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INCORPORATION OF IN VITRO METABOLISM DATA IN A PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL FOR CHLOROPRENE

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ABSTRACT

A physiologically based pharmacokinetic (PBPK) model for chloroprene in the mouse, rat and human has been developed that relies solely on *in vitro* studies for the estimation of model parameters describing tissue metabolism and partitioning. The PBPK model accurately predicts *in vivo* pharmacokinetic data from a 6-hr, nose-only chloroprene inhalation study conducted with female B6C3F1 mice, the most sensitive species/gender for lung tumors in the 2-year bioassays conducted with chloroprene. This PBPK model has been developed to support an inhalation cancer risk assessment for chloroprene using *in vitro* data on the metabolism of chloroprene to reactive epoxides in the lung target tissue of mice and humans. The approach for calculating target tissue (lung) dose metrics was based on the PBPK modeling performed in support of the inhalation cancer risk assessment for methylene chloride and represents the best available science for determining the impact of species differences in metabolism of chloroprene.

EXECUTIVE SUMMARY

- This document describes the development of a physiologically based pharmacokinetic (PBPK) model for chloroprene in the mouse, rat and human. The intended application of the model is to estimate target tissue dose metrics (total metabolism of chloroprene to reactive epoxides in the lungs) to support an inhalation risk assessment for lung cancer.
- The chloroprene model structure and dose metric selection are based on previous PBPK models for methylene chloride and vinyl chloride that were used in cancer risk assessments by the USEPA. As with methylene chloride and vinyl chloride, the observed carcinogenicity of chloroprene in the mouse is believed to be do the generation of a reactive metabolite in the target tissue. The chief difference from previous models is that the tissue metabolism parameters for chloroprene were based on the published results of *in vitro* studies using microsomes, rather than inferring the parameters indirectly by fitting the model to *in vivo* pharmacokinetic data.
- To assess the validity of the PBPK model, a new study was conducted with female B6C3F1 mice, the most sensitive species/gender for lung tumors in the 2-year bioassay conducted with chloroprene. The mouse PBPK model accurately predicted the *in vivo* pharmacokinetic data from a 6-hr, nose-only chloroprene exposure.
- It was not possible to confidently estimate metabolism parameters for the model in the human lung due to the low rate of metabolism observed in this tissue. Therefore, an alternative approach, previously used in the USEPA risk assessments for methylene chloride, was applied. In the case of methylene chloride, there were no data available to estimate human lung metabolism to the reactive metabolite that was considered to be responsible for carcinogenicity. Therefore, the lung metabolism parameters were based on the parameters for liver metabolism together with the ratio of liver and lung activity for a standard substrate in an *in vitro* assay. More recent evidence for lung and liver expression of the isozymes that metabolize chloroprene supports the *in vitro* activity ratio. Applying this approach to chloroprene provides a conservative (health-protective) estimate of human lung metabolism compared to the values that could be inferred from the highly uncertain *in vitro* data for chloroprene metabolism in human lung microsomes.
- The PBPK model was used to predict dose metrics – amounts of chloroprene metabolized in the lung per gram lung per day – in female mice and humans. The ratios of the human lung metabolism dose metrics to the lung metabolism dose metrics in the female mouse are roughly two orders of magnitude lower than those calculated on the basis of inhaled chloroprene concentration.
- Conclusions: The revised chloroprene PBPK model is based on the best available science, including a new test animal *in vivo* validation study, an updated literature review and a new Markov-Chain Monte Carlo analysis to assess parameter uncertainty. Inclusion of the best available science is especially important when deriving a toxicity value based on species extrapolation for the potential carcinogenicity of a reactive metabolite, since previous risk assessments have demonstrated that the default cross-species extrapolation using inhaled concentration is highly inaccurate for this mode of action.

1. INTRODUCTION

Chloroprene (CAS # 126-99-8) is a highly volatile chlorinated analog of 1,3-butadiene that is used in the manufacture of polychloroprene rubber (Neoprene). A cancer risk assessment for chloroprene conducted by the USEPA (2010) calculated an inhalation unit risk (IUR) of 5x10-4 per µg/m3 based on tumor incidence data from female mice exposed to chloroprene for 2 years (NTP 1998; Melnick et al. 1999). The USEPA (2010) assessment used a default cross-species extrapolation approach based on chloroprene exposure concentration, despite strong evidence of quantitative differences in chloroprene metabolism in mice and humans that would have a significant impact on the calculated risk (Himmelstein et al. 2004a, 2004b). The metabolism of chloroprene results in the formation of reactive epoxides that are considered to be responsible for its carcinogenicity in rodents (USEPA 2010).

To determine the potential impact of species-specific differences in the production of these epoxides, a physiologically based pharmacokinetic (PBPK) model was developed in a collaborative research effort between DuPont Haskell Laboratory and the USEPA National Health and Environmental Effects Research Laboratory (NHEERL). *In vitro* measurements of partition coefficients and metabolism parameters for chloroprene in mice, rats, hamsters and humans (Himmelstein et al. 2004a) were used in the PBPK model (Himmelstein et al. 2004b) to predict species-specific dose metrics for the production of epoxides in the lung, the most sensitive tissue in the mouse bioassay. The dose metric chosen for this comparison is consistent with the dose metrics used in previous PBPK-based risk assessments for methylene chloride and vinyl chloride, which are also metabolized to reactive metabolites that are considered to be responsible for the observed carcinogenicity in rodents. Closed-chamber exposures of mice, rats and hamsters were used to validate the PBPK model's ability to predict the pharmacokinetic behavior of chloroprene *in vivo*. The USEPA (2010), however, did not make use of the PBPK model from Himmelstein et al. (2004b) in their risk assessment, citing the lack of blood or tissue time course concentration data for model validation. In addition, USEPA indicated that they did not consider the comparisons of model predictions with the closed-chamber studies to be adequate because the data were limited to chloroprene vapor uptake from the closed chambers.

After the time of the USEPA (2010) evaluation, subsequent studies (IISRP 2009b) provided additional data for refining the PBPK model of Himmelstein et al. (2004b). To supplement the data in Himmelstein et al.(2004a) on liver and lung metabolism in male mouse, male rat, and pooled human cells, subsequent studies (IISRP 2009b) measured liver and lung metabolism in female mouse and female rat, as well as kidney metabolism in male and female mouse, male and female rat, and pooled human cells. The totality of the data from the Himmelstein et al. (2004a) and IISRP (2009b) *in vitro* metabolism studies was then used to refine the metabolism parameter estimates for the chloroprene PBPK model using Markov-chain Monte Carlo (MCMC) analysis. A comparison of lung dose metric estimates in mouse, rat and human was then performed using the updated metabolism parameters (Yang et al. 2012). These dose metrics were subsequently used in a study comparing genomic responses to chloroprene in the mouse and rat lung (Thomas et al. 2013) and a study comparing human risk estimates derived from mouse bioassay and human epidemiological data (Allen et al. 2014), but to date no *in vivo* blood or tissue time course concentration data have been published with which to evaluate the ability of the chloroprene PBPK model to predict *in vivo* kinetics.

The objectives of the present study were to: 1) characterize the *in vivo* pharmacokinetics of chloroprene via analysis of whole blood concentrations in female B6C3F1 mice during and

following a single 6-hour nose-only inhalation exposure, and 2) determine respiratory parameters (breathing frequency and tidal volume) during chloroprene exposure. We also demonstrate the ability of the refined chloroprene PBPK model to reproduce new *in vivo* validation data and calculate PBPK dose metrics that can be used to support an inhalation cancer risk assessment that properly considers species differences in pharmacokinetics and metabolism.

2. MATERIALS AND METHODS

Nose-only Exposure Study

Test Substance and Atmosphere Generation

The test substance, β-Chloroprene (CAS # 126-99-8) containing polymerization inhibitors, was supplied by the sponsor as a clear liquid. Exposure atmospheres were generated by metering saturated chloroprene vapor from a stainless-steel pressure vessel reservoir (McMaster Carr, Atlanta, GA) into the nose-only exposure chamber air supply. The concentrated chloroprene vapor was metered through a mass flow controller (MKS Instruments Inc., Andover, MA) and mixed with HEPA-filtered air approximately six feet upstream of the nose-only inlet. Chloroprene vapor was introduced counter-current to the dilution air to facilitate mixing of the vapors with the dilution air. Chloroprene concentrations were monitored on-line using a gas chromatography system with flame ionization detector (GC-FID). Calibration of the GC-FID for chloroprene analysis was conducted through the analysis of a series of calibration standards produced by introducing pure chloroprene into Tedlar® bags containing known volumes of nitrogen gas (nitrogen was metered into the bag using a calibrated flow meter).

Test Animals and Housing

Female B6C3F1 were purchased from Charles Rivers Laboratories, Inc (Raleigh, NC) at 8 weeks of age and acclimated to their surroundings for approximately two weeks prior to use. Following acclimation animals were assigned to a dosing group by randomization of body weights using Provantis NT 2000, assigned unique identification numbers, cage cards, and housed (1/cage) in polycarbonate cages with standard cellulose bedding. Animals were housed in a humidity and temperature controlled, HEPA-filtered, mass air-displacement room provided by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited animal facility at The Hamner Institutes. This room was maintained on a 12-hour light-dark cycle at approximately 64oC-79oF with a relative humidity of approximately 30-70%. Rodent diet NIH-07 (Zeigler Brothers, Gardners, PA) and reverse osmosis water was provided ad libitum except during exposures. Food and water were withheld from all animals during the chloroprene exposures. Prior to the start of the chloroprene exposure, animals were weighed, and their weights were recorded.

The Hamner Institutes for Health Sciences was fully accredited by the AAALAC during the time the study was performed. Currently acceptable practices of good animal husbandry were followed per the National Research Council Guide for the Care and Use of Laboratory Animals and were in compliance with all appropriate parts of the Animal Welfare Act. In addition, the study design and protocol were approved by The Hamner Institutes' Institutional Animal Care and Use Committee (IACUC) prior to the initiation of the study.

Inhalation Exposures

Inhalation exposures were conducted at 13, 32, and 90 ppm for 6 hours. Blood was collected by cardiac puncture at a total of 6 time-points, 0.5, 3, and 6 hours during exposure and 5, 10, and 15 minutes post-exposure. To support collection of whole blood during the exposures, nose only towers were fitted with specially designed nose only exposure tubes. These exposure tubes were manufactured from 50 mL polypropylene bulb irrigation syringes (Sherwood Medical, St. Louis, MO). Three elongated holes (0.625" x 1.125") were drilled into the wall of the syringe to allow access to the thorax of the mouse during chloroprene exposure. A second irrigation syringe was

cut to form a sleeve around the first syringe to provide an airtight barrier during the exposures. This sleeve was pulled back during the exposure to allow for the injection of pentobarbital (100 mg/kg) while the animal continued to inhale chloroprene. Blood was removed directly from the mouse via arterial-side cardiac puncture while the mouse was still housed in the syringe and breathing chloroprene.

Plethysmography

A total of 16 mice (4 per exposure group including air controls) were used for the purpose of collecting tidal volume and breathing frequency. Data were acquired using modified nose only Buxco plethysmograph tubes for pulmonary function monitoring. Data from control mice were collected prior to the first chloroprene exposure. Plethysmography data from both control and exposed mice were collected for 2-3 hours.

Blood Sampling

Whole blood was collected at 0.5, 3, and 6 hours during exposure and 5, 10, and 15 minutes post-exposure. Whole blood collection during chloroprene exposures (0.5, 3, and 6-hour time points) were done using the specially designed nose only exposure tubes described above.

Blood Analysis

Quantification of chloroprene in whole blood was conducted by headspace sampling with analysis by gas chromatography mass spectrometry (GC/MS). The sampling method to be used, headspace analysis, as well as the GC/MS method were based on the previously published method for the analysis of 1,3-butadiene in whole blood from mice and rats (Himmelstein et al. 1994).

Briefly, 200 µL of whole blood, obtained by cardiac puncture, was transferred into pre-labeled, capped, and weighed airtight headspace vials (1.5 mL autosampler vial). Sample vials were weighed to obtain an accurate estimate of sample size and allowed to equilibrate at room temperature for 2 hours. Once equilibration was complete, samples were analyzed using an Agilent 5973 mass spectrometer coupled to an Agilent 6890 gas chromatograph. The mass spectrum was run in electron impact mode with selective ion monitoring (instrumental conditions are listed below).

Calibration curves were prepared by spiking stock control whole blood with known amounts of chloroprene obtained as a certified standard solution of chloroprene in methanol. Quality control samples were prepared by spiking control rat plasma with a certified chloroprene standard. QC samples were spiked to low (near the first calibration point), medium (near the middle of the calibration curve), and high (near the highest point of the calibration curve) levels. Aliquots of the prepared QC's were placed in sealed GC vials (3 aliquots for each level, 9 total) and kept frozen at -80^{o}C until required (GC vials had a minimum of headspace prior to freezing). On the blood collection days, a low-, middle-, and high-level QC was thawed and allowed to come to room temperature for 4 hours. After this time, the QC samples were "sampled" with a syringe identical to those being used for the collection of whole blood, placed in a GC vial in a manner identical to that of the whole blood collection, and analyzed along with the samples and standards.

Additional details of the nose-only inhalation study can be found in IISRP (2009a).

Chloroprene PBPK Model

The development and documentation of the chloroprene PBPK model has been conducted in a transparent manner consistent with the WHO/IPCS (2010) guidance on PBPK modeling. The following sections describe the basis for the model structure and parameterization, as well and the methods used for sensitivity/uncertainty analysis and risk assessment application of the model.

Model Structure

The structure of the PBPK model used in this study (Figure 1) is based on the PBPK model of chloroprene described in Himmelstein et al. (2004b), as modified by Yang et al. (2012). As in previous models of volatile organic compounds (Ramsey and Andersen 1984; Andersen et al. 1987), the blood is described using a steady-state approximation and the model assumes blood-flow limited transport to tissues and venous equilibration of tissues with the blood. Metabolism is described in the liver, lung and kidney using Michaelis-Menten saturable kinetics.

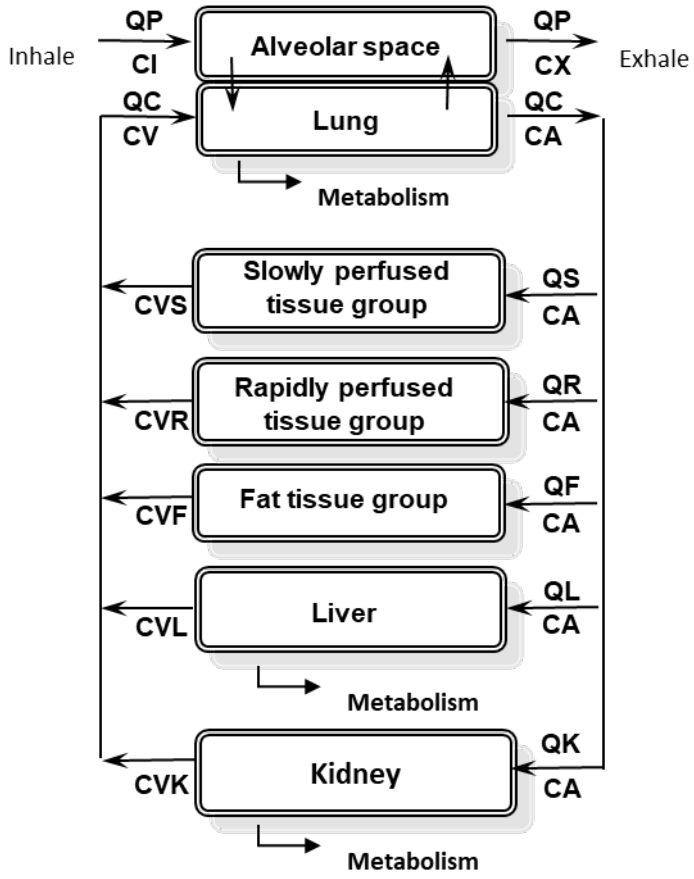

Figure 1. Chloroprene PBPK model diagram. Abbreviations: QP - alveolar ventilation; CI - inhaled concentration; CX - exhaled concentration; QC - cardiac output; CA - arterial blood concentration; CV - venous blood concentration; QS, CVS - blood flow to, and venous concentration leaving, the slowly perfused tissues (e.g. muscle); QR, CVR - blood flow to, and venous concentration leaving, the richly perfused tissues (most organs); QF, CVF - blood flow to,

and venous concentration leaving, the fat; QL, CVL - blood flow to, and venous concentration leaving, the liver; QK, CVK - blood flow to, and venous concentration leaving, the kidney.

Model Parameters

All physiological parameters in the model for mouse, rat and human (Table S-1 in Supplemental Materials A) are taken from Brown et al. (1997) except for the cardiac output in the mouse and the alveolar ventilation and cardiac output in the human. While the alveolar ventilation in the mouse is taken from Brown et al. (1997), relying on the value of cardiac output reported in Brown et al. (1997) would result in a value of 11.6 L/hr/bw3/4 for cardiac output (QCC). If used with the Brown et al. (1997) value of 29.1 L/hr/bw3/4 for alveolar ventilation (QPC), this would result in a serious mismatch between ventilation and perfusion (V/Q ratio >> 1). Andersen et al. (1987), the developers of the PBPK model for methylene chloride that was used in the USEPA (2011) IRIS assessment, argued that it would be more biologically realistic to assume that the V/Q ratio was close to 1 at rest, and stated that their previous experience with PBPK modeling of data on clearance of chemicals in the mouse under flow-limited metabolism conditions supported the use of a higher value for QCC. Therefore, the value of QCC in the current model was calculated by dividing the alveolar ventilation from Brown et al. (1997) by an estimate of V/Q = 1.45 for the mouse based on pharmacokinetic data for exposures to another volatile organic chemical, methylene chloride (Marino et al. 2006), which was used in the USEPA (2011) inhalation cancer risk assessment for that chemical. In the case of the human, it is more appropriate to use the default USEPA ventilation rate of 20 L/day, reflecting an average activity level, rather than a resting value (Clewell et al. 2001). Since the values for alveolar ventilation and cardiac output in Brown et al. (1997) are resting values, we used the values calculated for the PBPK model of vinyl chloride (Clewell et al. 2001), which was used in the USEPA (2000) cancer risk assessment for that chemical. The parameter values, which were calculated to be consistent with the USEPA default ventilation rate of 20 L/day, were QPC = 24.0 L/hr/ $bw^{3/4}$ and a QCC of 16.5 L/hr/ $bw^{3/4}$ (V/Q ratio of 1.45).

Apart from the physiological parameters, the model parameters are based entirely on *in vitro* data. 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), using the partition coefficients for muscle and kidney to represent the slowly and rapidly perfused tissues, respectively. To obtain the model parameters for metabolism in the liver, lung and kidney, the original *in vitro* chloroprene metabolism time-course data (Himmelstein et al. 2004a; IISRP 2009b) were re-analyzed using a MCMC analytical approach similar to the one performed in Yang et al. (2012). The key differences between the new analysis and the original Yang et al. (2012) analysis were: (1) the incorporation of an additional parameter in the analysis of the *in vitro* metabolism data (Kgl) to describe the rate of transfer of chloroprene from the headspace to the media in the metabolism studies, (2) the use of updated tissue microsomal protein concentrations for scaling the *in vitro* results to *in vivo* values appropriate for the PBPK model, and (3) the adoption of a previously published approach for estimating the metabolism parameters in the human lung (Andersen et al. 1987).

Re-estimation of *in vitro* metabolism parameters: Schlosser et al. (1993) suggested that mass transport limitations should be assessed when estimating metabolism from *in vitro* experiments conducted with volatile compounds where there is an air:liquid interface. Since the potential for a mass transport limitation was not addressed in the *in vitro* metabolism studies conducted with chloroprene (Himmelstein et al. 2004a; IISRP 2009b), a new experimental study was performed

by Denka Performance Elastomer LLC at the request of USEPA to estimate a Kgl for chloroprene following a protocol based on Schlosser et al. (1993). The new experimental study, which is described in Supplemental Materials B, resulted in an estimated value of 0.024 L/hr for Kgl, similar to the value previously reported for benzene (Schlosser et al. 1993). However, this experimentally estimated value of Kgl was not consistent with the high rates of liver metabolism observed at low concentrations of chloroprene; that is, the mass transport associated with a Kgl of 0.024 L/hr was too slow to support the observed rates of metabolism in the media.

We considered it likely that the much faster uptake of chloroprene in the metabolism studies than in the Kgl study was due to more effective mixing during the incubations, together with non-specific surface binding of chloroprene to the microsomes, which provide a lipophilic binding component in the aqueous media. No microsomes were present in the Kgl experiments for chloroprene or benzene (Schlosser et al. 1993). Although the rate of shaking in the metabolism studies (Himmelstein et al. 2004a; IISRP 2009b) was not reported, we were able to determine that these studies used a Gerstel MPS2 autosampler with an agitating heater, which was set to an agitation rate of 500 rpm (Himmelstein 2019, personal communication), in comparison to the 60 rpm agitation rate used in Schlosser et al. (1993) and the present study.

To account for this difference in agitation rates, it was suggested (Paul Schlosser 2019, personal communication) that the value of Kgl in the metabolism studies was likely to be higher than the value in the new experimental study by roughly the ratio of the mixing rates, that is, Kgl(metabolism studies) = Kgl(experimental study)×500/60 = 0.024×500/60 = 0.2 L/hr. To confirm this expectation, we conducted a new MCMC analysis to simultaneously estimate Kgl, Vmax and Km from the metabolism data for the male mouse (Himmelstein et al. 2004a), which provided the strongest information regarding the dose-response for the clearance of chloroprene in the vials. The resulting value of Kgl estimated from this analysis was 0.22 L/hr, with a 95% confidence interval of 0.19 – 0.33 L/hr, consistent with the estimated value. The estimated value was then used in the re-estimation of the metabolism parameters for all tissues (Supplemental Materials B). The results of the new *in vitro* metabolism parameter estimation are provided in Table S-3 in Supplemental Materials A.

Selection of tissue scaling parameters: Based on a review of the literature (Supplemental Materials C), an updated set of scaling parameters was chosen: 35, 40, and 40 mg protein/g liver for mice, rats, and humans, respectively, (Medinsky et al. 1994 for mouse, Medinsky et al. 1994 and Houston and Galetin 2008 for rat, Barter et al. 2007 for human). For the lung, 20 mg protein/g was used for all species (Medinsky et al. 1994 for rat and mice, Boogaard et al. 2000 for rat, mouse and human). A microsomal content of kidney of 18 mg protein/g was used for mouse and rat and 11 mg protein/g for human (Yoon et al. 2007 for mouse and rat; Scotcher et al. 2017 for human). The maximum velocity and 1st order clearance rate constants were scaled allometrically (mg/hr/$BW^{0.75}$ or L/hr/$BW^{0.75}$) using the species and sex specific time and survival weighted average BW from the control group reported in the chloroprene bioassay (NTP 1998) for mouse and rat and 70 kg for human. The *in vivo* metabolism parameters derived using these revised scaling parameters with the *in vitro* metabolism estimates in Yang et al. (2012) and with the results of the present re-analysis are listed in Table S-4 in Supplemental Materials A and the IVIVE calculations are provided in Supplemental Materials D.

Estimation of chloroprene metabolism in the human lung: Unfortunately, we found that the extremely low rates of chloroprene metabolism observed in the human lung (Himmelstein et al. 2004a) made parameter estimation for this tissue highly uncertain. Therefore, in the application of the model to calculate dose metrics we estimated the metabolism parameter for the human

lung using the approach applied in the USEPA (2011) risk assessment for methylene chloride, which relied on the PBPK model developed by Andersen et al. (1987), In that model, the Km for metabolism in the human lung was assumed to be the same as the Km in the human liver, and the Vmax in the human lung was calculated from the Vmax in the human liver using a parameter (A1) derived from the ratio of the specific activities for metabolism of 7-ethoxycoumarin, a well-studied CYP2E1 substrate, in liver and lung (Lorenz et al. 1984).

Model Simulations

The previously published version of the chloroprene PBPK model (Yang et al. 2012), which was written in the Advanced Continuous Simulation Language (ACSL), was translated into R, an open source programming language, to improve its portability. The R code for the model is included in Supplemental Materials E. The full model code, including the scripts for running the model, is provided separately.

To model the experimental data from the nose-only inhalation exposures reported here, only the alveolar ventilation and cardiac output were altered. The average ventilation rate measured in the mice during the study was used to calculate an alveolar ventilation for use in the model, assuming 2/3 of total ventilation is alveolar (Brown et al. 1997), and the cardiac output was then calculated by dividing the alveolar ventilation by the V/Q ratio from Marino et al. (2006), as described in the results.

Parameter Sensitivity Analysis

Parameter sensitivity analysis was conducted with the model under two scenarios: (1) the prediction of blood concentrations in the mouse nose-only study, and (2) the prediction of dose metrics for the mouse bioassay exposures and for the human at 1 ppm continuous exposure. The results were calculated as normalized sensitivity coefficients (fractional change in prediction divided by fractional change in parameter) for parameters with a coefficient greater than 0.1 in absolute magnitude. A positive coefficient indicates the direction of change of the prediction is the same as the direction of change of the parameter. The parameters were changed by 1%, one at a time.

Dose metric calculations

Consistent with previous PBPK modeling of chloroprene (Himmelstein et al. 2004b; Yang et al. 2012), the dose metric calculated with the PBPK model is micromoles of chloroprene metabolized in the lung per gram lung per day. This dose metric was chosen because the lung is the tissue with the highest tumor incidence in the chloroprene inhalation bioassays (NTP 1998) and the carcinogenicity of chloroprene in rodents is believed to result from its metabolism to reactive epoxides in the target tissue (Himmelstein et al. 2004a, 2004b). The dose metric selected for chloroprene is consistent with the dose metrics used in previous PBPK-based risk assessments for both vinyl chloride (Clewell et al. 2001; USEPA 2000) and methylene chloride (Andersen et al. 1987; USEPA 2011), which were also based on the production of reactive metabolites.

The PBPK model was first used to simulate the NTP (1998) bioassay exposures (12.8, 32 and 80 ppm; 6 hours/day, 5 days/week) and calculate the corresponding target tissue dose metrics (in this case, average daily production of epoxide metabolites in the lung per gram lung). The PBPK model was then used to estimate the same target tissue dose metric in a human exposed continuously to chloroprene at a concentration of 1 $\mu g/m^3$ for their lifetime. Due to the low rate of chloroprene metabolism in the human lung observed in the *in vitro* studies (Himmelstein et al.

2004a), the human lung metabolism parameters were estimated using the approach in the methylene chloride PBPK-based risk assessment (Andersen et al. 1987; USEPA 2011), where the affinity of lung metabolism was assumed to be the same as in the liver, and the relative capacity of lung to liver was based on *in vitro* data for a standard substrate, 7-ethoxycoumarin. As in the case of methylene chloride, this was done to provide a conservative (high-sided) estimate of the human dose metric, given the insufficiency of the *in vitro* chloroprene data for the human lung.

3. RESULTS

Chloroprene Exposure Atmospheres

Chloroprene concentrations were monitored in the nose only chambers during the 13, 32, and 90 ppm exposures, as well as in the control nose-only tower. All three target concentrations were well within 10% of their nominal levels.

Plethysmography

Figure 2 shows the measured minute volumes for the three exposure groups and controls. The data are represented as average values (circles) with standard deviation error bars. The data are provided in Table S-5 in Supplemental Materials A. There is no evidence of a concentration-related effect of short-term exposure to chloroprene on ventilation in mice. The average ventilation rate across all four exposure groups, including controls, was 56.2 mL/min. The average body weight for the mice in the study was 22g; therefore, this ventilation rate equates to a model parameter for alveolar ventilation (QPC) of 39.4 L/hr/bw3/4. The corresponding model value of QCC in this study is obtained by dividing QPC by the V/Q ratio of 1.45 for the mouse (Marino et al. 2006), yielding a value for QCC of 27.2 L/hr/bw3/4, which compares well with the QCC of 24.2 estimated for mouse exposures to methylene chloride (Marino et al. 2006).

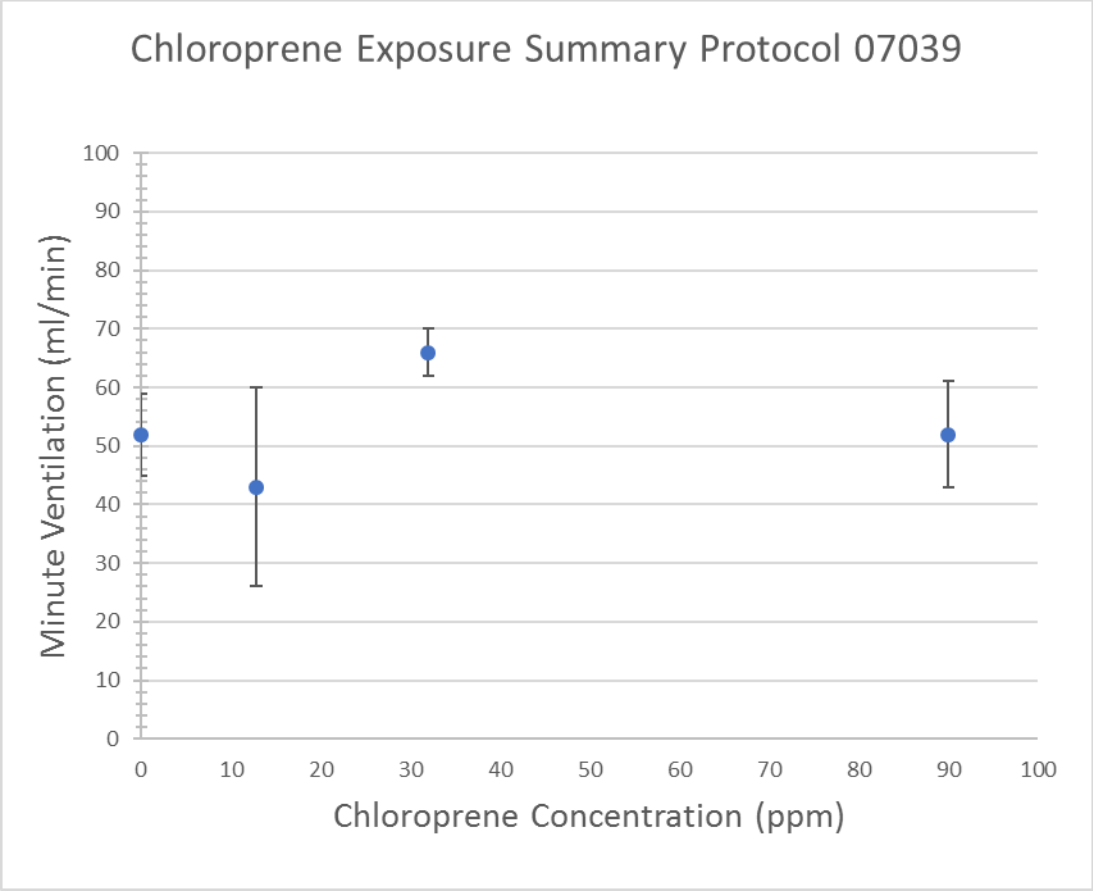

Figure 2. Measured minute ventilation during exposures.

Arterial Blood Chloroprene Concentrations

Figure 3 shows the average chloroprene (CD) blood concentrations at multiple timepoints for all three single day exposures (Data are provided in Table S-6 of Supplemental Materials A). Average blood chloroprene concentrations are represented by the symbols with standard deviations for each treatment group represented with error bars.

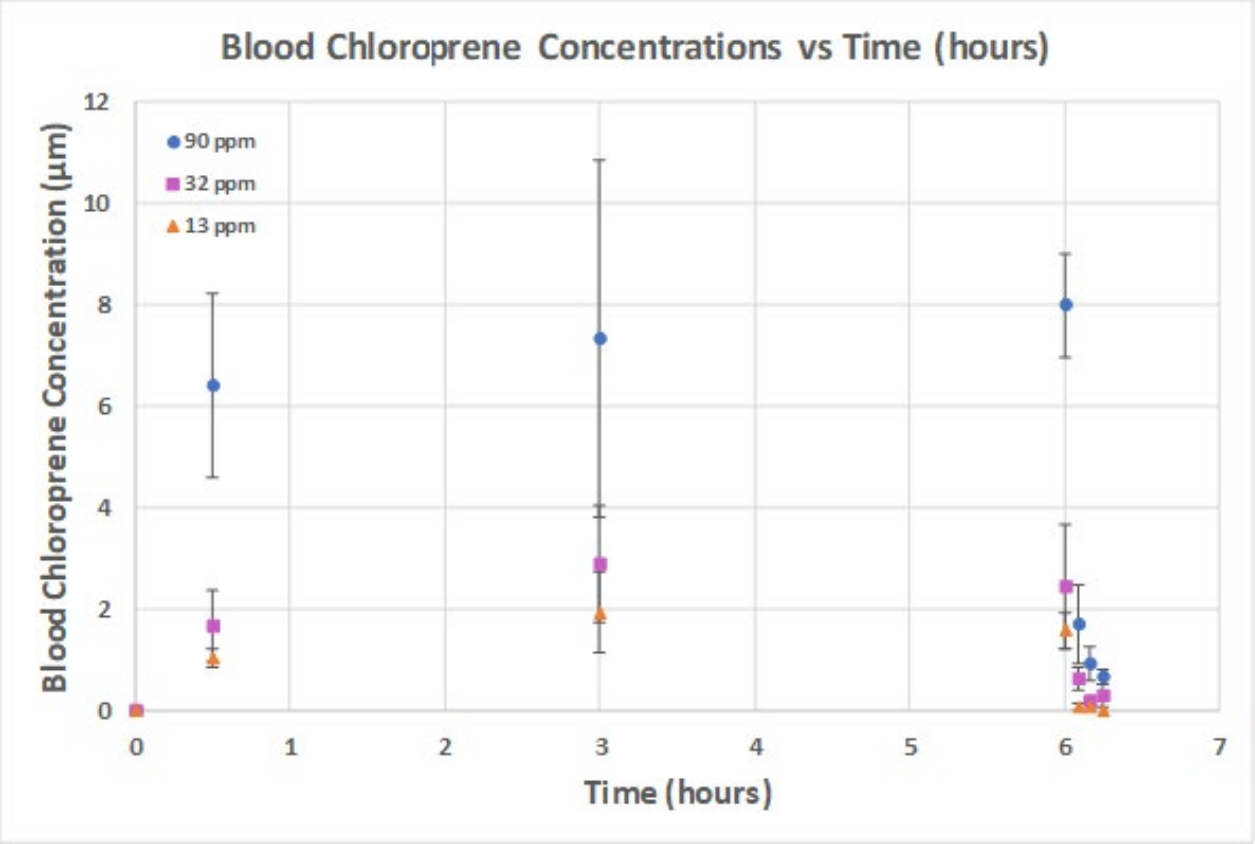

Figure 3. Arterial blood chloroprene concentrations during and following a single nose-only exposure of female B6C3F1 mice to chloroprene at 13, 32 or 90 ppm for 6 hours. Average blood chloroprene concentrations (symbols) and standard deviations (error bars) are shown for each treatment group.

Re-estimation of Metabolism Parameters

The mean and 95% confidence intervals for the *in vitro* metabolism parameters (Vmax and Km) resulting from the MCMC re-analysis are shown in Table S-3 of Supplemental Materials A and the scaled-up parameters for the PBPK model are listed in Table S-4. In our re-analysis of the *in vitro* metabolism data, we found that the extremely low rates of chloroprene metabolism observed *in vitro* (Himmelstein et al. 2004a; IISRP 2009b) made parameter estimation for several tissues highly uncertain: female mouse kidney, male and female rat lung, human kidney and lung. For the human lung, this uncertainty resulted from the very low rate of metabolism observed in these tissues during the *in vitro* studies conducted with chloroprene (Himmelstein et al. 2001; IISRP 2009b) compared to other sources of variability in the experiments. In particular, the data on loss of the chemical from control vials in the studies indicate that non-

metabolic losses contributed substantially to variability between assays. Unfortunately, the experimental protocol used in Himmelstein et al. (2001) and IISRP (2009b) did not include the use of internal controls to characterize non-metabolic losses in the vials in which metabolism was measured. The effect of the lack of internal controls cannot be completely overcome by *a posteriori* analysis.

Despite this experimental limitation, it was possible to reliably estimate the parameters for the capacity (Vmax) and affinity (Km) of metabolism in the majority of tissues because the enzymatic metabolism of chloroprene is known to be a saturable process (Michaelis-Menten kinetics), whereas the data from the control vials in the *in vitro* studies demonstrate that the other losses of the chemical from the vial are independent of concentration. Thus, whereas the other losses result in parallel lines on a log plot, metabolism results in downward concave curves with slopes that increase as the concentration decreases. Figure 4 shows the fit of the parameter estimates (curves) to the data (solid circles) for the *in vitro* metabolism studies in the mouse (left) and human (right) liver. It also shows the data from control vials that did not have any metabolism (open circles), which were only collected at some of the concentrations. The losses from the control vials are linear and parallel, while the rates of loss from the metabolism vials increase as the concentration decreases. 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.

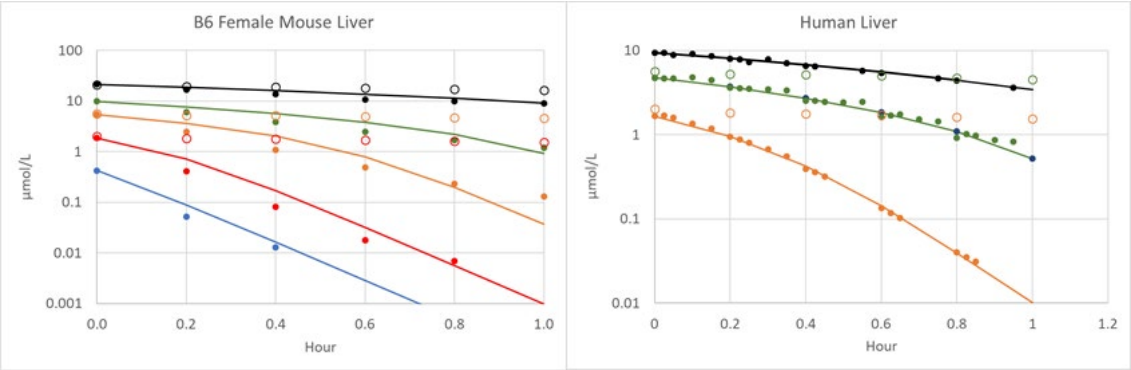

Figure 4. Predicted (curves) and measured (solid symbols) concentrations of chloroprene during *in vitro* metabolism experiments in the mouse (left) or human (right) liver. Open circles are data from control vials that lacked metabolic activity.

Metabolism in the female mouse lung (Figure 5) is much slower than in the liver, but the clearance from the metabolism vials (solid circles) is still clearly nonlinear, while the losses from the control vials (open circles) are linear. This systematic difference between the control and metabolism vials makes it possible to estimate both Vmax and Km.

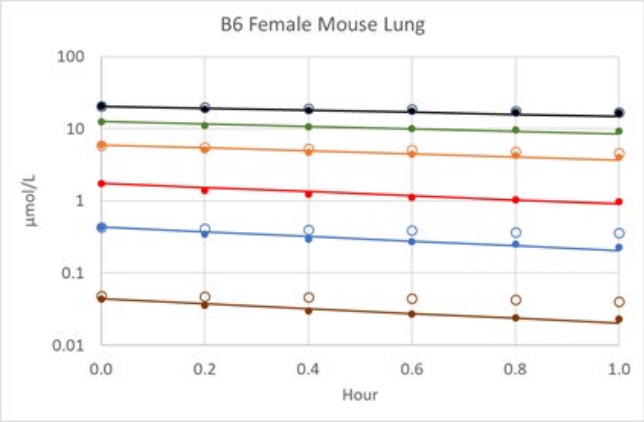

Figure 5. Predicted (curves) and measured (solid symbols) concentrations of chloroprene during *in vitro* metabolism experiments in the mouse lung. Open circles are data from control vials that lacked metabolic activity.

In the human lung, however, there is essentially no evidence of metabolism in the *in vitro* studies. Figure 6 shows the data from the metabolism vials (solid circles) along with the predictions (curves) from a model of the *in vitro* system that assumed there was no metabolism occurring. The slopes of each pair of lines represents the range of loss rates associated with taking samples from the vial headspace as well as losses associated with leakage through the vial septum after puncturing. The latter loss rates were estimated from all the control data in the *in vitro* studies (Supplemental Materials B). Controls for the human lung study were only performed at the lowest concentration (open symbols). The loss rate in the metabolism vial is within the range of loss rates in the control vials.

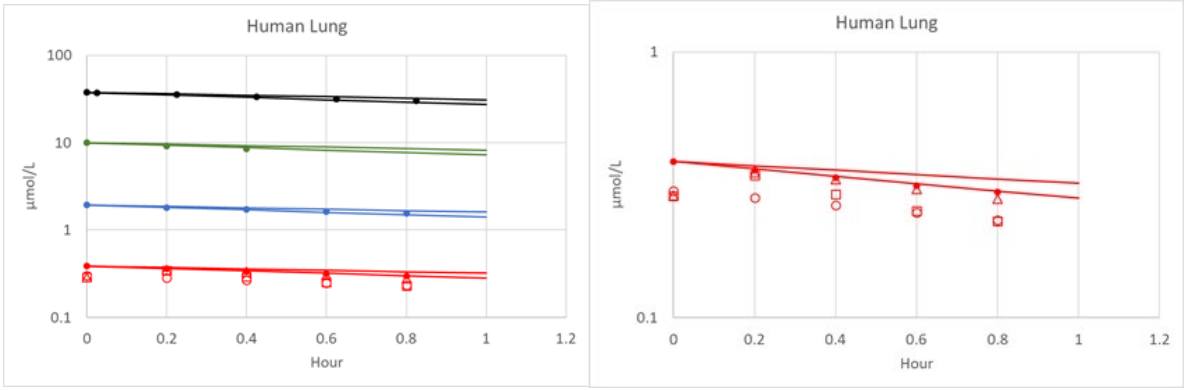

Figure 6. Measured concentrations of chloroprene during *in vitro* metabolism experiments in the human lung. (Solid symbols: metabolism vials; open symbols: control vials). Curves are model predictions assuming no metabolism is occurring.

In their analysis, Yang et al. (2012) attempted to estimate linear metabolism parameters in tissues where the MCMC analysis was unable to converge on estimates of both Vmax and Km. However, we have determined not to use that approach for two reasons: (1) estimation of a pseudo-linear metabolic parameter would only be appropriate for concentrations well below Km,

which for CYP2E1 is in the vicinity of 1 μM, but most of the data on chloroprene is at much higher concentrations, and (2) estimates of a linear metabolism component were unreliable due to confounding by other linear losses from the vials, as demonstrated by the high variability in controls.

Given the unreliability of the human lung data for chloroprene, we chose to estimate the metabolism parameters for the human lung using the same approach as the USEPA (2011) risk assessment for methylene chloride; that is, the Km for metabolism in the human lung was assumed to be the same as the Km in the human liver, and the Vmax in the human lung was calculated from the Vmax in the human liver using a parameter (A1) derived from the ratio of the specific activities for metabolism of 7-ethoxycoumarin, a well-studied CYP2E1 substrate, in liver and lung (Lorenz et al. 1984). Using the human value of A1 (0.00143), together with the estimated values of Vmax and Km in the human liver from the MCMC analysis (0.052 μmol/hr/mg protein and 0.32 μmol/L), results in a metabolic clearance in the lung of 0.16 L/hr/g microsomal protein. This human lung metabolism estimate is similar to the value of 0.32 L/hr/g microsomal protein previously estimated for chloroprene by Yang et al. (2012) and is within the confidence interval estimated by our new analysis of the *in vitro* data. In support of the applicability of A1 to chloroprene, the value of A1 in the male mouse (0.414) from Lorenz et al. (1984) is close to the ratio of the *in vitro* Vmax in the lung and liver of the male mouse in our new analysis (0.56, see Table S-3). The value of A1 is also consistent with the reported ratio of total CYP2E1 plus CYP2F1 mRNA expression in human lung and liver of 0.00059 (Nishimura et al. 2003), which is about a factor of two lower than A1.

For the tissues where metabolism was too slow to characterize (female mouse kidney, male and female rat lung, and human kidney), the model parameter for Vmax in that tissue was set to zero. Ignoring metabolism in these tissues did not perceptibly alter model predictions. In particular, it did not affect the predicted dose metrics in the female mouse lung.

PBPK Modeling of the Nose-Only Inhalation Study

The nose-only study described above was simulated with the chloroprene PBPK model using the parameters in Tables S1, S2, and S4, except for QPC and QCC, where the study-specific values derived from the plethysmography data were used. As shown in Figure 7, using only *in vitro*-derived metabolism and partitioning parameters the model predictions for blood concentrations during and after the 6-hr chloroprene exposures are in good agreement with the data collected in the study; consistent with the WHO/IPCS (2010) guidance on the use of PBPK modeling in risk assessment, model predictions are generally within roughly a factor of two of the means of the experimental data. It was not necessary to adjust any of the model parameters to provide agreement with the new data.

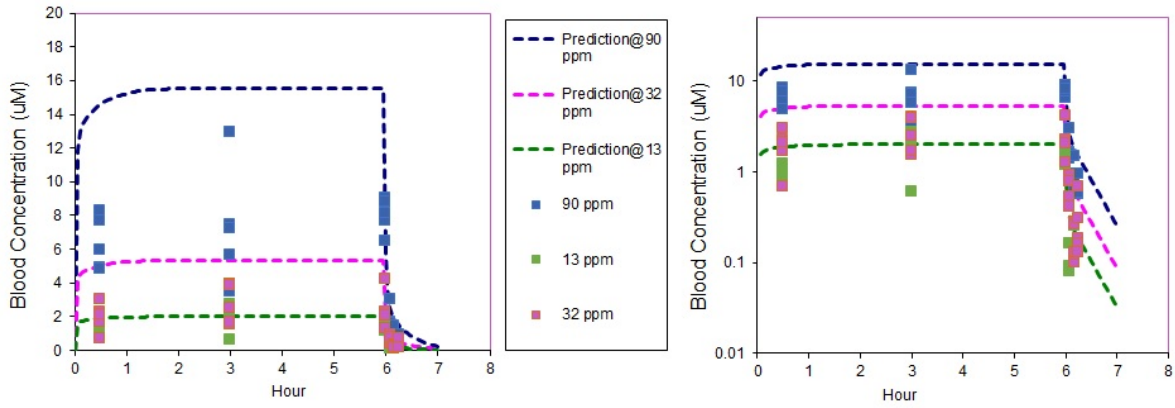

Figure 7. PBPK model predicted (dotted lines) and measured (symbols) blood concentrations during and following 6-hr exposures of B6C3F1 mice to chloroprene at 12.3 (green), 32 (fuchsia) or 90 (blue) ppm. The same data and model predictions are shown using a linear y axis (left) and a logarithmic y axis (right). The linear plot provides a better comparison for concentrations, whereas the logarithmic plot provides a clearer comparison for the post-exposure clearance.

PBPK Model Parameter Sensitivity

As shown in Figure 8, when simulating the nose-only exposures only 4 model parameters have sensitivity coefficients greater than 0.1 in absolute magnitude: alveolar ventilation, cardiac output, blood:air partition coefficient and fractional blood flow to liver. All these parameters were either directly measured or based on data from the literature, as described in the Methods, and can be considered to have low uncertainty. When predicting lung dose metrics in the female mouse (Figure 9), the sensitive parameters include the same parameters as those for the predictions of blood concentrations, with the addition of the parameters for lung metabolism and the body weight. The sensitive parameters for predictions of lung dose metrics in the human (Figure 10) are the same as those in the mouse, except that a single clearance parameter is used in the human due to the low rate of metabolism in the human lung. These analyses of the sensitivity of the model to uncertainty in its parameters suggest that performing a human *in vivo* validation study would be unlikely to provide a significant added value for model evaluation.

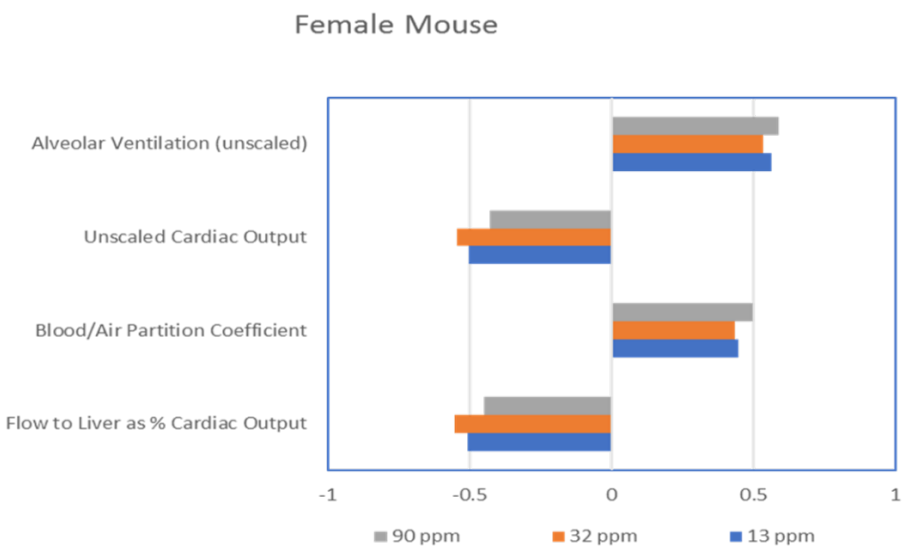

Figure 8. Parameter sensitivity coefficients for the chloroprene PBPK model for the prediction of arterial blood concentrations in the nose-only study.

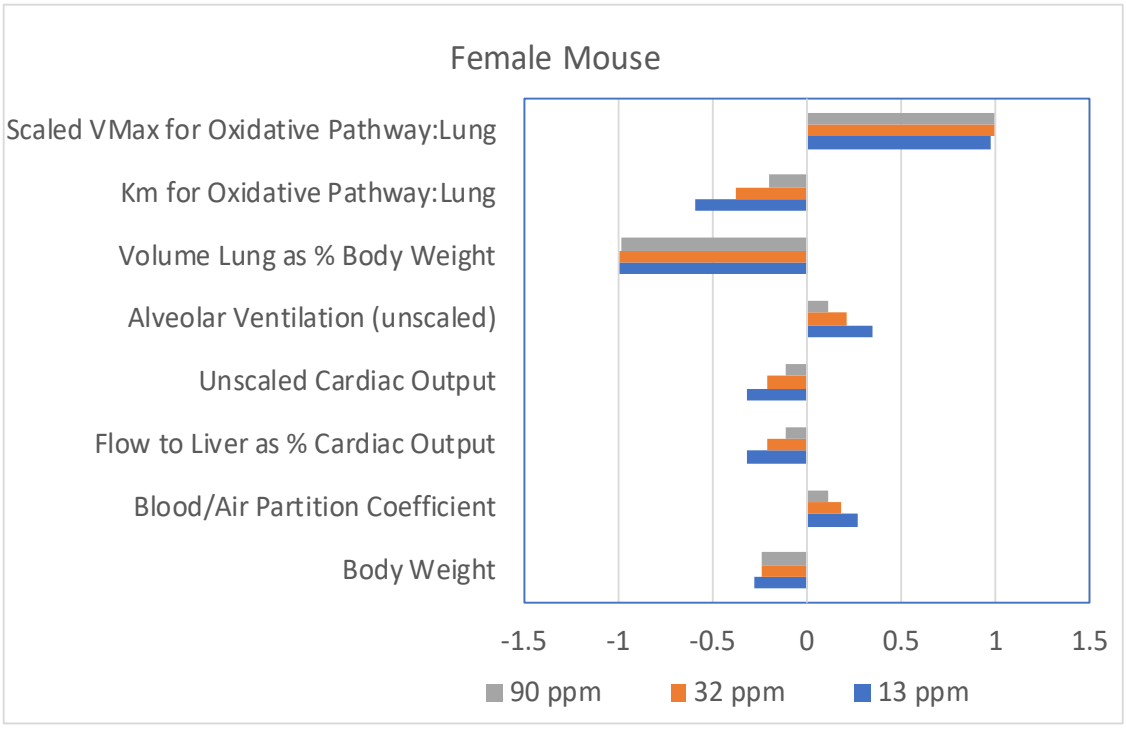

Figure 9. Parameter sensitivity coefficients for the chloroprene PBPK model for the prediction of lung dose metrics in the female mouse for exposures in the 2-year bioassay.

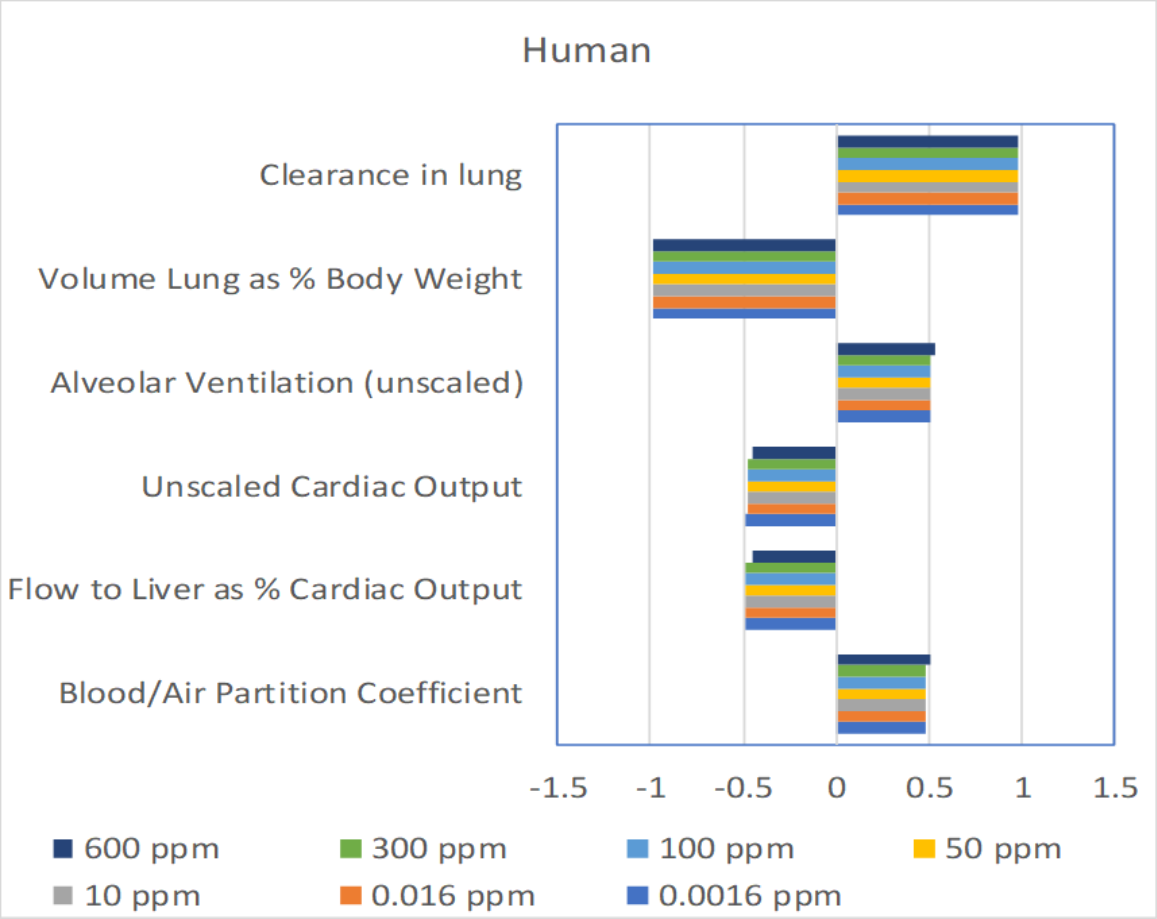

Figure 10. Parameter sensitivity coefficients for the chloroprene PBPK model for the prediction of lung dose metrics in the human for continuous exposure at 1 ppm.

PBPK-Based Dose Metrics for Chloroprene Lung Carcinogenicity

The dose metrics for lung metabolism in the female mouse bioassay and for human continuous exposure are shown in Table 1. These estimates were obtained with the chloroprene PBPK model using the parameters in Tables S1, S2, and S4.

Exposure	Concentration	Dose metric
Female mouse bioassay	12.8 ppm	1.00
	32 ppm	1.58
	80 ppm	2.15
Human continuous	100 ppm	3.48×10^{-2}
	10 ppm	2.76×10^{-2}
	1 ppm	9.00×10^{-3}
	0.1 ppm	1.16×10^{-3}
	0.01 ppm	1.19×10^{-4}
	1 ppb	1.20×10^{-5}
	1 µg/m^3	3.36×10^{-6}

Table 1. Dose metrics for lung metabolism (average mg metabolized per gram lung per day) in the female mouse bioassay and for human continuous exposures

As illustrated in Table 1, predicted dose metrics increase less than linearly above an inhaled chloroprene concentration of 1 ppm.

4. DISCUSSION

In this study, we characterized the time course blood concentrations of chloroprene in female B6C3F1 mice during and following a single 6-hour nose-only inhalation exposure over the range of concentrations used in the NTP (1998) bioassays. These data, including both arterial whole blood concentrations and respiratory parameters (breathing frequency and tidal volume) during and after these exposures provide a reliable basis for evaluating the ability of the chloroprene PBPK model to predict *in vivo* pharmacokinetics in the bioassays. We have then applied the PBPK model to calculate dose metrics to support a risk assessment that considers species differences in pharmacokinetics. The use of a PBPK model for this purpose is consistent with the conclusion of the National Academy of Science (NRC 1987) that: "relevant PBPK data can be used to reduce uncertainty in extrapolation and risk assessment." The application of the model is also consistent with recommended practice for the use of PBPK modeling in risk assessment (WHO/IPCS 2010).

It is important to note that, due to the low rates of metabolism in the *in vitro* assays for the rat and human lung, the original chloroprene model (Himmelstein et al. 2004b; Yang et al. 2012) used a linear description of metabolism in these tissues, which would only be appropriate in the concentration range below Km in the lung. Thus model-based metabolism predictions for human exposures significantly greater than 1 ppm would greatly overestimate the associated risk. Moreover, as described in the results section, estimates of linear metabolism from the *in vitro* data for chloroprene in the human lung are unreliable due to the high variability in other linear loss rates. One approach for dealing with the inability to estimate the parameters for saturable metabolism in the human lung is to use the value of Km estimated in the human liver, together with data on the ratio of metabolic activities in the liver and lung. This approach was applied by the USEPA in their risk assessment for methylene chloride using a PBPK model (Andersen et al. 1987) and in the present analysis. The impact of saturable metabolism on human dose metric predictions is shown in Figure 11. Without estimating a value for Km, the model-predicted risks above 1 ppm would continue to increase at a biologically implausible rate.

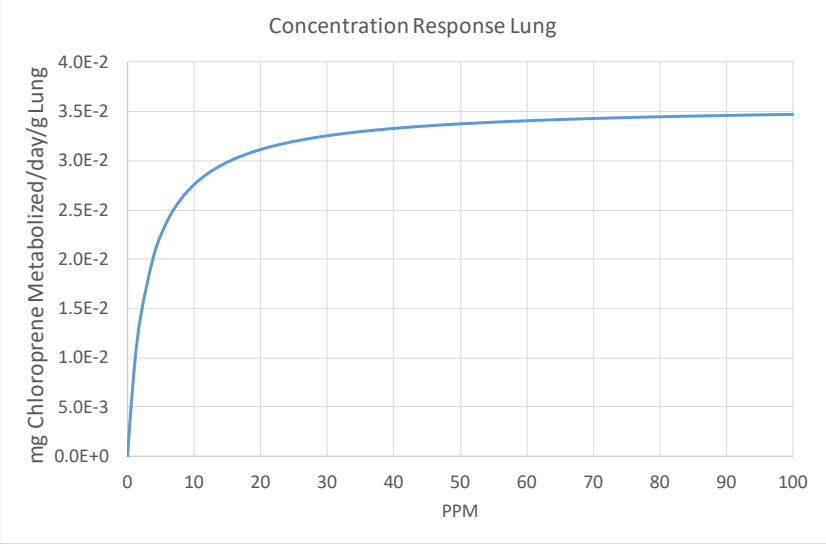

Figure 11. Inhaled concentration dependence of lung metabolism in the human for continuous exposures to chloroprene predicted with the PBPK model.

Interestingly, comparison of the Kms for chloroprene in liver and lung for male and female mice (Table S-3), which are based on the strongest data sets for estimating Kms, suggests that Km may be higher (lower affinity) in the mouse lung than in the mouse liver. This difference in apparent affinities in mouse liver and lung is consistent with differences in the relative tissue abundances of the murine CYP2E1 and CYP2F isozymes, both of which exhibit high affinities for chlorinated alkenes (Yoon et al. 2007). Whereas CYP2E1 is the predominant high affinity isozyme in the mouse liver, CYP2F is the predominant high affinity isozyme in the mouse lung (Yoon et al. 2007) and, consistent with the estimated Kms for chloroprene, the affinity of rCYP2E1 is roughly 3-fold higher (lower Km) than rCYP2F2 (Simmonds et al. 2004). However, since CYP2E1 is the predominant isozyme in both the lung and liver in the human (Nishimura et al. 2003), the estimation of human lung Km based on the human liver Km is appropriate.

It should be emphasized that the parameters in the chloroprene PBPK model represent estimates for an average mouse or human and this analysis does not address human inter-individual variability. The intention of the analysis conducted with the chloroprene PBPK model was to characterize the risk for an average individual. Previous evaluations of the impact of interindividual variability in pharmacokinetics on PBPK model-based risk estimates (Clewell and Andersen 1996) have suggested that the confidence interval for inter-individual variability in human internal dose is generally consistent with the default expectation of a factor of ten; that is, the ratio of a sensitive individual (95th percentile) to an average individual is on the order of a factor of 3. More recently, a MCMC evaluation of the variability in human risk estimates with the PBPK model for methylene chloride (David et al. 2006), which included consideration of a polymorphism for the metabolism of methylene chloride, found that the upper 95th percentile risk in the US population was within a factor of 3 of the mean risk estimate.

Selection of Dose Metric

The dose metric calculated with the PBPK model in this analysis is micromoles of chloroprene metabolized in the lung per gram lung per day (Himmelstein et al. 2004b; Yang et al. 2012). This dose metric was chosen because (1) the lung is the tissue with the highest tumor incidence in the chloroprene inhalation bioassays (NTP 1998) and (2) the carcinogenicity of chloroprene in rodents is believed to result from its metabolism to reactive epoxides in the target tissue (Himmelstein et al. 2004a, 2004b). The dose metric selected for chloroprene is consistent with the dose metrics used in previous PBPK-based risk assessments for both vinyl chloride (Clewell et al. 2001; USEPA 2000) and methylene chloride (Andersen et al. 1987; USEPA 2011), which were also based on the rate of production of reactive metabolites. The dose metric selected for the liver carcinogenicity of vinyl chloride was total mg vinyl chloride metabolized per kg liver per day, representing the production of the reactive chloroethylene epoxide. Due to the presence of chlorine in the epoxides generated from the metabolism of chloroprene, they are considered likely to have a reactivity comparable vinyl chloride (Haley 1978; Plugge and Jaeger 1979). The methylene chloride dose metric was average daily metabolism by the glutathione conjugation pathway in the lung per gram lung, which was selected based on evidence that the carcinogenicity of methylene chloride was associated with the local production of a reactive metabolite from the glutathione conjugate of methylene chloride. As with vinyl chloride and chloroprene, the assumption inherent in the dose metric was that the reactive metabolite would be completely consumed within the tissue where it was generated (Andersen et al. 1987).

Himmelstein et al. (2004b) have previously demonstrated that using the PBPK dose metric is able to harmonize the dose-responses for lung tumors in mice, rats and hamsters. However, they only had metabolism data for male animals. Figure 12 shows an update of the analysis from

Himmelstein et al. (2004b) that includes the results for the female mouse and rat. While the revised PBPK model is still able to demonstrate the consistency of the tumor incidence across male animals of different species and strains, female mice exhibit a higher tumor incidence than male mice at the same rate of lung metabolism.

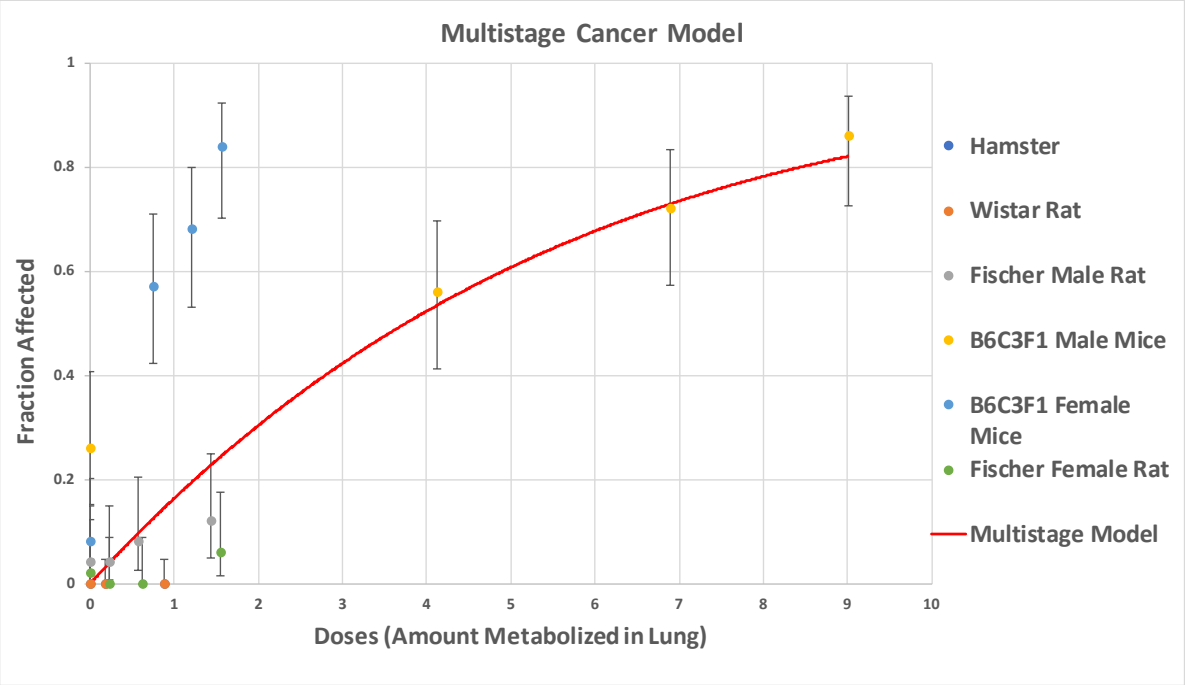

Figure 12. Comparison of Dose-Response for Lung Tumors in Chloroprene Bioassays in Rodents.

This discrepancy could indicate either of two possibilities: (1) the selected dose metric, rate of metabolism of chloroprene in the lung, is incorrect, or (2) the female mouse lung is more sensitive to the effects of chloroprene metabolites than the male mouse lung. Relatively few studies have been conducted to explore gender differences in the responses to chemical insult in the mouse lung. However, Yamada et al. (2017) provides evidence of a proliferative response of Club cells to the toxicity of permethrin in the female mouse lung that is not observed in the male mouse lung, and studies of naphthalene lung toxicity have demonstrated a greater sensitivity of the female mouse lung to both acute and repeated toxicity (Van Winkle et al. 2002, Sutherland et al. 2012). The greater susceptibility to a proliferative response to lung toxicity in the female mouse appears to result from gender differences in the tissue response to damage rather than metabolism (Laura Van Winkle, personal communication). A study of the genomic responses in the lungs of female mice and rats to inhaled chloroprene (Thomas et al. 2013) also demonstrated a greater pharmacodynamic sensitivity of the female mouse. In this study, female mice and rats were exposed for up to three weeks to inhaled chloroprene concentrations that were chosen to result in similar rates of epoxide production in the two species. The study found that while the most sensitive tissue responses occurred at similar values of the metabolism dose metric, transcriptional evidence of oxidative stress occurred at much lower concentrations in the female mouse. The more sensitive response of the female mouse to oxidative stress and to a proliferative response may underlie the apparent potency difference indicated by Figure 12. Using the metabolism dose metric appropriately considers the greater sensitivity of the female

mouse in a manner that is health protective, since the greater sensitivity of the female mouse results in a lower BMDL01 than would be obtained from the male mouse.

A steady-state analysis demonstrates that the chloroprene PBPK dose metric based on total metabolism per gram lung is conservative (i.e. a dose metric that also considered clearance would result in similar or lower risk estimates). The toxicity and associated carcinogenicity with chloroprene is believed to be related to the reactivity of the epoxides, 1-CEO and 2-CEO, that are formed by its metabolism (Himmelstein et al. 2004a). The use of a chloroprene PBPK dose metric that is based on total metabolism per gram lung represents a measure of the production of these metabolites but does not reflect any species differences that might exist in their clearance. However, due to the expectation that the rearrangement of 1-CEO and 2-CEO to reactive aldehydes is spontaneous and not enzymatically catalyzed, and that the reaction of the epoxides with glutathione is primarily related to direct GSH conjugation rather than enzymatic conjugation via glutathione transferases, clearance by these pathways would be expected to be identical across species, as was the case for methylene chloride, where the clearance of the chloromethyl glutathione metabolite was non-enzymatic and rapid (Andersen et al. 1987). The only other clearance pathway, enzymatic hydrolysis of chloroprene by epoxide hydrolase, has been shown to be slower in mouse lung compared to human lung in the case of 1-CEO (Himmelstein et al. 2004a), and it is reasonable to assume that relationship for 2-CEO, which is too reactive to measure *in vitro* (Himmelstein et al. 2004a), would be similar. Thus, the total clearance of both epoxides is expected to be similar or greater in the human compared to the mouse, and the use of the dose metric based solely on production would provide a health-conservative (similar or higher) estimate of human risk compared to a dose metric that also considered clearance.

The risk assessment for vinyl chloride (USEPA 2000) demonstrated that the use of a PBPK model to estimate target tissue dose (based on total metabolism per gram liver per day) was able to produce similar human risk estimates using data from animal bioassays and human occupational exposures. As a similar test of the chloroprene PBPK model to support cross-species extrapolation, Allen et al. (2014) used a statistical maximum likelihood approach to compare risk estimates obtained using external (air concentration) and internal (PBPK model estimated) metrics for the female mouse bioassay and human occupational exposures. The analysis concluded that if inhaled concentration was used as the dose metric, the estimates of human cancer risk using animal and human data were statistically significantly different, whereas using the PBPK metric consistent risk estimates were obtained across species. As with vinyl chloride, the use of the PBPK-based metric effectively reconciled the differences in mouse and human low-dose risk estimates.

Use of *In Vitro* Metabolism Data

The most notable aspect of the chloroprene PBPK model is that, apart from the physiological parameters, the parameters in the model are based on data derived solely from *in vitro* studies. The PBPK model for chloroprene is structurally similar to the PBPK model for methylene chloride (Andersen et al. 1987) and, just as in the case of the methylene chloride risk assessment, model predictions needed to support a risk assessment are critically dependent on parameters that can only be derived from *in vitro* metabolism experiments.

At the time the methylene chloride PBPK model was developed, the use of *in vitro* data to predict *in vivo* metabolism was a relatively new concept, but in the intervening years it has become common practice both for pharmaceuticals (Rostami-Hodjegan 2012) and environmental

chemicals (Yoon et al. 2012). While regulatory agency acceptance of PBPK models that are not based primarily on *in vivo* data still presents a challenge (EURL ECVAM 2017), "next generation" physiologically based modeling (NG PBK, Paini et al. 2019) has gained widespread acceptance for supporting regulatory decision making. In this regard, it is important to distinguish two forms of NG PBK: high-throughput IVIVE (HT-IVIVE) and chemical-specific PBPK/QIVIVE. In the HT-IVIVE methodology, a simplified generic pharmacokinetic model is applied across chemicals regardless of the potential impact of chemical-specific properties on the processes affecting their disposition and the nature of their metabolism. The simplified generic models used in HT-IVIVE necessarily ignore many factors that could be an important determinant of steady-state blood concentrations for a particular chemical, including incomplete absorption, pre-systemic intestinal metabolism, bypassing of hepatic pre-systemic metabolism by lymphatic uptake (in the case of lipophilic compounds), and active renal clearance or resorption. Due to the imprecision associated with this simplified generic approach (Wetmore et al. 2012; Wambaugh et al. 2015), HT-IVIVE is typically applied in screening approaches such as prioritization for further testing based on bioactivity concentrations from high-throughput testing. However, more exacting QIVIVE methods can be applied in chemical-specific PBPK modeling, and there are now many examples of published NG PBK models using these techniques to provide more accurate predictions *of in vivo* kinetics (Yoon et al. 2012; Paini et al. 2019). In the development of the chloroprene PBPK model, we have followed the PBPK/QIVIVE approach described in Yoon et al. (2012) and Paini et al. (2019). Going forward it will be important to develop a consensus on standard practices for IVIVE of metabolism in PBPK modeling in order to assist agencies in their evaluations.

Comparison of current MCMC analysis with analysis in Yang et al. (2012)

In their analysis of *in vitro* data on chloroprene metabolism, Yang et al. (2012) employed both a standard frequentist approach (referred to in their analysis as a "deterministic" approach) and an approach that used a Markov Chain Monte Carlo (MCMC) method (referred to as a "probabilistic" approach) with non-informative prior distributions for all estimated parameters. The use of non-informative priors allows this Bayesian approach to be interpreted from a frequentist perspective. As stated in the Yang et al. (2012) document, the two methods were compared to demonstrate that they provided consistent estimates of metabolic parameter values. Yang et al. (2012) then relied on the MCMC-based estimates for developing dose metrics for chloroprene exposures in mouse, rat and human. Because it seeks a global optimum using a probabilistic direct search algorithm, MCMC is less likely than deterministic search algorithms to converge on a local optimum. Moreover, when used with non-informative priors, as in Yang et al. (2012), the posterior distribution represents the likelihood distribution for the parameter, and the mode of the distribution represents the maximum likelihood estimate (MLE). As pointed out in Chiu et al. (2007), the Bayesian approach, in principle, yields a more global characterization of parameter uncertainty than the local, linearized variance estimates provided by traditional optimization routines, which should be viewed as lower bound estimates of true parameter uncertainty. Because of its superior properties, we have also relied on the MCMC approach in our re-analysis of the original *in vitro* metabolism data.

The key difference between the MCMC analysis performed in this study and the original analysis (Yang et al. 2012) was that this re-analysis included an additional parameter (Kgl) for the *in vitro* experiments, representing the potential for a mass transport limitation for uptake of chloroprene from the air in the metabolism vials. To evaluate the impact of our re-analysis of the *in vitro* metabolism data using Kgl on predicted risk estimates, the PBPK model was also used to calculate dose metrics using the previously published metabolism parameters from Yang et al.

(2012). Again, due to the high uncertainty of the human lung metabolism parameter, the approach using A1 from Andersen et al. (1987) was applied. The results with the two parameterizations are compared in Table 2. Using the new parameters estimated under the assumption of an air:liquid transport limitation in the *in vitro* studies, the mouse dose metrics increase by roughly 30-40% and the human dose metrics increase by roughly 40%, but the mouse/human ratios are similar, providing additional evidence of the robustness of the PBPK model.

Exposure	**Concentration**	**Dose Metric Yang et al. 2012 parameters**	**Dose Metric Re-estimated parameters**
Female mouse bioassay	12.8 ppm	0.75	1.00
	32 ppm	1.20	1.58
	80 ppm	1.57	2.15
Human continuous	1 μg/m^3	2.7x10^{-6}	3.36x10^{-6}

Table 2: Comparison of daily lung metabolism dose metrics in the mouse bioassay and for a human continuous exposure to 1 μg/m^3 chloroprene using either the parameters from Yang et al. (2012) or the newly estimated parameters in this study.

PBPK modeling has now been applied in risk assessments for a variety of environmental chemicals by regulatory agencies worldwide. The development of these models has typically required the use of *in vivo* experimental animal and/or human data to estimate key kinetic parameters such as uptake, metabolism and elimination. Some agencies also require the use of separate *in vivo* data to demonstrate model validity. However, it has become increasingly difficult to conduct controlled exposures of human subjects to chemicals of concern, other than for pharmaceuticals. The need for live animal studies is also being challenged, particularly in the EU, due to both ethical and practical (cost, throughput) concerns. Therefore, requirements for *in vivo* testing will increasingly limit the potential application of PBPK modeling in risk assessment, and agencies will need to consider whether *in vivo* validation data are truly necessary for assessing the fitness of a model for the specific purpose of its use in a particular risk assessment. To support these decisions, PBPK model evaluations should make greater use of uncertainty analyses to estimate the potential reduction in model uncertainty associated with the collection of additional data; that is, to determine the added value of a proposed study (Clewell et al. 2008; Keisler et al. 2013; Wilson 2015).

The original chloroprene PBPK model (Himmelstein et al. 2004b) was not used by USEPA (2010) because the agency considered it necessary to have blood or tissue time course concentration data from an *in vivo* study to adequately validate the model. The study reported here was conducted to address this requirement and we have now demonstrated that the chloroprene PBPK model accurately simulates these *in vivo* blood time course validation data.

No *in vivo* validation data for chloroprene are available in the human, and it is unlikely that such a study could be performed given the current classification of chloroprene as "likely to be a carcinogen" (USEPA 2010). However, the sensitivity analyses reported here suggest that such a study would not provide significant added value for demonstrating that the PBPK model is fit for

purpose for a chloroprene risk assessment. The validity of the model instead derives from the biological validity of the physiological and biochemical underpinnings of the model structure and parameters. The key parameters for performing a risk assessment for chloroprene are those for lung metabolism, and a human *in vivo* study would not be able to provide informative data for those parameters. As shown in Figure 8, blood concentrations of chloroprene associated with inhalation are insensitive to lung metabolism, and depend only on alveolar ventilation, cardiac output, blood:air partition coefficient and fractional blood flow to liver, which serves as the primary site of metabolic clearance.

The limited value of human *in vivo* data for determining whether a PBPK model is fit for purpose in a risk assessment based on target tissue metabolism was also an issue during the development of the PBPK model of methylene chloride (Andersen et al. 1987), where a similar dose metric was used: average daily metabolism of methylene chloride by glutathione transferase (GST) in the lung per gram lung. Although the model accurately reproduced blood and exhaled air concentration time-course data from multiple studies with human subjects, the *in vivo* data were not adequate to estimate the rates of GST metabolism in the liver and lung. Instead, it was necessary to estimate the rate of GST metabolism in the human liver by allometric scaling from animal data (Andersen et al. 1987), and to then estimate the rate of GST metabolism in the human lung using the ratio of specific activities for GST metabolism in liver and lung measured *in vitro* by Lorenz et al. (1984). This same approach was used in the chloroprene modeling documented in this report.

5. CONCLUSION

A PBPK model of chloroprene that relies solely on data from *in vitro* studies for its metabolism parameters accurately predicts the *in vivo* time course for chloroprene in the blood of female mice exposed by nose-only inhalation at the 3 concentrations used in the chloroprene 2-year cancer bioassay. This PBPK model has been used to estimate dose metrics for the metabolism of chloroprene to reactive epoxides in the lung target tissue of mice and humans to support an inhalation cancer risk assessment for chloroprene. Large differences between PBPK-based risk estimates and estimates based on inhaled concentration have been seen in previous inhalation risk assessments for chemicals where toxicity results from the production of reactive metabolites (Andersen et al. 1987; Clewell et al. 2001). The present PBPK model follows the same approach used in these previous PBPK models used in risk assessments by the USEPA and incorporates the best available science to describe the impact of species differences in metabolism on the potential cancer risk associated with chloroprene inhalation.

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Supplemental Materials

A. Supplemental Tables

B. Re-estimation of Metabolism Parameters

C. IVIVE Literature Review

D. Metabolism Parameter Calculations

E. PBPK Model Equations