYP2E1 mRNA levels poorly correlate with CYP2E1 protein levels (Ohtsuki et al., 2012; www.doi.org/10.1124/dmd.111.042259).

Annie Lumen, Ph.D.

Vmax for an enzyme as I understand is a product of an enzyme's catalytic turnover rate and the total enzyme levels available. If CYP2E1 is shown to be the only enzyme metabolizing Chloroprene (please gather sufficient evidence from the literature or other sources to verify this, **Tier 1**) and if 7-ethoxycoumarin is a CYP2E1 specific substrate (please gather sufficient evidence from the literature or other sources to verify this, **Tier 1**) then assuming that Km and the enzyme catalytic rate are the same (please gather sufficient evidence from the literature or other sources to verify this assumption, **Tier 1**) and that the in vitro activity translates well in vivo (please gather sufficient evidence from the literature or other sources to verify this assumption, **Tier 1**) – the ratio of Vmax in human liver and lung for 7-ethoxycoumarin can be

used to estimate the proportional enzyme expression levels between the two tissues (each of the verification criteria stated above are of equal ranking in my opinion to confidently use the 7-ethoxycoumarin relative activity to predict chloroprene metabolism in human lung. Appropriate uncertainty analysis can be undertaken if some of the verification criteria are not met. Overall, I consider this verification exercise and associated uncertainty evaluation to be **Tier 1** recommendation for your consideration). This ratio can then perhaps be used to scale the Vmax for chloroprene oxidative metabolism from human liver to human lung.

Preliminary literature research indicates 7-ethoxycoumarin may be a substrate not only for CYP2E1 but also for other CYPs. If it can be evaluated that 7-ethoxycoumarin is a selective substrate or that any differential expression levels of CYPs that 7-ethoxycoumarin might interact with in the human lung and liver are proportional (the latter might be hard to establish or verify hence I'm marking this as only **Tier 3** information to find in support of this approach), then I would think that the use of this relative term to estimate average rate of chloroprene oxidative metabolism in the human lung would have higher confidence and if not perhaps moderate confidence.

The value A1 calculated based on this approach has been further supported based on its similarity to the reported ratio of total CYP2E1 plus CYP2F1 mRNA expression in the human lung and liver. The validity of using mRNA expression ratio to support enzyme activity ratio was discussed in detail as part of the meeting. Panel members who have expertise in this area provided supporting references that suggests that this could be true for some enzymes but for CYP2E1 the mRNA expression correlates very poorly to its protein expression levels and that protein expression levels are better correlated to CYP2E1 activity than mRNA expression levels (Ohtsuki et al. 2012; Sadler et al 2016). Based on this information my suggestion (**Tier 2**) is perhaps to not rely on the mRNA expression ratios to support the choice of A1 value. And if possible, other approaches be sought or the associated uncertainties in this value be appropriately evaluated (**Tier 2**).

Ohtsuki, S., Schaefer, O., Kawakami, H., Inoue, T., Liehner, S., Saito, A., ... & Terasaki, T. (2012). Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual human liver: comparison with mRNA levels and activities. Drug metabolism and Disposition, 40(1), 83-92.

Sadler, N. C., Nandhikonda, P., Webb-Robertson, B. J., Ansong, C., Anderson, L. N., Smith, J. N., ... & Wright, A. T. (2016). Hepatic cytochrome P450 activity, abundance, and expression throughout human development. Drug Metabolism and Disposition, 44(7), 984-991.

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and experience and I have no comments to add.

Kan Shao, Ph.D.

I have no expertise in this area and thus am unable to comment.

Jordan Ned Smith, Ph.D.

I think there are unproven assumptions being made to conduct these extrapolations. If these assumptions hold true, then this approach is appropriate. However, more evidence is needed to support assumptions.

Any substrate marker activity used for metabolism extrapolations needs to be evaluated to determine if the same enzymes that are responsible for the substrate marker activity also metabolize chloroprene. Yamazaki et al (1996) suggests that cytochrome P450s (CYP) 2E1, 1A2, and 2B6 are primarily responsible for 7-ethoxycoumarin activity in humans. Ramboll (2020) suggests that CYPs 2E1 and 2F are enzymes that "exhibit high affinities for chlorinated alkenes" (Page 23, Paragraph 1). On Page 8, Paragraph 2 of Supplemental Material C, CYP2A6 is also implicated based on its ability to metabolize butadiene, a similar compound to chloroprene. Ramboll does not provide specific experimental data supporting these enzymes role in chloroprene metabolism and assumes these enzymes are involved based on metabolism of similar compounds. Based on the limited evidence provided, I do not think 7-ethoxycoumarin activity is appropriate due to poor enzyme overlap with assumed enzymes involved with chloroprene metabolism.

Tier 1: Key Recommendation: If this approach is going to be used to extrapolate extrahepatic metabolism, I recommend that Ramboll experimentally determine which enzymes are responsible for chloroprene metabolism.

Tier 1: Key Recommendation: I also recommend that a substrate marker activity is then selected based on which enzymes are identified . For example, an alternative substrate marker for CYP2E1 may be chlorzoxazone activity, which is commonly used by vendors to assess CYP2E1 activity in commercially available samples.

Additionally, if there is only one or few major enzymes involved with chloroprene metabolism, I would expect similar Km values across tissues within species. Measured Km values range 0.46-1.72 µM in mice, 0.348-0.841 µM in rats, and 0.316 µM in humans (Table S-3). These measured values may be evidence of several enzymes involved with chloroprene metabolism as discussed on Page 8, Paragraph 2 of Supplemental Material C.

CYP2E1 is an inducible enzyme. If CYP2E1 is the primary metabolizing enzyme of chloroprene, Ramboll should consider the implications of induced levels of CYP2E1 in scenarios of repeated chloroprene exposures to animal models or in human simulations. If CYP2E1 is induced during repeated chloroprene exposures, metabolism estimates could be significantly underestimated. Repeated exposures have not demonstrated significant changes in blood kinetics of chloroprene; however, CYP2E1 levels could be induced in the lung altering proposed dose metrics of interest and not impact overall blood kinetics of chloroprene.

Tier 2 Suggestion: I recommend that CYP2E1 induction be evaluated in lung tissue, if it is determined that this enzyme is primarily responsible for chloroprene metabolism.

Raymond S.H. Yang, Ph.D.

The use of 7-ethoxycoumarin as a surrogate is unnecessary and inappropriate because there are sufficient information available on a close analog, 1,3-butadiene. Furthermore, as discussed below in the "Post-Meeting Thoughts", there were actually availability of kinetic data on chloroprene itself which was not known to this Reviewer and others on the Panel.

The Ramboll/Denka presentation of the IVIVE is too simplistic concentrating on chloroprene disappearance and epoxide formation without consideration of downstream metabolic pathways (Kohn and Melnick, 2000; 2001) and potential chemical chain-propagation reactions related to oxidative stress (Halliwell, 1989). These biochemical and chemical reactions, subsequent to the formation of the monoepoxide, will undoubtedly affect the pharmacokinetics of chloroprene, some probably rather significantly. This might provide explanation to the anomalies to the disproportionate plasma levels as the exposure levels of chloroprene increased (Figs. 3 and 7, Ramboll Report and elsewhere). Several issues are discussed below:

Kohn and Melnick (2001) reported not only complicated metabolic pathways but mass balance of at least 8 metabolites in the mice and rats. In the mice, glutathione S-transferase (GST) catalyzed conjugation reaction of epoxybutene is a major detoxication mechanism. If such a mechanism were taken into consideration for chloroprene, the pharmacokinetics of the "internal dose" (i.e., epoxide) estimated in the mice in the Ramboll Report would undoubtedly be affected. From a different perspective, Kohn and Melnick (2000) proposed a "Privileged Access" of epoxides formed in situ to epoxide hydrolase within the microsomal membrane as a metabolic clearance of 1,3-butadiene and other epoxide-forming chemicals. The absence of such a mechanistic incorporation into a PBPK model for 1,3-butadiene, Kohn and Melnick (2000) reported "…resulted in an order of magnitude overprediction of circulating epoxides regardless of the choice of values for the parameters with conflicting measurements." These findings, on a close analog of chloroprene, reflect the inadequacy of lack of consideration of pharmacokinetics, in vivo, beyond the disappearance of the parent compound, chloroprene, in the Ramboll Report.

Since Ramboll's use of 7-ethoxycoumarin was for the eventual derivation of a "dose metric" for risk assessment of chloroprene, an entirely new angle of biochemical and chemical reactions in the body under oxidative stress need to be discussed. Thus, another probable major impact on toxicokinetics is related to the probable in vivo initiation of propagating chain reactions, such as the reactive species production by lipid peroxidation discussed above through the initial attacks by the chloroprene epoxides and other ROS to cellular membranes (Halliwell, 1989; Schneider et al., 2008; Ayala et al., 2014). Such propagating chain reactions would produce the "ultimate carcinogens", thus the true dose metric. Their different rates of formation, as well as the efficiencies of repair mechanisms, between humans and animals would be the ultimate determinants of the species difference in human risk assessment.

I am bringing in this area of science in for discussion for the following reasons:

a) Many of chloroprene's metabolites are highly reactive oxygen species.
b) "It has long been recognized that high levels of free radicals or reactive oxygen species (ROS) can inflict direct damage to lipids." (Ayala et al., 2014).

c) Lipid peroxidation is a result of lipid damage by free radicals or ROS; once it is initiated, a propagation of chain reactions will take place until termination products are produced (Halliwell, 1989; Ayala et al., 2014). Such reactions are the mechanistic basis for radiation oncology (Halliwell, 1989).
d) A very active research area of carcinogenesis in recent years is the key toxic products of lipid peroxidation; among them, 4-hydroxynonenal (4-HNE) has been the center of attention (Schneider et al., 2008; Ayala et al., 2014). In fact, 4-HNE has been considered as a major bioactive marker of lipid peroxidation and a signaling molecule involved in regulation of several transcription factors related to stress, cell proliferation, and/or differentiation, cell survival, autophagy, senescence, apoptosis, and necrosis (Schneider et al., 2008; Ayala et al., 2014).
e) Both the NTP mouse inhalation chronic toxicity/carcinogenicity studies on chloroprene and 1,3-butadiene (NTP 1993; 1998) reported tumor formation at multiple sites distant from the lung, the route of exposure. It could mean that these reactive chemicals (i.e., chloroprene and 1,3-butadiene) were stable enough to travel to these remote sites and then activated by CYP2E1 in these organs to expoxide to initiate carcinogenesis as suggested by Ramboll scientists during the meeting. However, two other possibilities involving lipid peroxidation propagation of chain reactions are likely: (i) chloroprene or 1,3-butadiene travels to remote sites and attacks lipid in cellular membranes, initiating propagation chain reactions in situ, and the resulting ROS starting carcinogenesis; and (ii) chloroprene or 1,3-butadiene attacks lung cellular lipid and initiating propagation of chain reactions which produce more stable ROS such as 4-HNE which then travels to remote site and initiating carcinogenesis.

I am not advocating undergoing extensive new research to address these issues prior to chloroprene risk assessment. I simply want to point out that the issues involved in chloroprene toxicokinetics and toxicodynamics are much more complicated than the first enzymatic reaction as the Ramboll/Denka approach reflected.

References quoted for this section:

Andersen ME, Clewell III HJ, Gargas ML, Smith FA, Reitz RH (1987) Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. Toxicol. Appl. Pharmacol. 87:185-205.

Ayala A, Muñoz MF, Argüelles S. (2014) Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative Medicine and Cellular Longevity. Volume 2014, Article ID 360438, 31 pages.

Halliwell B (1989) Current Status Review: Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. Br. J. Exp. Path. 70:737-757.

Kohn MC, Melnick RL (2000) The Privileged Access Model of 1,3-Butadiene Disposition. Environ. Health Perspect. 108(Supple. 5):911-917.

Kohn MC, Melnick RL (2001) Physiological modeling of butadiene disposition in mice and rats. Chem. Biol. Interactions 135-136:285-301.

NTP (1993) Toxicology and carcinogenesis studies of 1,3-butadiene (CAS No. 106-99-0) in B6C3F1 mice (inhalation studies). NTP TR 434.

NTP (1998) Toxicology and carcinogenesis studies of chloroprene (CAS No. 126-99-8) in F344 rats and B6C3F1 mice (inhalation studies). NTP TR 467.

Schneider C, Porter NA, Brash AR (2008) Routes to 4-hydroxynonenal: Fundamental issues in the mechanisms of lipid peroxidation. JBC http://www.jbc.org/cgi/doi/10.1074/jbc.R800001200

Yiliang Zhu, Ph.D.

No response provided

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Question 8

Please comment on the possible use of a parallel approach, based on the relative activity of 7-ethoxycoumarin or another marker CYP2E1 substrate, to estimate the rate of metabolism in the rat lung and the human kidney.

Leslie Z. Benet, Ph.D.

If it is hypothesized to work for the lung, it should also work for other tissues, as suggested on page 23 of the US EPA Supplement.

Jeffrey J. Heys, Ph.D., P.E.

As stated previously in my comments, I am concerned about the significant physical and chemical differences between 7-ethoxycoumarin and chloroprene so I would not recommend using relative activity levels for either the lung or kidney (i.e., using A1 and A2), but I also acknowledge that an estimate of relative activity is better than an incomplete model.

Jochem Louisse, Ph.D.

As indicated in the previous comment, the approach (scaling of Vmax) would only be possibly valid if 7-ethoxycoumarin O-deethylation and chloroprene would be converted by exactly the same enzymes, which is not the case.

To my opinion such a scaling approach may work if chloroprene would be metabolized only by CYP2E1 and when data on another specific CYP2E1 substrate would be available for the scaling. These data for another CYP2E1 substrate should preferably have been obtained within the same experiment, as inter-experimental variation can have a large impact on the value obtained for scaling the Vmax of the liver to a Vmax of another organ. Also, scaling of liver data may be possible if data on protein expression of CYP2E1 in the liver vs other organs of interest are available, assuming that maximal velocity in the organs is directly related to the expression of CYP2E1 at the protein level.

The table below provides an example of reported metabolism data of the CYP2E1 substrate butadiene obtained with human liver and human lung microsomes in the same study. The related

scaling factor from that data amounts to 0.13 (91 times higher than the factor obtained based on the 7-ethoxycoumarin O-deethylation data). It must be noted that the reported Km value for butadiene slightly differs between liver and lung microsomes.

CYP2E1 substrate	Kinetic constants liver	Kinetic constants lung	Reference	Scaling factor
Butadiene	Vmax: 1180 ± 40[a] Km: 5.14 ± 2.59 µM	Vmax: 150 ± 40[a] Km: 2.00 ± 0.15 µM	Csanady et al. (1992) www.doi.org/10.1093/carcin/13.7.1143	150/1180 = 0.13

[a] pmol/min/mg microsomal protein

Recommendation (Tier 1):
Perform a literature study to obtain data on the in vitro conversion of CYP2E1 substrates in human liver and human lung tissue fractions (see as example above butadiene), also including other organs of interest. These data may provide insight into whether the derivation of a factor to scale the Vmax obtained with liver microsomes to a Vmax for lung microsomes (and other tissue fractions) may be feasible. If these analyses provide values for A1 that largely differ, this would indicate that this approach is not valid. If these data are limited to do such a comparative assessment, one should be cautious using the approach. In that case, the recommendation below would become a Tier 1 recommendation. In any case, in order to obtain the most reliable estimation of chloroprene oxidation in the lung, I would highly recommend to perform the studies as indicated below.

Perform in vitro biotransformation studies with microsomes and determine the time- and concentration-dependent formation of metabolites (epoxide metabolites), instead of using a substrate depletion approach, to derive kinetic constants for chloroprene oxidation.

Recommendation (Tier 3): It is recommended to determine the detoxification of epoxide metabolites, as the amount of epoxide metabolites available for causing toxicity (DNA binding) depends on both bioactivation and detoxification reactions, which should be described in the PBPK model. In the report, it is indicated that detoxification is faster in humans than in mouse, suggesting that not including this process in the PBPK model may be a conservative approach.

Annie Lumen, Ph.D.

Please see my comments on the parallel approach in response to Q7 in general and in extension to rat lung and the human kidney. For rats it would be good to confirm that other enzymes such as CYP2F does not contribute to chloroprene metabolism (please gather sufficient evidence from the literature or other sources to verify this, **Tier 1**) and if found to be the case please evaluate if CYP2E1 substrate is a good choice to estimate relative activity between tissues in that specific species (**Tier 1**) . My only additional comment is that if the parallel approach is agreed to be appropriate for use in estimating human lung metabolism from liver values then it seems reasonable that the same approach will be applied to other metabolically relevant tissues as well (**Tier 1**).

In my preliminary comments I had indicated that a consensus be reached on what is the lowest limit of metabolism below which we accept that no metabolism needs to be described for that particular tissue (**Tier 2**). During the meeting this was clarified that if by using MCMC analysis Vmax and Km values were found to be identifiable from the low metabolism data then metabolism parameters were derived from that data.

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and experience and I have no comments to add.

Kan Shao, Ph.D.

I have no expertise in this area and thus am unable to comment.

Jordan Ned Smith, Ph.D.

See response to Question 7. Any substrate marker activity used for extrapolations needs to be evaluated for correlation to chloroprene metabolism or evaluated as co-substrates for the same enzyme. If these assumptions are achieved, then this approach could be used to extrapolate Vmax values to extrahepatic tissues within species. An alternative substrate marker for CYP2E1 may be chlorzoxazone activity, which is commonly used by vendors to assess CYP2E1 activity in commercially available samples.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

No response provided

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<u>IVIVE Calculations for Chloroprene</u>

IVIVE extrapolation is summarized in the *Model Parameters* section of the Ramboll (2020) report, with details on scaling factors in Supplemental Material C of Ramboll (2020) and results in Table S-4 of Supplemental Material A. (Calculations are provided in an Excel workbook, Supplemental Material D of Ramboll (2020). The U.S. EPA performed a quality-assurance evaluation of the workbook to assure the calculations are as described in the report text and tables.) Wood et al. (2017) evaluated the ability of IVIVE to predict clearance for oral dosing of a number of pharmaceutical compounds with data in rats and humans and reported a systematic bias towards under-prediction with increasing clearance. However, the Wood et al. (2017) results may not be relevant to chloroprene because of differences in the route of exposure, chemical properties, metabolizing enzymes, and rate-determining processes for the set of compounds analyzed. In particular, Wood et al. (2017) evaluated IVIVE for oral dosing of drugs, but not for

the inhalation of volatile compounds like chloroprene. While, IVIVE for oral exposure to drugs may be more difficult and is subject to additional sources of uncertainty compared to inhalation of volatile compounds due to variability in intestinal absorption and metabolism (Yoon et al., 2012; Liao et al., 2007), analysis of Wood et al. (2017) specifically focuses on predictions of hepatic *clearance* of drugs, for which metabolism in the liver is a significant component. Thus, the analysis of Wood et al. (2017) may be considered relevant to chloroprene since it addresses the ability to predict metabolic clearance via IVIVE, not oral absorption. The U.S. EPA is not aware of a systematic evaluation of IVIVE accuracy like that of Wood et al. (2017) but focused on volatile organic (chlorinated) compounds like chloroprene for the inhalation route.

Question 9.
Please evaluate the choices of extrapolation factors and formulas used for the IVIVE calculations. Please discuss the soundness of the metabolic parameters in Table S-4 as estimates for average adult female and male mice and rats, and average adult humans (combined sexes).

Leslie Z. Benet, Ph.D.

There are some very strange unrealistic assumptions in the Ramboll model, but consistent with PBPK approaches if the model fits the data and provides useful predictions, these assumptions are accepted. The main unusual assumption is that the extensive metabolism in the liver to the hypothesized reactive (toxic species) is irrelevant in predicting toxicity. That is, measured loss of chloroprene to potential reactive metabolites in the blood is irrelevant. Second, it is assumed that the rate of this irrelevant metabolism in the liver predicts the relevant metabolism in the lung. Third, metabolism to the reactive species in the lung is not a function of the amount of the reactive species formed, but rather whether the reactive species is formed in a female mouse versus a male mouse. The Ramboll proposed lung metabolic clearance in the male mouse is more than 8 fold greater than the metabolic clearance in the female mouse (5.1 L/hr/kg male mouse vs 0.62 L/hr/kg calculated from the values in Table S-4). As shown in Fig. 12, page 24, female B6C3F1 female mice exhibit significant greater multistage cancer outcome than male B6C3EF1 mice for a much lower dose metabolized. In contrast female Fisher rats exhibit 32% more metabolism but have about half the multistage cancer outcome as male Fischer rats (although these cancer outcomes are very low for both and not significantly different between sexes). Fourth, throughout the analyses in Ramboll and in the comments of EPA, the investigators seem to downplay that it is clearance that drives formation of the reactive metabolites, wherever they are hypothesized to occur. There are no sensitivity analyses of the clearance predictions in the animal species, with a great concentration on Vmax, although the sensitivity analysis of Km shows exposure dependence. Fifth increasing inhalation exposure results in less than linear increases in arterial blood concentrations in the B6C3EF1 mice as opposed to what one would expect from saturable metabolism. This could suggest that there are other loss pathways or inhibition of uptake that are not considered in the model, which is mentioned but left at that.

On pages 12-13 of the Instructions, the Wood et al. (2017) analysis is discussed. Our Sept 2020 paper referenced in Section I analyzes these data of Wood et al. showing that for 60-80% of the drugs evaluated, it would be impossible to obtain a correct IVIVE outcome since the data violate

the boundary conditions of the relationship between in vivo clearance and the in vivo intrinsic clearance scaled up from in vitro metabolism incubations.

On September 22, Chloroprene PBPK reviewers were informed "that EPA has been saying, including in the charge, that they are not aware of an evaluation of IVIVE of PBPK models for VOCs…Well now there is one" and that Dr. Paul Schlosser will likely address this article in his presentation. I have carefully reviewed this manuscript and communicated via Tracey with Dr. Schlosser. One point of agreement is that although protein binding is a very important consideration in drug metabolism IVIVE, both for systemic measurements and for in vitro metabolic incubations, VOCs have very low if any protein binding and appropriate measures of in vitro intrinsic clearance and the prediction of in vivo intrinsic clearance and total in vivo clearance can be made using measured concentrations. (This should also be considered in my response to Charge Question 2 as to how microsomal protein may be able to facilitate an increase rate of chloroprene mass transfer between the air and liquid phases in the in vitro system.) The Sept 15, 2020 paper of Kenyon et al. in Toxicology In Vitro "Comparison of in vivo derived and scaled in vitro metabolic rate constants for several volatile organic compounds (VOCs)" evaluated how well metabolic rate parameters derived from in vitro data predict overall in vivo metabolism for a set of environmental chemicals for which well validated and established methods exist. Values of VmaxC derived from in vivo vapor uptake studies were compared with estimates of VmaxC scaled up from in vitro hepatic microsomal metabolism studies for VOCs for which data were available in male F344 rats. For 6 of 7 VOCs, differences between the in vivo and scaled up in vitro VmaxC estimates were less than 2.6-fold and only for bromodichloromethane was the in vivo derived VmaxC approximately 4.4-fold higher than the in vitro derived and scaled up VmaxC. I don't disagree with the analysis but question whether it has any relevance to the present chloroprene PBPK prediction. First, it appears that all VOCs are very high clearance substrates for liver metabolism and therefore clearance will be rate limited by blood flow to the liver independent of any measures of VmaxC and Km. This is acknowledged in the Himmelstein, Yang and Ramboll reports. Second as noted above in my response to Charge Question 6, the only markedly changed in vitro metabolic parameter of relevance in the Ramboll analysis compared to the Yang analysis is Km.

In correspondence to the committee and EPA staff after the first day I made the following comments concerning the Kenyon analysis, which included the attachment labeled Fig. 2.

I apologize for continuing to harp on this point, but I need to make you aware of what has been done in the Kenyon et al. analysis. I noticed that the In Vivo VmaxC for 1,1 DCPe was not correctly plotted in Fig. 2 of the recently published Kenyon et al. paper that was sent to the panel by Tracey after we received the other documents. Dr. Kenyon replotted that value and we were all sent the revised Fig. 2 as attached. I rounded-off some of the numbers when I was compiling the data from the Supplementary material of Kenyon et al. as per the attached table. But if I run the regression on my rounded off numbers my regression $Y = 0.020X + 3.45$ ($r^2 = 0.0011$) is very close to the regression on the revised Fig. 2 $Y=0.019X + 3.46$, with a very, very poor r^2. But look at what is being done in Fig. 2. The regression is calculated for 6 VmaxC values and one VmaxC/Km value. That is not valid. Everyone should know that you can't combine different measurements in a regression. And the only point that is close to the one-to-one regression line is the invalid clearance measurement for 1,2 DCP. If you carry out the regression on the 6 VmaxC

values only, the equation is Y = -0.26X + 5.60 (r^2 = 0.182) and it is very obvious from the attached table that the correlation of in vivo VmaxC/Km to in vitro VmaxC/Km is very, very different that the VmaxC in vitro to in vivo correlation. And the poor correlation is not just due to inclusion of BDCM. If you run the regression on the 5 VmaxC in vivo-in vitro numbers excluding BDCM the resulting equation is Y = -0.26X + 6.18 (r^2= 0.076), no change in slope, intercept is 10% higher and the correlation is poorer. As can be seen from the differences of the slopes from 1 and the poor r^2 values, there is no good correlation of in vivo VmaxC with in vitro VmaxC. But as pointed out by Drs. Clewell and Kenyon, looking at all 5 values may be useful. Even though each of the 5 differ by less than 2.6, if you just looked at one set of in vivo and in vitro VmaxC values, say toluene, you would predict an in vivo Vmax value of 18.5 mg/hr/kg for 1,1DCPe rather than the measured 4.35. There is no useful predictable correlation between in vivo VmaxC and in vitro measurements (slope -0.26), it just depends on how many compounds you look at. And since all of the actual values do not differ that much, don't run any in vitro or in vivo studies, just assume VmaxC is always 5.60 mg/hr/kg from the intercept above and every in vivo and in vitro VmaxC value, including those for BDCM, will be within 2.7 fold. Dr. Mel Andersen in response to my analysis above provided the following comment with references.

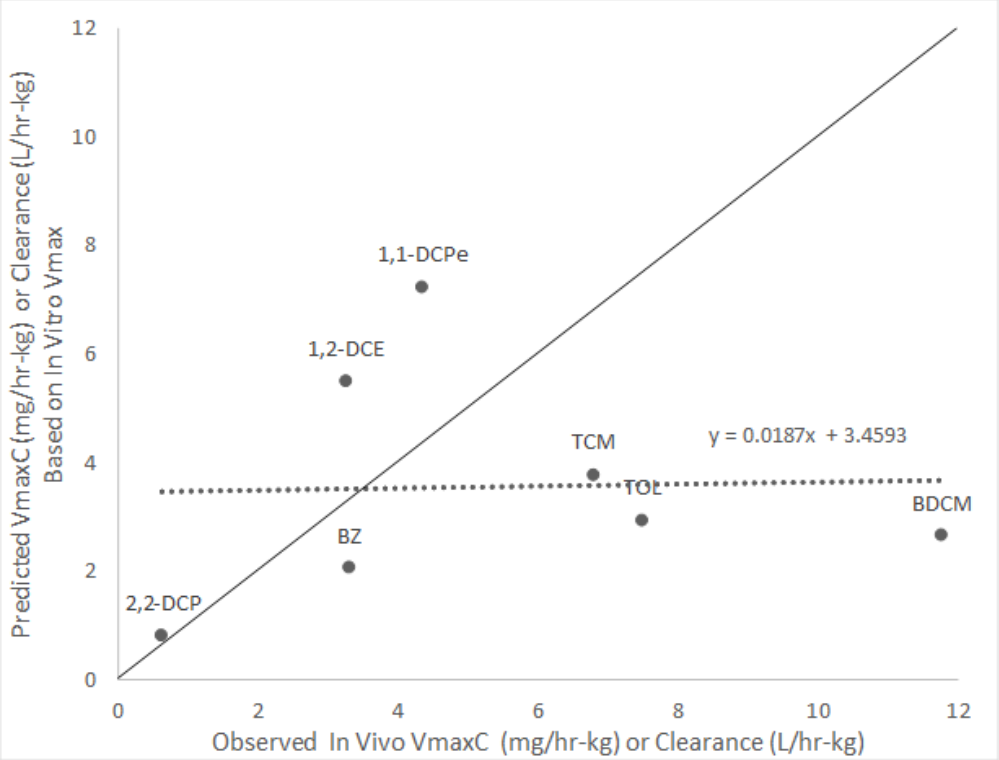

Figure 2. Revised and Rounded-off Parameters from Kenyon et al. 2020.

Rounded-off Kenyon et al. Parameters

VOC	In Vivo VmaxC	In Vivo Km	In Vivo VmaxC/Km	In Vitro VmaxC	In Vitro Km	In Vitro VmaxC/Km
	mg/hr/kg	mg/L	L/hr/kg	mg/hrkg	mg/L	L/hr/kg
1,2 DCE	3.25	0.25	13	5.50	1.40	3.93
BDCM	11.77	0.5	23.5	2.65	2.49	1.06
Chloroform	6.8	0.25	27.2	3.77	0.12	31.4
Toluene	7.5	0.30	25	2.93	0.95	3.08
Benzene	3.3	0.30	11	2.05	0.62	3.31
1,1 DCP	4.35	---	---	7.22	0.28	25.8
2,2 DCP			0.59			0.80

Metabolic parameters for VOCs:

The Vmax and Km values of most of the VOCs we examined fell into a fairly narrow range. We first described the gas uptake system for evaluating kinetic constants of metabolism (Gargas et al., 1986a). This paper discussed three well-metabolized compounds (dichloroethylene, bromochloromethane and diethyl ether) and two poorly metabolized compounds (methyl chloroform and carbon tetrachloride). The Vmax's of the well-metabolized were 20 to 30 µmol/hr and of the poorly metabolized between 0 and 1 µmol/hr. Km values for the well metabolized compounds were 2 to 4 µM. The Vmax's were for a 225-g rat and not weight adjusted as in other studies. Table II from a larger study (Gargas et al., 1988) has kinetic constants for 27 compounds. Of these methyl chloride is a bit misleading since it is not directly metabolized by oxidation (Kornbrust and Bus, 1983) and cis- and trans-dichloroethylene are more difficult to assess because they are suicide inhibitors of CYP2E1 (Lilly et al., 1998). Of the group of well metabolized compounds, the Vmax ranged from about 30 to 80 µmol/hr and the Km from 2 to about 6 µM. Km values tended to be higher with ethanes that had chlorines on both carbons, but the differences though systematic were small. The poorly metabolized group were carbon tetrachloride, tetrachloroethylene, 1,1,1-trichloroethane and hexachloroethane - all compounds lacking a hydrogen on a chlorine containing carbon atom. We also studied various other compounds and published papers that were more targeted to groups of compounds such as the dihalomethanes (Gargas et al., 1986b). Overall, we found that there was little variation of Vmax or Km among the well-metabolized CYP2E1 VOC substrates. We also developed an alternative approach to assessing Km in studies evaluating the inhibition of metabolism in mixtures of two well-metabolized VOCs, 1,1-dichloroethylene and trichloroethylene (Andersen et al., 1987). With the mixtures, the competitive binding constants were estimated as 1.03 and 1.91 µM for 1,1-DCE and TCE, respectively. The original estimate of the Km for 1,1-DCE (Gargas et al., 1986a).

Partition coefficients:

We also cataloged blood:air partition coefficients for blood from rat and human (Gargas et al., 1989). Figure 4 in this paper showed that human blood:air partition coefficients tended to be 1.5 to 2.0-fold greater than rat blood:air partition coefficients. As I mentioned at the meeting, one possibility for the species differences in Pblood:air might be different alkane binding sites on hemoglobin for the two species (Wishnia, 1969). However, no detailed work has probed species differences in these binding sites. Finally, with some VOCs with very low blood:air partition coefficients, achieved blood concentrations at equilibrium/steady state are lower than expected based on measured blood:air partition coefficients. This behavior was evident with hexane where the Pblood:air was about 2.3 while the achieved blood/air ratio was closer to 1.0 (unpublished results presented at an Air Force conference in Dayton, Ohio). With some poorly soluble siloxanes – hexamethyldisiloxane and octamethylcyclotetrasiloxane – we relied on optimizing the value of Pblood:air based on the time-course data rather than direct measurement in of the parameter in vial equilibration experiments. This behavior of the Pblood:air might explain the discrepancy in the predictions of blood chloroprene rather than posing much larger liver blood flows.

Why do we find he similar values for Vmax and Km?

The catalytic region site of CYP2E1 is downstream of a channel guiding the substrate to the active site. Once the substrates gain access, CH bonds are readily oxidized, mostly through second order reaction processes rather than lock and key binding. Vmax would then mostly represent access site accessibility and be limited by the size of the substrate. We investigated Km by examining isotope effect for the oxidative metabolism of deuterated dichloromethane (Andersen et al., 1994). There was a marked isotope effect on Km - nearly 14-fold -but none for Vmax. The specific kinetic mechanism for the isotope effect on Km is not clear. Nonetheless, it apparent that Km is not a conventional binding affinity constant but an aggregate constant related to reaction rate constants associated with breaking the C-H bond.

Andersen, M.E., Clewell, H.J., 3rd, Mahle, D.A., Gearhart, J.M., 1994. Gas uptake studies of deuterium isotope effects on dichloromethane metabolism in female B6C3F1 mice in vivo. Toxicol Appl Pharmacol 128, 158-165.

Andersen, M.E., Gargas, M.L., Clewell, H.J., 3rd, Severyn, K.M., 1987. Quantitative evaluation of the metabolic interactions between trichloroethylene and 1,1-dichloroethylene in vivo using gas uptake methods. Toxicol Appl Pharmacol 89, 149-157.

Gargas, M.L., Andersen, M.E., Clewell, H.J., 3rd, 1986a. A physiologically based simulation approach for determining metabolic constants from gas uptake data. Toxicol Appl Pharmacol 86, 341-352.

Gargas, M.L., Burgess, R.J., Voisard, D.E., Cason, G.H., Andersen, M.E., 1989. Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. Toxicol Appl Pharmacol 98, 87-99.

Gargas, M.L., Clewell, H.J., 3rd, Andersen, M.E., 1986b. Metabolism of inhaled dihalomethanes in vivo: differentiation of kinetic constants for two independent pathways. Toxicol Appl Pharmacol 82, 211-223.

Gargas, M.L., Seybold, P.G., Andersen, M.E., 1988. Modeling the tissue solubilities and metabolic rate constant (Vmax) of halogenated methanes, ethanes, and ethylenes. Toxicol Lett 43, 235- 256.

Kenyon, E.M., Eklund, C., Pegram, R.A., Lipscomb, J.C., 2020. Comparison of in vivo derived and scaled in vitro metabolic rate constants for several volatile organic compounds (VOCs). Toxicol In Vitro 69, 105002.

Kornbrust, D.J., Bus, J.S., 1983. The role of glutathione and cytochrome P-450 in the metabolism of methyl chloride. Toxicol Appl Pharmacol 67, 246-256.

Lilly, P.D., Thornton-Manning, J.R., Gargas, M.L., Clewell, H.J., Andersen, M.E., 1998. Kinetic characterization of CYP2E1 inhibition in vivo and in vitro by the chloroethylenes. Arch Toxicol 72, 609-621.

Wishnia, A., 1969. Substrate specificity at the alkane binding sites of hemoglobin and myoglobin. Biochemistry 8, 5064-5070.

Returning to the comments in the Instructions related to Wood et al. (2017), what we are trying to predict in drug metabolism is the in vivo clearance and for very high clearance drugs the actual values of Vmax and Km are irrelevant. However, as noted on page 12 of the Instructions, Wood et al. "reported a systemic bias toward under-prediction with increasing clearance". We also addressed this issue in our Sept 2020 paper and suggested that this underprediction for high clearance compounds could be explained by recognizing that actual hepatic flow in the well-stirred model utilized to predict in vivo clearance from in vitro methods may not be the flow rate limiting clearance that could be potentially 2.5 fold greater than measured.

Jeffrey J. Heys, Ph.D., P.E.

I have limited experience with PBPK modeling, but I did not see anything that concerned me in reviewing the extrapolation factors and formulas.

Jochem Louisse, Ph.D.

Extrapolation factors

For tissue scaling, limited data are available in the literature on microsomal protein yield for organs other than the liver. Based on the limited data available, I am of the opinion that the values for microsomal protein yield used for tissue scaling are valid for liver, lung and kidney, as substantiated by the literature study as presented in Supplemental Material C. As the model outcome of interest (mg chloroprene metabolized/day/g lung) largely depends on the value used for microsomal protein yield in the lung (not presented in the sensitivity analysis, since scaling is

not part of the model code (model code in Supplemental Material E)), especially the value used for microsomal protein yield in the lung is important. The document states that for all species the same value (20 mg protein/g tissue) is used for microsomal protein yield in the lung. Since the model will be applied for assessing the relative difference between mice and humans, and no difference in microsomal protein yield between mouse lung vs human lung is expected (?), the exact value chosen is of minor importance.

To my opinion, scaling of in vitro data to values applied in the PBPK model is in general adequate. However, possible differences in the free fraction available for metabolism in vitro (microsomal incubations) vs the free fraction available in vivo (unbound blood concentration in liver) are not taken into account. As protein binding is expected to be limited for chloroprene, corrections may not significantly affect prediction and therefore not be required.

Annie Lumen, Ph.D.

The formula as shown in Eq. 1 in Pg. 5 of Supplemental Material C for IVIVE from microsomes looks okay to the best of my knowledge. The choice of extrapolation factors particularly the mg microsomal protein per gram of tissue to represent average values in each tissue and species also seem reasonable and well justified. Given that quality assurance has been done for the IVIVE calculations I don't have any comments on the absolute values determined in the current analysis in Table S-4. One thought that I had when comparing the values between the current analysis and the Yang et al. 2012 was: In Pg. 8 of Supplementary Material B, when using the Kgl approach and parameter, it was shown that the estimated Km values for male mouse liver were halved from Yang et al. 2012 value of 1.34 µM to 0.62 µM in the current analysis. In my preliminary comments I wondered why such a drastic difference in Km is not evident in other tissues or species. This was also raised by another panel member and it was concluded that it is not clear why exactly this is the case and that this could possibly be a function of the Bayesian analysis and the data informing it. Such open-endedness was a little unsettling. Therefore I think it is worth exploring (as a **Tier 3** suggestion) to gain some understanding of the effect of Kgl on final metabolic parameter estimates or at the very least to have a grasp on why some numbers turn out to be the way they are and if that is reasonable.

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and experience and I have no comments to add.

Kan Shao, Ph.D.

I have no expertise in this area and thus am unable to comment.

Jordan Ned Smith, Ph.D.

Methods and parameters used for IVIVE seem appropriate and are consistent with many PBPK models in the field.

Raymond S.H. Yang, Ph.D.

Professor Benet's recent publication (Benet and Sodhi, 2020) on the theoretical basis for in vitro - in vivo extrapolation (IVIVE) and providing directions for future experimental pathways to improve IVIVE practices is comprehensive and reflects the current state-of-science. This paper included the re-analyses of the datasets of drugs from Obach (1999), Wood et al. (2017) and Riccardi et al. (2019); it also offered point by point discussions on the parameters relevant to IVIVE. In the Ramboll/Denka Public Comment/Report (2020), their scientists dismissed EPA's use of Woods et al. (2017) paper for evaluation of the IVIVE approach presented by Ramboll/Denka as irrelevant because the chloroprene studies were inhalation exposure. I would urge Ramboll/Denka scientists to study the Benet and Sodhi (2020) paper seriously, particularly regarding the probable involvement of transporters and protein binding. Once again, this goes back to my continued emphasis on the consideration of the global picture of the in vivo metabolism and pharmacokinetics rather than focusing only on one enzymatic reaction in such an important process as human risk assessment impacting Public Health. I would also urge the Ramboll/Denka scientists to pay particular attention to the repeatedly mentioned 2-3 fold discrepancies in IVIVE in the Benet and Sodhi (2020) paper.

References quoted for this section:

Benet LZ, Sodhi JK (2020) Investigating the theoretical basis for in vitro – in vivo extrapolation (IVIVE) in predicting drug metabolic clearance and proposing future experimental pathways. Journal???: In press.

Obach RS (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: an examination of in vitro half-life approach and nonspecific binding to microsomes. Drug Metab. Dispos. 27:1350-1359.

Ramboll/Denka Public Comments/Report dated August 2020. Comments on the technical materials for the external peer review of a report on physiologically based pharmacokinetic (PBPK) modeling for chloroprene and a supplemental analysis of metabolite clearance (July 2020).

Riccardi KA, Tess DA, Lin J, Patel R, Ryu R, Atkinson K, Di L, Li R (2019) A novel unified approach to predict human hepatic clearance from both enzyme – and transporter – mediated mechanisms using suspended human hepatocytes. Drug Metab. Dispos. 47:484-492.

Wood FL, Houston JB, Hallifax D (2017) Clearance prediction methodology needs fundamental improvement: Trends common to rat and human hepatocytes/microsomes and implications for experimental methodology. Drug Metab. Dispos. 45:1178-1188.

Yiliang Zhu, Ph.D.

No response provided

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PBPK Model Structure, Physiological Parameters, and Partition Coefficients

Question 10.
Please discuss the appropriateness of the PBPK model structure presented by* Ramboll (2020) *for estimation inhalation dosimetry in an EPA Toxicological Review of chloroprene. Please consider in particular the model structure for the kidney, liver, and lung; i.e., tissues in which chloroprene metabolism is predicted by the model.

Leslie Z. Benet, Ph.D.

The fact that Kenyon et al. showed how well metabolic rate parameters derived from in vitro data predict overall in vivo metabolism for a set of environmental chemicals for which well validated and established methods exist seems to be inconsistent with the nonlinearity of arterial blood concentrations in B6C3F1 mice with increasing inhalation exposure as shown in Fig. 3 of Ramboll (2020). I did not examine the referenced studies in detail, but I would expect that the Kenyon analysis of the 7 VOCs was not limited to only one exposure for each VOC.

It appears from page 8 of the Background Description for Chloroprene PBPK Modeling that a key question of the EPA in terms of chloroprene lung metabolism is the Ramboll assumption that the Vmax in the lung is predicted by multiplying the in vitro mouse liver IVIVE Vmax by A1 [Vmax(lung)/Vmax(liver) using 7-ethoxycoumarin in vitro human values]. But in looking at Table S-3, this essentially gives the same results as utilized previously by Yang et al. (2012) based on in vitro data for chloroprene in liver and lung of humans, so I am having difficulty in understanding the issue with respect to Ramboll.

Figure 7 in Ramboll compares the measured chloroprene blood concentrations following 6 hr exposure of B6C3F1 mice to chloroprene at 12.3, 32 and 90 ppm with the Ramboll model predictions. It appears that the model overpredicts the concentrations by about 2-fold. The Instructions state “A factor to consider is the high variability with large standard deviations for many of the data points as illustrated in Figure 3 of Ramboll (2020).” But the counter to that concern is that the Ramboll model is already predicting higher concentrations, not lower concentrations, and these higher concentrations on which to base potentially toxicity issues exceed the standard deviations of the measured data.

During the discussion I noted the finding from our recent IVIVE paper, cited above, that the effective hepatic blood flow of the clearance model analyzing the Wood et al. (2017) data set was about twice the measured hepatic blood flow for very high clearance drugs in humans. On the simplest level for the Ramboll analysis in Fig. 7, I suggested that doubling the liver clearance because of increased apparent blood flow as proposed in my paper would result in decreased predicted chloroprene blood concentrations in female B6C3F1 by half in Fig 7 of the Ramboll report, which would be more consistent with the measured values. This is my only **Tier 3** suggestion.

Jeffrey J. Heys, Ph.D., P.E.

I have limited experience with PBPK model structure, but the model appears consistent with other IVIVE models for inhalation to organic molecules (e.g., Andersen, Clewell et al. (1987)). I would note that according to the IRIS database entry on chloroprene, tumor site(s) include reproductive, ocular, gastrointestinal, and dermal. Adding additional compartments to the model, beyond just the liver, lung, and kidney, could be beneficial.

Jochem Louisse, Ph.D.

The model has been developed by knowledgeable scientists having many years of experience in the development and application of PBPK models for inhalation exposure to volatile chemicals. The general model structure is in line with other PBPK models in the literature for the assessment and description of the kinetics of volatile chemicals upon inhalation exposure.

The overall PBPK model structure is adequate, describing the relevant organ for uptake (lung). Regarding metabolic clearance, inclusion of the liver is of importance. Since intrinsic clearance of the liver is so high, liver clearance is mainly driven by liver blood flow. Therefore, for prediction of blood concentrations of chloroprene (data used for model evaluation), precise estimation of these parameters is not crucial for adequate description of lung dosimetry in the PBPK model, i.e. regarding the in vitro kinetic data, the accurate estimation of parameters for chloroprene oxidation in the lung is most important (as also indicated by the sensitivity analyses).

Lung and kidney are also described as organs in which chloroprene is converted. Conversion in these organs has limited effect on total chloroprene clearance. These compartment are therefore included as target organs to estimate chloroprene oxidation in those organs, assumed to be relevant for the toxicity of the chemical and applied for estimation of species differences in toxicity related to differences in toxicokinetics. I understand from the documents that the PBPK model-predicted chloroprene oxidation in the lung will be used for the species comparison, so I wonder why the kidney has been included as compartment in this model. On the other hand one could argue that all organs in which (primary) tumors have been found in the animal studies should be included. Of course, this would only be relevant if reliable data would be available on metabolic activation in those organs.

The model also includes an adipose tissue compartment, allowing partitioning of the lipophilic chloroprene to adipose tissue. The remaining organs are lumped into a slowly perfused or a rapidly perfused compartment, which is adequate if these organs are not of interest as possible target organs.

The metabolite(s) formed upon chloroprene oxidation is(are) assumed to be unstable and to directly bind to DNA (the term ‘totally consumed’ is used in the report). Since tumors have been observed in organs for which biotransformation is in general assumed to be limited, one may ask the question whether the ultimate carcinogenic metabolite is able to travel in the blood to such tissues (not described in the present model) and/or whether these organs possess biotransformation capacity enabling bioactivation in the tissue itself.

It must be noted that the model only describes total concentrations in blood and tissues and not unbound (free) concentrations. This is in line with various other PBPK models for volatile chemicals in the literature. This has worked when chemical-specific parameter values (such as Km and Vmax) were estimated by fitting these to adequately describe the in vivo kinetic data. In the present case, in vitro kinetic data are used as input for the PBPK model (IVIVE), making the situation a bit different, so one may need to consider whether addition of the description of plasma protein binding (and blood:plasma ratio) would be required for adequate incorporation of the in vitro kinetic data in the PBPK model. In that case, only the free fraction in tissue blood would be available for metabolism (and not the total venous blood concentration as described in the current model). Corrections may then also need to be made taking unspecific binding of chloroprene to the microsomes into account. For chemicals with limited protein binding, effects on predicted kinetics using this approach may be negligible, but it would be of interest to assess whether the current model predictions would change by adding these processes to the PBPK model. This would provide more insight into the uncertainties of the predictions by the current PBPK model.

It must also be noted that no further biotransformation of the toxic metabolites is described in the PBK model, including detoxification reactions. Such reactions are expected to also determine the amount of reactive metabolite binding to the DNA, and there may be species differences in these reactions.

Recommendation (Tier 2): Perform plasma binding studies to provide insight into whether chloroprene plasma protein binding is limited and whether description of plasma protein binding is indeed not needed in the PBPK model, or whether description of plasma protein binding should be included in the PBPK model.

Annie Lumen, Ph.D.

In general, the overall model structure and the parameterization on the physiological parameters were clear and reflective of known physiology and its relevance to chloroprene disposition.

Few points for consideration are as below:

- One minor critique is that throughout the document it was blurry as to for which conclusive enzyme (CYP2E1 or CYP2F across species) and which specific metabolic pathway (1-CEO/2-CEO, vs. others) the IVIVE calculations are attributed to for subsequent parameterization or inclusion in the PBPK model structure and whether or how this differed among the various species and tissues tested.

- Approximately, 36% of mouse body weight and 24% of human body weight doesn't seem to be contributing to chemical disposition. This seems to be a rather large fraction of body weight to remain unperfused. Please verify (**Tier 1**). This might not have a significant impact on the predictions given that the partition coefficient of slowly perfused tissues is not drastic, and neither is it a sensitive parameter but might be worth checking in the interest of model completeness.

- I suppose the assumption here is that all of chloroprene is free of plasma protein binding or erythrocyte partitioning and available for disposition. I couldn't find any reports that suggest either; however, just pointing out that this component is missing in the model though it might be fitting for volatile compounds such as Chloroprene.

- The choice of the amount of metabolites formed per gram of tissue per day as dose-metric is reasonably justified in the report. I initially had concerns about the lack of description of the downstream metabolite clearance as a part of the model structure particularly since species-specific differences in this step has been noted. When I learnt that the epoxide hydrolase pathway might be the only major enzyme-dependent metabolite clearance pathway and that even with enantiomeric selectiveness the clearance rate is faster in the mouse vs. human (liver particularly and by extension to the lung), the proposed dose-metric made sense.

However, in thinking about any scenario in which this dose-metric might not hold to be health-protective, one thought that came to mind is even if the metabolite production rate is low in human lung compared to mouse lung is there a possibility (at least theoretically) for epoxide hydrolase to be saturated at higher exposure levels resulting in 1-CEO metabolite accumulation in human with time? In my preliminary comments, I had suggested a proof of concept modeling evaluation for epoxide hydrolase activity using available literature data as an added component to the current model (**Tier 2**). In the public meeting, when discussing this comment, it was mentioned that 1-CEO might only constitute 4-5% of the total metabolites produced and that other metabolites might be more reactive. Therefore, I'll leave this suggestion here as a lower tiered one for consideration if potential concern for downstream metabolite accumulation is seen to be likely (**Tier 2**).

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and I have only limited experience with the form of PBPK models. From what I have seen in other PBPK models I have reviewed this model displays the expected structure with logical modification to accounts for the specifics of how the body is expected to respond to chloroprene exposures.

Kan Shao, Ph.D.

I have no expertise in this area and thus am unable to comment.

Jordan Ned Smith, Ph.D.

The structure of the chloroprene PBPK model follows a conventional structure based on similar compounds. The model consists of 6 compartments: lungs, liver, kidney, fat, slowly perfused tissues, and rapidly perfused tissues. Absorption occurs through the inhalation of chloroprene. Distribution is assumed to be flow limited. Protein binding of chloroprene to plasma protein is assumed to be weak based on similar compounds. Metabolism is described in the lung, liver, and kidney compartments. I assume that metabolism in the kidney is to predict chloroprene dosimetry in a target organ rather than a major clearance route due to relatively slow kidney metabolic rates compared to the liver. Elimination is facilitated through exhalation or metabolic

clearance. This structure captures the major target tissues and sites of metabolism. Considering the proposed dose metric and known pharmacokinetics of chloroprene, this model structure seems appropriate.

The dose metric selected by Ramboll (amount of chloroprene metabolized in 1 day in target tissues) is probably the best available dose metric given the current assumptions and model structure. In a perfect world, a better dose metric would be some derivative/variant of the actual ultimate toxicant: the epoxide metabolite. The epoxide metabolite will be subjected to detoxification pathways (e.g. epoxide hydrolase) that may differ among animal models and humans. More so, there may be some delivery and loss due to distribution of non-target-tissue-originated epoxide metabolite (e.g. formed in liver and distributed to the lung). Although, due to the reactive nature of epoxides, it could be possible that these additional sources and losses could be relatively small and inconsequential compared to locally formed epoxide metabolite. Regardless, an accurate model of the epoxide metabolite kinetics and respective dose metrics would capture the net result of all these processes and provide a better dose metric for risk assessment.

Tier 3 Future work: I suggest creating a parallel model to account for the epoxide metabolite dosimetry. Creating a parallel model for the epoxide metabolite would require additional experiments, resources, and time to properly develop and parametrize the model.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

No response provided

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Arterial blood concentrations in B6C3F1 mice after inhalation exposures to chloroprene are shown in Figure 3 of Ramboll (2020). In particular, it is noted that when chloroprene exposure was increased 2.5-fold from 13 to 32 ppm, the mean arterial concentration increased less than 1.5-fold. Further, the mean arterial concentrations from 90 ppm exposure, which is seven (7) times higher than 13 ppm, are only about 4 times higher than those measured at 13 ppm. These data might indicate that some process not included in the PBPK model may have reduced chloroprene uptake or somehow increased metabolic efficiency at 90 and 32 ppm relative to 13 ppm. A factor to be considered is the high variability with large standard deviations for many of the data points, as illustrated in Figure 3 of Ramboll (2020). The PBPK model structure implies that blood levels should increase in proportion to exposure as long as blood concentrations remain below the level of metabolic saturation and should increase at a faster rate above saturation, unless there is some other exposure-related change in model parameters. However, the plethysmography data evaluated do not show a clear or significant dose-response Ramboll (2020). Figure 7 of Ramboll (2020) presents the extent of agreement of the model predictions with the blood concentrations in mice following inhalation exposure. It is noted that the

inhalation PK data are from a single exposure (animals were not previously exposed to chloroprene) and the non-proportionality is evident by the 3-hour time-point.

Question 11.
Given these data, please evaluate the likelihood that changes in respiration rate or metabolic induction might be factors in the observed PK relationship between exposure and internal dose. Please comment on any other physiological or biochemical mechanisms that might be explanatory factors in the apparent discrepancy or whether experimental variability in the data may explain these differences.

Leslie Z. Benet, Ph.D.

Yes, changes in respiration rate and induction could increase the possibility of greater toxicity. I have already listed in my response to Charge Question 9 the anomalies in the present model, all of which are ignored in the present model. I do not view the present model as providing the explanations of what is actually the mechanisms leading to the increased toxicity sensitivity in the female mice. It is a model that provides a safety margin concerning what are believed to be potentially toxic responses.

Jeffrey J. Heys, Ph.D., P.E.

No comment.

Jochem Louisse, Ph.D.

I do not have experience with inhalation studies. I would not expect that the relatively low blood levels upon 32 and 90 ppm exposure (compared to the blood levels upon 13 ppm exposure) are due to the induction of metabolic enzymes in this short time frame. Possibly, the model assumption of immediate chloroprene partitioning from air to blood is not valid at relatively high doses and uptake into the blood in the lungs may be limited.

It is of interest to note that the blood concentrations upon 90 vs 32 ppm seem to differ ~ 3-fold (Figure 3 of Ramboll report), as expected with linear kinetics (~ 3-fold difference in the dose). The difference in blood concentrations between the 32 and 13 ppm doses (2.5-fold difference in dose) seems to be only ~ 1.5-fold. From that perspective, one may consider the concentrations in blood at the 13 ppm dose to be relatively high.
I assume that no technical issues related to the quantification of the samples with GC/MS play a role here, but one may consider carefully reanalyzing these data.

Annie Lumen, Ph.D.

Blood flow to the liver is identified as the primary rate limiting step for Chloroprene elimination from systemic circulation rather than liver enzyme-mediated clearance (as shown in Figure 8, Pg. 19, in Ramboll report). As such, it is doubtful whether induction if modeled as further increase in rate of liver-enzyme mediated metabolism will have any substantial impact on reconciling the existing discrepancies.

The variabilities in the experimentally measured respiration rates are rather narrow so I'm not sure if changing that would be the plausible reasoning. Overall experimental variability could be a plausible explanation for the discrepancy but the systematic overprediction of the model with increasing exposures indicates that this might not be the only reason.

Model predictions are only shown as an average value. I realize inter-individual variabilities in laboratory mice might not be drastic. But it might be worth considering a Monte Carlo simulation accounting for confounded parameter variabilities and uncertainties for at least the most sensitive parameters to see if that would be sufficient to explain the observed differences (**Tier 3**).

Based on the current model structure, to understand the plausible mechanistic reasoning, perhaps it might be useful to run a time-dependent sensitivity analysis (particularly between 0-3hrs) to see if any unsuspected change in parameter sensitivities are observed at early timepoints across exposure levels (**Tier 2**). This could only be useful to generate hypothesis challenging the current assumptions of chloroprene uptake (e.g. need for saturable uptake kinetics at higher exposures for example) but might not provide a solution to the discrepancy directly.

Kenneth M. Portier, Ph.D.

Physiological and biochemical mechanisms of action is not my area of expertise.

Kan Shao, Ph.D.

I have no expertise in this area and thus am unable to comment.

Jordan Ned Smith, Ph.D.

It is concerning that the model is overpredicting chloroprene concentrations in blood of higher exposures by ~2-fold causing the observed disproportionalities of chloroprene concentrations in blood compared to the air, although these overpredictions are withing an acceptable range per ICPS PBPK modeling guidance. These overpredictions give the appearance that some important process may be missing.

Several factors could influence these overpredictions. If higher concentrations of chloroprene depressed ventilation rates, I would expect lower predicted concentrations of chloroprene in blood as observed. However, according to Ramboll (2020) Page 13, Paragraph 2, higher chloroprene concentrations in the air did not impact ventilation rates in mice. Given acute exposures and disproportionalities of chloroprene concentrations are observed in as little as 0.5 hr, I would not expect enzyme induction to play a significant role. Saturable plasma protein binding process could result higher unbound chloroprene in blood, faster chloroprene metabolism, and the observed disproportionalities of chloroprene concentrations at higher exposure chloroprene conditions. Ramboll assumes that chloroprene is weakly bound to plasma proteins.

Tier 3: Future work: It may be prudent to experimentally evaluate protein binding.

As far as the question does experimental variability of the data explain these differences: the model predictions appear outside of the standard deviations of the measured data, suggesting model overpredictions are not explained by data variability. A Monte Carlo type analysis using distributions for uncertainty of sensitive parameters could provide confidence interval ranges of model simulations to compare to the variability of the data. This would provide a better assessment of if the model overpredictions can be explained by data variability.

Tier 2 Suggestions: I suggest an uncertainty analysis, such as a Monte Carlo type approach, to quantify model uncertainty. This would allow quantitative evidence to better assess if the model overpredictions are explained by data variability.

Raymond S.H. Yang, Ph.D.

I have comments and a question on the possible changes in respiration rate. The nose only exposure system I was familiar with is basically a tube for each animal with an opening for the nose. The animal, in this case a mouse, is restricted without much movement at all in this tube throughout the experimental period. Therefore, the mouse is under a great deal of stress. This would likely cause changes in respiration rate over time, particularly if the mice have to breath the vapor of a nasty chemical. I didn't see any time-course Plethysmography data in Table S-5 in Supplemental Materials. Are there such time-course data? Does the respiration rate change over the experimental period?

Yiliang Zhu, Ph.D.

The Structure of the PBPK model appears to be reasonable to me. However, the over-estimation of blood concentrations during and following 6-h exposure of B6C3F1 mice to Chloroprene raises questions regarding its reliability of model prediction and robustness toward key kinetic parameters. A systematic approach to sensitivity analysis involving the parameters of this high dimension would be useful. Sensitivity/robustness should be one criterion for selecting the kinetic parameters for prediction purposes (**Tier 2**).

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In *Model Parameters* section of the Ramboll (2020), the authors describe the apparent discrepancy between the rate constant for cardiac output (QCC) from Brown et al. (1997) and other data. The sensitivity of the predicted blood concentration to unscaled cardiac output is shown in Figures 5 and 6 of the report.

Question 12.
Please comment on the analysis presented here and the proposed choice of QCC for the mouse.

Leslie Z. Benet, Ph.D.

I feel that Ramboll adequately addressed this issue and provided support from other VOC studies.

Jeffrey J. Heys, Ph.D., P.E.

Page 8 in Ramboll (2020) provides the discussion for not using the Brown et al. (1997) value for cardiac output. The argument for abandoning the Brown value is acceptable. Essentially, the report argues that the cardiac output value from Brown would result in a physiologically unlikely imbalance between ventilation and blood perfusion. However, the report provides almost no justification for the value that was used instead, relying on a single previous publication.

Jochem Louisse, Ph.D.

Although not within my area of expertise, I feel that this issue is adequately addressed.

Annie Lumen, Ph.D.

The logic of reducing any large discrepancy between ventilation rate and cardiac output and to have them match is reasonable. One recommendation is that since cardiac output value in a mouse model would most likely be a well-studied parameter, a secondary check from a source external to Brown et al. 1997 or the current Ramboll estimates/references could be useful as a confirmation to validate if the selected QCC is reflective of an average cardiac output for mouse models (**Tier 2**).

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and experience and I have no comments to add.

Kan Shao, Ph.D.

It seems to me that the proposed choice of QCC are well supported by the evidence and arguments presented in the report under review, but I have limited knowledge on this topic.

Jordan Ned Smith, Ph.D.

Ramboll (2020) makes a convincing argument for using the alternative QCC. Brown et al. (1997) values would lead to a QPC:QCC ratio of 2.5. Our group has linked QCC and QPC in PBPK models for volatile chemicals using QPC:QCC ratios of 1.20 for rats, 1.6 for male humans, and 1.4 for female humans (Smith, et al. 2020). Assuming a QPC:QCC of 1.45 for mice as previously used by Andersen et al. (1987) seems reasonable and has previous precedent for risk assessment of similar compounds.

Smith JN, Tyrrell KJ, Smith JP, Weitz KK, Faber W. 2020. Linking Internal Dosimetries of the Propyl Metabolic Series in Rats and Humans Using Physiologically Based Pharmacokinetic (PBPK) Modeling. *Regulatory Toxicology and Pharmacology*. 110:104507. DOI: 10.1016/j.yrtph.2019.104507.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

No response provided

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Question 13.
Given the specific considerations above, please comment on the appropriateness of the values selected for the physiological parameters in Table S-1 and partition coefficients in Table S-2, for prediction of chloroprene dosimetry.

Leslie Z. Benet, Ph.D.

I probably don't know enough to comment here, but I found the values reasonable**.**
As stated above I do believe that the model meets the EPA's rule of thumb. I was impressed with the sensitivity analysis depicted in Figure 8 and agree that it appears that these data and specific predictions are not sensitive to the estimated metabolic parameters. Lung dose metrics are sensitive to the estimated metabolic parameters but are apparently within safe limits. As noted above there is no sensitivity analysis of lung metabolic clearance, the driver of potential toxicity, although sensitivity coefficients for Km of the oxidative pathway in the lung are exposure dependent.

Jeffrey J. Heys, Ph.D., P.E.

Other than my concerns expressed in prior comments, I have no additional comments.

Jochem Louisse, Ph.D.

Table S-1:
Brown et al. (1997) is a commonly used source for physiological parameters for PBPK models for chemicals. This suggests that the choice of physiological parameter values is adequate.
It must be noted that the Brown et al. (1997) document contains different values (in different tables) for certain physiological parameters. Table S-1 in Supplemental Material A provides good referencing to the specific Tables of the Brown et al. (1997) document in which the underlying data can be found. One should also realize that the Brown et al. (1997) document is more than 20 years old and newer data may be available.

Table S-2:
I did not find any information in the documents how the partition coefficients have been determined, other than '*The partition coefficients (Table S-2 in Supplemental Materials A) were calculated from the results of in vitro vial equilibration data reported by Himmelstein et al.*

(2004b)'. The data presented in that Himmelstein et al. (2004b) paper do not allow evaluation of the appropriateness of the experiments to determine partition coefficients.

Given the results of the sensitivity analysis, adequate estimation of the Blood/Air partition coefficient is important, especially since, according to Table S-2, Blood/Air partition coefficients differ between mice and humans, directly affecting the PBPK model output of interest (mg chloroprene metabolized/day/g lung).

Although the lung/blood partition coefficient affects the total concentration in the lung, it does not affect the venous blood concentration leaving the lung (CVL, defined as CL/PL, according to model code presented in Supplemental Material E). Given that chloroprene oxidation in the lung is dependent on the CVL, a change in PL hardly affects the predicted chloroprene oxidation in the lung.

Annie Lumen, Ph.D.

Please see response to Q10 about higher proportion of unperfused tissue volumes. Brown et al. 1997 is a very well cited reference for physiological parameters and unless newer research contributed substantially to updating those estimates or there is a reason for any discrepancies as in the case of QCC, I would think it is reasonable for use in PBPK model parameterization.

Tissue-to-blood partition coefficients calculated based on tissue-to-air partition coefficients and presented in Table S-2 seems straight forward and reasonable for use in PBPK model. The use of the partition coefficient value of muscle for slowly perfused tissues and kidney for richly perfused tissues is reasonable. I did want to note that the blood-to-air partition coefficient, a sensitive parameter determining of chloroprene blood concentration, is determined in humans based on a sample size of 3 healthy male adult volunteers (Himmelstein et al 2004 PartII). However, the values do seem to be tight and less variable so I'm not sure if there is any room for uncertainties here but changing this parameter considerably changes the model predictions of blood concentrations (as shown by the sensitivity analysis in Pg. 19 of the Ramboll report). It might be worth verifying that there is no room for uncertainties in the blood-to-air partition coefficient (**Tier 2**).

Kenneth M. Portier, Ph.D.

This topic is outside my area of expertise and experience and I have no comments to add.

Kan Shao, Ph.D.

I have no expertise in this area and thus am unable to comment.

Jordan Ned Smith, Ph.D.

Consistent with many PBPK models, Ramboll cites most physiological parameters from a standard source (Brown et al. 1997). Ramboll uses human physiological parameters that are either male or a blend/central measure of male and female parameters.

Tier 1: Key recommendation: I suggest that male and female physiological parameters are implemented independently to ensure that physiologies of both sexes are adequately considered. ICRP (2002) could serve as a reference for male and female physiologies across various life-stages.

I was surprised to observe significant differences between the partition coefficients of animal models (mice and rat were within 25% of each other) and humans. Human tissue:blood partition coefficients were 15-80% higher than those of rodents (Table S-2). Considering humans have higher lipid content in blood compared to rodents and chloroprene is moderately lipophilic (Log Kow 2.2), I would have expected lower tissue:blood partition coefficients and higher blood:air partition coefficient in humans compared to rodents. In fact, the human blood:air partition coefficient is ~40% less than rodents. Himmelstein et al. (2004 B) measured partition coefficients, providing confidence in the parameter values. Himmelstein et al. (2004 B) observed that these human-rodent relationships were consistent with other volatile organic chemicals (Gargas, et al. 1989); however, it is not clear what drives this. Ramboll (2020) reported that the blood:air partition coefficients were moderately sensitive (sensitivity coefficient <0.5) in respect to the amount of chloroprene metabolized in the lung in mice or humans. Regardless, experimental origins of these parameters provide confidence.

ICRP, 2002. Basic Anatomical and Physiological Data for Use in Radiological Protection Reference Values. ICRP Publication 89. Ann. ICRP. 32.

Gargas, M. L., Burgess, R. J., Voisard, D. E., Cason, G. H., and Andersen, M. E. (1989). Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. Toxicol. Appl. Pharmacol. 98, 87–99.

Raymond S.H. Yang, Ph.D.

I have questions and comments on some parameters in Tables S-1 and S-2 regarding the application of PBPK Modeling to Risk Assessment, specifically regarding populations vs. individuals; thus, these questions/comments are for Ramboll/Denka colleagues, as well as for the EPA colleagues.

First, Human BW of 70 kg (Table S-1). This assignment reflects the past centuries' bias of scientists toward obtaining information on white, Caucasian, males. Since women are not merely "little men," shouldn't there be PBPK model simulations for "Female Humans"? After all, you have rightly provided data on male and female rats and mice.

Second, VFC, the % body fat, for rats and mice were fixed at 10%. While it might be appropriate for the early part of the lives of the animals, wouldn't the animals gaining weight through fat increase because they basically sat around and ate all they wanted to? This is particularly relevant when cancers are usually induced later in their lives, say 12 months or later?
I know that EPA does take into consideration of these issues post-PBPK modeling applications in risk assessment process. However, the above questions basically raise the issue that when we use PBPK modeling for human risk assessment, we really ought to do simulations for different genders, life stages, …etc. in the assessment of internal dose metrics. They should happen at the

stage of PBPK modeling! Since Mel Andersen brought the use of PBPK modeling for risk assessment in 1987 with their work on methylene chloride, it's been 33 years! Shouldn't we think about improving the utility of this technology?

Tier 2 recommendation for EPA: Please take the lead and initiating an effort to improve PBPK modeling for subpopulations taking into considerations of gender, life-stages, and improve physiological parameters to reflect current human data (e.g., 1/3 US adult population are obese).

Yiliang Zhu, Ph.D.

See comments to Q11.

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Overall PBPK Model Soundness and Applicability

Model-predicted doses in model tissue compartments corresponding to tissues in which neoplasm were observed in the rat and mouse bioassay, with corresponding cancer incidence for 80 ppm chloroprene inhalation exposure, are provided in the EPA background document. In potential application to human health risk assessment, the relative risk of tumors in human liver and lung will depend on the relative rate of metabolism predicted in those tissues, compared to the mouse or rat (as well as the relative rate of clearance). Estimation of risks for tissues other than liver and lung could depend on the relative estimates of chloroprene venous blood or tissue concentration. An evaluation of the model's applicability and degree of uncertainty should consider both the absolute model predictions (i.e., does the model accurately predict the absolute rates of metabolism and blood/tissue concentrations in each species?) and also the ability to predict the relative rate of metabolism or relative concentration in human vs. rodent tissues, though some inaccuracy in the absolute values may exist. See "Background for the Peer Review" document for additional context.

Demonstration of the PBPK model's ability to predict *in vivo* PK data is shown by the level of agreement between model predictions and chloroprene venous blood concentrations in Figure 7 of Ramboll (2020). For reference, where there are data, and as a rule of thumb, EPA often seeks dosimetric estimates from a model that are within a factor of two of empirical results. The results of the sensitivity analysis shown in Figure 8 for arterial concentrations indicate that these data and specific predictions are not sensitive to the estimated metabolic parameters: a relatively large range in the estimated metabolic parameters (such as the apparent difference between male and female mouse parameters) would yield similar predictions of blood concentrations. However, as demonstrated in Figure 9, the estimation of lung dose metrics is sensitive to the estimated metabolic parameters.

Question 14.
Please comment on the capacity of the PBPK model to provide sound estimates of chloroprene inhalation dosimetry in mice, rats, and humans. In particular, please comment on the reliability of model predictions of the rate of chloroprene metabolism in liver and lung for use in animal-to-human extrapolation. Please also comment on the reliability and uncertainty of

model predictions of chloroprene concentrations in blood and other tissues from inhalation exposures. Please provide your scientific judgement about the potential order of magnitude of quantitative uncertainty in these estimates.

Leslie Z. Benet, Ph.D.

I will not respond to Charge Questions 14-19. I have no experience or expertise in evaluating uncertainty analyses. I do believe that the PBPK model proposed can provide sound estimates of chloroprene inhalation dosimetry in mice, rats and humans, and that the model allows animal to human extrapolation.

Jeffrey J. Heys, Ph.D., P.E.

I have limited experience with PBPK modeling so I will not comment.

Jochem Louisse, Ph.D.

Although the PBPK model can be considered to adequately predict chloroprene blood concentrations upon inhalation exposure, the fit of PBPK-model predicted blood concentrations to these in vivo data does not necessarily indicate that the rate of chloroprene metabolism in liver and especially lung is adequately described.

It is of interest to note that when omitting liver metabolism in the PBPK model, predicted blood concentrations would be around 2-fold higher, showing a worse fit of the PBPK model predictions to the in vivo data (outside the accepted factor of 2 between model prediction and in vivo data). This may suggest that liver clearance is adequately described in the PBPK model. As noted before, liver clearance is in this model mainly dependent on the blood flow to the liver and description of intrinsic liver clearance can therefore be considered to be adequate.

Omitting lung metabolism in the PBPK model hardly affects predicted blood concentrations, indicating that a possible correct prediction of chloroprene blood concentrations does not provide information whether chloroprene metabolism in the lung is adequately described in the current PBPK model. Therefore, correct description of lung dosimetry (or of any target organ) cannot be evaluated with this in vivo dataset. Therefore, it is of utmost importance that the in vitro data on lung metabolism of chloroprene is reliable. As indicated in responses to questions 7 and 8, this is especially a concern for the human lung data. As indicated before, it would be better to determine kinetic parameters for lung metabolism using a metabolite formation approach. For reactive metabolites these may be quantified using an approach in which the reactive metabolite is trapped, e.g. by DNA nucleotides, as has been shown before for the reactive metabolite 1-sulfooxyestragole (Punt et al., 2007; doi.org/10.1021/tx600298s). Such approaches can be used to predict in vivo adduct formation with the PBPK model, which can be evaluated in animals. Such an approach has been used for the alkenylbenzene estragole (with the ultimate carcinogenic metabolite 1-sulfooxyestragole) (Paini et al., 2012; www.doi.org/10.1093/mutage/ges031).

To my opinion, most uncertainty lies in the estimation of lung dosimetry in humans. Based on the approach presented in the report, the scaling factor A1 (scaling of liver metabolism to lung

metabolism) amounts to 0.00143. A scaling factor based on the metabolism of another CYP2E1 substrate (see question 8) would amount to 0.13, indicating that based on the metabolism data of these different substrates, large differences in PBPK model-predicted chloroprene oxidation in the human lung would be obtained. I do not have the human model code to assess the differences in PBPK model-predicted oxidized chloroprene/day/g lung using these different values for A1.

Annie Lumen, Ph.D.

The comparison of model predictions to the in vivo data of blood concentrations in mice after nose-only exposure of Chloroprene offers a degree of validation to the predictive performance of the PBPK model. However, this only specifically speaks to the ability of the PBPK model to capture inhalation and species-specific physiologies and their influence on the circulating concentrations of chloroprene. Since blood flow to the liver is identified as the primary determinant of the overall elimination of Chloroprene and no influence of tissue-specific metabolism is noted to affect blood concentrations, the predictive evaluation using blood concentration data should not be weighted to validate the extrapolative performance of IVIVE, its related PBPK parameterization, and subsequent overall predictive performance of tissue-dose metrics (parameters that influence blood concentrations have normalized sensitivity coefficients < 0.5 in influencing lung dosimetric) (**Tier 1**).

Based on the model validation using in vivo data, I think the PBPK model is capable of providing reasonable estimates of Chloroprene blood concentrations in the various species tested with high confidence at lower exposures and with moderate confidence at higher exposures since there were some unexplained discrepancies between the model predictions and observations with increasing exposure levels. It is noted in the Ramboll report (Pg. 17) that the model predictions are roughly within a factor of 2 of the means of the experimental data. However, perhaps this needs to be seen in context that the average measured blood concentrations between 13ppm and 90ppm are only 3.8-fold and 5.1-fold apart at 3h and 6h respectively (Table S-6). That said, from a health-protective stand point, an overprediction of blood concentration I suppose in better than an under-prediction. Nevertheless, this (the observed discrepancy in model predictions and observations) is a model uncertainty and needs to be evaluated as such (**Tier 1**).

The rate of chloroprene metabolism in the liver and lung, as we know, is dependent on two components, the metabolic capacities of the tissue and the circulating concentrations of chloroprene presented by tissue flow, equilibrated, and available and for metabolism in that tissue. My preceding comments related to the reliability of PBPK model to predict the latter, which in the case of liver, is more determinant of the amount of metabolite produced. However, for the lung tissue, the amount of metabolite produced is more dependent on the tissue-specific metabolic capacities and therefore the reliability is tied to the uncertainties associated with IVIVE across tissues and species fairly independent of the performance of the whole-body PBPK.

Based on the current model structure, except for the tissues that are metabolically relevant (comments above), I think the PBPK model can reliably predict tissue concentrations accounting for blood concentrations and tissue partitioning. For the tissues that are metabolically relevant but did not have sufficient data to inform metabolic parameters (in respective species), the model

can predict the blood concentrations reaching those tissues but without estimates of tissue-specific metabolism it might not be predictive of tissue concentrations of chloroprene or the associated dose-metric of metabolites formed with high confidence. I recommend caution be exercised when making predictions of tissue-dosimetric (rate of metabolite production) in other metabolically relevant tissues with only blood concentrations (**Tier 1**).

And with regards to the order of magnitude, I would say perhaps no more than one order of magnitude on top of the uncertainties estimated for IVIVE due to the model uncertainty in predicting observations at higher exposure levels and the uncertainties associated with tissues for which metabolism is anticipated but not parameterized.

Kenneth M. Portier, Ph.D.

The capacity of the PBPK model to provide sound estimates of chloroprene inhalation dosimetry in mice, rats, and humans:

Given that predictions within a factor of 2 of the means of the experimental data can be deemed acceptable under WHO/IPCS (2020) guidance, then the model does seem to have the ability to provide sound estimates for mice (the target organism for which observed and predicted blood concentrations are presented in Figure 7.) Conclusions in human would have been better supported if a similar diagram had been presented for rats and that showed that predictions in rats were also withing a factor of 2. Figure 7 does demonstrate that the PBPK model does overpredict blood concentrations, seemingly for all tested concentrations of chloroprene, and that for the highest dose group, predicted results are almost twice observed average. This would have been clearer if the data points had been slightly "jittered" in time on the graph (see for example https://ggplot2.tidyverse.org/reference/position_jitter.html). Also, time is a repeated measurement on each mouse so the dots for each mouse should be connected.

Recommendation (Tier 3): Modify Figure 7 in the Ramboll (2020) report to better illustrate the overprediction of blood concentrations and to illustrate the repeated measurement nature of the data.

The reliability of model predictions of the rate of chloroprene metabolism in liver and lung for use in animal-to-human extrapolation.

A reliable model is one that performs consistently well and whose predictions can be depended upon to be accurate. This analysis attempts to substitute in vitro data on chloroprene metabolism in liver and lung in humans and/or in rodents for in vivo data. The reliability issue is whether the information obtained from in vitro studies of rodent and human liver and lung microsomal uptake of chloroprene reliably predicts the rate of chloroprene metabolism in vivo in rodents and humans. Having only limited rodent in vivo data and no human in vivo data against which to measure predictions, the assessment of reliability is difficult if not impossible.

As mentioned above, this analysis demonstrates the capacity of the approach to produce adequate predictions. I would think that to demonstrate reliability would require demonstrating adequacy

of prediction against measurements in at least one additional experiment designed to challenge the predictions. I am not certain what that experiment should look like.

The reliability and uncertainty of model predictions of chloroprene concentrations in blood and other tissues from inhalation exposures.

The answer to this is similar to the previous paragraph.

Scientific judgement about the potential order of magnitude of quantitative uncertainty in these estimates.

I think this model is capable of predicting rates of metabolism and concentrations in blood and other tissue from inhalation exposures to chloroprene to within 2 orders of magnitude or less.

Kan Shao, Ph.D.

My comments are focused on technical issues related to statistical and quantitative analysis. A few technical issues undermine the overall quality of the PBPK model, including how to justify the necessity and validity of introducing a new parameter "Kgl" to quantify the air and liquid mass-transfer, the lack of detailed analytic results prevent better evaluating the statistical approaches' ability to characterize uncertainty and variability, etc. (**Tier 2**). Therefore, better addressing and more clearly explaining these issues will certainly improve the quality of the report.

Jordan Ned Smith, Ph.D.

The chloroprene PBPK model has the potential to be useful for human risk assessment. Sensitivity analyses identified metabolism parameters, partition coefficients, and a handful of physiological parameters (e.g. ventilation rates, blood flow to liver, cardiac output) as the most important parameters for determining chloroprene internal dosimetry. Most of these parameters have been measured using in vitro assays providing confidence in the parameter values. Mouse to human extrapolation of lung metabolism is based 7-ethoxycoumarin activity assuming that CYP2E1 is the primary enzyme responsible for metabolizing chloroprene. This assumption needs additional evidence (e.g. identification of enzymes involved with metabolism, assess potential of enzyme induction) to support it (see Question 7). The model predicts concentrations in blood from 13 ppm inhalation exposures to mice reasonably well, but overpredicts higher exposures (32-90 ppm) by ~2-fold. No data from repeated chloroprene exposures were provided as evidence of potentially altered kinetics due to enzyme induction. No chloroprene concentration data in tissues was presented. The model was used to extrapolate human exposures of 0.0003 ppm (1 $\mu g/m^3$) or 4.5 orders of magnitude lower than exposures used with mice. This magnitude of differences in exposure creates some uncertainty as well. The model can be used to translate known uncertainties in parameters to simulated dose metrics. Due to integration of many measured aspects of chloroprene pharmacokinetics (e.g. metabolism, portioning, etc.) and physiology (e.g. ventilation rates, body weights, etc.) into a model capability of extrapolating dosimetry across species, and quantitatively integrating uncertainty, this model offers an improved risk assessment tool compared to traditional 10× uncertainty factors.

Raymond S.H. Yang, Ph.D.

The approach Ramboll/Denka adopted using the same strategy which worked successfully for the revision of methylene chloride (dichloromethane) risk assessment left many holes such that the capacity and reliability of the PBPK modeling/IVIVE approach in the present petition are weak at best. The essence of my objection are given below:

1) The total disregard of the metabolic processes downstream from the first epoxidation reaction. I presented the related detail discussions under Charge Questions 6 and 7.
2) Probable Oxidative Stress-Related Chain-Propagation Reactions discussions under Charge Question 7.
3) Problems of Using Methylene Chloride IRIS Risk Assessment to Support the Present Petition in Ramboll/Denka Report

Toxicologically, chloroprene is an entirely different "beast" from methylene chloride. A simple illustration is to compare the dose levels used in the NTP inhalation studies leading to the IRIS risk assessments for the following three chemicals: chloroprene, 1,3-butadiene, methylene chloride.

For chloroprene, NTP (1998) used 0, 12.8, 32, or 80 ppm for B6C3F1 mice 2-year studies.
For 1,3-butadiene, NTP (1993) used 0, 6.25, 20, 62,5, or 200 ppm for B6C3F1 mice 2-year studies.
For methylene chloride, NTP (1986) used 0, 2000, or 4000 ppm for B6C3F1 mice 2-year studies.
There were evidences for carcinogenicity at the lowest level of exposures in all three studies. Thus, a rough estimate (and a good rough estimate because of the care and rigor for the NTP protocol to select doses for chronic toxicity/carcinogenicity studies) revealed that chloroprene is about 2000/12.8 = 156 times more potent a carcinogen than methylene chloride. We haven't even accounted for the many more sites of tumors observed with chloroprene. The above information also indicates that chloroprene and 1,3-butadiene are in the same league (i.e., same "beasts") as far as carcinogenicity goes.

Also, in the case of methylene chloride, the lung tumors were caused by the reactive metabolite formed by the enzyme glutathione S-transferase (GST); thus, it is an intoxication reaction. Whereas, in the case of 1,3-butadiene, GST played very important detoxication roles by conjugating with reactive species (Kohn and Melnick, 2000; 2001). By analogy, GST was highly likely to play a role in detoxication in chloroprene as well,

Given the above discussion, the argument in the Ramboll/Denka Public Comments/Report dated August 2020 "…using the previously accepted approach from the USEPA (2011) IRIS assessment for methylene chloride" to support their petition is shaky.

References quoted for this section:

Kohn MC, Melnick RL (2000) The Privileged Access Model of 1,3-Butadiene Disposition. Environ. Health Perspect. 108(Supple. 5):911-917.

Kohn MC, Melnick RL (2001) Physiological modeling of butadiene disposition in mice and rats. Chem. Biol. Interactions 135-136:285-301.
NTP (1986) Toxicology and carcinogenesis studies of dichloromethane (methylene chloride) (CAS No. 75-09-2) in F344/N rats and B6C3F1 mice (inhalation studies). NTP TR 306.
NTP (1993) Toxicology and carcinogenesis studies of 1,3-butadiene (CAS No. 106-99-0) in B6C3F1 mice (inhalation studies). NTP TR 434.
NTP (1998) Toxicology and carcinogenesis studies of chloroprene (CAS No. 126-99-8) in F344 rats and B6C3F1 mice (inhalation studies). NTP TR 467.
Ramboll/Denka Public Comments/Report dated August 2020. Comments on the technical materials for the external peer review of a report on physiologically based pharmacokinetic (PBPK) modeling for chloroprene and a supplemental analysis of metabolite clearance (July 2020)

Yiliang Zhu, Ph.D.

Overall the PBPK model (Ramboll, 2020) appear to be flexible and capable for predicting tissue specific dosimetry (oxidative metabolites). It remains critical to calibrate the model for different scenarios (species, tissue, gender, etc.) given that many of the key kinetic and metabolic parameters can influence prediction accuracy and precision. A comprehensive analysis of sensitivity is highly valuable in order to better understand the order of variability and uncertainty. EPA is developing schemes for assessing uncertainties. (**Tier 3**) It is important to assess the cascading effect and propagating effect of kinetic parameters on metabolic parameter, reaching eventually downstream to influence prediction of tissue specific dose. This remains an area of further research that PBPK models face.
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Proposed Uncertainty Analysis of *In Vitro* Metabolic Data and PBPK Model Predictions

The U.S. EPA seeks input on initial analyses that it has conducted, its proposed approach to evaluate quantitative uncertainty of the metabolic parameters estimated from *in vitro* data, and its proposed approach to incorporate the metabolic parameter uncertainty into an estimate of uncertainty in the PBPK model predictions U.S. EPA (2020).

Question 15.
Please comment on the analysis and statistical assumptions for control data from* Yang et al. (2012) *as an approach for evaluating the underlying experiments, data, and distribution of RLOSS for use in subsequent uncertainty analyses of the metabolic data.

Leslie Z. Benet, Ph.D.

I will not respond to Charge Questions 14-19. I have no experience or expertise in evaluating uncertainty analyses. I do believe that the PBPK model proposed can provide sound estimates of chloroprene inhalation dosimetry in mice, rats and humans, and that the model allows animal to human extrapolation.

Jeffrey J. Heys, Ph.D., P.E.

The analysis and statistical assumption appeared to be valid in my opinion, but I have limited experience with uncertainty quantification.

Jochem Louisse, Ph.D.

I do not have the expertise to provide any input on these analyses.

Annie Lumen, Ph.D.

I do not have the statistical expertise to comment on the assumptions and analysis conducted but do think it is a good idea to capture the distribution of any loss parameter from the control data for subsequent analysis of metabolic parameters across all tissues and species.

Kenneth M. Portier, Ph.D.

I cannot really comment on the appropriateness of the laboratory methodology used to generate the data used in the estimation of measurement uncertainty and RLOSS.

Statistically, the approach used in estimating RLOSS using the control data from Yang et al. (2012) assumes the following:

1. Some measurement uncertainty in assessing metabolic rates is due to vial-to-vial variability.
2. Additional uncertainty in assessing metabolic rates is due to the instantaneous rate of background loss, assumed to be proportional to the concentration of chloroprene in the media with constant of proportionality given by the parameter for the rate of background loss (RLOSS).
3. Measurement uncertainty is independent of within individual/tissue variability or among individual variability in metabolism rates for which estimates cannot be obtained from the in vitro experimental data of Yang et al. (2012).

From a statistical point of view, these assumptions are reasonable given the methodology of the experiment.

Kan Shao, Ph.D.

Generally, the statistical assumptions and analyses presented in EPA's supplemental Uncertainty Analysis for control data from Yang et al (2012) were adequate. However, a few minor issues need some additional attention or explanation:

(1) Both OLS and MLE methods were applied to analyze the Yang et al 2012 data, and generated similar results. Which set of results will be used eventually? The MLE method is usually preferred but typically sensitive to the initial values specified in the algorithms. How sensitive the MLE results to the initial values? (**Tier 3**)

(2) The statement "This implies that the uncertainty and variability in the rates of background loss were comparable, but …" is not necessarily accurate. (**Tier 2**) Although

the standard deviation estimated from the two studies are numerically similar, the magnitude of the variance is quite different (i.e., the coefficient of variance, stdev/mean, is different) indicating that the standard deviation obtained from Himmelstein et al (2004) data has larger magnitude in its own data set.

(3) The prior specified on Page 12 is quite unclear. How was this prior applied in the MCMC algorithm? Why is this prior "vaguely informative"? (**Tier 2**)

(4) Why was the sigma (σ) value set at 0.006? (**Tier 3**)

Jordan Ned Smith, Ph.D.

This is outside my expertise, and I do not have a comment.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

EPA clearly described an analysis plan for uncertainty in the estimate of RLOSS using the control data from Yang et al (2012). EPA's approach consisted of three steps. EPA first conducted a least-squares regression for each of the 30 vial samples separately, to explore the validity of a normal likelihood function for the key parameters q=(RLOSS, A0). EPA demonstrated the need for log-transformation of concentration to use normal distribution approximation. EPA should explicitly present the functional form for the predicted concentration $C_{mod}(t; q)$ at time t. EPA recognized that air samples were taken in sequence from each vial. However the likelihood did not incorporate a serial correlation (equation 4) between these samples. As a result, the estimates of the key parameters (RLOSS, A0) were likely artificially more precise (smaller standard error) than they really were. Further, the rational for EPA to fit one regression model for each vial to obtain a total 30 sets of estimates of q appeared to be based on the assumption that serial data from each vial followed a different distribution. One may reason that the 30 vials were replications of the same experiment. The 30 serial samples would be different because of experimental errors. Statistically, we should assume them to be 30 independent samples following the same underlying distribution. Consequently, a single regression analysis should be done for all 30 sets of vial samples simultaneously. There are well established statistical advantages for joint analysis as opposed to one regression for each of the 30 sets.

In the second step EPA used MCMC to generate posterior samples based on the likelihood function (equation 4) and a semi non-informative prior for (A0, RLOSS, σ) for each of the 30 sets. Again, this is not a recommended approach. A single MCMC chain is sufficient and advantageous, using all 30 sets of samples simultaneously.

In the third step EPA used a kernel estimate to average over the 30 MCMC posteriors and derived an overall posterior distribution for RLOSS. This kernel estimation can be avoided altogether if in steps 1 and 2 EPA did joint analysis of 30 sets of vial samples.

The following is a **Tier-1** recommendation:

It is highly recommended that EPA conducts a joint analysis of all 30 sets of vial samples instead one analysis for each of the 30 sets. Analysis of single sample was neither necessary nor efficient. Non-convergence in MCMC for some sets was evidence of statistical inefficiency because a single set of data may not provide sufficient amount of information about the underlying parameters, compared with using all 30 sets simultaneously.

Joint analysis of 30 independent vial samples would be statistically more powerful, and computationally more economical. Variation between vial sets can be characterized with random effects, one for each vial, in the regression model (likelihood) if so desired. The random effects can be incorporated to MCMC as another random variable.

It is important that the MCMC posterior samples depict the joint distribution for all three parameters (RLOSS, A0, σ). See also recommendation to Q3 for implementation of MCMC. In describing variation or uncertainty of RLOSS on the basis of the posterior distribution, EPA may consider the conditional posterior distribution of RLOSS given the estimated value of (A0, σ). A conditional posterior in the current context is preferred to the marginal posterior over σ and/or A0, projecting a different a different description of the uncertainty of RLOSS. Therefore EPA should thought through this process, and if necessary compare the conditional approach with the marginal approach. In reporting the uncertainty of RLOSS, EPA should, at a minimum, include percentiles of the posterior distribution in conjunction with graphic displays. See also comments to Q3.

During the penal discussion, a suggestion was made to include RLOSS in equation (1) of the 2-compartment kinetic model. This appears to be reasonable. Equation needs clarification and more precise notation so that Kf and the Michaelis-Menten kinetic component would not be in the equation concurrently.

Additional Clarifications Requested from EPA

After review of the draft post-meeting comment report, EPA had some additional clarifying questions regarding Dr. Zhu's comments on the supplemental EPA uncertainty analysis.

EPA's additional clarifications and Dr. Zhu's responses are listed below:

In response to Charge Question 15, Dr. Yiliang Zhu stated:

- "Joint analysis of 30 independent vial samples would be statistically more powerful, and computationally more economical. Between vial sets variation can be characterized with random effects, one for each vial, in the regression model (likelihood), if so desired. The random effects can be incorporated to MCMC as another random variable."

EPA: Could Dr. Zhu clarify if this means one should represent "RLOSS" as a random variable rather than as a parameter in the differential equation model?

Dr. Zhu's response: Regarding the first point, RLOSS is specified as a parameter in the differential equations.
Specifically, RLOSS_i=RLOSS+a_i (i=1,2,…,30) is for the ith vial and a_i is the random effect that captures the between-vial variation (experimental error).
Our objective is to estimate the common underlying RLOSS (mean of RLOSS_i).
A main difference is that this way we conduct a joint analysis of 30 vial samples that share a common RLOSS rather than a separate analysis of a single vial sample.
One limitation that has been observed in the separate analysis is difficulty in convergence as a single vial may generate somewhat "less typical" data that deviates from the expected model.

- "In describing variation or uncertainty of RLOSS on the basis of the posterior distribution, EPA may consider either the conditional posterior distribution of RLOSS given the estimated value of (A0, σ), or marginal over σ and/or A0. The choice could lead to different magnitude of the uncertainty of RLOSS, therefore should be thought through and be compared."

EPA: Could Dr. Zhu provide specific suggestions for tools and methods for computing these conditional and marginal distributions?

Dr. Zhu's response: Regarding the second point, my personal preference is towards conditional posterior distribution of RLOSS, conditioning on a plausible range of (A0, σ) because RLOSS and (A0, σ) are likely to be correlated. Where there are strong evidences of the plausible region for (A0, σ), MCMC can be conducted directly for the conditional posterior of RLOSS by fixing (A0, σ) within the region. I suspect the determination of the region and choice of the value of (A0, σ) can be a difficult one.

In the absence of such evidence for (A0, σ), it might be desirable to have two phases for MCMC. In the first phase we generate samples from the joint posterior for RLOSS, A0, and σ. Upon learning about key values of about (A0, σ) based on their marginal posterior distribution (e.g. mode, median, mean, 25- and 75-percentiles), we may conduct second phase of MCMC in which we draw posterior samples intensively for RLOSS conditioning on these key values. (The conditional posterior distribution is proportional the ratio of joint likelihood function to the marginal likelihood of (A0, σ).) This contrasts with the traditional MCMC where joint samples are drawn such that the posterior samples for RLOSS may not be sufficiently intense conditioning on these key values of (A0, σ), yet there are MCMC samples of RLOSS in association with less plausible values of (A0, σ) which may not be of interest.

Graphical displays as well as key percentiles can then be employed for the conditional posterior distribution of RLOSS conditioning on the key value of (A0, σ) (e.g. mode, median, quartiles etc.) Finally, theoretical form of the conditional posterior of RLOSS may be available (at least approximately) when a normal distribution is assumed for the prior of RLOSS and A0.
For quantifying uncertainties I feel graphic and descriptive tools (e.g. percentiles) for the conditional posterior of RLOSS would be adequate.

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Question 16.
Considering the preliminary results for RLOSS provided, please provide any specific suggestions you may have for how the analyses methods might be improved.

Leslie Z. Benet, Ph.D.

I will not respond to Charge Questions 14-19. I have no experience or expertise in evaluating uncertainty analyses. I do believe that the PBPK model proposed can provide sound estimates of chloroprene inhalation dosimetry in mice, rats and humans, and that the model allows animal to human extrapolation.

Jeffrey J. Heys, Ph.D., P.E.

I do not have any suggestions for improving the analysis.

Jochem Louisse, Ph.D.

I do not have the expertise to provide input here.

Annie Lumen, Ph.D.

Aside the statistical aspect, I cannot think of any useful suggestions to what has already been done.

Kenneth M. Portier, Ph.D.

The method used to estimate uncertainty due to RLOSS involves balancing model predictions of headspace concentrations against measured concentrations in replicate vials over time and assigning uninformative (minimally non-informative) priors to the distribution of A0, RLOSS and σ across the 30 replicate vials. A three-stage approach is used:

- Stage 1: use of OLS to estimate initial value of A0, RLOSS and residuals,
- Stage 2: use of ML estimation of A0, RLOSS and σ for each replicate vial assuming normally distributed differences between natural log transformed observed and model predictions.
- Stage 3: use of MCMC and kernel density estimation (KDE) to determine posterior distribution of A0, RLOSS and σ.

The estimated posterior distributions of A0, RLOSS and σ are subsequently used to inform measurement uncertainties.
Figure 6 and Figure 7 of U.S. EPA (2020) illustrate expected variability in RLOSS using generated samples from the posterior distribution of RLOSS for the Yang et al. (2012) data. Figure 9 and Figure 10 of U.S. EPA (2020) illustrate expected variability in RLOSS using generated samples from the posterior distribution of RLOSS for the Himmelstein et al. (2004) data.

In comparing the results from the two studies, applying KDE (kernel density estimation) to the means (or medians) of the individual vial RLOSS posterior distributions would have been informative. Subtracting means (or medians) from the individual RLOSS distributions and then applying KDE to the "residuals" would have been illustrative as well. The plot of the means illustrates expected average RLOSS for a vial replicate. The plot of the residuals would illustrate the uncertainty in expected RLOSS.

During Reviewer discussion at the virtual 2-day meeting, it was pointed out that there is no need to divorce the MCMC sub-step from the KDE fitting sub-step in Stage 3 of this process. The analysis model used at the MCMC step can be two-leveled, allowing assignment of a prior distribution to RLOSS estimates from the control incubation samples (in one analysis, the 30 Yang et al. (2012) samples and in the other analysis the 5 Himmelstein et al. (2004) samples). This recommendation by another Reviewer seems logical and should be attempted.

Kan Shao, Ph.D.

A few suggestions that may be helpful for improving the quality of the uncertainty analysis, including:

(1) It's better to show the trace plot of the posterior sample. The trace plot together with the PSRF value will be more helpful to determine if the results from non-satisfactory convergence can be used for analysis (**Tier 2**).
(2) To analyze the uncertainty and variability among the 30 or 5 control incubation data sets, Bayesian hierarchical model is more statistically plausible than analyzing each data set individually. It is definitely worth trying the Bayesian hierarchical model as an alternative to analyze the data sets (**Tier 2**).

Using the "KDE" density estimation function is good to generate the density plot for us to have an impression of the shape of the distribution. However, the results from the "KDE" (essentially a non-parametric algorithm) are not necessarily good for following analysis, just like the negative values for RLOSS generated from the algorithm. One potential solution is to fit a parametric distribution to the data, lognormal and gamma distribution can be a plausible choice (**Tier 2**).

Jordan Ned Smith, Ph.D.

I do not have suggestions for improvement.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

See comments to Q15.

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A similar analysis was conducted using data from five control incubations obtained by Himmelstein et al. (2004). Comparison of the results for RLOSS based on Yang et al. (2012) control data vs. Himmelstein et al. (2004) control data indicates that the value of RLOSS may have been lower in the Himmelstein et al. (2004) study. The two sets of experimental *in vitro* studies were conducted in the same laboratory by the same principle investigator (Matthew Himmelstein), but given the period of time between the two studies, the applicability of non-concurrent control data is a source of uncertainty.

Question 17.
Please comment and provide any specific suggestions you have on the possible use of either:

Question 17.a. separate distributions of RLOSS obtained from the* Yang et al. (2012) *vs.* Himmelstein et al. (2004) *studies when analyzing the uncertainty for the different metabolic parameters obtained with data from the respective studies; or

Leslie Z. Benet, Ph.D.

I will not respond to Charge Questions 14-19. I have no experience or expertise in evaluating uncertainty analyses. I do believe that the PBPK model proposed can provide sound estimates of chloroprene inhalation dosimetry in mice, rats and humans, and that the model allows animal to human extrapolation.

Jeffrey J. Heys, Ph.D., P.E.

No comment.

Jochem Louisse, Ph.D.

I do not have the expertise to provide input here.

Annie Lumen, Ph.D.

In my preliminary comments I had shared that based on my understanding that this method seems preferable since the values are different between the studies and that there are differences in vial volumes. And that I agree that perhaps using only the RLOSS parameter Yang et al. data to analyze all data could lead to underestimation of metabolic capacities in the human lung for example. However, during the public meeting I learnt that answering this question needs a lot more statistical inputs and therefore I defer this question to other panel members who have the necessary statistical expertise to address this.

Kenneth M. Portier, Ph.D.

See response to Questions 17.b. below.

Kan Shao, Ph.D.

In my opinion, using separate distributions of RLOSS from the Yang et al 2012 and Himmelstein et al 2004 for uncertainty analysis is not preferred (**Tier 2**). The difference in the estimated distributions of RLOSS may further complicate the resulting analysis outcomes, and make the results even more difficult to interpret.

Jordan Ned Smith, Ph.D.

This is outside my expertise, and I do not have a comment.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

There were recorded experimental differences (VINJ, vial volume, and chloroprene concentration) between Yang et al (2012) and Himmelstein et al (2004). There were also potential difference masked by temporal distance between the two studies. Initial analysis also indicated a 2-fold difference in RLOSS estimate. Thus experiment itself appears to be a source of uncertainty. MCMC analysis showed that the posterior means of the two experiments are within each other's 2.5-97.5 percentile range. One approach to combine the two experiments is to consider a hierarchical Bayes model in which the likelihood involves RLOSS=RLOSS_0+b1*experiment, where experiment=0 or 1 to represent Yang et al (2012) and Himmelstein et al (2004) respectively. Similarly we may consider A0=a0+b2*experiment if warranted by the data. Therefore the joint posterior distribution involves additional parameters b1 and b2. The posterior distribution of b1 quantify the experimental uncertainty. At a moderate computational expense, this approach in more quantitative for uncertainty analysis. This would be a preferred approach. An alternative is to develop a posterior distribution for RLOSS, one for each experiment, and then use sampling weight to create a mixture of the two posteriors. This mixture distribution could be bi-modal. The median of this mixture would be a sort of weight average of the two medians. These are options for combining the data while retaining the distinction of the two experiments statistically. Data combination here should not be interpreted as pooling the data and ignoring experimental difference, which is not advisable.

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Question 17.b. combining the control incubation data and analysis to obtain a distribution applicable to all metabolic data.

Leslie Z. Benet, Ph.D.

I will not respond to Charge Questions 14-19. I have no experience or expertise in evaluating uncertainty analyses. I do believe that the PBPK model proposed can provide sound estimates of

chloroprene inhalation dosimetry in mice, rats and humans, and that the model allows animal to human extrapolation.

Jeffrey J. Heys, Ph.D., P.E.

No comment.

Jochem Louisse, Ph.D.

I do not have the expertise to provide input here.

Annie Lumen, Ph.D.

This might need some statistical inputs that I'm unable to offer.

Kenneth M. Portier, Ph.D.

First, I contend that while estimating RLOSS is necessary, statistically it is a "nuisance" parameter when thinking about the need to propagate uncertainty in the key metabolism parameters of Km, Kgl and Vmax. In the final analysis, RLOSS variability needs to be "handled", "eliminated", "averaged over" or "integrated out".

The two differential equations (equations 1 and 2) in US EPA (2020, Supplement: Uncertainty Analysis) purport to fully describe the mass balance for air and liquid phases in the experimental vials. If these models are correct, then the Yang et al. (2012) and Himmelstein et al. (2004) control incubation study results reporting time changes in the respective amounts of chloroprene in the air and media for each vial should be fully described by estimating values for equation parameters unique to each vial. There are no parameters in these equations that specifically relate experimental design factors such as vial dimensions, mixing rates, etc. other than the initial conditions specified for each vial (e.g. A0 – initial amount of chloroprene in the vial). That is for example the parameters R_{loss}, P, V_{max} and K_m are not specified as functions of say the cross-sectional diameter of the vial. If indeed RLOSS is a function of some factor of the experimental set-up, then with only two experimental conditions, there is the opportunity when specifying a prior for RLOSS to add one parameter to shift the location or scale of the RLOSS distribution between the two studies.

Assuming equations 1 and 2 are correct, it seems feasible that the results of the Yang et al. (2012) and Himmelstein et al. (2004) study data, generated as they were from the same methodology but at admittedly different times can be combined and the analysis redone to provide a (combined) estimate for the posterior distribution of RLOSS. The text on lines 14-22, page 22 of U.S. EPA (2020, Supplement: Uncertainty Analysis), indicate there is concern with experimental method differences between the Yang et al. (2012) and Himmelstein et al. (2004) studies that seem to lead to different estimated RLOSS distributions. If the combined plots proposed in my comments to Question 16 were generated the validity of these concerns could be illustrated.

It is not clear to me that after accounting for A0 differences among the vials that RLOSS values are sufficiently different to preclude combining results. In addition, I do not follow the logic behind choosing the analysis of the Himmelstein et al. (2004) data to define the prior distribution for RLOSS over the analysis of the Yang et al. (2012) data which demonstrated a broader range of uncertainty. I am not sure how the lack of concurrency of the control data in the Yang et al. (2012) study impacts this.

Recommendation (**Tier 2**): Clarify the logic behind choosing the analysis of the Himmelstein et al. (2004) data to define the prior distribution for RLOSS over the analysis of the Yang et al. (2012) data.

Kan Shao, Ph.D.

This is a preferred approach. Applying a Bayesian hierarchical model to get an integrated distribution with consideration of uncertainty and variability among the data sets is more statistically plausible and will make the following analysis and results more consistent (**Tier 2**).

Jordan Ned Smith, Ph.D.

This is outside my expertise, and I do not have a comment.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

See comments on 17.a.

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U.S. EPA (2020) describes intended methods for evaluating the uncertainty in the metabolic parameters obtained from the *in vitro* data, given the distribution in RLOSS already obtained. The analysis is particularly focused on the human liver and lung data, which were obtained with pooled microsomes from 15 individuals for liver microsomes and 5 individuals for lung microsomes.

Question 18.
Please evaluate the planned analysis as an appropriate statistical approach for evaluating the uncertainty in the metabolic parameters for the pooled tissue samples. Note any additional quantitative factors whose uncertainty you believe would not be addressed by this approach. Please provide any specific suggestions you have on how the analysis should be modified.

Leslie Z. Benet, Ph.D.

I will not respond to Charge Questions 14-19. I have no experience or expertise in evaluating uncertainty analyses. I do believe that the PBPK model proposed can provide sound estimates of chloroprene inhalation dosimetry in mice, rats and humans, and that the model allows animal to human extrapolation.

Jeffrey J. Heys, Ph.D., P.E.

The outlined approach appears appropriate to me, but I have limited experience with uncertainty quantification.

Jochem Louisse, Ph.D.

I do not have the expertise to provide input here.

Annie Lumen, Ph.D.

If not planned already and as I mentioned earlier, it would be good to evaluate the uncertainties in Kgl and A1 parameters and their effects on metabolic parameter estimates (**Tier 1**).

I understand that the reason for modeling loss from the media compartment as indicated in the supplemental analysis document and not from both media and air compartments. This was discussed in the public meeting and it was mentioned that the loss term although originally was modeled to account for loss from both the media and air compartment – it was later chosen to be only modelled in the media compartment so that the values can be compared to the estimates derived from the Yang et al study. Difficulties in the identifiability of two separate loss parameters was also indicated.

Because of the comprehensive nature of the proposed analysis, one overall suggestion is to see if there is a statistical or mathematical way to ensure that too much information is not asked off of the available data (**Tier 1**).

Since the A1 ratio-based approach indicated in Ramboll report and the KF approach indicated in EPA's supplemental analysis both rely on limited and challenging tissue-specific in vitro data for humans without any corresponding in vivo tissue data for model verification: I wondered if both analyses be conducted and used for cross verification at lower exposure levels (**Tier 2**) and at higher exposure levels the one that provides the metabolic parameters that yields the most health-conservative estimate of the tissue dose-metric be selected for subsequent determinations (**Tier 2**).

Kenneth M. Portier, Ph.D.

The planned analysis is laid out logically and seems reasonable.

I suspect that the posterior joint distribution for TLOSS, RLOSS, A0 and σ will display a high degree of collinearity. If this is the case, the posterior marginal distributions would not fully specify the joint distribution, and credibility intervals based on the estimated marginal distributions might not be as useful in subsequent Monte Carlo analyses of model uncertainty as would be the estimated joint posterior distributions. In particular, using marginal distributions instead of the joint posterior distribution could lead to inappropriate credibility intervals for VMAX which is a function of KM, TLOSS and RLOSS [note that VMAX is defined as KM*(TLOSS-RLOSS)].

Line 14-22, page 21 of U.S.EPA, 2020, Supplement Uncertainty Analysis, indicates that an a priori distribution will be assigned to the parameter vector $\boldsymbol{\theta}$ to ensure appropriate non-negative values, assign higher probability to smaller values of each σ_i and relative probabilities to RLOSS according to the combined distributional estimate of RLOSS from the analysis on control vials. What is not clear is the extent to which this prior will also account for expected high covariability.

To elaborate on this. KM will have its own prior (based on the central estimate obtained in Ramboll (2020) with suitably wide bounds). It looks like the prior distribution of TLOSS will depend on one aspect of the RLOSS prior, the KM prior and the derived VMAX (lines 10-13, page 21 U.S.EPA, 2020, Supplement Uncertainty Analysis – $TLOSS_{max}$, which will be calculated as the sum of an upper bound for the RLOSS distribution and an upper bound for VMAX/KM – but VMAX/KM =TLOSS-RLOSS). The posterior distribution of VMAX depends on the prior distributions of KM, TLOSS and RLOSS. Statistically this is quite complex, and it is not at all clear what the impact of this will be on the final model predictions. Concerning to me is the likelihood that these "constraints" on a priori distributions will lead to narrowed and possibly biased posteriori distributions for the prediction of blood concentrations.

With all the above constraints/relationships I cannot see how the promise of line 25, page 21 U.S.EPA, 2020, Supplement Uncertainty Analysis that "These 61 distributional parameter estimates will be generated simultaneously…" can be accomplished.

During the 2-day virtual meeting, one Reviewer recommended the use of a hierarchical model for the MCMC analysis which eliminated the need to estimate the RLOSS distribution separately using KDE (see also comments to Question 16). This approach has the potential to dramatically reduce the number of distributional parameters to which prior parameters need to be generated and should help accomplish convergence in the MCMC for estimation of the in vitro kinetic parameters. If I have this model correct, the 15 TLOSS parameters, 15 RLOSS parameters, 15 A0 parameters and 15 σ_i parameters would be reduced to 8 hyper-parameters specifying four prior distributions.

Recommendation (Tier 2): Be clear on how constraints/relationships are incorporated among the distributional parameter estimates and illustrate how these impact expected collinearities among parameters.

Kan Shao, Ph.D.

Generally, the proposed analysis and statistical approach for evaluating uncertainty in metabolic parameters are logically reasonable and clearly outlined. However, the proposed estimation of in vitro kinetic parameters for the human liver and lung confuses me. The Ramboll (2020) report assumed that the Km for metabolism in human lung and liver are the same, but the uncertainty of these parameters will be evaluated separately based on different data sets (15 vs 5). This proposed approach should be better justified and presented (**Tier 2**).

Jordan Ned Smith, Ph.D.

This is outside my expertise, and I do not have a comment.

Raymond S.H. Yang, Ph.D.

No response provided

Yiliang Zhu, Ph.D.

EPA outlined a number of assumptions that underlying the planned uncertainty analysis for the metabolic parameters. One of them, assumption c) states that

"Since the exact amount of enzyme added to each incubation vial and the activity of enzyme in the sample may vary between vials, the Vmax or Kf for a given incubation type is assumed to be different between vials, hence a different distribution for Vmax and Kf will be fitted to each vial."

I would assume that it was the experiment error that the exact amount of enzyme added was not the same between vials. As discussed in comments to Q15 and Q16, this assumption counters to the statistical principle of replications. The multiple vials of the same type of incubations were intended to be replications. Variation in the amount of enzyme and enzyme activity between the vials is exactly why an experiment needs replications. It is not expected in any experiment that the enzyme activity to be exactly the same between individual vials. Thus it is a convention that replications are assumed to be independent samples following the same underlying distribution. In other words, Vmax and Kf across vials follow along the same distribution.

Tier-1 recommendation:

If the amount of enzyme added was different per experimental protocol, the correct approach is to assume Vmas and Kf follows a distribution whose mean depends on the amount of enzyme added to the vial, e.g. Vmax=Vmax0+b*vial. A hierarchical Bayes model would incorporate the coefficient b into the MCMC.

If the experimental error is such that between-vial error is of concern, a conventional statistical approach is to include random effects for vials. This will lead to inclusion a distribution for the random effects which involve additional parameters as a part of the posterior.

To conclude, separate MCMC for RLOSS of individual vial is unnecessary and undesirable. See comments to Q15.

Assumption d) gives rise to the same concern as assumption c).

Assumption e) needs clarification. If measurement uncertainty is the source of intra-vial uncertainty, what is the source of the uncertainty for w given vial?

Assumption f) is that of parameter non-identifiability as is seen in equation 2 and the re-parameterization in equation 6. Re-parameterization may help, other constraints derived from kinetics would also help (e.g. Kf=Vmax/Km).

As commented in Q3, for estimation of in vitro metabolic parameters for the human liver and lung, EPA should generate a single posterior joint distribution using all vials (joint likelihood of 15 vials for liver and 5 for lung)) instead one vial at a time. I have made a tier-1 recommendation for generating multivariate posterior for all kinetic parameters to Q5. The recommendation applies equally here. EPA should develop a step-by-step MCMC implementation procedure for joint estimation of Km, A0, RLOSS, TLOSS, and σ.

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U.S. EPA (2020) describes intended methods for evaluating the uncertainty in the PBPK model predictions for the rate of metabolism in liver, lung, and kidney, and in predictions of chloroprene venous blood concentrations. Since the analysis is focused on estimation of population average doses, uncertainty in human physiological parameters would be quantified as uncertainty in the *mean* values for a healthy adult, rather than overall population variance. For model predictions based on the parameter A1 (lung:liver metabolic ratio obtained from data for 7-ethoxycoumarin) and a similar parameter for the kidney (A2), uncertainty in A1 or A2 based upon variance in tissue-specific values reported for the corresponding *in vitro* studies will be included.

Question 19.
Please comment on whether the planned analysis for PBPK-predicted dose metrics as outlined by* U.S. EPA (2020) *is an appropriate approach for evaluating quantitative uncertainty in the estimated internal doses. Please provide any specific suggestions you have on how the analysis could be improved.

Leslie Z. Benet, Ph.D.

I will not respond to Charge Questions 14-19. I have no experience or expertise in evaluating uncertainty analyses. I do believe that the PBPK model proposed can provide sound estimates of chloroprene inhalation dosimetry in mice, rats and humans, and that the model allows animal to human extrapolation.

Jeffrey J. Heys, Ph.D., P.E.

It appears that the uncertainty analysis is focused on the prediction of metabolism rates. This approach may fail to include uncertainty associated with delivery or dosing rates. Differences in airway geometry, respiratory parameters, and overall lung health would lead to differences in the amount of chloroprene absorbed in the lungs even with a known concentration in the air. These differences may be negligible, but I worry they might be significant. Beyond this concern, the planned analysis appears appropriate based on my limited familiarity with uncertainty quantification.

Jochem Louisse, Ph.D.

I do not have the expertise to provide input here.

Annie Lumen, Ph.D.

The planned analysis for PBPK-predicted dose metrics overall is reasonable.

Although I understand that the goal of this exercise is to estimate a population average dose-metric, I'm not entirely sure that not evaluating population variabilities in metabolic capacities is a solid approach particularly when tissue-specific metabolic parameter estimates in humans are informed by limited invitro samples, with no corresponding in vivo data for model verification, and the implications of the dose-metric is to be reflective of the overall population's public health. Therefore, I think the uncertainty of whether the average metabolic parameters measured in vitro is truly reflective of the population average particularly due to enzyme polymorphisms should be evaluated in a bit more detail (as it applies to Chloroprene metabolism specifically) (**Tier 1**). Perhaps some suggestions can be gleaned from the pharmaceutical sector on the polymorphic nature (or lack thereof) of the CYP2E1 enzymes to determine if there is any considerable concern if this uncertainty is not captured (**Tier 2**).

Kenneth M. Portier, Ph.D.

The planned analysis is laid out logically and seems reasonable. I have concerns related to the approach proposed.

Line 20, page 23 indicates that in the Monte Carlo sampling for the model simulations to assess uncertainty in PBPK predictions, metabolism parameters will be treated as "independent random variables". In particular, KM, KF, and VMAX will have their own (marginal) uncertainty distributions. But as has been mentioned previously there is a high likelihood that these parameters are not independent, and the estimated posterior joint distribution will display a high degree of collinearity. As mentioned in a previous question, properly specifying the uncertainty in VMAX will conditionally depend on the chosen values for KM and/or KF. Similarly, if A1 and A2 metabolic ratio estimates are used, it is quite possible that these two parameters are correlated because they use the same numerator. In this case the marginal distributions do not fully describe the joint distribution.

Using the product of marginal distributions to describe a (correlated) joint distribution in a Monte Carlo experiment results in some combinations of parameter values being generated with higher probability that would be expected if using a true joint distribution. When the impact of this is propagated through the PBPK model, some extreme outputs might be represented with the wrong probability. The bottom line is that the bounds on uncertainty in the outputs are likely to be wrong (resulting in improperly high or improperly low estimates).

Recommendation (Tier 1): Justify better the use of independent distributions for the uncertainty in metabolism parameters that are likely to be jointly distributed with high correlations.

Kan Shao, Ph.D.

The proposed plan seems reasonable and is presented clearly.

Jordan Ned Smith, Ph.D.

Overall, the uncertainty analysis seems very comprehensive. Full disclosure: I do not fully understand the Bayesian approach used by EPA and defer to experts in Bayesian methods regarding the appropriateness of EPA's methods. However, the concept of identifying uncertainty in model parameters and evaluating uncertainty of various parameters using a Monte Carlo to assess overall PBPK model uncertainty seems very appropriate and useful. Perhaps the only further suggestion I may add is this process could be simplified, in that it may not be needed for every parameter within the model. Sensitivity analyses identify the most important parameters for simulations of interest. Focusing uncertainty analyses on sensitive parameters may help simplify uncertainty analyses by eliminating the need to evaluate every parameter in the model.

Raymond S.H. Yang, Ph.D.

The lead scientist at Ramboll, Dr. Harvey Clewell, and the lead scientist at the EPA, Dr. Paul Schlosser, are both experts and highly reputable scientists in this field. Therefore, there is no reason to question the accuracy of the working details (e.g., model code, biological descriptions, mass balance, …etc.), particularly the two teams had obviously checked on each other carefully.

Other than that, I only wish to make three specific recommendations:

First, because I, too, believe that PBPK modeling is a useful tool for human risk assessment, I would urge and recommend the Ramboll colleagues not to dismiss all the science conducted by others as discussed above in this meeting; rather, they should carefully design and incorporate all the available metabolic and pharmacokinetic information discussed above and elsewhere into the PBPK model for chloroprene to make it more robust and state-of-the-science for their purpose. It would be a real contribution to the toxicology and risk assessment community. A possible scenario would be that the Ramboll scientists compare the simulation results of three PBPK modeling approaches: (i) their present approach modified and refined with consideration of downstream detoxication processes; (ii) PBPK modeling using kinetic constants estimated using data from Cottrell et al. (2001) and Munter et al. (2003) with consideration of downstream

detoxication processes; and (iii) PBPK modeling using kinetic constants from 1,3-butadiene data from Csanady et al. (1992) and Kohn and Melnick (2000; 2001) with consideration of downstream detoxication processes (**Tier 1** recommendation).

Second, I urge the EPA to always consider IRIS IUR for 1,3-butadiene carefully if any update of IRIS IUR for chloroprene is to be implemented. These two IURs should not be too far apart. The principal reason that I made this recommendation is that the similarity of the two NTP mouse inhalation studies (NTP 1993; 1998) suggest common toxicokinetics and toxicodynamics between chloroprene and 1,3-butadiene (**Tier 1** recommendation).

Third, as Bayesian population PBPK modeling and Markov Chain Monte Carlo (MCMC) simulation seemed to have been used more often by the EPA in its IRIS Risk Assessment since late 2006, I do have one recommendation (next paragraph) which if carried out by either the Ramboll Team or the EPA Team would be a great service and contribution to the PBPK modeling community.

Currently in the toxicology and risk assessment communities, software with MCMC capabilities are MCSim, the no-longer supported acslX, Magnolia, some computer programs in the R community, and possibly MATLAB. Researchers use these software without questions and trusted their accuracies and performances. Since no one knows if there are bugs in any of the software, I would recommend that either the Ramboll Team or the EPA Team do a systematic evaluation of the PBPK Modeling/MCMC performance of the available software much the same way as carried out in 2017 for PBPK modeling software by Lin et al. (2017). (**Tier 1** recommendation)

Reference quoted for this section:

Cottrell L, Golding BT, Munter T, Watson WP (2001) In vitro metabolism of chloroprene: Species differences, epoxide stereochemistry and a de-chlorination pathway. Chem. Res. Toxicol. 14:1552-1562.

Csanady GA, Guengerich FP, Bond JA (1992) Comparison of the biotransformation of 1,3-butadiene and its metabolite, butadiene monoepoxide, by hepatic and pulmonary tissues from humans, rats and mice. Carcinogenesis 13:1143-1153.

Kohn MC, Melnick RL (2000) The Privileged Access Model of 1,3-Butadiene Disposition. Environ. Health Perspect. 108(Supple. 5):911-917.

Kohn MC, Melnick RL (2001) Physiological modeling of butadiene disposition in mice and rats. Chem. Biol. Interactions 135-136:285-301.

Lin Z, Jaberi-Douraki M, He C, Yang RSH, Fisher JW, Riviere JE (2017) Performance assessment and translation of physiologically based pharmacokinetic models from acslX™ to Berkeley Madonna™, MATLAB®, and R language: Oxytetracycline and gold nanoparticles as case examples. Toxicol. Sci. 158:23-35.

Munter T, Cottrell L, Golding BT, Watson WP (2003) Detoxication pathways involving glutathione and epoxide hydrolase in the in vitro metabolism of chloroprene. Chem. Res. Toxicol. 16:1287-1297.

NTP (1993) Toxicology and carcinogenesis studies of 1,3-butadiene (CAS No. 106-99-0) in B6C3F1 mice (inhalation studies). NTP TR 434.

NTP (1998) Toxicology and carcinogenesis studies of chloroprene (CAS No. 126-99-8) in F344 rats and B6C3F1 mice (inhalation studies). NTP TR 467.

Yiliang Zhu, Ph.D.

EPA outlined a plan for evaluating uncertainties of PBPK model predicted oxidative metabolites as tissue dose in liver, lung, and kidney as well as predicted venous blood concentration. EPA's plan focuses on the impact of the upstream physiological/metabolic parameters that are directly involved in IVIVE, including Vmax for the liver and Kf for the lung (or relative activity coefficient A1 of the lung to the liver or A2 of the kidney to the liver) as well as microsomal protein in the liver and lung and fraction of the liver, lung, and kidney to body weight. EPA is aware that only a single pool of tissue/species specific microsomes were used in the experiment, range of variation in microsomal protein is unknown.

EPA's priority on these direct parameters makes practical sense. Not addressing the impact of uncertainty of other indirect parameters on model prediction remains an important source of overall uncertainty.

The EPA's strategy centers on generating a (random) sample for each parameter under consideration, from either a posterior distribution or range of values based on literature review as appropriate. This approach is implementable if the objective is to generate a distribution of uncertainty for prediction in liver, lung, kidney or venous blood. The computation will be very extensive because to cover key combinations of all these parameters, a sufficiently large sample is needed for each parameter. However, some combinations may be less likely (as seen in the joint posterior distribution for metabolic parameters which can be highly correlated). The sensitivity analysis that the EPA proposes is likely to generate a range of variation that actually includes both variability and uncertainty. The following recommendations might improve this analysis.

Tier-1 Recommendation:

- For parameters such as fraction of body weight for the liver, kidney, lung, microsomal content, EPA should conduct a literature search/review as systematically as practically feasible to ensure transparency and consistency. Consider adopting a Systematic Review approach if justified.
- Bayes samples for key physiological/kinetic parameters should be taken from their joint posterior distribution instead of marginal distribution. One apparent advantage is that less likely combinations of these parameters will be less likely sampled, making sampled values more plausible and computation more efficient. Accepting rules for sampled values can also be established to reject those that are less relevant.

If the objective is to quantify the range of uncertainty of model prediction, random sampling of the parameters can be replaced by a grid of key percentile (e.g. 5, 25, … 95 percentiles) of each parameter, minus those points corresponding to less likely combinations of the parameters.
>>

V. SPECIFIC OBSERVATIONS

Specific observations or comments on a draft report on physiologically based pharmacokinetic (PBPK) modeling for chloroprene (Ramboll, 2020) entitled, "Incorporation of In Vitro Metabolism Data in a Physiologically Based Pharmacokinetic (PBPK) Model for Chloroprene."

Leslie Z. Benet, Ph.D.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		I have no specific observations that I have not already addressed in my responses above.

Jeffrey J. Heys, Ph.D., P.E.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		References Andersen, M. E., H. J. Clewell, M. L. Gargas, F. A. Smith and R. H. Reitz (1987). "Physiologically Based Pharmacokinetics and the Risk Assessment Process for Methylene-Chloride." Toxicology and Applied Pharmacology **87**(2): 185-205 Clewell, H. J., J. L. Campbell, C. Van Landingham, A. Franzen, M. Yoon, D. E. Dodd, M. E. Andersen and P. R. Gentry (2019). "Incorporation of in vitro metabolism data and physiologically based pharmacokinetic modeling in a risk assessment for chloroprene." Inhalation Toxicology **31**(13-14): 468-483. Csanady, G. A., F. P. Guengerich and J. A. Bond (1992). "Comparison of the Biotransformation of 1,3-Butadiene and Its Metabolite, Butadiene Monoepoxide, by Hepatic and Pulmonary Tissues from Humans, Rats and Mice." Carcinogenesis **13**(7): 1143-1153. Galaction, A. I., D. Cascaval, C. Oniscu and M. Turnea (2004). "Prediction of oxygen mass transfer coefficients in stirred bioreactors for bacteria, yeasts and fungus broths." Biochemical Engineering Journal **20**(1): 85-94 Himmelstein, M. W., S. C. Carpenter and P. M. Hinderliter (2004). "Kinetic modeling of beta-chloroprene metabolism: I. In vitro rates in liver and lung tissue fractions from mice, rats, hamsters, and humans." Toxicological Sciences **79**(1): 18-27. Karimi, A., F. Golbabaei, M. R. Mehrnia, M. Neghab, K. Mohammad, A. Nikpey and M. R. Pourmand (2013). "Oxygen mass transfer in a stirred tank bioreactor using different impeller configurations for environmental purposes." Iranian Journal of Environmental Health Science & Engineering **10.** Ramboll (2019). Incorporation of in vitro metabolism data in a physiologically based pharmacokinetic (PBPK) model for chloroprene.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		Schlosser, P. M., J. A. Bond and M. A. Medinsky (1993). "Benzene and Phenol Metabolism by Mouse and Rat-Liver Microsomes." Carcinogenesis **14**(12): 2477-2486. Seader, J. D. and E. J. Henley (2006). Separation process principles. Hoboken, N.J., Wiley. U.S. EPA (2019). In Vitro to In Vivo Extrapolation (IVIVE) of Metabolism and Non-Enzymatic Conjugation of (1-chloroethenyl)oxirane (1-CEO) and Estimation of Total 1-CEO Clearance in the Liver and Lung of Mice, Rats, and Humans. Vantriet, K. (1979). "Review of Measuring Methods and Results in Nonviscous Gas-Liquid Mass-Transfer in Stirred Vessels." Industrial & Engineering Chemistry Process Design and Development **18**(3): 357-364. Versteeg, G. F., P. M. M. Blauwhoff and W. P. M. Vanswaaij (1987). "The Effect of Diffusivity on Gas-Liquid Mass-Transfer in Stirred Vessels - Experiments at Atmospheric and Elevated Pressures." Chemical Engineering Science **42**(5): 1103-1119.

Jochem Louisse, Ph.D.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
2	Abstract	I wonder whether one can say that data was used on 'the metabolism of chloroprene to reactive epoxides', since no data on epoxide formation, but data on chloroprene depletion was used in the PBPK model.
3	3rd bullet	The model validity has been assessed for the prediction of blood concentrations, not necessarily for the formation of reactive metabolites in the target tissue (amount of chloroprene metabolized in the lung per gram lung per day).
11	1st paragraph	Is the assumption that the Km in the liver is equal to the Km in the lung substantiated by data?
11	1st paragraph	Is CYP2E1 the major enzyme responsible for 7-ethyxycoumarin O-deethylation in liver and lung?
12	1st paragraph	Based on what is concluded that the human dose metric is a conservative estimate? Are differences in the sensitivity of the in vitro methods used to assess 7-ethoxycoumarin O-deethylation vs chloroprene depletion taken into account?
17	1st paragraph	Is the Km mentioned for CYP2E1 ('in the vicinity of 1 μM') for chloroprene? How was that Km determined? Based on data derived with liver microsomes or with recombinant CYP2E1?
18	1st paragraph	It is stated that 'a single clearance parameter is used in the human', whereas Table S-4 indicates that both a Km (0.028 mg/L) and a Vmax (0.0029 mg/h/kg^0.75) are included in the human model to describe

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		metabolism in the lung. Has the sensitivity analysis been performed for the human model with a Km and Vmax for lung metabolism?
22	1st paragraph	What is meant with 'pharmacokinetics' in the sentence '... reliable basis for evaluating the ability of the chloroprene PBPK model to predict *in vivo* pharmacokinetics in the bioassays'? If this refers to prediction of chloroprene blood concentrations upon inhalation exposure, I would agree, but this cannot be concluded for the prediction of the lung dosimetry of interest I think.
22	1st paragraph	'The model-based predictions for human exposure greater than 1 ppm would greatly overestimate the associated risk'. Does that refer to the human cancer risk based on the mouse bioassay data?
23	1st paragraph	Are data available that support that the role of CYPs other than CYP2E1 can be neglected?
23	3rd paragraph	What is meant with 'completely consumed' in the sentence '..., the assumption inherent in the dose metric was that the reactive metabolite would be completely consumed within the tissue where it was generated'? Does that refer to complete binding to DNA? Besides species differences in bioactivation (as predicted by the current PBPK models) one may expect species differences in detoxification, which is not taken into account in the current PBPK models.
24		Could differences between male and female mice also be related to possible differences in detoxification of the reactive metabolite between males and females, and if yes, should detoxification be described in the PBPK models when performing species extrapolation?

Annie Lumen, Ph.D.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		The reviewer provided no specific observations/comments.

Kenneth M. Portier, Ph.D.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
14	1	Sample sizes for the experiment that provided Figure 3 in the Ramboll (2020) report are not provided. Is the n=4/exposure group used in the plethysmography study? If so, it would be nice to see the actual individual measurements displayed in Figure 3 so that we could see whether the large variation is the result of an unusual value for one of the 4 animals in the 90 ppm exposure group at 3 hours. Indeed, the experiment is essentially a repeated measures in time study so Figure 3 should actually show the 16 time-profiles color coded to exposure group.

Kan Shao, Ph.D.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		The reviewer provided no specific observations/comments.

Jordan Ned Smith, Ph.D.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		When I run the provided model code, the model simulates a 3-week exposure and plots data from days 1, 5 and 19. I did not find a description of data from days 5 and 19 in the report. Is there a description of this data available for evaluation?

Raymond S.H. Yang, Ph.D.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		See numerous observations/comments under the Charge Questions.

Yiliang Zhu, Ph.D.

Page	Paragraph	Comment or Question on the draft report PBPK modeling for chloroprene (Ramboll, 2020)
		Figures in Supplement B often do not have adequate legends. For example, no explanation of the color-coded data and the model in Figure B-1; unclear if the scatter plot in Figure B-3 is the MCMC samples for Kgl and Km.
		Throughout the report, authors used the term 95% confidence interval to describe presumably the mid-95 percentile range of the posterior distribution derived from MCMC analysis. Such a range is NOT a confidence interval.
15	last	Clearance from metabolism vials (solid circles) appear to be straight lines with fast declining than controls (open circles). The statement "nonlinear" curve for metabolic clearance is not supported by the data in Figure 5.
16	Figure 6	Notes are needed to explain right panel.
Supp B	Figures B.3 and B.4	B.3. uses log10 and B4 uses ln. Be consistent
Supp B P7	Figure B-4	Reported estimate of Kgl is 0.22 (95% CI 0.19-0.33. These results are not supported by the density of Kgl whether on take log10 or ln.
	Figure B-4	The posterior densities do not support the reported MCMC estimates of Km, Vmax, and Kgl: when the distribution is extremely skewed, mode or median is preferred to mean. Should report 95% percentile range. Mean plus minus 2 SD should not be used.
P 9	Table B-2	Using log-normal(1,1) as a prior for SD is extremely unusual, requires strong justification
Supp B P3	Figure B-1	The color codes are confusing. Legends are needed. P2 indicates 12 vials or 12 replications at each of the 10 contacting time. Then steps 7 states replacing 5 second contact sample by 600 second contact sample at R-15. What was the true number of replications?

Specific observations or comments on a supplemental analysis of metabolite clearance (U.S. EPA, 2020) entitled, "Supplement: Uncertainty Analysis of In Vitro Metabolic Parameters and of In Vitro to In Vivo Extrapolation (IVIVE) Used in a Physiologically Based Pharmacokinetic (PBPK) Model for Chloroprene."

Leslie Z. Benet, Ph.D.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
		I have no specific observations that I have not already addressed in my responses above.

Jeffrey J. Heys, Ph.D., P.E.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
		The reviewer provided no specific observations/comments.

Jochem Louisse, Ph.D.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
		The reviewer provided no specific observations/comments.

Annie Lumen, Ph.D.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
		The reviewer provided no specific observations/comments.

Kenneth M. Portier, Ph.D.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
		The reviewer provided no specific observations/comments.

Kan Shao, Ph.D.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
		The reviewer provided no specific observations/comments.

Jordan Ned Smith, Ph.D.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
		The reviewer provided no specific observations/comments.

Raymond S.H. Yang, Ph.D.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
		See numerous observations/comments under the Charge Questions.

Yiliang Zhu, Ph.D.

Page	Paragraph	Comment or Question on the supplemental analysis of metabolite clearance (U.S. EPA, 2020)
14	Figure 7	Title and y-lab are inconsistent with the y-axis of the plot: the latter is frequency. Change y-axis to fraction or relative frequency would make them consistent.
18	Figure 10	Same as above
18	Figure 10	The KDE estimate suggest a non-negligible fraction <0 was truncated. This raises a concern either on the experimental data or on the method in which we generated and accepted the posterior samples.
19	Lines 3- 6	Kf=Vmax/Km. Unable to determine Km due to low enzyme activity does not imply Vmax=0. In the last sentence “with Vmx=0” is confusing needs clarification.
19	Line 18-19	So the example suggests the rate of metabolism and non-specific loss was about the same according the data.

Appendix A. List of Peer Reviewers

External Peer Review Meeting for
Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolite Clearance (U.S. EPA, 2020)

October 5-6, 2020

LIST OF REVIEWERS

Leslie Z. Benet, Ph.D.
University of California San Francisco
Expertise: Metabolic rates in vitro

Jeffrey J. Heys, Ph.D., P.E.
Montana State University
Expertise: Mass transport, fluid dynamics, and molecular diffusion

Jochem Louisse, Ph.D.
Wageningen Food Safety Research (WFSR),
part of Wageningen University and Research, the Netherlands
Expertise: PBPK modeling; Metabolic rates in vitro

Annie Lumen, Ph.D.
U.S. Food and Drug Administration, National Center for Toxicological Research (NCTR)
Expertise: PBPK modeling

Kenneth M. Portier, Ph.D. (Chair)
Independent Consultant
Expertise: Statistics; PBPK modeling

Kan Shao, Ph.D.
Indiana University
Expertise: Statistics

Jordan Ned Smith, Ph.D.
Pacific Northwest National Laboratory (PNNL)
Expertise: PBPK modeling; Metabolic rates in vitro

Raymond S. H. Yang, Ph.D.
Colorado State University
Expertise: PBPK modeling

Yiliang Zhu, Ph.D.
University of New Mexico School of Medicine
Expertise: Statistics

Appendix B. Meeting Agenda

AGENDA

External Peer Review Meeting for Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolite Clearance (U.S. EPA, 2020)

October 5, 2020 9:00 AM to 5:00 PM (EDT)
October 6, 2020 9:00 AM to 5:00 PM (EDT)

(434) 329-7637 (833) 752-1787 (Toll-free)	Code: 649 256 371#

Webinar Link: Join Microsoft Teams Meeting

October 5, 2020

9:00 AM EDT	**Welcome, Goals of Meeting, and Introductions** David Bottimore, Versar, Inc.
9:25 AM EDT	**EPA Introduction to the Meeting** Kris Thayer, U.S. EPA, Director, Chemical and Pollutant Assessment Division, Center for Public Health and Environmental Assessment (CPHEA)
9:35 AM EDT	**Presentation(s) on the Model** Robinan Gentry/Harvey Clewell, Ramboll
10:05 AM EDT	**EPA Uncertainty Analysis Overview and Context** Paul Schlosser/Dustin Kapraun, U.S. EPA, CPHEA
10:20 AM EDT	*Break**
10:30 AM EDT	**Observer Comment Session**
11:30 AM EDT	**Chair's Introduction and Review of Charge** Ken Portier, Chair
11:50 AM EDT	*Lunch Break**
12:45 PM EDT	**Round Table General Overview Comments**
1:30 PM EDT	**Discussion - Response to Charge Questions (Initial Question(s))**
3:00 PM EDT	*Break****
3:15 PM EDT	**Discussion - Response to Charge Questions (Continued)**
5:00 PM EDT	Adjourn

AGENDA

External Peer Review Meeting for Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolite Clearance (U.S. EPA, 2020)

October 5, 2020 9:00 AM to 5:00 PM (EDT)
October 6, 2020 9:00 AM to 5:00 PM (EDT)

(434) 329-7637 (833) 752-1787 (Toll-free)	Code: 649 256 371#

Webinar Link: Join Microsoft Teams Meeting

October 6, 2020

9:00 AM EDT **Agenda for Continuing Peer Review Meeting**
David Bottimore, Versar, Inc.
Ken Portier, Chair

9:15 AM EDT **Discussion – Response to Charge Questions (continued)**

10:30 AM EDT *Break**

10:45 AM EDT **Discussion – Response to Charge Questions (continued)**

12:00 PM EDT *Lunch Break**

1:00 PM EDT **Discussion – Response to Charge Questions (continued)**

3:00 PM EDT *Break**

3:15 PM EDT **Discussion – Response to Charge Questions (continued)**

4:45 PM EDT **Wrap-up/Next Steps**
David Bottimore, Versar, Inc.
Ken Portier, Chair

5:00 PM EDT Adjourn

Appendix C. List of Observers and Commenters

LIST of OBSERVERS and COMMENTERS
October 5 and 6, 2020

External Peer Review Meeting for Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolite Clearance (U.S. EPA, 2020)

Name	Affiliation	Oral Comments
Bruce Allen	Bruce Allen Consulting	
Melvin Andersen	Andersen ToxConsulting LLC	
James Avery	U.S. EPA	
Jeremy Bernstein	Inside EPA	
Joseph Bruno	Bruno & Bruno	
Joelle Burnham	Wadsworth Center, NYS DOH	
Christine Cai	U.S. EPA	
Jerry Campbell	Ramboll	
Andrea Candara	NYS Dept of Health	
Wayne Cascio	U.S. EPA	
Harvey Clewell	Ramboll	
Tokesha Collins-Wright	Louisiana Chemical Association	Yes
Theron Cooper	Louisiana Chemical Association	
Sivanesan Dakshanamurthy	Georgetown University	
Lou D'Amico	U.S. EPA	
J. Allen Davis	U.S. EPA	
Qiaoxiang Dong	CDPR	
Ingrid Druwe	U.S. EPA	
Julija Filipovska	Independent	
Amanda Fitzmorris	U.S. EPA	
Nobuhiko Fujii	Denka Company Ltd.	
Robinan Gentry	Ramboll	Yes
Lydia Gerard	Community Member	
Mary Hampton	Community Member	
Suzanne Hartigan	American Chemistry Council	
Dale Hattis	Clark University	Yes1
Esther Haugabrooks	PCRM	
Maria Hegstad	Inside EPA	
Tammy Houston	N/A	
Nan-Hung Hsieh	CDPR	
Terry Hyland	Chemical Watch	
Lindsey Jones	U.S. EPA	

Name	**Affiliation**	**Oral Comments**
Samantha Jones	U.S. EPA	
Marco Kaltofen	Boston Chemical Data Corp	
Zachary Kanzler	Critical Visualization/Media Lab at Tulane	
Dustin Kapraun	U.S. EPA	
Muhammad Karimi	U.S. EPA	
Athena Keene	Afton Chemical Corporation	
Alexander Kliminsky	GHD	
Hugh Lambert	The Lambert Firm, PLC	
David LaPlante	N/A	
David Leong	Spectra Health & Safety Consulting	
Pinpin Lin	National Health Research Institutes	
Yu-Sheng Lin	U.S. EPA	
Summer Lingard-Smith	U.S. GAO	
Michelle Mabson	Earthjustice	Yes
Brian Mersman	The Lambert Firm, PLC	
Viktor Morozov	U.S. EPA	
Anuradha Mudipalli	U.S. EPA	
Keeve Nachman	Johns Hopkins BSPH	
Ruhan Nagra	University Network for Human Rights	
Robert Nocco	Self	
Jennifer Orme-Zavaleta	U.S. EPA	
Cayce Peterson	The Lambert Firm, PLC	
Margaret Pratt	U.S. EPA	
Siva Ramoju	Risk Sciences International	
Kelley Raymond	U.S. EPA	
Flora Ratpan	NOVA Chemicals	
Don Reichert	Bruno & Bruno, LLP	
Bruce Rodan	U.S. EPA	
Fakhralzoha Rousta	University student	
Katerine Saili	U.S. EPA	
Joshua Salley	Ramboll	
Jennifer Sass	NRDC	Yes
Sonja Sax	Ramboll	
Paul Schlosser	U.S. EPA	
Rachel Shaffer	U.S. EPA	
Dahnish Shams	U.S. EPA	
Tracy Sheppard	U.S. EPA	
Darcie Smith	U.S. EPA	
Babasaheb Sonawane	Georgetown University	
Larry Sorapuru	Retired	
Vicki Soto	U.S. EPA	

Name	**Affiliation**	**Oral Comments**
Wilma Subra	Louisiana Environmental Action Net	Yes2
June Sutherlin	Louisiana DEQ	
Robert Taylor	Community Member	
Kris Thayer	U.S. EPA	
Joanne Trgovcich	ICF	
Cynthia Van Landingham	Ramboll	
John Vandenberg	U.S. EPA	
Victor Idinmachukwu Vincent Anoliefo	Eco-G	
Patrick Walsh	Denka Performance Elastomer	
Geraldine Watkins	Community Member	
Paul White	U.S. EPA	
Kelly Widener	U.S. EPA	
Miglena Wilbur	DPR, CalEPA	
John Wilhelmi	ERG	
Linda Wilson	NYS OAG	

[1] Dale Hattis provided oral comments on behalf of Marco Kaltofen and Keeve Nachman.
[2] Wilma Subra provided oral comments on behalf of the Concerned Citizens of St. John.

Appendix D. Meeting Presentations

Peer Review of the Report on Physiologically Based Pharmacokinetics (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolite Clearance (U.S. EPA, 2020)

October 5-6, 2020

David Bottimore
Versar, Inc.

VERSAR
Your Needs. Our Mission.

1

Overview of Peer Review Meeting

- Purpose of peer review
- Peer review meeting:
  (1) Overview of peer review process
  (2) Presentations of documents, model, other materials
  (3) Observer comments
  (4) Chair's introduction and review of charge
  (5) Discuss recommendations and suggestions by charge question
- Next steps

2

Agenda for Peer Review Meeting

- October 5, 2020
  - Welcome, Goals, Introduction of Reviewers
  - EPA Introduction
  - Presentation on the Model (Ramboll)
  - Uncertainty Analysis Overview and Context (EPA)
  - Observer Comment Session
  - Chair's Introduction and Review of Charge
  - Round Table – General Overview Comments
  - Reviewer Responses to Charge Questions (1 to ?)
- October 6, 2020
  - Reviewer Responses to Charge Questions (? to 19)
  - Wrap Up/Next Steps


AGENDA

External Peer Review Meeting for
Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolic Clearance (U.S. EPA, 2020)

October 5, 2020 9:00 AM to 1:00 PM (EDT)
October 6, 2020 9:00 AM to 1:00 PM (EDT)

October 5, 2020

9:00 AM EDT — Welcome, Goals of Meeting, and Introductions: David Bottimer, Versar, Inc.

9:25 AM EDT — EPA Introduction to the Meeting: Kris Thayer, U.S. EPA, Director, Chemical and Pollutant Assessment Division, Center for Public Health and Environmental Assessment (CPHEA)

9:55 AM EDT — Presentation(s) on the Model: Rebuna Gentry/Harvey Clewell, Ramboll

10:05 AM EDT — EPA Uncertainty Analysis Overview and Context: Paul Schlosser/Dustin Kapraun, U.S. EPA, CPHEA

10:20 AM EDT — *Break**

10:30 AM EDT — Observer Comment Session

11:30 AM EDT — Chair's Introduction and Review of Charge: Kris Porter, Chair

11:50 AM EDT — *Lunch Break**

12:45 PM EDT — Round Table General Overview Comments

1:30 PM EDT — Discussion - Response to Charge Questions (Initial Question(s))

3:00 PM EDT — *Break**

3:15 PM EDT — Discussion - Response to Charge Questions (Continued)

5:00 PM EDT — Adjourn

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Selection of Expert Peer Reviewers

EPA Develop Selection Criteria	• PBPK, statistics, IVIVE/metabolic rates, mass transport, fluid dynamics, molecular diffusion
Identified Experts	• Independent search by Versar
Evaluated Credentials	• Reviewed scientific and technical expertise
Conducted COI Screening	• Reviewed professional and financial information
Developed Interim List	• Narrowed list to 19 candidates • Public comment on interim list

Selected Final 9 Reviewers

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Peer Review Process

- Individual pre-meeting reviews by each expert
- Discussion during peer review meeting
- Individual comments: everyone participates
- Consensus is not necessary/actively sought
- Chair facilitates discussion, lead reviewers per question
- Revisions and additions to premeeting comments and preparation of final peer review report


DRAFT
Pre-Meeting Peer Review Summary Report

External Peer Review of a Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolite Clearance (U.S. EPA, 2020)

October 2, 2020

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Ground Rules for Meeting

- Keep discussion focused on subject, scope, and timing
- Focus on scientific content of documents, model, and data
- Peer review among the 9 reviewers is the primary activity – not a dialogue with EPA and/or observers
- Transparency – Discussions are to take place on the virtual call, not during breaks, lunch, etc.
- Chair's prerogative – timing, breaks, etc.

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Introduction of Reviewers

Kenneth M. Portier (*Chair)*
Independent Consultant

Leslie Z. Benet
University of California
San Francisco

Jeffrey J. Heys
Montana State University

Jochem Louisse
Wageningen Food Safety Research, part of Wageningen University and Research, Wageningen, the Netherlands

Annie Lumen
Food and Drug Administration
National Center for Toxicological Research

Kan Shao
Indiana University

Jordan Ned Smith
Pacific Northwest National Laboratory

Raymond S. H. Yang
Colorado State University

Yiliang Zhu
University of New Mexico

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Housekeeping

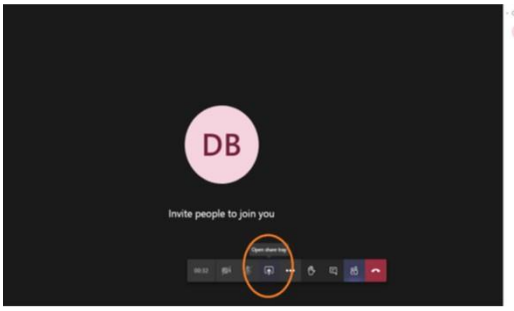
chloroprenepbpk@versar.com

"Presenters" – share screen, unmuted

"Attendees" – Muted, listen-only mode

*6 to unmute phone

Observer Commenters
- Unmuted for session 10:30-11:30
- share screen



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Agenda

AGENDA

External Peer Review Meeting for
Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020) and a Supplemental Analysis of Metabolite Clearance (U.S. EPA, 2020)

October 5, 2020 9:00 AM to 5:00 PM (EDT)
October 6, 2020 9:00 AM to 5:00 PM (EDT)

October 5, 2020

9:00 AM EDT	**Welcome, Goals of Meeting, and Introductions** David Bottimore, Versar, Inc.
9:25 AM EDT	**EPA Introduction to the Meeting** Kris Thayer, U.S. EPA, Director, Chemical and Pollutant Assessment Division, Center for Public Health and Environmental Assessment (CPHEA)
9:35 AM EDT	**Presentation(s) on the Model** Robinan Gentry/Harvey Clewell, Ramboll
10:05 AM EDT	**EPA Uncertainty Analysis Overview and Context** Paul Schlosser/Dustin Kapraun, U.S. EPA, CPHEA
10:20 AM EDT	*Break**
10:30 AM EDT	**Observer Comment Session**
11:30 AM EDT	**Chair's Introduction and Review of Charge** Ken Portier, Chair

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EPA Introduction to the Meeting

Kris Thayer, EPA

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External Peer Review for a Report on Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene and a Supplemental Analysis of Parameter and Model Uncertainty: Introduction

Kristina Thayer
Director, Chemical & Pollutant Assessment Division (CPAD)
Center for Public Health and Environmental Assessment (CPHEA)
Office of Research and Development
U.S. Environmental Protection Agency

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- **IRIS assessments contribute to decisions across EPA and other health agencies.**
- **Toxicity values**
  - Noncancer: Reference Doses (RfDs) and Reference Concentrations (RfCs).
  - Cancer: Oral Slope Factors (OSFs) and Inhalation Unit Risks (IURs).
- **IRIS assessments have no direct regulatory impact until they are combined with**
  - Extent of exposure to people, cost of cleanup, available technology, etc.
  - Regulatory options.
  - Both of these are the purview of EPA's program offices.

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Chloroprene Background and Timeline

- **2010:** EPA finalizes the IRIS Toxicological Review of Chloroprene. EPA classifies chloroprene as a "Likely to be carcinogenic to humans" via the inhalation route of exposure.
  - Multiple tumor sites were identified in animals during EPA's evaluation of chloroprene.
- **2015:** EPA published the National Air Toxics Assessments (NATA). Updated assessment indicated high levels of chloroprene around the Denka Performance Elastomer LLC (DPE) plant in LaPlace, La.
- **2017:** EPA received a Request for Correction (RFC) provided on behalf of DPE under EPA's Information Quality Guidelines.
- **2018:** EPA denied DPE's Request for Correction. EPA concluded that the underlying information and conclusions presented in the IRIS Chloroprene assessment are consistent with the EPA's Information Quality Guidelines.
  - EPA conducted an evaluation of the literature published since the 2010 finalization of the IRIS Assessment of Chloroprene.
  - EPA did not identify evidence that would change its conclusions, including the availability of a physiologically based pharmacokinetic (PBPK) model published in 2012. EPA evaluated the suitability of the model for inclusion into the assessment, but uncertainties were identified that prevent its application to an IRIS assessment.

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Chloroprene Background and Timeline (Cont'd)

- **2018:** DPE submitted a Request for Reconsideration of Denial of Request for Correction (RFR) with regard to EPA's decision. DPE entered discussions with EPA to address uncertainties identified by EPA regarding PBPK modeling for chloroprene.
- **2019:** DPE conducted additional laboratory studies and modeling work to address uncertainties identified by EPA.
- **2020:** EPA initiates an independent peer review on the documents, *Physiologically Based Pharmacokinetic (PBPK) Modeling for Chloroprene (Ramboll, 2020)* and *Supplement: Uncertainty Analysis of In Vitro Metabolic Parameters and of In Vivo Extrapolation (IVIVE) Used in a Physiologically Based Pharmacokinetic (PBPK) Model for Chloroprene (U.S. EPA, 2020)*.
- **2020:** Once the peer review report is finalized, EPA will evaluate the recommendations provided by the reviewers and respond to the DPE's Request for Reconsideration (RFR).
  - If the RFR is granted, EPA will evaluate the impact of the model on the conclusions presented in the finalized 2010 IRIS assessment of Chloroprene.

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Presentation(s) on the Model

Robinan Gentry/Harvey Clewell, Ramboll

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RAMBOLL PBPK MODEL AND MODEL VALIDATION

Harvey Clewell, PhD, DABT, FATS

P. Robinan Gentry, PhD, DABT Jerry

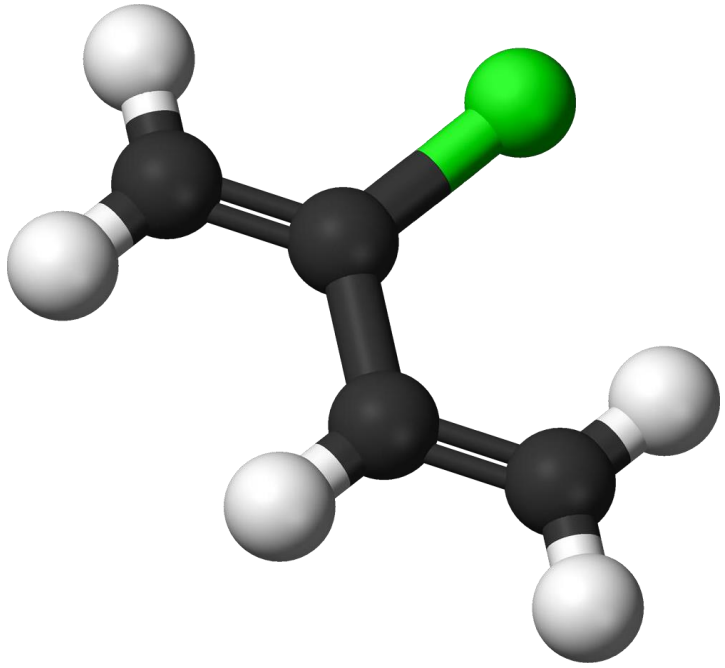
Campbell, PhD

Cynthia Van Landingham, MS

Melvin Andersen, PhD

Bruce Allen, MA

Sonja Sax, ScD



RAMBOLL

OVERVIEW

- Background: History of interacting with USEPA on the chloroprene PBPK model as part of the Request for Correction
- Evidence demonstrating the need for a PBPK correction
- Why use a PBPK model?
- Mode of action considerations
- The updated PBPK model for chloroprene
- Model testing and validation
- Uncertainty analysis
- Conclusions

RAMBOLL

2016

- Initial Denka Performance Elastomer (DPE)/Ramboll meeting with USEPA to discuss updating the 2010 IRIS Assessment, which included the Himmelstein (2004a,b) model.

2017

- USEPA conducted a quality review of an earlier published PBPK model (Yang et al. 2012) as part of the Request for Correction, raising concerns regarding its reliance on *in vitro* data.

2018

- Submitted DPE/Ramboll updated PBPK model to USEPA, and addressed questions raised during the Request for Correction review, including the reliance on *in vitro* data.
- Developed protocol for a USEPA-requested experiment to determine a chloroprene mass-transport parameter (Kgl).

2019

- Conducted the Kgl experiment with DPE based on an USEPA-approved protocol.
- Modified the Ramboll PBPK model to incorporate Kgl, considering discussions and recommendations from Dr. Schlosser.

2020

- January: Revised chloroprene PBPK model published in Inhalation Toxicology (Clewell et al. 2020).
- February: Chloroprene weight of evidence analysis published in print in Risk Analysis (Sax et al. 2020).
- April: Submitted chloroprene PBPK model documentation (Ramboll 2020) to USEPA for peer review.

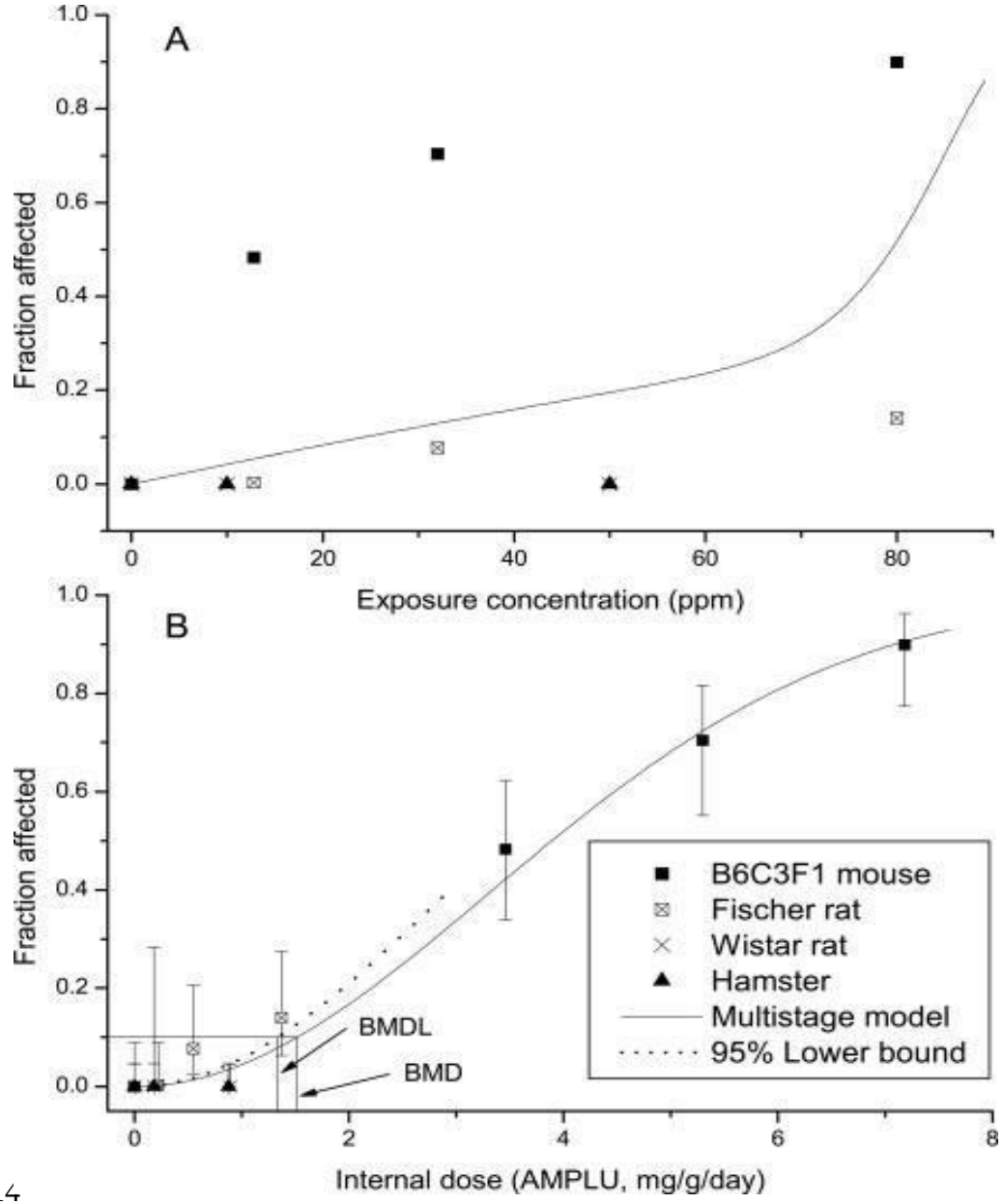
EVIDENCE DEMONSTRATING THE NEED FOR A PBPK CORRECTION

- Application of the PBPK model explains the differences across animal species (Himmelstein et al. 2004b), as well as the differences between animals and humans (Allen et al. 2014).
- USEPA (2010) IRIS Assessment for Chloroprene notes that "a PBPK model for the internal dose(s) of the reactive metabolite(s) would decrease some of the quantitative uncertainty in interspecies extrapolation."
- The USEPA (2005) "Guidelines for Carcinogen Risk Assessment" note that toxicokinetic or PBPK modeling is the preferred approach for estimating dose metrics from exposure.
- PBPK-derived estimates are necessary so that results are more consistent with cancer incidence observed in occupational studies; Marsh et al. (2007) found no evidence of excess cancer risk in a cohort of over 12,000 workers (1,100 from the Louisiana plant alone) i.e., none of the observed cancers were shown to be associated with chloroprene compared to local county cancer rates.
- Lung cancer incidence rates reported by the Louisiana Tumor Registry for St. John the Baptist Parish (where DPE is located) are lower than state cancer rates, indicating no excess lung cancers in the communities around the plant; other cancer rates are also lower or no different than state rates (Maniscalco et al. 2020).
- All the lines of evidence are outlined in Sax et al. (2020) and indicate a need for PBPK correction.

WHY USE A PBPK MODEL?

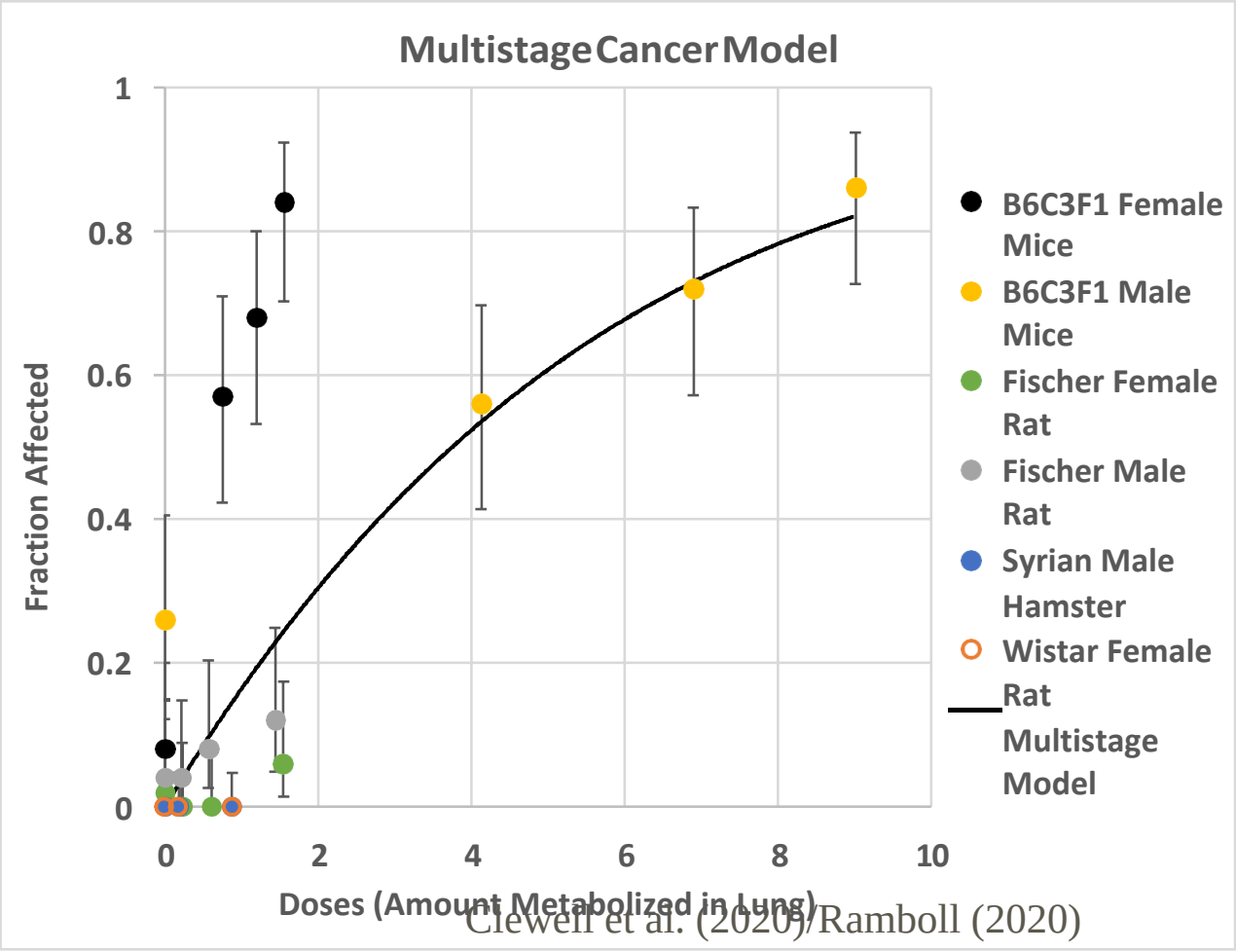
- Inhaled chloroprene concentration does not correlate with observed lung tumor incidence for different species in the chloroprene bioassays (top figure).
- The use of a PBPK model to predict total metabolism of chloroprene in the lung provided a consistent prediction of the lung tumor incidence in mice, rats and hamsters.
- The use of a dose metric based on tissue metabolism is consistent with the mode of action for chloroprene i.e., metabolic production of reactive epoxides.

Because mode of action matters!



RAMBOLL

SENSITIVITY OF THE FEMALE MOUSE

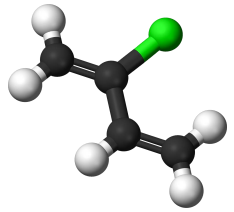


Clewell et al. (2020)/Ramboll (2020)

- The revised PBPK model confirms the results from Himmelstein et al. 2004b, but indicates that, based on target tissue dose, the female mouse is more susceptible to the effects of the chloroprene epoxides compared to male mice and other species.
- The female mouse lung also demonstrated a more sensitive genomic response to oxidative stress from chloroprene than the female rat lung (Thomas et al. 2013).
- Studies with other chemicals provide evidence of a proliferative response to toxicity by Club cells in the female mouse lung that is not observed in the male mouse lung (Yamada et al. 2017) and is not explained by differences in metabolism (Van Winkle et al. 2002, Sutherland et al. 2012).
- Using the internal dose metrics from the highly susceptible female mouse results in a more conservative (higher) risk estimate.

RAMBOLL

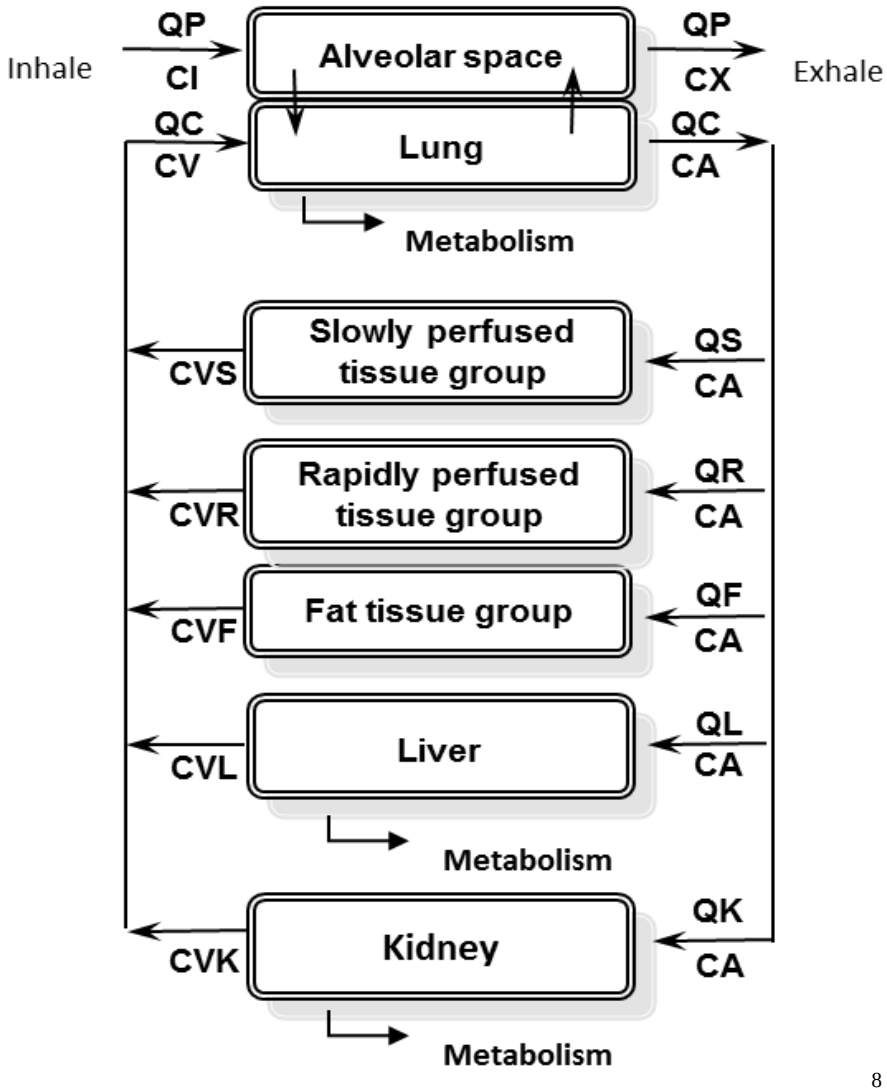
MODE OF ACTION CONSIDERATIONS



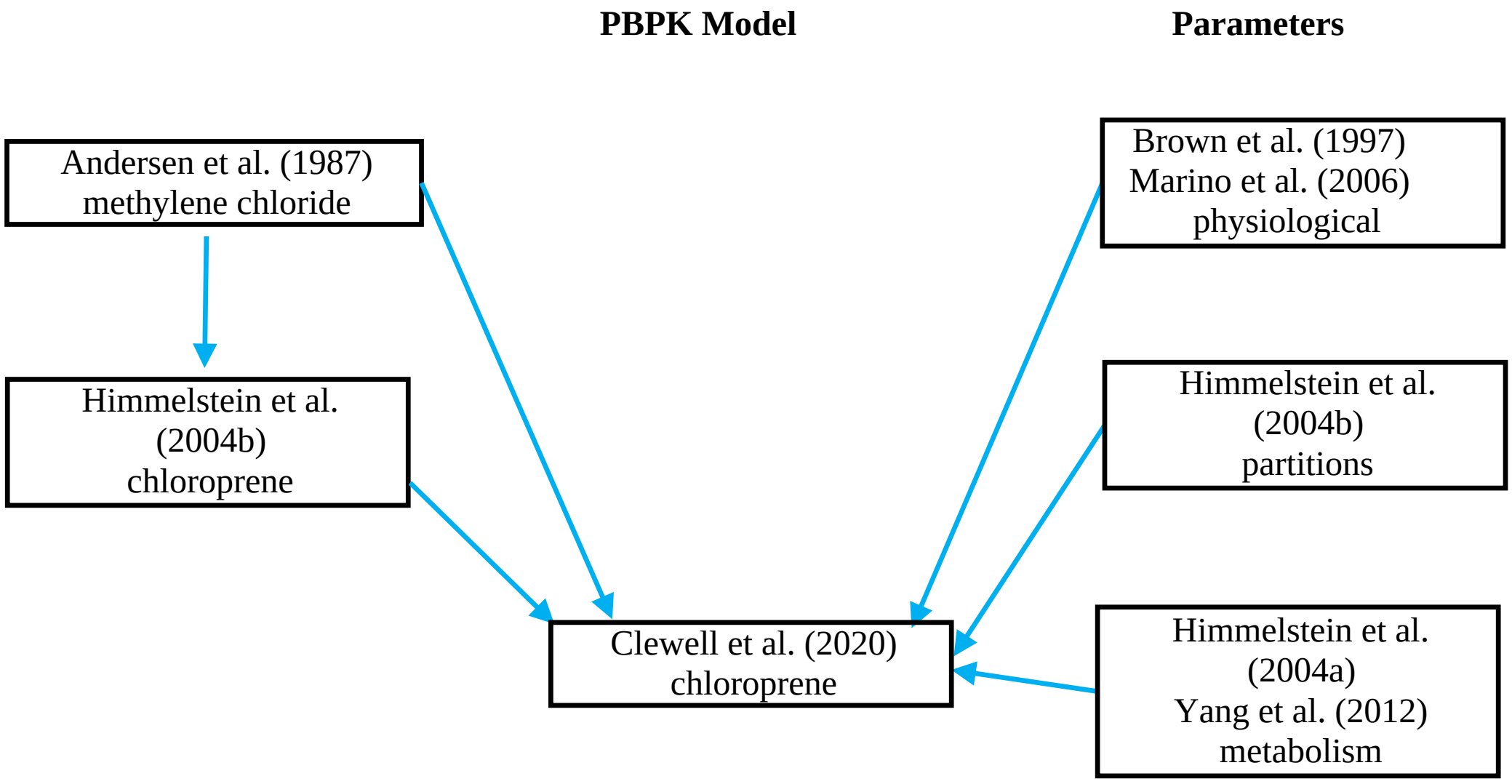
- Chloroprene is not in itself carcinogenic; tissue metabolism of chloroprene to highly reactive chloroalkyl epoxides is responsible for tumors observed in the cancer bioassays.
- The high reactivity of the chloro-epoxides limits their effects to the tissue in which they are generated.
- In contrast to the stable alkyl epoxides produced by the metabolism of chemicals like ethylene and butadiene, where clearance is by further metabolism and blood flow, the clearance of the chloroalkyl epoxides is by direct chemical reaction and is species invariant.
- Therefore, the appropriate dose metric is the total daily production of epoxides in the tissue of concern divided by the tissue volume (Andersen et al. 1987).
- Dose metrics based on chloroprene concentrations, whether in the inhaled air, blood or tissues, are not consistent with the mode of action and provide seriously erroneous estimates of risk for chemicals with the same mode of action as chloroprene (e.g., methylene chloride and vinyl chloride).

UPDATED CHLOROPRENE PBPK MODEL

- Structure based on PBPK model of methylene chloride (Andersen et al. 1987).
- Parameters obtained from the literature:
  - Physiological parameters: Brown et al. (1997)
  - Partition coefficients: Himmelstein et al. (2004b)
  - Metabolism parameters: Himmelstein et al. (2004a) and Yang et al. (2012)
- Code: R programming language
  - R-scripts for running mouse validation study and dose metrics in mouse, rat and human.
  - Documentation provided for all parameters.



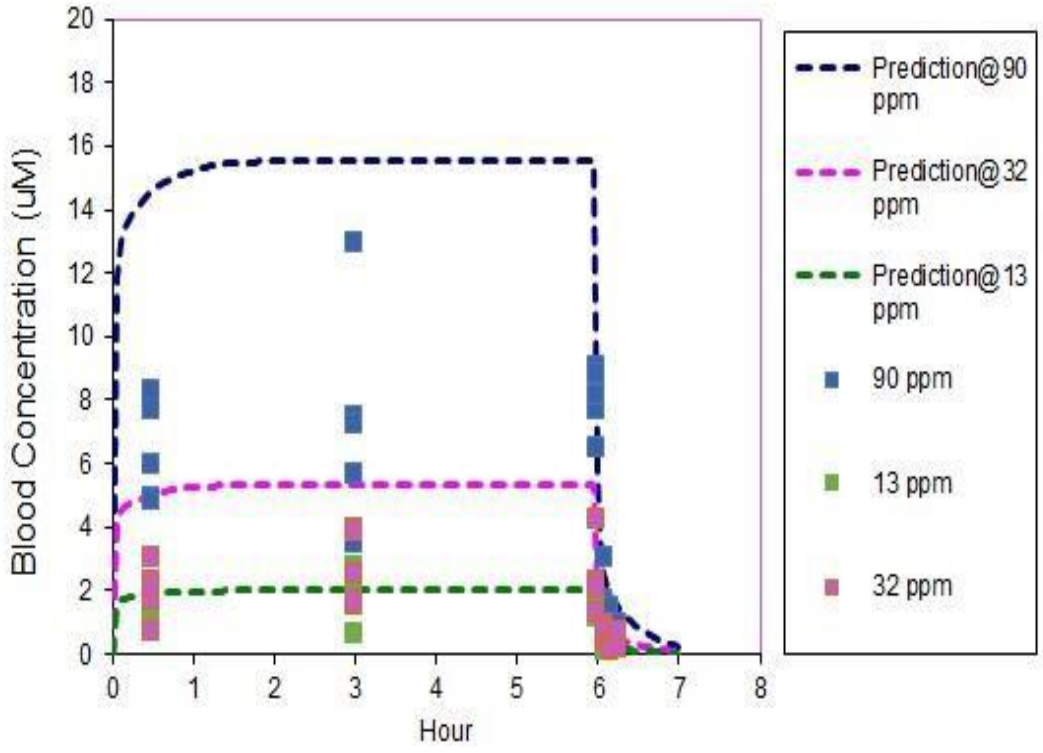
RAMBOLL


PBPK Model
Parameters
Andersen et al. (1987)
methylene chloride
Himmelstein et al.
(2004b)
chloroprene
Clewell et al. (2020)
chloroprene
Brown et al. (1997)
Marino et al. (2006)
physiological
Himmelstein et al.
(2004b)
partitions
Himmelstein et al.
(2004a)
Yang et al. (2012)
metabolism

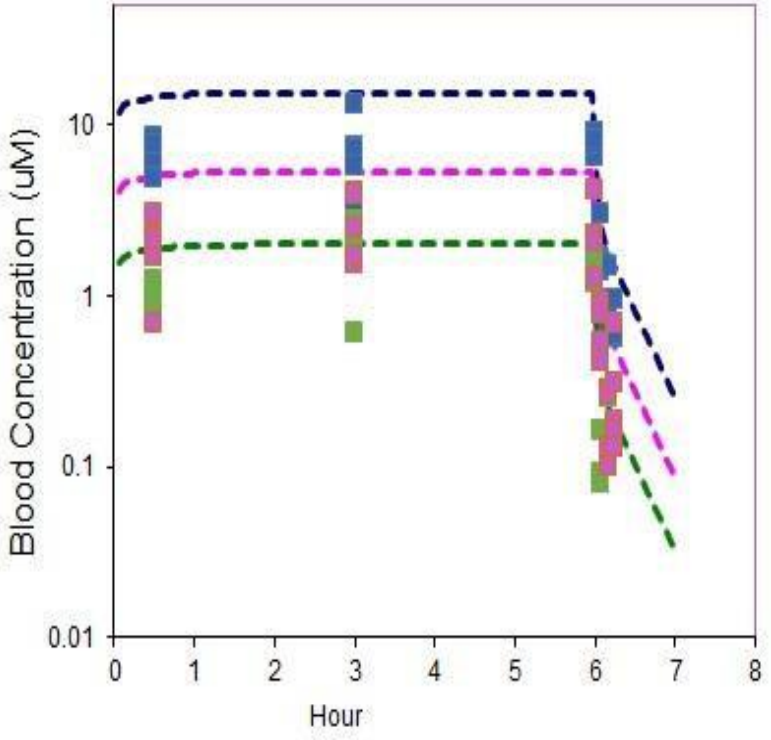
- Validation against the *in vivo* data
  - Ramboll tested the chloroprene PBPK model and found it was able to reproduce the blood concentrations reported in both the single and repeated exposure *in vivo* studies.
  - Ramboll evaluated the minute ventilation data from the chloroprene single exposure study and the metabolism induction data from the repeated exposure study and determined that there was no evidence of reduced ventilation or induction of metabolism in response to chloroprene exposure.
- Re-estimation of model parameters and consistency across tissues and genders
  - At the request of USEPA, Ramboll investigated the impact of re-estimating the published estimates from Yang et al. (2012) using an additional estimated mass transport parameter (Kgl) suggested by USEPA.
  - Ramboll conducted an analysis of the impact of the alternative parameter estimates on resulting dose metrics.
- Scale-up of in vitro data
  - A metabolism expert, Dr. Miyoung Yoon (now with USFDA), collaborated with Ramboll on the approach for conducting quantitative *in vitro* to *in vivo* extrapolation of the *in vitro* metabolism data.

VALIDATION OF THE MODEL

- 6-hour inhalation exposures of female mice to chloroprene (Clewell et al. 2020).
- The model predictions fit the in vivo results very well (within a factor of 2 of the means of animal data) with no adjustment of parameters.



Linear plot (concentrations)

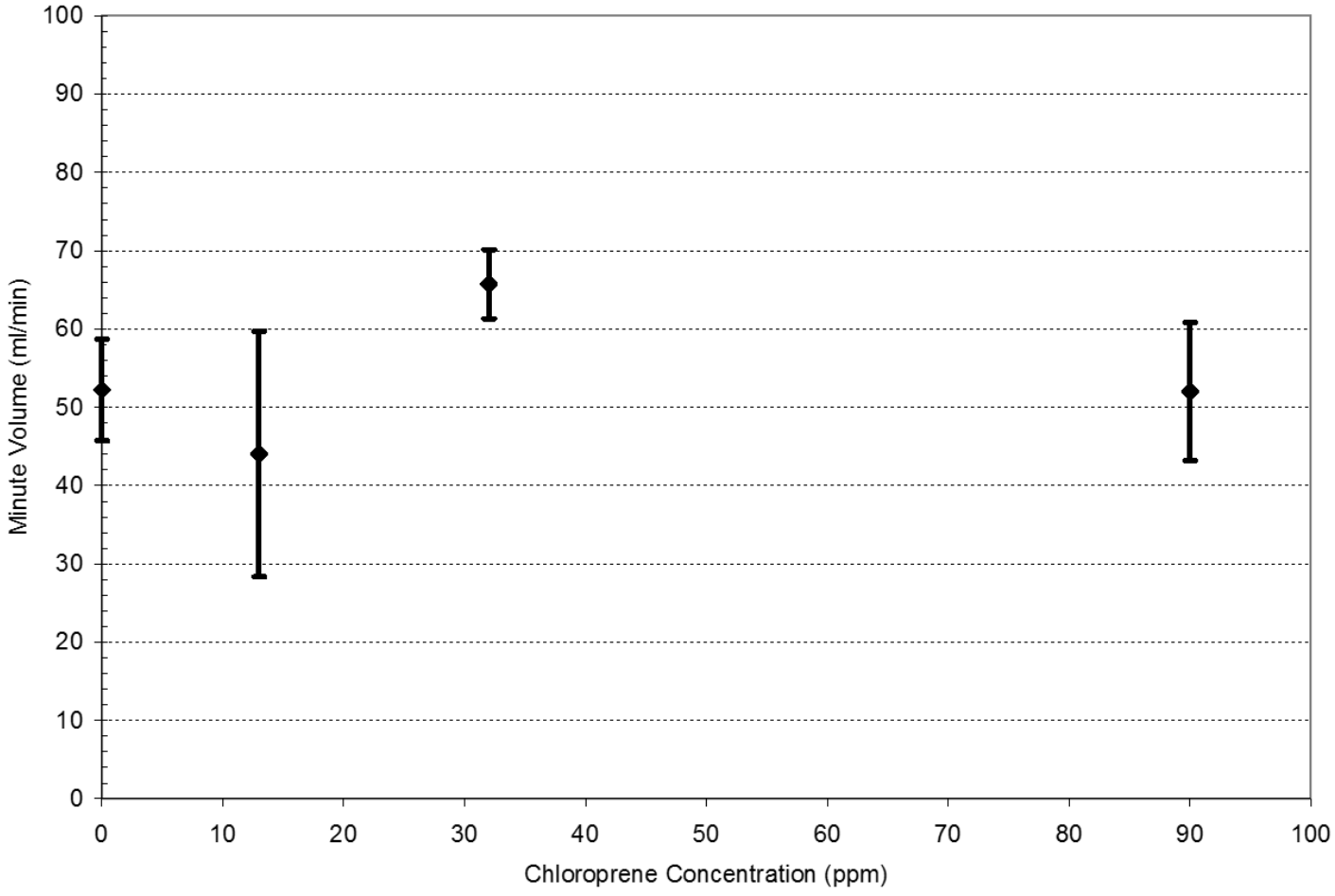


Log plot (rates)

VALIDATION OF THE MODEL

Minute ventilation during 6-hour inhalation exposures of female mice to chloroprene (Clewell et al. 2020)

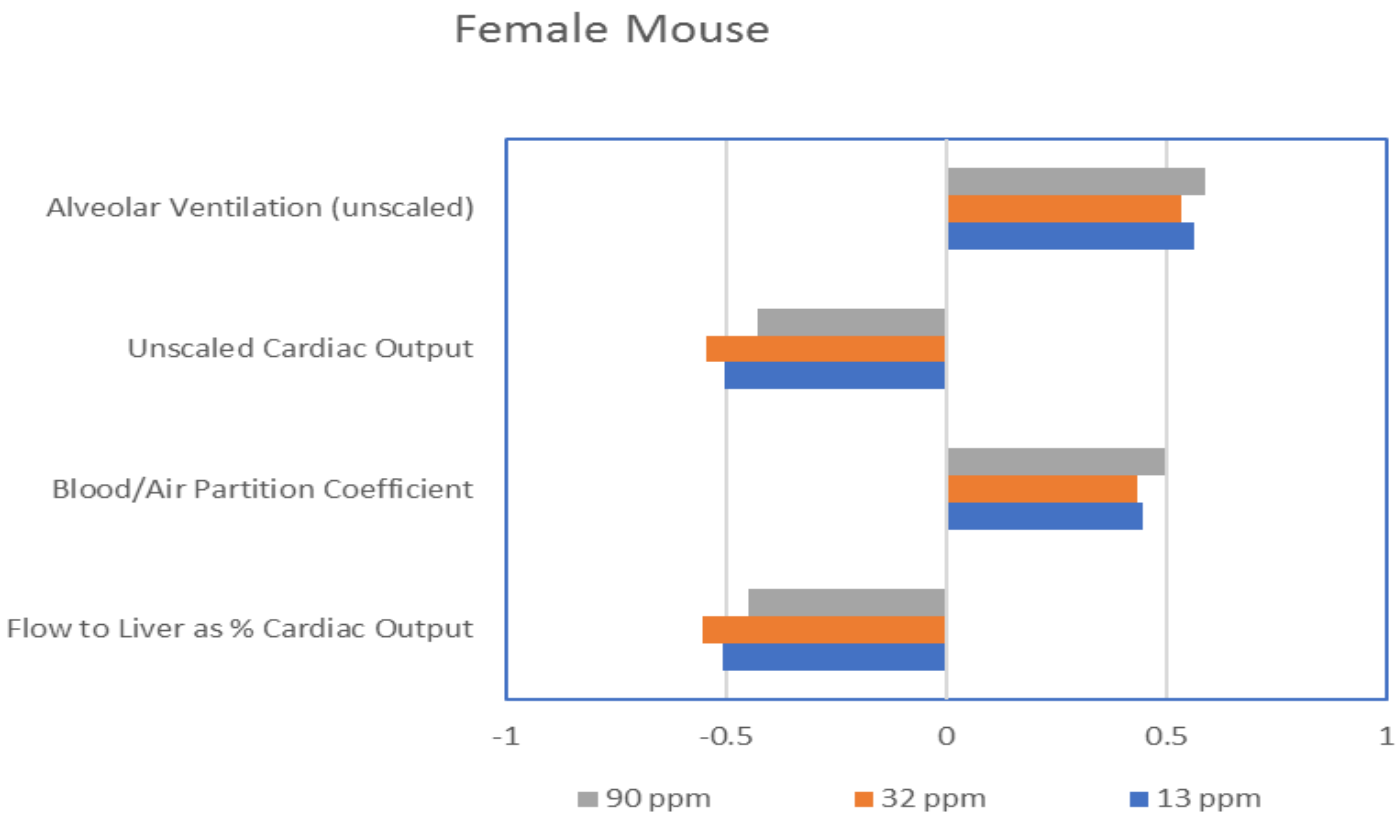
- Plot shows measured pulmonary ventilation (ml/min) as a function of chloroprene concentration.
- Results show that minute volume is not associated with chloroprene concentrations.
- This suggests that respiratory depression was not an issue.
- Alveolar ventilation used in PBPK model corresponds to average measured value.



RAMBOLL

MODEL PARAMETERS: SENSITIVITY OF BLOOD CONCENTRATION

(CVLC) TO CHANGES IN THE MODEL PARAMETERS

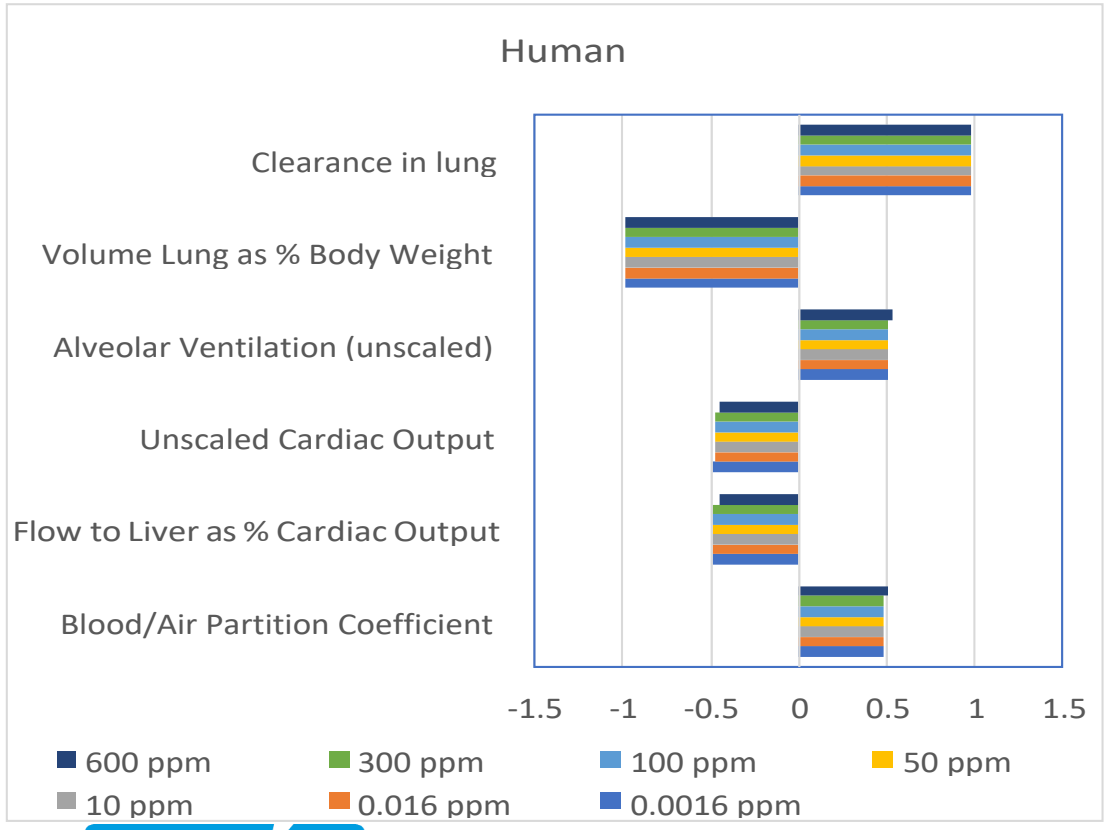


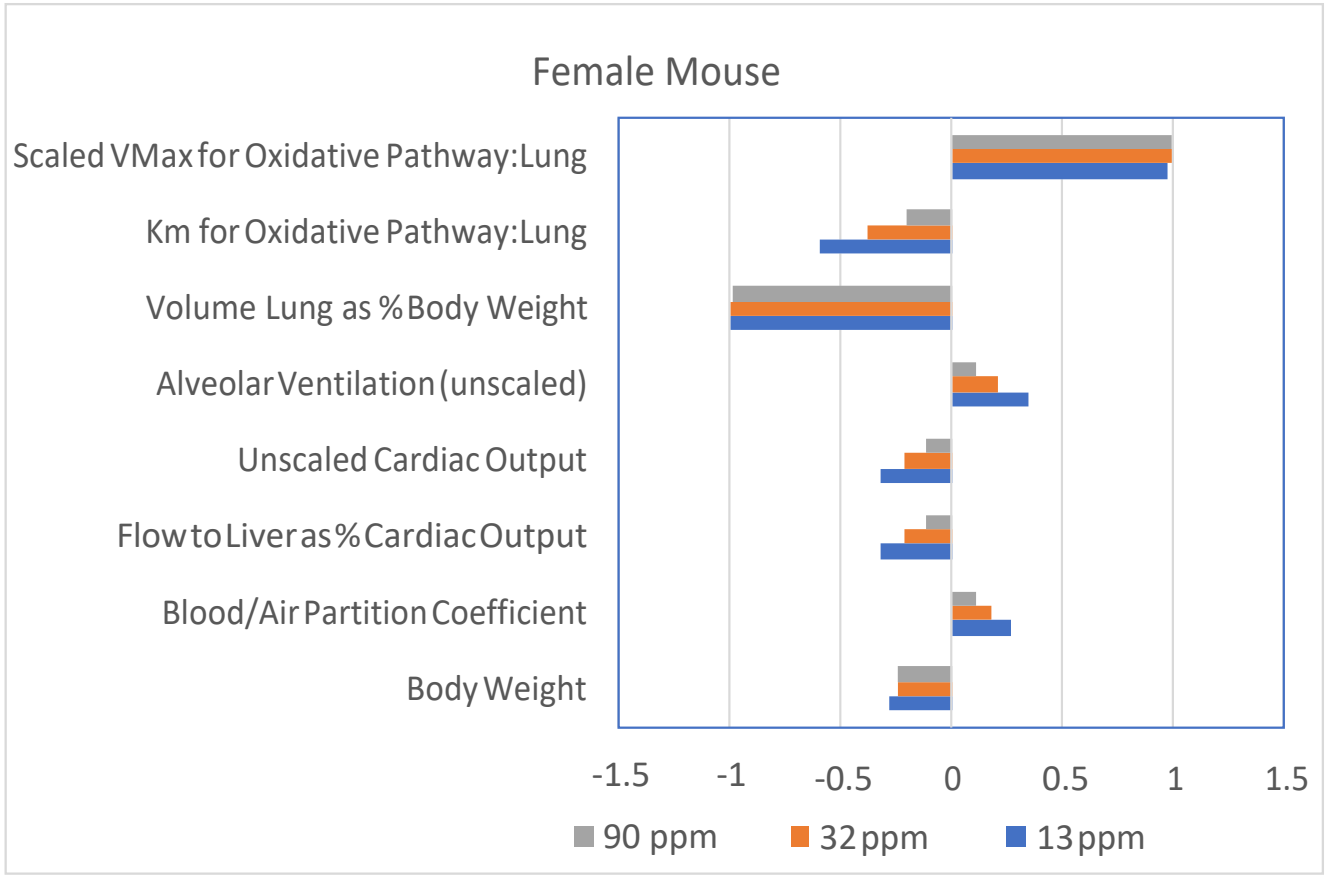
All sensitive parameters are either:

- directly measured (ventilation, blood/air partition) or
- obtained from physiological literature (cardiac output, liver blood flow)

MODEL PARAMETERS: SENSITIVITY ANALYSIS OF AMOUNT METABOLIZED IN THE LUNG DAILY PER GRAM OF TISSUE (AMPLU) TO CHANGES IN THE MODEL PARAMETERS

As expected, the lung dose metric is sensitive to the same parameters as the in vivo study, plus lung metabolism and lung volume.





RAMBOLL

INVESTIGATION OF TRANSPORT LIMITATION (KGL) DURING IN VITRO METABOLISM STUDIES

- USEPA raised questions regarding the transfer of chloroprene from the air to the media (Kgl) in the vials and how this could have affected the observed clearance rates reported in Himmelstein et al. (2004a) metabolism studies.

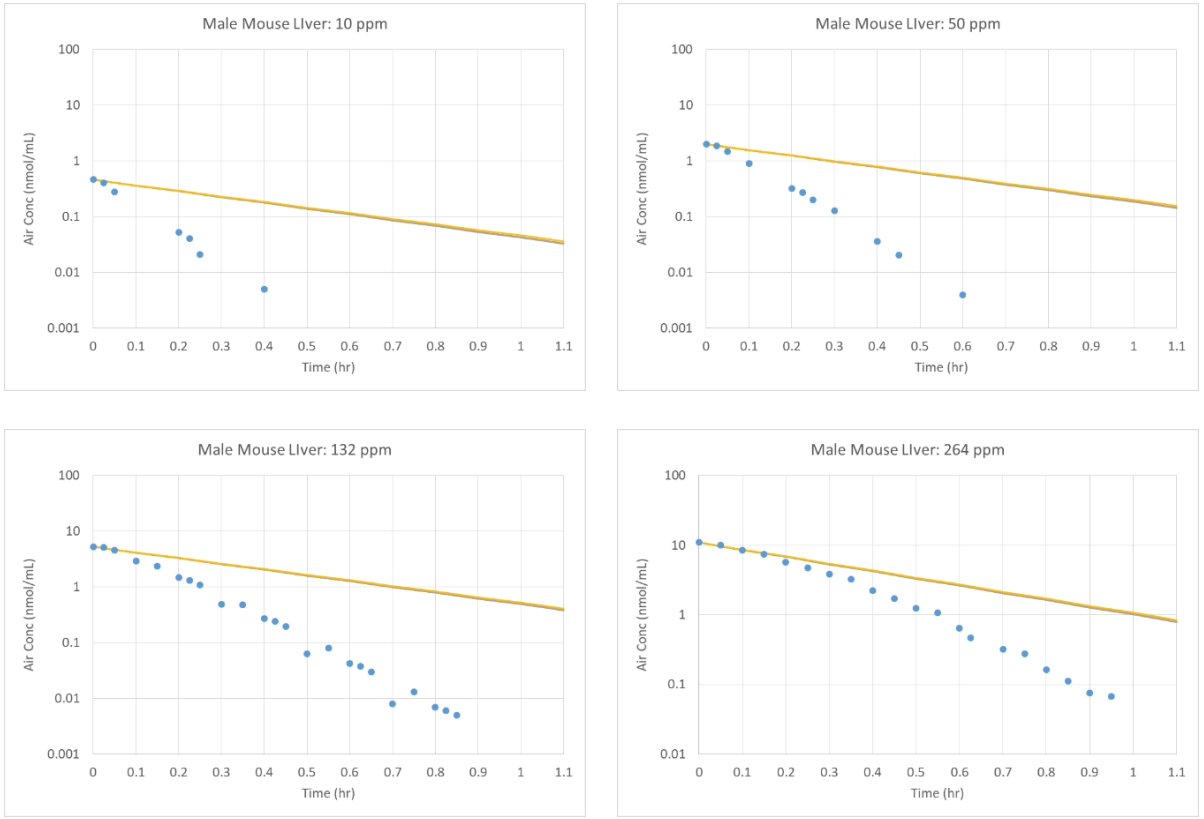
- At the request of USEPA, a new experimental study was performed to estimate a Kgl for chloroprene, following a protocol based on a benzene study conducted by Schlosser et al. (1993).

- The application of these data into the model demonstrated that the experimental value of Kgl obtained in this study was inconsistent with the high rates of liver metabolism reported in Himmelstein et al. (2004a).

- Therefore, Ramboll re-estimated the Kgl from the metabolism study data using an approach suggested by Dr. Schlosser (personal communication), which is based on the ratio of the mixing rates in the new Kgl study and the Himmelstein et al. (2004a) study.

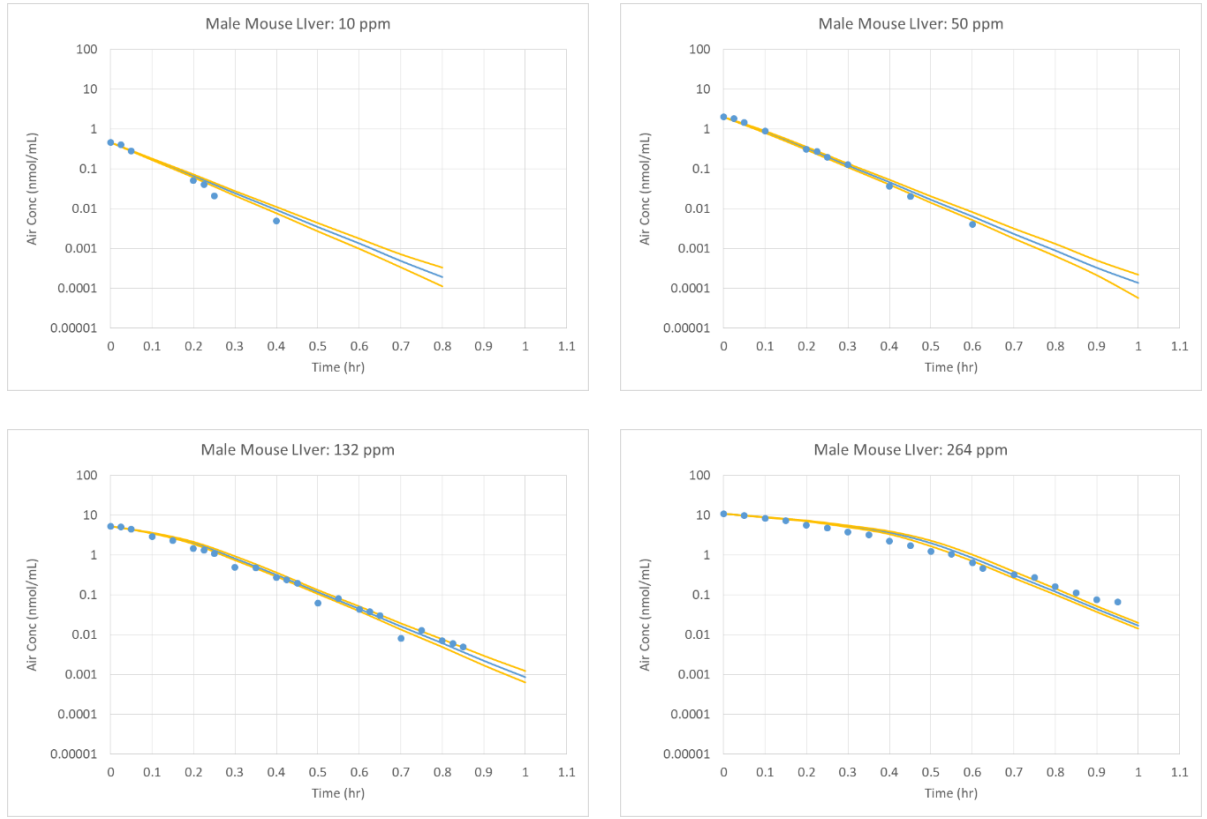
TRANSPORT LIMITATION (KGL) MCMC EVALUATION

Experimental Kgl = 0.020 L/hr (95% CI. = 0.015 – 0.036)



* Cannot fit metabolism data if Kgl < 0.11 L/hr

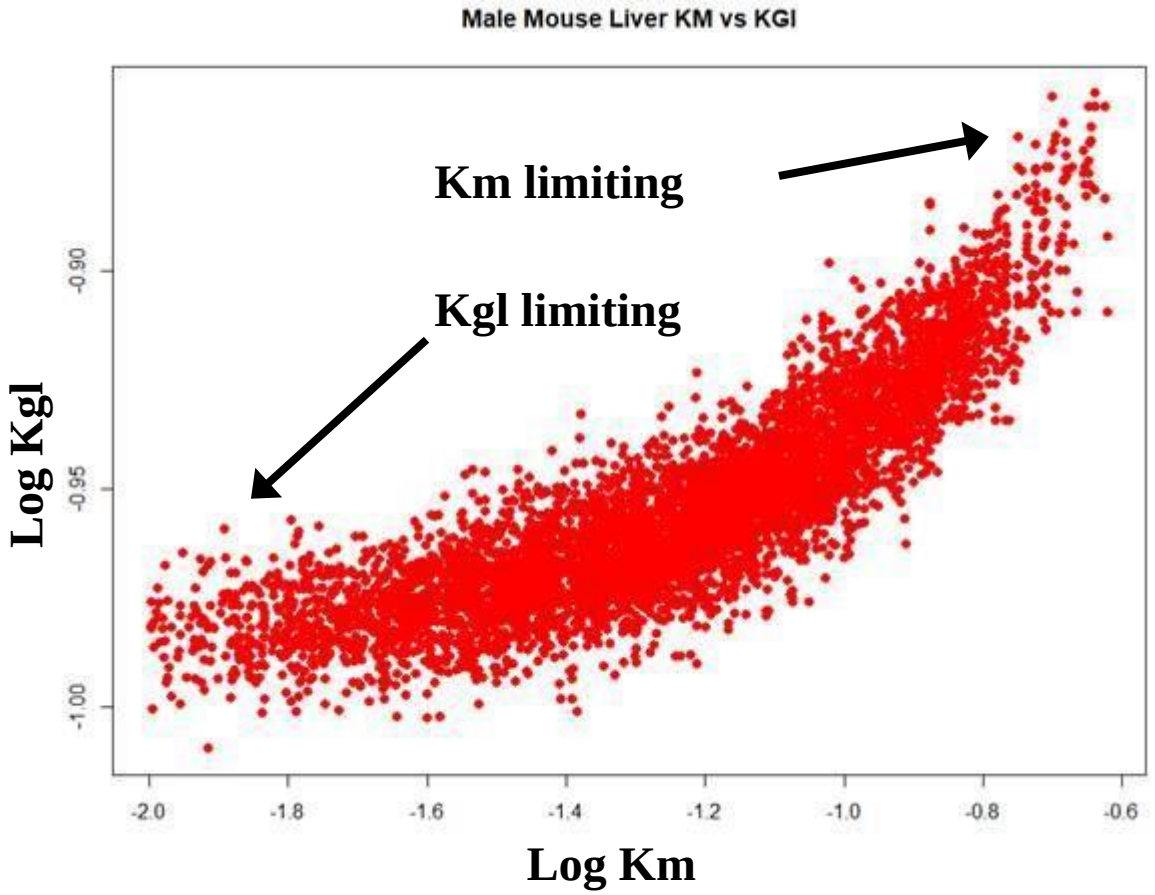
Estimated Kgl = 0.45 L/hr* (95% CI. = 0.34 – 0.65)



* Estimated from male mouse liver metabolism data, with Km = 1 μM

EFFECT OF ASSUMING A TRANSPORT LIMITATION (KGL)

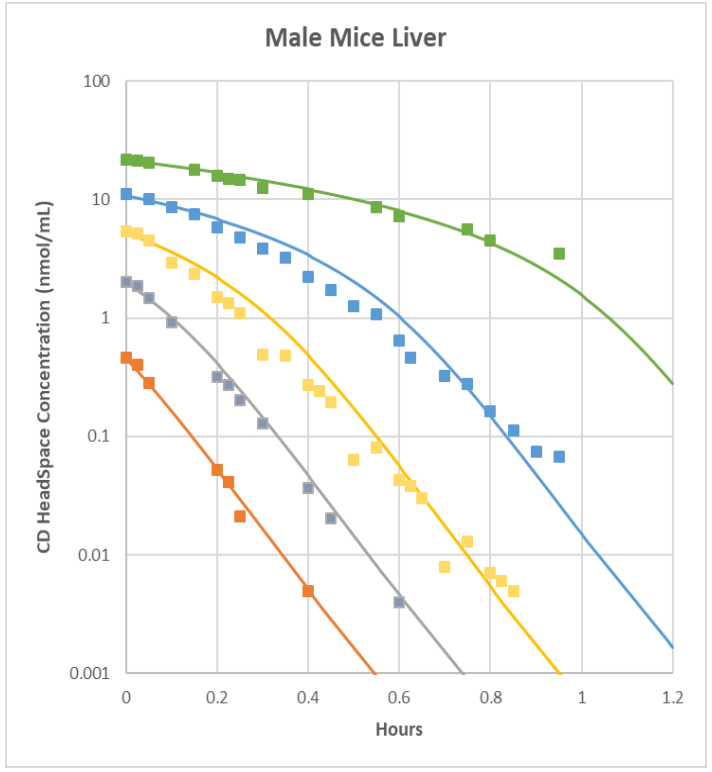
- As Kgl decreases, it competes with metabolism, decreasing clearance of chloroprene in the vial.
- The effect of introducing Kgl into the metabolism parameter estimation is to reduce the estimated Kms in the tissues to implausible values, much lower than the range of 1-7 µM observed *in vivo* for other CYP2E1 substrates.



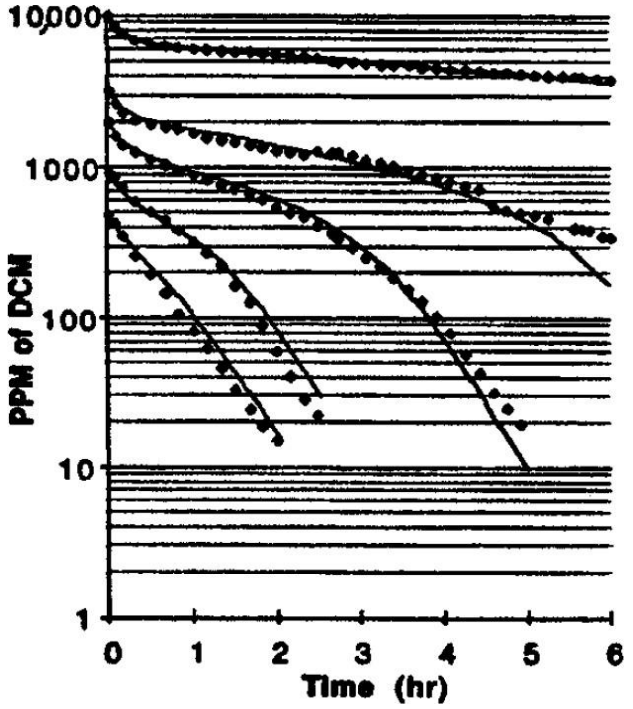
CONSIDERATIONS REGARDING KGL

- An experimental Kgl is critically dependent on the nature of mixing. It is difficult to apply a Kgl estimated from one experimental design to another, different design.
- As mixing increases, the transition from diffusion to laminar convection and then to turbulent convection increases the rate of mass transfer in a nonlinear manner.
- Based on the experimental metabolism data, we believe that more effective mixing and non-specific binding to microsomes increased the rate of transport of chloroprene in those studies.
- The investigator who conducted the study considered the possibility of slow mixing when he designed the study and is confident that the system was well mixed.
- We were able to obtain an acceptable fit to the data without using Kgl.
- Incorporating this additional, unsupported parameter (Kgl) results in a more uncertain analysis.

Chloroprene (2020) In Vitro Data



Methylene Chloride (1987) Closed Chamber Data



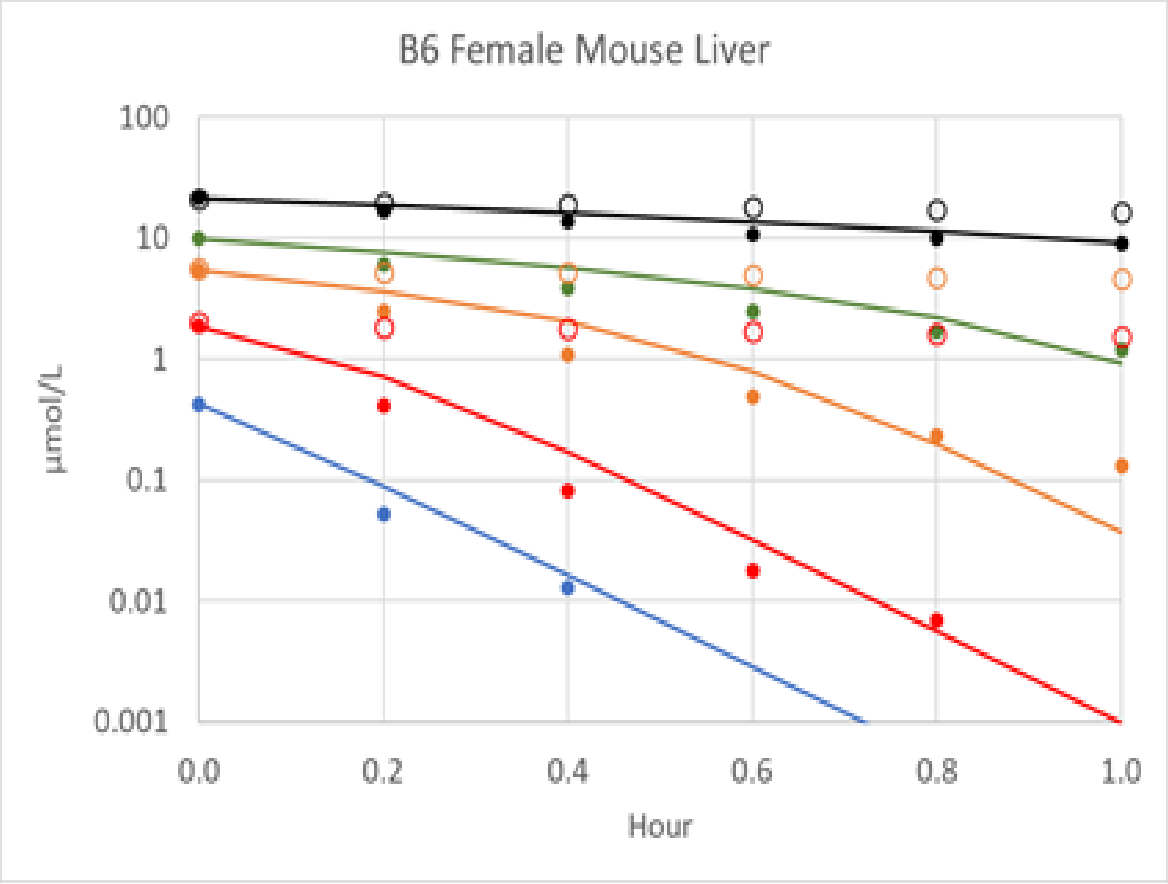
Andersen et al. (1987)

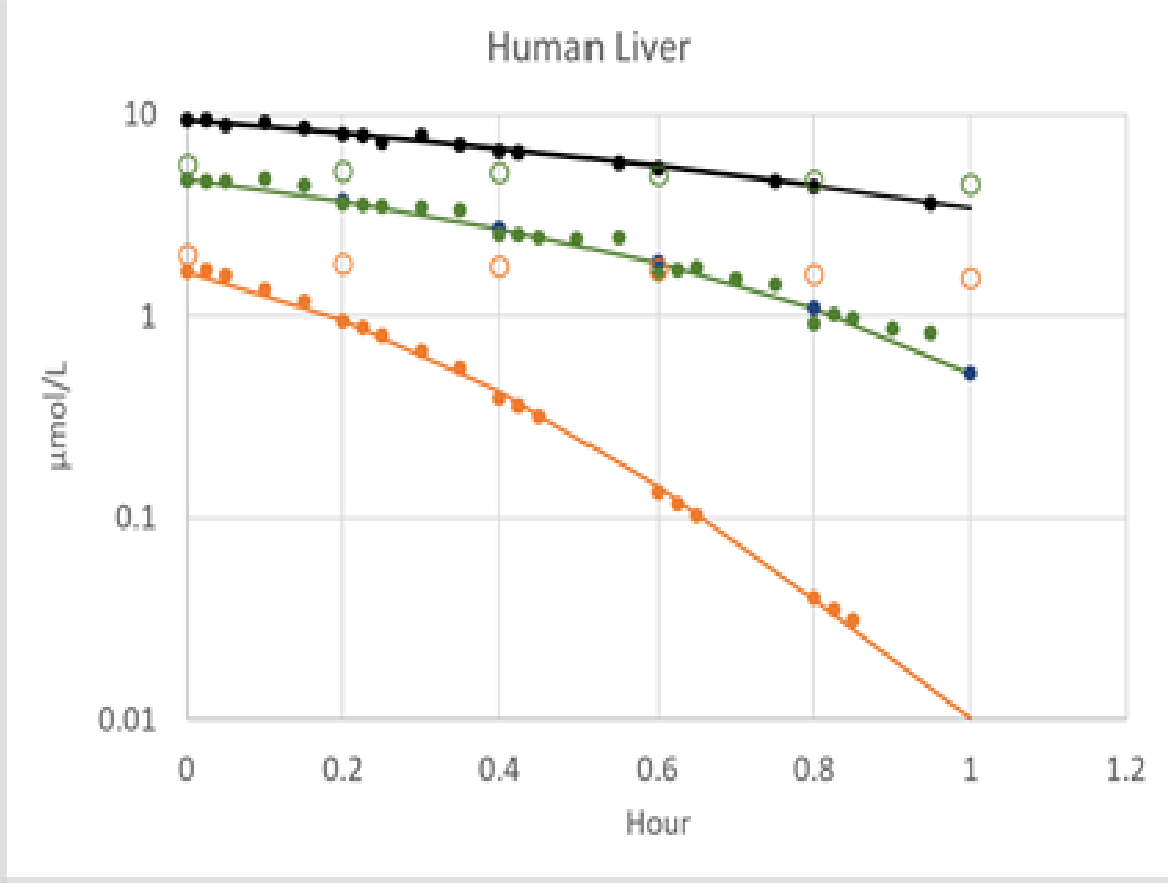
Both *in vitro* and *in vivo* data can be used to estimate metabolism

RAMBOLL

KINETIC ANALYSIS OF IN VITRO DATA: MOUSE AND HUMAN LIVER

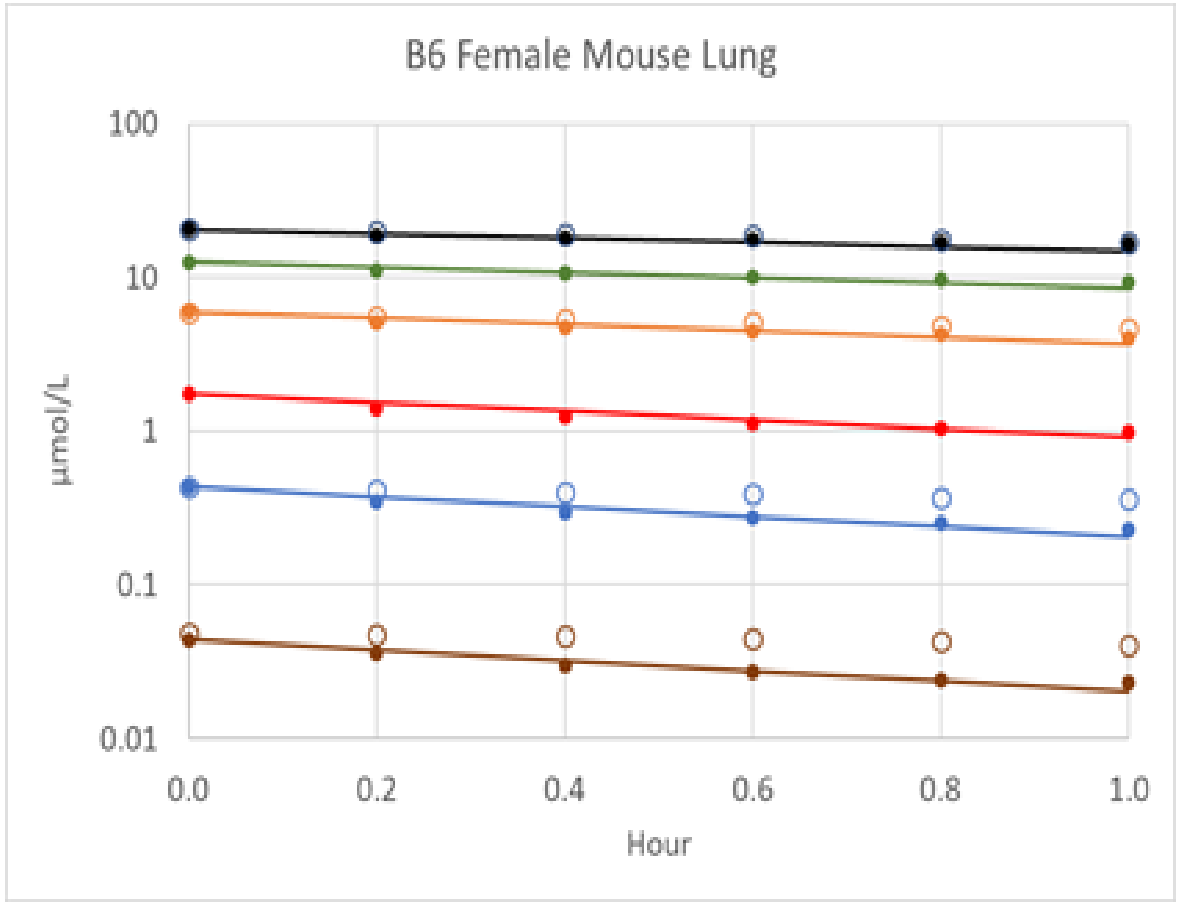
Because the data spans concentrations from above to below saturation it was possible to estimate reliable values of both the capacity (Vmax) and affinity (Km) of metabolism.





KINETIC ANALYSIS OF IN VITRO DATA: MOUSE LUNG

Because the data spans concentrations from above to below saturation it was possible to estimate reliable values of both the capacity (Vmax) and affinity (Km) of metabolism.



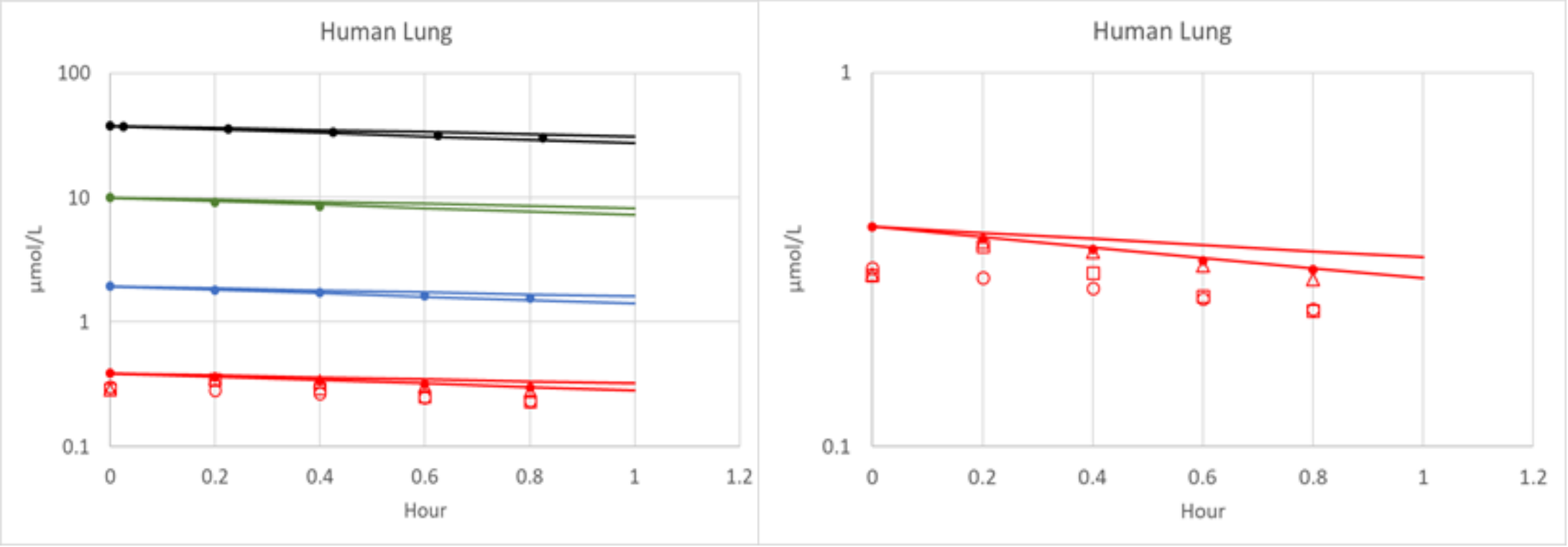
Ramboll (2020)

Open circles: control vials; Solid symbols: metabolism vials

KINETIC ANALYSIS OF IN VITRO DATA: HUMAN LUNG

Metabolism in the human lung is so slow that it was not possible to estimate reliable values of both the capacity (Vmax) and affinity (Km) of metabolism.

Rate of loss in metabolism vials is less than in controls.



Open symbols: control vials; Solid symbols: metabolism vials

Ramboll (2020)

The EPA proposed uncertainty analysis of the human lung metabolism is not needed and will not be reliable

RAMBOLL

- The approach used in this effort was designed by Dr. Miyoung Yoon (now with USFDA), an internationally recognized expert in IVIVE, and reflect the state of the art for quantitative *in vitro* to *in vivo* extrapolation (QIVIVE).
- QIVIVE should not be confused with rapid screening IVIVE approaches such as the USEPA *httk* modeling software, which is designed to make rapid predictions with minimal data to support interpretation of HTS results.
- The USEPA Office of Pesticides has accepted the use of PBPK models using IVIVE of microsomal metabolism data to support their evaluations of early life sensitivity to pesticides.
- The FDA routinely accepts microsomal metabolism data and PBPK modeling to predict drug-drug interactions *in vivo.*
- Uncertainty in the human lung metabolism of chloroprene was addressed using the approach from the USEPA (2011) methylene chloride IRIS assessment, which used a measure of the relative CYP abundance in human liver and lung (Andersen et al. 1987).

THERE IS UNCERTAINTY IN METABOLISM PARAMETERS DERIVED FROM *IN VIVO* STUDIES TOO

Development of a Physiologically Based Pharmacokinetic Model of Trichloroethylene and Its Metabolites for Use in Risk Assessment

CLEWELL ET AL. (2000)

Table 1. Parameter values used in the PBPK model for TCE.

Parameter	Abbreviation	Units	Mouse	Rat	Human
TCE metabolism					
Capacity	VMC	mg/hr[a]	39* (39.–60.)	12* (12.–20.)	10* (6.–10.)
Affinity	KM	mg/L	0.25	0.25* (0.25–18.)	1.5* (1.5–3.)
Fraction TCA	PO	–	0.035* (0.035–0.1)	0.02* (0.02–0.06)	0.08

Different metabolism parameters were required to fit each study.

UNCERTAINTY ANALYSIS

- An uncertainty analysis was conducted on the PBPK model and the results are presented in the publication documenting the application of the model in a risk assessment for lung tumors (Clewell et al. 2020).
- The Ramboll (2020) report does not estimate quantitative uncertainty in the PBPK model because the USEPA specifically requested that the report not include any discussion related to estimation of risks.
  - It is our understanding that the USEPA intends to conduct additional uncertainty analyses to evaluate the impacts on the cancer unit risk estimate.
- For this review we performed a comparison of the dose metric predictions obtained with the newly revised chloroprene PBPK model against those obtained using the original published model (Yang et al. 2012).
  - Despite major differences in the approaches taken for metabolism parameter estimation and *in vitro* to *in vivo* extrapolation, the two model versions produce almost identical dose metrics, demonstrating the robustness of the PBPK model predictions.

COMPARISON OF DOSE METRIC PREDICTIONS

Exposure	Concentration	Ramboll 2020 Dose Metric*	Yang et al. 2012 Dose Metric*
Female Mouse Bioassay	12.8 ppm	1.00	0.75
	32 ppm	1.58	1.2
	80 ppm	2.15	1.57
Human Continuous Exposure	1 $\mu g/m^3$	3.36×10^{-6}	2.7×10^{-6}

* average mg metabolized per gram lung per day

CONCLUSIONS

- PBPK modeling is the preferred approach for cross-species extrapolation (USEPA 2005) because it considers the large pharmacokinetic differences demonstrated between mice and humans for chemicals such as chloroprene.
- A validated PBPK model has been developed and documented, and the results have been published in a peer-reviewed journal (Clewell et al. 2020).
- The Ramboll team appreciates the interaction with USEPA scientists to further validate and improve the model and make it accessible to others.
- We have high confidence in the chloroprene PBPK model due to its similarity to previously accepted PBPK models and the robustness of the *in vitro* data on which it is based. It is likely better than data you would obtain from in vivo studies. Kenyon et al. (2020) have shown that *in vitro* estimates of metabolism for similar volatile organic compounds are generally within a factor of two to three of estimates inferred from *in vivo* studies
- Ramboll has determined that the impact of uncertainties in the PBPK model is small compared to the impact associated with ignoring important species differences in target tissue dosimetry (i.e. relying on default assumptions).
- The PBPK model indicates a need for revising the 2010 IRIS assessment to provide a corrected cancer unit risk based on the best available science.

THANK YOU

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EPA Uncertainty Analysis Overview and Context

Paul Schlosser/Dustin Kapraun, EPA

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Supplemental Analysis of Parameter and Model Uncertainty*: Overview & Context

- Focus of this peer review and possible next steps
- Rationale & context for EPA UA
- Chemical-specific vs. non-specific data
- Newly published papers of interest

*Supplement: Uncertainty Analysis of In Vitro Metabolic Parameters and of In Vitro to In Vivo Extrapolation (IVIVE) Used in a Physiologically Based Pharmacokinetic (PBPK) Model for Chloroprene

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Focus of Peer Review

- Focus of current peer review: Is the chloroprene PBPK model sufficiently reliable for use in an IRIS Toxicological Review?
  - Prediction of chloroprene tissue concentrations in mice, rats, and humans
  - Prediction of chloroprene oxidative metabolism in lung, liver, and kidney of mice, rats, and humans
- If the model is found sufficiently reliable, then details of "how" it is applied would be addressed in a subsequent EPA analysis.
- That subsequent EPA analysis would include consideration of:
  - Multiple tumor sites identified and considered in the 2010 IRIS assessment
  - Appropriateness of specific internal dose metrics vs. use of external exposure to extrapolate risk for each site
  - Factors to address other possible animal-human differences
- For now, EPA seeks to evaluate the broadest possible use of the model
  - Prediction of metabolism depends on accurate tissue concentration

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Rationale & Context for EPA UA

- **Expectation of quantitative UA for PBPK models**
  - When PBPK models are used in IRIS Toxicological Reviews and other EPA assessments there is an increasing expectation for a quantitative UA
  - Rigorous UA requires robust measures of uncertainty for all parameters
- **Observations re. confidence intervals (CIs) presented in Ramboll report**
  - CIs appear to be very narrow
    - Example: Vmax for female mouse liver has 95% CI ~ ± 20% of mean
  - Experiments involve repeated measures (samples) from incubation vials
    - If 5 vials are each sampled 6 times, those are not 30 independent measurements
    - Variability between vials may occur due to exact volume of microsomes added, temperature (location in incubator), septum seal, etc.
  - Ramboll analysis appears to treat each data point as independent, which *could* result in overly narrow CIs

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Rationale & Context for EPA UA (2)

- **Difference between Himmelstein et al. (2004) and Yang et al. (2012)**
  - Vmax for male vs. female mouse lung appears quite different
    - Male data: Himmelstein et al. (2004); female: Yang et al. (2012)
    - Same lab, same experimental PI (M. Himmelstein), but 8 years apart
  - Similar tumor response rate for male vs. female mice in the NTP bioassay
    - If risk is proportional to metabolic rate, then tumor response rates should be different
  - Could be due to pharmacodynamic differences between males & females
  - But could this reflect an underlying uncertainty in the in vitro metabolism results, due to unrecognized differences in the two sets of experiments?
- **Ramboll analysis assumes control incubation data from Yang et al. (2012) are applicable to Himmelstein et al. (2004) studies**
  - Apparent significance of difference between male & female mice depends on assumptions in the statistical modeling

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Rationale & Context for EPA UA (3)

- **U.S. EPA Uncertainty Analysis (UA) (initial results shown)**
  1. Parameters fit separately to data for each vial (experimental unit)
  2. Vial-to-vial variation assumed due to irreducible sources of experimental variation → uncertainty in parameters
  3. Parameters for each vial then combined in a way that maintains this measure of uncertainty
  4. *Considering* option of analyzing Himmelstein et al. (2004) data using only concurrent controls (limited #) vs. all controls (much more data)
- **Technical public comment on likelihood derivation (Ramboll)**
  - EPA considered & responded to comment on a prior version
  - Additional slides available, if requested, with details of EPA's derivation

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Chemical-Specific vs. Non-Specific Data

- **Ramboll report suggests use of relative CYP activity in human lung vs. liver to estimate human lung metabolism for chloroprene (parameter "A1")**
- **This approach was used previously by the U.S. EPA for dichloromethane (DCM)**
- **However, A1 is determined using data for a marker substrate, 7-ethoxycoumarin**
  - Relative affinity/activity of various CYPs for this substrate is likely to be different from chloroprene and DCM
- **For DCM, chemical-specific data was not available for human lung metabolism**
  - Use of A1 was best alternative
- **For chloroprene, we have chemical-specific Himmelstein et al. (2004) data**
  - All else being equal, chemical-specific data are preferred
  - But these data are challenging to analyze because activity is close to background
  - EPA believes this challenge can be overcome through appropriate, careful statistical analysis
  - Estimation of background losses (and the uncertainty in those), depends on which control incubation data are used
- **Ultimately, EPA's approach should provide an alternate estimate, *and upper confidence bound*, of the human lung metabolic rate, and all other metabolic parameters**

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New IVIVE Studies

- **Additional studies have recently been published on IVIVE**
  - Benet, L.Z., Sodhi, J.K. Investigating the Theoretical Basis for *In Vitro–In Vivo* Extrapolation (IVIVE) in Predicting Drug Metabolic Clearance and Proposing Future Experimental Pathways. *AAPS J* **22,** 120 (2020). https://doi.org/10.1208/s12248-020-00501-9
  - Kenyon EM, Eklund C, Pegram RA, Lipscomb JC. Comparison of in vivo derived and scaled in vitro metabolic rate constants for several volatile organic compounds (VOCs). Toxicol In Vitro. 2020 Sep 15:105002. https://doi.org/10.1016/j.tiv.2020.105002
- **These papers were brought to attention of the peer review panel for their consideration**
- **EPA scientists are evaluating these studies and what they might suggest for the chloroprene IVIVE PBPK model**

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Relevance of New Studies

- **Benet and Sodhi (2020)**
  - Focus on clearance of drugs
  - Binding to serum proteins is a significant factor
  - Distinguishes between net hepatic clearance (CL_H) and intrinsic clearance (CL_{nt}):

$$CL_H = Q_H \cdot f_{u,B} \cdot CL_{nt} / (Q_H + f_{u,B} \cdot CL_{int})$$

    where Q_H = hepatic blood flow and $f_{u,B}$ = fraction unbound in blood.
  - Suggests that commonly used values for Q_H may be too low
  - While serum binding is significant for some environmental chemicals (e.g., PFAS), it is not a factor for chloroprene and other VOCs ($f_{u,B} = 1$)
  - Use of reported blood perfusion rates in PBPK models has generally been found to provide adequate predictions of VOC distribution and hepatic clearance
  - Significantly increasing Q_H is likely to lead to an over-prediction of hepatic clearance for VOCs

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Relevance of New Studies (2)

- **Kenyon et al. (2020)**
  - Term "clearance" used in paper corresponds to intrinsic clearance
  - PBPK model only has metabolic clearance in the liver, but...
  - Compares hepatic Vmax or CL_{int} = Vmax/Km estimated by IVIVE to value obtained by fitting gas-uptake data from in vivo exposures
  - While in vivo CL_H also depends on hepatic clearance, a key focus of this review is the IVIVE estimation of Vmax
  - For the *lung*, metabolic clearance is generally less dependent on blood-flow:

$$CL_{lung} \approx Vmax(lung)/Km$$

  - For chloroprene metabolism in the *lung* two options are being considered:
    - $Vmax(lung) = A1 * Vmax_{liver,in\text{-}vitro}$(IVIVE) [Ramboll's primary proposal]
    - $Vmax(lung) = Vmax_{lung,in\text{-}vitro}$(IVIVE) [alternate being considered by EPA]
  - Both options depend on the accuracy of IVIVE predictions for Vmax
  - Kenyon et al. (2020) evaluates Vmax(IVIVE) vs. Vmax(in vivo) in rats, for which extra-hepatic oxidative metabolism (including in the lung) is low.

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Clarifications on Statistical Likelihood Derivation Comments Submitted by Ramboll

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Comment on Likelihood

During the public comment period, Ramboll submitted the following comment:

> p. 11, equation 4. The equation should have a factor of Cdat(tj) in the denominator on the right-hand side of that equation. The basis for that statement is in the line previous to that equation where it is stated that the data are {(tj, Cdat(tj)): j ϵ {1, …, N}}. As noted in Equation 3, USEPA specifies that log[Cdat(t)] is assumed to be normally distributed. Note that the integral over the support for any given data point should equal 1. The support for Cdat(t) is from 0 to infinity. The integral based on the expression given in Eq. 4 does not equal 1. An easy fix to this discrepancy might be to state that the data are {(tj, log[Cdat(tj)]): j ϵ {1, …, N}}. The support for log[Cdat(t)] is negative infinity to infinity, and integration over that support does indeed equal 1 as desired.

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Comment on Likelihood (2)

- U.S. EPA (2020) states that the *residuals,* or differences between observations and model estimates, which are represented by ε in Eq. 3, are normally distributed. (Justification for this assumption is provided earlier in the U.S. EPA document, notably in Figures 3 and 4.)

$$\log[C_{dat}(t)] = \log[C_{mod}(t;q)] + \varepsilon \quad \text{(Eq. 3)}$$

$$\Updownarrow$$

$$\varepsilon = \log[C_{dat}(t)] - \log[C_{mod}(t;q)]$$

- U.S. EPA (2020) does not state, as Ramboll asserts, that the *observations* $(\log[C_{dat}(t)])$ are normally distributed.

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Comment on Likelihood (3)

- The likelihood function defined in Eq. 4 of U.S. EPA (2020) has been correctly stated based on the error model stated in Eq. 3.

$$\mathcal{L}(\theta|\mathcal{D}) = \prod_{j=1}^{N} \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-\frac{\left(\log\left|C_{dat}(t_j)\right| - \log\left|C_{mod}(t_j;q)\right|\right)^2}{2\sigma^2}\right] \quad \text{(Eq. 4)}$$

- If one makes the substitution $\varepsilon_j = \log[C_{dat}(t_j)] - \log[C_{mod}(t_j;q)]$ in Eq. 4, then the term in the product is, by definition, the probability density function for $N(0,\sigma)$ evaluated at ε_j. If one integrates the expression in the product over the domain of possible values of ε_j ($-\infty$ to ∞) the value of the definite integral is one, as shown here*.

*The hyperlink is to https://www.wolframalpha.com with the site search string: "integral(1/sqrt(2*pi*s^2) * exp(-x^2/(2*s^2))) from negative infinity to infinity"

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Observer Comment Session

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Observer Comment Session

Jennifer Sass (3 minutes)
NRDC

Mel Anderson (3 minutes)
Andersen ToxConsulting LLC

Dale Hattis (10 minutes)
Clark University

Wilma Subra (10 minutes)
Louisiana Environmental Action Network
On behalf of the Concerned Citizens of St. John

Michelle Mabson (3 minutes)
Earthjustice

Tokesha Collins-Wright (3 minutes)
Louisiana Chemical Association

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Cancer Hazard from Chloroprene

Dale Hattis, Ph.D

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Background

- Multiple Somatic Mutation Mechanism of Cancer
- Metabolic Activation of Chloroprene to Mutagenic Metabolites
- Implications of Saturable Metabolism for Low Dose Response Relationship
- Indicated Dosimeter for Interspecies Projection

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Metabolic Activation of Chloroprene to Mutagenic Metabolites

- Oxygen is Added Across the Double Bond to Form an Epoxide
- Epoxides are Capable of Reacting With DNA, and Can be Expected to Reach DNA in Some Proportion
- Adducted DNA Can Give Rise to Mutations if Present the Next Time the DNA is Copied
- Repair Processes Can Reduce the Amount of Initially-Generated Damage, but Must In General Be Imperfect—Leaving Some Unrepaired Damage Available to Give Rise to Permanent Changes

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Implications of Saturable Metabolism for Low Dose Dose Response Relationship

Standard Michaelis-Menten Equation for Enzyme-Mediated Reactions--

Where [C] is the Concentration of the Substrate,

$$\text{Reaction Rate} = V_{max}[C]/(K_m + [C])$$

At low doses the [C] in the denominator is small relative to the constant Km, meaning that although the equation approaches saturation at high doses, at low doses it approaches linearity

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Indicated Dosimeter for Interspecies Projection

Concentration of DNA-Reactive Metabolite(s) X

Time Inside Cells Near DNA Before Being Lost

(Where Loss Rates are Typically Slower for Humans Compared to Small Rodents, Approximately in Proportion to Body Weight$^{-1/4}$—for details see Boxenbaum H 1982, **Interspecies scaling, allometry, physiological time, and the ground plan of pharmacokinetics J. Pharmacokinetics Biopharm. 10(2):201-27)**

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Responses to Sax et al. (2020)

- Assumption of passive, rather than active, elimination of chloroprene mutagenic metabolites is unsupported by empirical evidence.
- Implicit assumption of exclusively local metabolism in the lung (rather than systemic activating metabolism in the liver and elsewhere) is unsupported.

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Selected References

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Chair's Introduction and Review of Charge

Ken Portier, Chair

40

Charge Question Topic Areas

- Estimation of Mass Transfer Resistance in the In Vitro Metabolism Experiments
- Estimation of Metabolic Parameters from In Vitro Metabolism Experiments
- IVIVE Calculations for Chloroprene
- PBPK Model Structure, Physiological Parameters, and Partition Coefficients
- Overall PBPK Model Soundness and Applicability
- Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

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Charge Questions

VERSAR

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Charge Format

- Charge is organized into topic areas addressing the major components of the modeling analyses to be reviewed
- Prior to each set of questions, text is provided to provide some background and context, and to help identify specific parts of the documents being addressed by the questions.
  - Summary results may be in the main report with details in supplements
- In the slides that follow the full text is provided, in case there are questions that reviewers have identified.

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Charge Questions

Topic 1: Estimation of Mass Transfer Resistance in the In Vitro Metabolism Experiments

- A model of the *in vitro* incubation system was used to estimate the metabolic parameters from the *in vitro* data. This model is based on certain assumptions and physical parameters, such as the volume of the *in vitro* incubation vials and volumes of air and liquid media in the vials.

- The model of the *in vitro* system initially used for the analysis of the *in vitro* experiments to estimate the corresponding metabolic parameters (Yang et al., 2012; Himmelstein et al., 2004) assumed that the chloroprene in the air and liquid (incubation medium) phases was at equilibrium at all times after the start of the experiment; i.e., concentration in the medium was set equal to the concentration in the air times the equilibrium partition coefficient (CM = CA*P). At EPA's suggestion, the model was changed to explicitly describe separate air and liquid media compartments, with a mass-transfer coefficient (Kgl) limiting the rate of distribution between them, as described by Kreuzer et al. (1991) and others.

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Two-Compartment vs. One-Compartment In Vitro Metabolism Model

Two-Compartment Model

Alternate forms for rate of mass transfer:

$k_{12} \cdot y_1 \cdot V_1 - k_{21} \cdot y_2 \cdot V_2 = Kgl \cdot (y_1 - y_2/P)$

Kgl = gas-liquid mass-transfer coefficient,

P = liquid-gas equilibrium partition coefficient

Two ODEs, one for each compartment

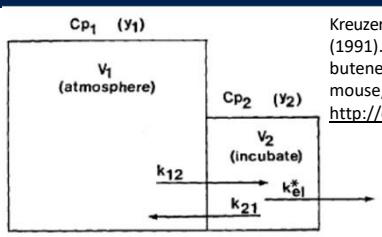


Fig. 2. Kinetic two-compartment model and differential equations for the exposure system.
Differential equations related to the kinetic model:
Atmosphere: $V_1 \times (dy_1/dt) = -k_{12} \times V_1 \times y_1 + k_{21} \times V_2 \times y_2$
Incubate: $V_2 \times (dy_2/dt) = k_{12} \times V_1 \times y_1 - (k_{el}^* + k_{21}) \times V_2 \times y_2$

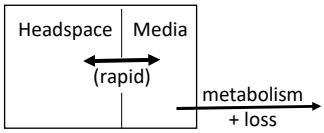
Kreuzer, PE; Kessler, W; Welter, HF; Baur, C; Filser, JG. (1991). Enzyme specific kinetics of 1,2-epoxy-3-butene in microsomes and cytosol from livers of mouse, rat, and man. Arch Toxicol 65: 59-67. http://dx.doi.org/10.1007/BF01973504

One-Compartment Model

Assumes atmosphere (incubation vial headspace) concentration is always at equilibrium with "incubate" (liquid media):

$y_1 = y_2/P$

One ODE for concentration in liquid media



Himmelstein, MW; Carpenter, SC; Hinderliter, PM. (2004). Kinetic modeling of beta-chloroprene metabolism: I. In vitro rates in liver and lung tissue fractions from mice, rats, hamsters, and humans. Toxicol Sci 79: 18-27. http://dx.doi.org/10.1093/toxsci/kfh092

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Charge Questions

Topic 1: Estimation of Mass Transfer Resistance in the In Vitro Metabolism Experiments

- **Question 1:** Please evaluate the validity and uncertainties of these two approaches to estimation of the kinetics in the vitro system and therefore in the estimation of metabolic parameters:

  a) treating the air and liquid phases as always being at equilibrium (original model); or

  b) treating the air and liquid phases as distinct compartments with the rate of transfer limited and determined by a mass-transfer constant (Kgl).

  Materials:

  Re-estimation of in vitro metabolism parameters, pp. 9-10, Ramboll (2020)

  Experimental Determination of Mass Transport Limitation, pp. 2-8, Supp Matt B (details related to following questions as well)

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Charge Questions

Topic 1: Estimation of Mass Transfer Resistance in the In Vitro Metabolism Experiments

- Experiments were conducted to determine the Kgl for the *in vitro* system, however the value of Kgl obtained from those experiments is not consistent with some of the observed metabolic data (Ramboll (2020) Supplemental Material B), and Kgl would need to be at least 8 times higher to obtain results consistent with those data and to obtain a Km consistent with metabolic parameters reported for other VOCs. This inconsistency may exist because the experiments conducted to estimate Kgl used an incubator mixing speed of 60 rpm while the experiments of Himmelstein et al. (2004) and Yang et al. (2012) used 500 rpm. Also, the experiments to measure Kgl were performed without microsomal protein and the report hypothesizes that the presence of microsomal protein (1–3 mg/mL) in the metabolic experiments could increase mass transfer. It is noted that the mean value of the partition coefficient, P, estimated from the Kgl data in the absence of microsomal protein was 0.48 (Ramboll (2020) Supplemental Material B) while that reported by Himmelstein et al. (2001) for chloroprene equilibration with media containing heat-inactive protein was 0.69, 44% higher. To be clear, simulations of the metabolically active experiments used to estimate the metabolic parameters used P = 0.69, so have accounted for the difference in the equilibrium partition coefficient but are still not consistent with the highest activity data when using the value of Kgl obtained from the 60 rpm data.

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Charge Questions

Topic 1: Estimation of Mass Transfer Resistance in the In Vitro Metabolism Experiments

- **Question 2:** Please comment on the likelihood that either the presence of microsomal protein (1–3 mg/mL) or that the higher mixing speed used in the metabolic experiments (500 rpm) vs. the mass transfer experiments (60 rpm) would increase the rate of chloroprene mass transfer between the air and liquid phases in the in vitro system by a factor of 8 or greater, relative to the rate observed in the mass-transfer experiments.

Materials: Supp Matt B, p. 4 (paragraph after Fig. B-2)

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Charge Questions

Topic 1: Estimation of Mass Transfer Resistance in the In Vitro Metabolism Experiments

- An analysis provided in Supplemental Material B of Ramboll (2020) demonstrates that estimates of the metabolic parameter Km depend strongly on the value of Kgl. Two approaches were used to estimate the value of Kgl:

  a) the measured Kgl was increased by (500/60), the ratio of mixing speeds in the metabolic experiments vs. Kgl experiments, yielding Kgl = 0.2 L/h; and

  b) a Bayesian analysis used to estimate Kgl from the metabolic data yielded a mean Kgl = 0.22 L/h.

  Materials:
  Experimental Determination of Mass Transport Limitation in Supp Matt B, pp. 2-8

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Charge Questions

Topic 1: Estimation of Mass Transfer Resistance in the In Vitro Metabolism Experiments

- **Question 3:** Given the two-compartment in vitro model structure, please comment on the two approaches for estimating Kgl and whether the value obtained is sufficiently reliable to support valid estimates of metabolic parameters and assess the uncertainties in those estimates.

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Charge Questions

Topic 2: Estimation of Metabolic Parameters from In Vitro Metabolism Experiments

- The following questions address the robustness of the available metabolic data for application in the model. The questions are written with the assumption that the choice of Kgl is appropriate. Using this value of Kgl while evaluating the remaining analysis of *in vitro* metabolic data as described in Supp Mat B, Ramboll (2020) results in parameter values listed in Table S-3 of Supp Mat A, Ramboll (2020). For the chloroprene *in vitro* experiments, the human liver microsome samples were obtained from a pool of 15 donors while the human lung microsomes were obtained from a pool of 5 individuals (Himmelstein et al., 2004). For the 7-ethoxycoumarin *in vitro* experiments used to estimate the relative lung:liver metabolic activity, represented by the parameter A1, tissue samples were not pooled; activity was measured in liver microsomes obtained from 10 donors while the human lung activity was measured using microsomes from 12 donors (Lorenz et al., 1984). Other information on the specific microsomal samples, preparation methods and *in vitro* experiments are in Lorenz et al. (1984), Himmelstein et al. (2004) and Yang et al. (2012).

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Charge Questions

Topic 2: Estimation of Metabolic Parameters from In Vitro Metabolism Experiments

- **Question 4:** Please comment on the pool sizes for the human microsomes used to estimate chloroprene metabolic rates in vitro, and the number of tissue samples (donors) evaluated for 7-ethoxycoumarin activity, for the estimation of average metabolic activity for human adults.
  Materials: See previous slide and supporting references for information on number of donors.

- **Question 5:** Discuss the appropriateness of the data used and the statistical modeling approach with regard to representing average (or mean) adult human, mouse, and rat metabolic parameters. In particular, please comment on whether a sufficient number of microsomal samples (incubations) were analyzed to represent the average values and to characterize metabolic variation across species, sexes, and tissues.
  Materials:
  Pool sizes: preceding slide,
  3-5 incubation vials per tissue pool (tissue / species / sex) and concentration level; details in Himmelstein et al. (2004) & Yang et al. (2012)
  Re-estimation of In Vitro Metabolism Parameters in Supp Matt B, pp. 8-15 for details of statistical analysis

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Charge Questions

Topic 2: Estimation of Metabolic Parameters from In Vitro Metabolism Experiments

- **Question 6:** Considering the experimental and computational methods, please comment on the potential order of magnitude and direction of bias of the quantitative uncertainties in the estimated in vitro metabolic rates that may be related to these factors, collectively.

Materials:

Re-estimation of Metabolism Parameters, pp. 14-17, Ramboll (2020)

Supp Matt B

Results in Supp Matt A, Table S-3

Lorenz et al. (1984), Himmelstein et al. (2004) and Yang et al. (2012)

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Charge Questions

Topic 2: Estimation of Metabolic Parameters from In Vitro Metabolism Experiments (continued)

- Additional discussion on the estimation of lung metabolic parameters in rats and humans is provided in:
  IVIVE for first order metabolic clearance in rat and human lung, Supp Mat C, p.8.
- However, the metabolic rate parameter values for the human lung were ultimately selected as described in the main report in:
  Estimation of chloroprene metabolism in the human lung, pp. 10-11, and p. 17, Ramboll (2020), because the *in vitro* chloroprene experiments with human lung microsomes showed minimal metabolism.

Question 7: Please comment on the use of the relative 7-ethoxycoumarin activity in human lung vs. liver tissue to predict the average rate of chloroprene oxidative metabolism in the human lung.

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Charge Questions

Topic 2: Estimation of Metabolic Parameters from In Vitro Metabolism Experiments

- **Question 8:** Please comment on the possible use of a parallel approach, based on the relative activity of 7-ethoxycoumarin or another marker CYP2E1 substrate, to estimate the rate of metabolism in the rat lung and the human kidney.

Material: Option suggested on U.S. EPA (2020) Supplement, pp. 23;
approach would be the same as for use of A1 to estimate lung metabolism.

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Charge Questions

Topic 3: IVIVE Calculations for Chloroprene

- IVIVE extrapolation is summarized in the *Model Parameters* section of the Ramboll (2020) report, with details on scaling factors in Supplemental Material C of Ramboll (2020) and results in Table S-4 of Supplemental Material A. (Calculations are provided in an Excel workbook, Supplemental Material D of Ramboll (2020). The U.S. EPA performed a quality-assurance evaluation of the workbook to assure the calculations are as described in the report text and tables.) Wood et al. (2017) evaluated the ability of IVIVE to predict clearance for oral dosing of a number of pharmaceutical compounds with data in rats and humans and reported a systematic bias towards under-prediction with increasing clearance. However, the Wood et al. (2017) results may not be relevant to chloroprene because of differences in the route of exposure, chemical properties, metabolizing enzymes, and rate-determining processes for the set of compounds analyzed. In particular, Wood et al. (2017) evaluated IVIVE for oral dosing of drugs, but not for the inhalation of volatile compounds like chloroprene. While, IVIVE for oral exposure to drugs may be more difficult and is subject to additional sources of uncertainty compared to inhalation of volatile compounds due to variability in intestinal absorption and metabolism (Yoon et al., 2012; Liao et al., 2007), analysis of Wood et al. (2017) specifically focuses on predictions of hepatic *clearance* of drugs, for which metabolism in the liver is a significant component. Thus, the analysis of Wood et al. (2017) may be considered relevant to chloroprene since it addresses the ability to predict metabolic clearance via IVIVE, not oral absorption. The U.S. EPA is not aware of a systematic evaluation of IVIVE accuracy like that of Wood et al. (2017) but focused on volatile organic (chlorinated) compounds like chloroprene for the inhalation route.

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Charge Questions

Topic 3: IVIVE Calculations for Chloroprene

- **Question 9:** Please evaluate the choices of extrapolation factors and formulas used for the IVIVE calculations. Please discuss the soundness of the metabolic parameters in Table S-4 as estimates for average adult female and male mice and rats, and average adult humans (combined sexes).

Materials, Ramboll (2020):

Selection of tissue scaling parameters, p. 10

Supp Mat C

Supp Mat D (Excel spreadsheet with all IVIVE calculations)

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Charge Questions

Topic 4: PBPK Model Structure, Physiological Parameters, and Partition Coefficients

- **Question 10:** Please discuss the appropriateness of the PBPK model structure presented by Ramboll (2020) for estimation inhalation dosimetry in an EPA Toxicological Review of chloroprene. Please consider in particular the model structure for the kidney, liver, and lung; i.e., tissues in which chloroprene metabolism is predicted by the model.

Model Structure, pp. 8-9, Ramboll (2020)

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Charge Questions

Topic 4: PBPK Model Structure, Physiological Parameters, and Partition Coefficients

- Arterial blood concentrations in B6C3F1 mice after inhalation exposures to chloroprene are shown in Figure 3 of Ramboll (2020). In particular, it is noted that when chloroprene exposure was increased 2.5-fold from 13 to 32 ppm, the mean arterial concentration increased less than 1.5-fold. Further, the mean arterial concentrations from 90 ppm exposure, which is seven (7) times higher than 13 ppm, are only about 4 times higher than those measured at 13 ppm. These data might indicate that some process not included in the PBPK model may have reduced chloroprene uptake or somehow increased metabolic efficiency at 90 and 32 ppm relative to 13 ppm. A factor to be considered is the high variability with large standard deviations for many of the data points, as illustrated in Figure 3 of Ramboll (2020). The PBPK model structure implies that blood levels should increase in proportion to exposure as long as blood concentrations remain below the level of metabolic saturation and should increase at a faster rate above saturation, unless there is some other exposure-related change in model parameters. However, the plethysmography data evaluated do not show a clear or significant dose- response Ramboll (2020). Figure 7 of Ramboll (2020) presents the extent of agreement of the model predictions with the blood concentrations in mice following inhalation exposure. It is noted that the inhalation PK data are from a single exposure (animals were not previously exposed to chloroprene) and the non- proportionality is evident by the 3-hour time-point.

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Charge Questions

Topic 4: PBPK Model Structure, Physiological Parameters, and Partition Coefficients

- **Question 11:** Given these data, please evaluate the likelihood that changes in respiration rate or metabolic induction might be factors in the observed PK relationship between exposure and internal dose. Please comment on any other physiological or biochemical mechanisms that might be explanatory factors in the apparent discrepancy or whether experimental variability in the data may explain these differences.

Ramboll (2020) (primary report)
Model Structure, p. 14
Figure 3, p. 14
PBPK Modeling of the Nose-Only Inhalation Study, pp. 17-18
Figure 7, p. 18

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Charge Questions

Topic 4: PBPK Model Structure, Physiological Parameters, and Partition Coefficients

In

Model Parameters, p. 9, Ramboll (2020),

the authors describe the apparent discrepancy between the rate constant for cardiac output (QCC) from Brown et al. (1997) and other data. The sensitivity of the predicted blood concentration to unscaled cardiac output is shown in

Figures 5 and 6, pp. 18-20.

- **Question 12** Please comment on the analysis presented here and the proposed choice of QCC for the mouse.

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Charge Questions

Topic 4: PBPK Model Structure, Physiological Parameters, and Partition Coefficients

- **Question 13:** Given the specific considerations above, please comment on the appropriateness of the values selected for the physiological parameters in Table S-1 and partition coefficients in Table S-2, for prediction of chloroprene dosimetry.

Previous materials reviewed

Supp Mat A, Tables S-1 and S-2

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Charge Questions

Topic 5: Overall PBPK Model Soundness and Applicability

- Model-predicted doses in model tissue compartments corresponding to tissues in which neoplasm were observed in the rat and mouse bioassay, with corresponding cancer incidence for 80 ppm chloroprene inhalation exposure, are provided in the EPA background document. In potential application to human health risk assessment, the relative risk of tumors in human liver and lung will depend on the relative rate of metabolism predicted in those tissues, compared to the mouse or rat (as well as the relative rate of clearance). Estimation of risks for tissues other than liver and lung could depend on the relative estimates of chloroprene venous blood or tissue concentration. An evaluation of the model's applicability and degree of uncertainty should consider both the absolute model predictions (i.e., does the model accurately predict the absolute rates of metabolism and blood/tissue concentrations in each species?) and also the ability to predict the relative rate of metabolism or relative concentration in human vs. rodent tissues, though some inaccuracy in the absolute values may exist. See "Background for the Peer Review" document for additional context.
- Demonstration of the PBPK model's ability to predict *in vivo* PK data is shown by the level of agreement between model predictions and chloroprene venous blood concentrations in Figure 7 of Ramboll (2020). For reference, where there are data, and as a rule of thumb, EPA often seeks dosimetric estimates from a model that are within a factor of two of empirical results. The results of the sensitivity analysis shown in Figure 8 for arterial concentrations indicate that these data and specific predictions are not sensitive to the estimated metabolic parameters: a relatively large range in the estimated metabolic parameters (such as the apparent difference between male and female mouse parameters) would yield similar predictions of blood concentrations. However, as demonstrated in Figure 9, the estimation of lung dose metrics is sensitive to the estimated metabolic parameters.

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Charge Questions

Topic 5: Overall PBPK Model Soundness and Applicability

- **Question 14:** Please comment on the capacity of the PBPK model to provide sound estimates of chloroprene inhalation dosimetry in mice, rats, and humans. In particular, please comment on the reliability of model predictions of the rate of chloroprene metabolism in liver and lung for use in animal-to-human extrapolation. Please also comment on the reliability and uncertainty of model predictions of chloroprene concentrations in blood and other tissues from inhalation exposures. Please provide your scientific judgement about the potential order of magnitude of quantitative uncertainty in these estimates.

Ramboll (2020), all materials provided.

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Charge Questions

Topic 6: Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

- The U.S. EPA seeks input on initial analyses that it has conducted, its proposed approach to evaluate quantitative uncertainty of the metabolic parameters estimated from *in vitro* data, and its proposed approach to incorporate the metabolic parameter uncertainty into an estimate of uncertainty in the PBPK model predictions U.S. EPA (2020).

Supplement: Uncertainty Analysis of In Vitro Metabolic Parameters and of In Vitro to In Vivo Extrapolation (IVIVE) Used in a Physiologically Based Pharmacokinetic (PBPK) Model for Chloroprene, U.S. EPA (2020)

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Charge Questions

Topic 6: Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

- **Question 15:** Please comment on the analysis and statistical assumptions for control data from Yang et al. (2012) as an approach for evaluating the underlying experiments, data, and distribution of RLOSS for use in subsequent uncertainty analyses of the metabolic data.

- **Question 16:** Considering the preliminary results for RLOSS provided, please provide any specific suggestions you may have for how the analyses methods might be improved.

(1a) Analysis of Uncertainty of Background Loss in the In Vitro Experimental System, pp. 5-14, U.S. EPA (2020)

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Charge Questions

Topic 6: Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

- A similar analysis was conducted using data from five control incubations obtained by Himmelstein et al. (2004). Comparison of the results for RLOSS based on Yang et al. (2012) control data vs. Himmelstein et al. (2004) control data indicates that the value of RLOSS may have been lower in the Himmelstein et al. (2004) study. The two sets of experimental *in vitro* studies were conducted in the same laboratory by the same principle investigator (Matthew Himmelstein), but given the period of time between the two studies, the applicability of non-concurrent control data is a source of uncertainty.

Estimation of RLOSS Using Himmelstein et al. (2004) Control Data, pp. 14-18, U.S. EPA (2020)

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Charge Questions

Topic 6: Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

- **Question 17:** Please comment and provide any specific suggestions you have on the possible use of either:

  a) separate distributions of RLOSS obtained from the Yang et al. (2012) vs. Himmelstein et al. (2004) studies when analyzing the uncertainty for the different metabolic parameters obtained with data from the respective studies; or

  b) combining the control incubation data and analysis to obtain a distribution applicable to all metabolic data.

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Charge Questions

Topic 6: Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

- U.S. EPA (2020) describes intended methods for evaluating the uncertainty in the metabolic parameters obtained from the *in vitro* data, given the distribution in RLOSS already obtained. The analysis is particularly focused on the human liver and lung data, which were obtained with pooled microsomes from 15 individuals for liver microsomes and 5 individuals for lung microsomes.

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Charge Questions

Topic 6: Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

- **Question 18:** Please evaluate the planned analysis as an appropriate statistical approach for evaluating the uncertainty in the metabolic parameters for the pooled tissue samples. Note any additional quantitative factors whose uncertainty you believe would not be addressed by this approach. Please provide any specific suggestions you have on how the analysis should be modified.

(1b) Assessment of Uncertainty in Metabolic Parameters, pp. 18-22, U.S. EPA (2020)

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Charge Questions

Topic 6: Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

- U.S. EPA (2020) describes intended methods for evaluating the uncertainty in the PBPK model predictions for the rate of metabolism in liver, lung, and kidney, and in predictions of chloroprene venous blood concentrations. Since the analysis is focused on estimation of population average doses, uncertainty in human physiological parameters would be quantified as uncertainty in the *mean* values for a healthy adult, rather than overall population variance. For model predictions based on the parameter A1 (lung:liver metabolic ratio obtained from data for 7-ethoxycoumarin) and a similar parameter for the kidney (A2), uncertainty in A1 or A2 based upon variance in tissue- specific values reported for the corresponding *in vitro* studies will be included.

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Charge Questions

Topic 6: Proposed Uncertainty Analysis of In Vitro Metabolic Data and PBPK Model Predictions

- **Question 19:** Please comment on whether the planned analysis for PBPK-predicted dose metrics as outlined by U.S. EPA (2020) is an appropriate approach for evaluating quantitative uncertainty in the estimated internal doses. Please provide any specific suggestions you have on how the analysis could be improved.

  (2) Assessment of Uncertainty in PBPK Model Prediction of Metabolic Rates and Venous Blood Concentrations, pp. 22-23, U.S. EPA (2020)

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Wrap Up and Next Steps
David Bottimore, Versar

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Wrap Up/Next Steps

- Virtual public peer review meeting 10/5-6/20
- Post-meeting revised written comments 10/16/20
- Draft post-meeting peer review report
- EPA comments and questions on draft report
- Final peer review report

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