Using the concordance of *in vitro* and *in vivo* data to evaluate extrapolation assumptions

Gregory S. Honda\(^{1,2}\), Robert G. Pearce\(^{1,2}\), Ly L. Pham\(^{1,2}\), R. W. Setzer\(^1\), Barbara A. Wetmore\(^3\), Nisha S. Sipes\(^4\), Jon Gilbert\(^5\), Briana Franz\(^6\), Russell S. Thomas\(^1\), John F. Wambaugh\(^*\)

\(^1\) National Center for Computational Toxicology, U.S. EPA, Research Triangle Park, North Carolina, United States of America, \(^2\) Oak Ridge Institute for Science and Education, Oak Ridge, Tennessee, United States of America, \(^3\) National Exposure Research Laboratory, U.S. EPA, Research Triangle Park, North Carolina, United States of America, \(^4\) Division of the National Toxicology Program, NIEHS, Research Triangle Park, North Carolina, United States of America, \(^5\) Cyprotex, Watertown, MA, United States of America

* wambaugh.john@epa.gov

Abstract

Linking *in vitro* bioactivity and *in vivo* toxicity on a dose basis enables the use of high-throughput *in vitro* assays as an alternative to traditional animal studies. In this study, we evaluated assumptions in the use of a high-throughput, physiologically based toxicokinetic (PBTK) model to relate *in vitro* bioactivity and *rat in vivo* toxicity data. The fraction unbound in plasma (\(f_{up}\)) and intrinsic hepatic clearance (\(Cl_{int}\)) were measured for rats (for 67 and 77 chemicals, respectively), combined with \(f_{up}\) and \(Cl_{int}\) literature data for 97 chemicals, and incorporated in the PBTK model. Of these chemicals, 84 had corresponding *in vitro* ToxCast bioactivity data and *in vivo* toxicity data. For each possible comparison of *in vitro* and *in vivo* endpoint, the concordance between the *in vivo* and *in vitro* data was evaluated by a regression analysis. For a base set of assumptions, the PBTK results were more frequently better associated than either the results from a “random” model parameterization or direct comparison of the “untransformed” values of AC\(_{50}\) and dose (performed best in 51%, 28%, and 21% of cases, respectively). We also investigated several assumptions in the application of PBTK for IVIVE, including clearance and internal dose selection. One of the better assumptions sets—restrictive clearance and comparing free *in vivo* venous plasma concentration with free *in vitro* concentration—outperformed the random and untransformed results in 71% of the *in vitro-in vivo* endpoint comparisons. These results demonstrate that applying PBTK improves our ability to observe the association between *in vitro* bioactivity and *in vivo* toxicity data in general. This suggests that potency values from *in vitro* screening should be transformed using *in vitro-in vivo* extrapolation (IVIVE) to build potentially better machine learning and other statistical models for predicting *in vivo* toxicity in humans.

Introduction

Relatively few chemicals in commercial use have been fully evaluated for hazard, in part due to the resource intensive nature of *in vivo* animal testing \([1–4]\). To address concerns over the
Cyprotex provided no independent funding in support of this work. Funding for G. S. Honda, R. Pearce, and L. Pham was supported by the Research Participation Program at the National Center for Computational Toxicology, U.S. Environmental Protection Agency, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U. S. Department of Energy and EPA. The National Institute of Environmental Health Sciences provided funding for N. S. Sipes. The specific roles of these authors are articulated in the ‘author contributions’ section.

Competing interests: B. Franz and J. Gilbert are employed by Cyprotex. This does not alter our adherence to PLOS ONE policies on sharing data and materials, per contractual agreements with the United States Environmental Protection Agency regarding data availability and transparency of publicly funded research.
	potential health effects of data-poor chemicals, new approach methodologies for chemical toxicity testing based on high-throughput in vitro and computational tools are being developed by researchers from government, industry, and academia [5]. High-throughput screening assays, such as those used in the Tox21 and ToxCast programs, provide in vitro bioactivity data that may inform the potential hazard of a chemical [6, 7]. To link in vitro assays with particular in vivo endpoints, statistical and machine learning models have been developed that select and weigh the potency and hit call data from relevant assays [8–10]. Using toxicokinetics (TK) may potentially improve performance of such models and elucidate the general correlation between in vitro bioactivity and in vivo toxicity data [11–13]. Since the probability of a biochemical interaction is proportional to the chemical concentration of ligand at the receptor [14, 15], the 2007 National Academies of Sciences, Engineering, and Mathematics report “Toxicity Testing in the 21st Century” proposed that dose-response modeling using physiologically-based TK (PBTK) models is needed to use high-throughput screening data to estimate chemical risk [16]. TK describes the mathematical relationship between external dose and internal concentrations, accounting for processes including absorption, distribution, metabolism, and excretion of a chemical [13]. Utilizing TK, an in vitro bioactive concentration that is suggestive of potential hazard can be extrapolated to an administered equivalent dose (AED) on a mg/kg body mass/day basis, allowing for subsequent comparison to estimated exposure rates [2, 5, 17–21].

Although TK is important for transforming in vitro bioactive concentrations to estimate in vivo doses, a large majority of chemicals are without TK data [19, 20], including hundreds of chemicals detected in many U.S. citizens by the Centers for Disease Control and Prevention National Health and Nutrition Examination survey [22]. To overcome this limitation, high-throughput methods have been developed that use in vitro approaches to determine important TK parameters such as plasma protein binding and first order hepatic clearance. In vitro TK parameters can then be scaled to in vivo situations in a process referred to as TK in vitro-to-in vivo extrapolation (IVIVE) [21, 23–26]. More broadly, IVIVE methods are critical in the interpretation of in vitro toxicity results and enable those results to be understood in the context of risk posed to human health [17, 19, 20]. Wetmore et al. 2013 evaluated the effect of including a TK model on the ability of a statistical classification analysis to predict in vivo results from in vitro toxicity data for the rat [27]. The authors measured in vitro rat intrinsic hepatic clearance (Clint) and fraction unbound in plasma (fu) for 59 chemicals and used in a TK model for IVIVE. A classical TK model was used to convert ToxCast assay AC50 (the concentration at 50% of maximum activity) values to AED for comparison to the lowest dose in which any effect was observed from traditional in vivo toxicity studies. In general, the in vitro assay with the lowest AED was less than the corresponding lowest dose in which any effect was observed for a given compound, demonstrating the potential of TK in estimating hazard. However, the performance of a statistical classification analysis was only slightly improved by incorporating TK. The authors suggested that this could have been due to assumptions in the application of the TK, the interpretation of the in vitro assay results, variability in the in vivo results, or other factors. Regarding the TK, the authors noted that the use of steady state values, assumption of restrictive clearance (i.e., hepatic clearance dependent on fu) [28], inability to characterize active renal transport, inaccurate bioavailability assumptions, and extrahepatic metabolism routes may have influenced results. Additionally, assuming a nominal AC50 concentration to be bioactive ignores the disposition of the chemical to in vitro assay components (e.g., cells, media, aqueous, plastic walls) [11, 29].

One critical assumption in the PBTK model is whether hepatic clearance is dependent or independent of fu (i.e., restrictive or nonrestrictive) [19]. Restrictive clearance assumes that bound chemical is not made readily available for metabolism and imparts a delay on chemical
clearance rates. In contrast, non-restrictive clearance assumes that the off-rates for the plasma protein-bound parent chemicals are sufficiently rapid to instantly replenish the pool of unbound parent in the plasma as needed. Regarding selection of predicted internal concentrations derived from in vivo bioassay doses, assumptions are made about which concentration determined from the PBTK model (plasma vs. tissue, mean vs. maximum, total vs. free) would be most comparable to the AC\textsubscript{50} \cite{11}. Assumptions must also be made regarding the chemical concentration (nominal vs. free) that is bioactive in the in vitro assay, and whether additional calculations for distribution of the chemical in the assay are necessary \cite{11}. Rather than use steady-state solutions from a classical 3-compartment model \cite{27}, a time-dependent, PBTK model allows for comparison of concentration from a tissue compartment corresponding to the cell type or target organ of an assay \cite{11, 13}.

In this current work, the ability of a high-throughput PBTK model to elucidate the general association between in vitro bioactivity and in vivo toxicity data is evaluated. Performance of the application of the PBTK model in this respect is determined relative to results from a randomization test (i.e., random parameterization of the model) and direct comparison of the untransformed values of dose and AC\textsubscript{50}. Additionally, the influence of some of the main assumptions in the application of the high-throughput PBTK model are investigated. New TK parameters of rat-based in vitro measured \textit{f}_\text{up} and \textit{Cl}_\text{int} are reported and were incorporated in the PBTK modeling. Evaluations were carried out for 84 chemicals having TK parameter data and corresponding in vitro bioactivity and in vivo toxicity data. The in vitro bioactivity data used were from 242 ToxCast assay endpoints having observed bioactivities for any of the corresponding chemicals. These were compared to in vivo data for rat in two separate analyses: 1) the "endpoint level" analysis used doses at which 68 specific pathologies were observed in ToxRefDB for different study types while 2) the "point of departure" (POD) level analysis used lowest observed effect level (LOEL) and lowest observed adverse effect level (LOAEL) values reported on the EPA’s CompTox dashboard \cite{30}. Regarding the latter dataset, the definition of POD in this work is meant for research purposes and suggests doses that might be benchmarks for the minimum effect in repeat dose toxicity studies.

To thoroughly evaluate the application of PBTK for IVIVE, the model was used for both forward and reverse dosimetry. In forward dosimetry, in vivo endpoint level doses for specific pathologies in rat and study level POD were converted to internal concentrations (e.g., mean plasma concentration) for comparison with in vitro AC\textsubscript{50} values. In reverse dosimetry \cite{13, 31}, AC\textsubscript{50} values were converted to AED values \cite{17, 32} for comparison with doses from the endpoint level and POD level data. While one might expect that the TK that works in the reverse application should perform similarly for the forward solution, the comparison of dose vs. AED (reverse dosimetry) is not equivalent to the comparison of AC\textsubscript{50} and predicted concentration (forward dosimetry) because the transformation between dose and concentration varies across chemicals and in vivo study treatment regimens (e.g., the number of doses). The performances of the reverse and forward solutions were evaluated by a series of orthogonal regressions of the standardized log\textsubscript{10} transforms of the variables. In general, applying the PBTK model elucidates the association between in vitro bioactivity and in vivo endpoint level toxicity data. Several assumption sets appeared to perform well, including the assumption set of restrictive clearance with the selection of in vivo mean free concentration in venous plasma and the in vitro free concentration predicted by an in vitro disposition model. Additionally, this latter assumption set demonstrated less bias with respect to input model parameters (e.g., \textit{f}_\text{up} and \textit{Cl}_\text{int}) than the biases exhibited by otherwise similarly performing assumptions sets.
Methods

In this work, the parameters $f_{up}$ and $C_{int}$ were measured in vitro for rat and incorporated in a PBTK model. These data were combined with previously published rat-specific in vitro TK data collected in the R package httk [23]. A PBTK model was used to evaluate two dosimetry approaches for comparing high throughput screening in vitro bioactivity and rat in vivo toxicity data. A reverse dosimetry approach transformed in vitro concentrations to predicted administered equivalent doses. Conversely, a forward dosimetry approach transformed in vivo doses to predicted plasma concentrations. We restricted our evaluation to chemicals for which effects were observed for both in vitro bioactivity and in vivo toxicity data. For each combination of in vitro bioactivity and in vivo endpoint a regression analysis [24, 33, 34] was used to evaluate the performance of the PBTK model relative to the untransformed values and randomized PBTK results. Results were summarized based on the count of the number of times the PBTK model performed better than both a randomized result (y-randomized TK parameters) and comparison of the untransformed values (in vitro AC$_{50}$ vs. in vivo dose). Processed data and models are provided in the R package httk [23] version 1.9 (https://cran.r-project.org/web/packages/httk/). All analyses were performed in R version 3.5.1. Input data and scripts for analysis are available in S1 File. A list of the abbreviations used in this work is included in S2 File.

New in vitro measured TK parameters

New In vitro TK parameters for rat of $f_{up}$ and $C_{int}$ measured in this work are reported in S1 Table; 67 chemicals had measurable values for $f_{up}$ and 77 chemicals had measurable values for $C_{int}$. Collectively, 65 chemicals had measured values for both $f_{up}$ and $C_{int}$. In general, experimental procedures similar to those previously described in [12, 17, 19, 20, 27] were employed for this work.

Chemical samples

Neat chemicals along with supporting certificates of analysis were obtained from commercial sources by the ToxCast chemical library management contractor (Evotec, San Francisco, CA). Test substances were provided to Cyprotex (Watertown, MA) by Evotec in vials as solids. The solids were dissolved in Dimethyl sulfoxide (DMSO) at 50 mM stock concentration and further diluted as necessary. During chemical concentration analytical method development attempts were made to identify the presence of the chemical peak and to assess the presence of any background peaks. Only chemicals with verifiable presence and minimal background were analyzed for protein binding and/or metabolic stability.

Chemical analysis

The in vitro methods for both $f_{up}$ and $C_{int}$ require the development of chemical-specific analysis methods to determine relative concentration. This requirement is in contrast to many assays used for high throughput screening in which a single reporter, such as bioluminescence, allows rapid analysis of the degree of perturbation across many chemicals [35]. The methods used here did not develop a calibration curve to quantitate the chemical but rather relied on a percent remaining approach (ratio of chemical peak areas).

For LC-MS/MS the signal is optimized for each compound by electrospray ionization positive or negative ionization mode. A full mass scan is used to optimize the fragmentation voltage and precursor ion m/z. A product ion analysis is used to identify the best fragment for analysis and the optimal collision energy. A test injection is then performed using a standard
C18 and/or HILIC column with a water/acetonitrile plus 0.1% formic acid gradient. Samples are analyzed by LC-MS/MS using an Agilent 6410 or AB Sciex 5500 mass spectrometer coupled with an Agilent HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent) or Analyst (AB Sciex). Instrumentation details and parameters for the chemical analysis are also provided in S2 Table and S3 Table.

For some chemicals, peaks could be identified in the hepatocyte incubation media, but could not be separated from the background caused by the presence of plasma protein in the assay for \( f_{up} \) or vice versa. This was possibly due to matrix effects (e.g., differential recovery from the plasma matrix compared to the hepatocyte media matrix) that impacted sensitivities in detecting chemicals. As such, \( f_{up} \) and \( Cl_{int} \) were not necessarily determinable for the same chemical.

### Plasma protein binding data analysis (\( f_{up} \))

The fraction of the chemical unbound in the presence of plasma protein (\( f_{up} \)) for rats was measured using rapid equilibrium dialysis (RED) [36] in which the free fraction of the chemical was calculated based on the measured chemical concentrations in two chambers separated by a dialysis membrane [17, 19, 27, 36]. Whenever \( f_{up} \) was below the limit of detection, a default value of 0.005 was assumed. This default value was estimated from half the minimum detectable level and previous experience with the RED assay [17]. New measured values for \( f_{up} \) for 67 chemicals are included in the \( htk \) R package version 1.9 and are available in S1 Table. For use in the models, a correction for non-specific binding in the RED assay was applied following previous methods [24].

Positive controls used in the RED assay were Warfarin (Sigma, Part A2250) and (±)-propranolol hydrochloride (Sigma, Part P0884). DMSO was obtained from Fisher Scientific (Part D128). Assay preparation used acetonitrile (Fisher Scientific, Part A955), water (Fisher Scientific, Part W6), methanol (Fisher Scientific, Part A45), Potassium phosphate buffer pH7.4 (Corning, Part 451201), hydrochloric acid 1N (HCl, Fisher Scientific, Part SA48), and sodium hydroxide (NaOH, Fisher Scientific, Part SS266). Rat plasma was purchased from BioIVT (formerly Bioreclamation; Westbury, NY). Approval by an Institutional Animal Care and Use Committee or equivalent animal ethics committee was not needed.

The plasma was thawed at 37°C using a water and pH adjusted to pH 7.4 ± 0.1 using a concentrated stock solution of either NaOH or HCl. This mixture was dialyzed in a RED Device (Pierce) per the manufacturers’ instructions against phosphate-buffered solution (PBS) and incubated on an orbital shaker. The test compound solutions were diluted into plasma, 5 \( \mu \)M final concentration, where the DMSO concentration did not exceed 1%. The assay was initiated with the addition of 50 µL PBS containing 1% DMSO in the white chamber and 300 µL of the chemical-spiked plasma in the red chamber of the RED device, samples were run in duplicate. The RED device plate was then sealed and incubated in a 37°C incubator with gentle rotating shaking for 4 hours.

The recovery determination plate, or \( T_0 \), was immediately prepared after the start of the 4-hour incubation. For recovery determination, 20 µL of the initial non-incubated plasma was transferred to a 96-well plate and mixed with 50 µL blank PBS containing 1% DMSO followed by the addition of 150 µL cold methanol containing internal standard (1 µM bucetin and 1 µM diclofenac). Blanks were prepared for background subtraction and run in duplicate and prepared by quenching 20 µL plasma containing 1% DMSO and 50 µL PBS containing 1% DMSO into 150 µL internal standard. The quench plate was kept on ice for 10 minutes then centrifuged at ~6000x relative centrifugal force at 4°C for 20 minutes.
After the 4-hour incubation, each well was mixed and 200 μL aliquoted to a transfer plate. Aliquots from both plasma and PBS sides are collected, an equal amount of PBS is added to the plasma sample, and an equal volume of plasma is added to the PBS sample. Methanol containing internal standard is added to precipitate the proteins and release the test agents. From the transfer plate 20 μL of each plasma sample and 50 μL of each PBS sample were aliquoted into a 96-well quench plate. The samples were matrix matched (i.e. 50 μL PBS containing 1% DMSO were mixed with plasma samples and 20 μL plasma containing 1% DMSO were mixed with PBS samples). The samples in the quench plate all received 150 μL of cold methanol containing internal standard. The quench plate was kept on ice for 10 minutes then centrifuged at ~6000x relative centrifugal force at 4°C for 20 minutes. The supernatant was then transferred to a liquid chromatography tandem mass spectrometry (LC-MS/MS) plate or gas chromatography-mass spectrometry (GC-MS) vial for analysis. Each assay was run with propranolol and warfarin as controls.

**Intrinsic hepatic clearance (Cl\textsubscript{int}) assay**

The *in vitro* intrinsic hepatic clearance (i.e., rate of first order metabolic clearance of the parent compound normalized to cell number) in primary rat hepatocytes was measured and analyzed similar to previously described methods \[19, 27\] at concentrations of 1 μM and 10 μM for each chemical. If the chemical was measurable but disappearance of parent compound was not apparent during the two hours over which the assay was performed, then the clearance was assumed to be zero. For the subsequent calculations the clearance at 1 μM was used, if measured successfully, otherwise the clearance at 10 μM was used. New measured values of rat Cl\textsubscript{int} for 77 chemicals are reported in the *httk* R package version 1.9 and are available in S1 Table. *In vivo* clearance is estimated from the *in vitro* measured Cl\textsubscript{int} following methods in Wambaugh et al. \[26\], including division by the estimated unbound fraction in the *in vitro* clearance assay \[37\], correction using a well-stirred model \[38\], and scaling by the number of cells in the liver.

For the Cl\textsubscript{int} assay, reference chemicals used were (+)-verapamil hydrochloride (Sigma, Part V4629) and Warfarin. Pooled male cryopreserved rat hepatocytes were purchased from BioIVT. Characterization of hepatocytes, including enzyme expression levels, was performed by the vendor (BioIVT). 500 mL of William’s E medium, 5 mL of 200 mM L Glutamine (final concentration 2 mM) and 3 g of HEPES (final concentration 25 mM) were added to the hepatocytes. The media was mixed by inversion, warmed to 37°C, and adjusted to pH 7.4 before each use. The cells were thawed, viable cells counted, and then equilibrated according to the supplier’s directions. Pooled male cryopreserved rat hepatocytes were added to an incubation plate and kept at 37°C in the incubator. After 30 min equilibration with gentle agitation, 250 μL of test compound solutions were aliquoted in triplicate into wells containing the cells to give the desired final concentration of 1 or 10 μM. The cell suspension was incubated at 37°C as above. At 15, 30, 60 and 120 minutes, 100 μL aliquots from the incubation plate were removed and precipitated into a quench plate containing an equal volume of cold methanol containing internal standard (1 μM bucatin, 1 μM propranolol, and 1 μM diclofenac). The 0 minute sample was prepared from aliquoting 50 μL hepatocytes into a quench plate containing 100 μL internal standard followed by the addition of 50 μL from the compound plate. Blanks were prepared for background subtraction and run in duplicate and prepared by quenching 50 μL hepatocytes and 50 μL media into 100 μL internal standard. The quench plates were kept on ice for 10 minutes then centrifuged at ~6000x relative centrifugal force at 4°C for 20 minutes. The supernatant was then transferred to a LC-MS/MS plate or GCMS vial for analysis. Each assay was run with verapamil, midazolam, and 7-OH 4-trifluoromethyl coumarin as controls.
Literature data on in vitro TK

The prior version 1.8 of the R package htk included literature data on rat-specific measurements of $f_{up}$ and $Cl_{int}$ for 97 chemicals. For $f_{up}$ these data were composed of 58 chemicals from Wetmore et al. 2013 [27], 19 chemicals from Wood et al. 2017 [39], 13 chemicals from Pearce et al. 2017 [23], and 7 chemicals from Naritomi et al. 2003 [40]. For $Cl_{int}$ these data were composed of 59 chemicals from Wetmore et al. 2013 [27], 35 chemicals from Wood et al. 2017 [39], and 3 chemicals from Naritomi et al. 2003 [40].

Toxicity data

Two sets of in vivo data were analyzed in this work: one set for an “endpoint level” analysis and another for a “POD level” analysis. For the analysis of in vivo endpoint level data, doses corresponding to observation of specific pathology endpoints were determined for each chemical, and study types were analyzed separately. For the analysis of POD level data, minimum doses were determined for each chemical from combined a dataset of LOEL and LOAEL values determined across studies and study types. While allowing for simultaneous comparisons for a larger number of chemicals, a potential lack of concordance between doses across study types may be expected in the POD analysis. This serves as a point of comparison for the endpoint level analysis. Data for in vivo doses used in the endpoint level analysis and the POD level analysis are available in S4 Table and S5 Table, respectively.

In vivo endpoint level data

In vivo data for rat were accessed from the Toxicity Reference (ToxRef) database [41–44] version 1. Much of the data in ToxRefDB v1 was derived from studies or study summaries for study designs compliant with or similar to the EPA OCSPP 870 series guidelines [45]. ToxRefDB v1 is a “positives-only” database, and in vivo data were reported as the nominal dose at which an effect (not necessarily critical) was observed for a particular endpoint (e.g., nonneoplastic liver change, change in body weight gain, etc.), along with additional information including the chemical used and study descriptors. The analysis in this work included chronic (2 year), subchronic (90 day), and developmental (parental and fetal generations) study types, which were analyzed separately. Multigenerational studies were not evaluated as they were not directly amenable to treatment with the PBTK model as the time units were given in generations. Entries with rat in vivo data were selected for analysis. In vivo endpoints were defined for each response pathology, as described by each unique combination of study type, effect-category, effect-type, and effect-target. For a given combination of study, chemical, and response endpoint, the lowest dose at which a response was observed was taken as the endpoint level dose. In this respect, the analysis was restricted to positive effects and multiple endpoints from a given study were represented for a given chemical. The doses may therefore differ from LOEL and LOAEL values taken across the study and should not be considered study-level LOEL or LOAELs as they are instead endpoint specific. Also, some chemicals had results from multiple studies. Both sexes and all rat strains were used. The list of in vivo endpoints in the analysis are reported in S6 Table.

In vivo POD level data

In contrast to the endpoint specific in vivo data, we also evaluated POD level data using LOEL and LOAEL maintained on the CompTox Chemicals dashboard (https://comptox.epa.gov/dashboard). A table of the values used in this analysis (accessed March 2018) is provided in S5 Table. Rat data were selected, entries without usable dose units (mg/kg/day) or duration of
study were filtered out, and the study types included in the evaluation were subacute, subchronic, and chronic. Evaluation was further restricted to those doses that were orally administered and had non-zero values. For each chemical, the minimum doses from a combined set of LOEL and LOAEL values across the available data was determined and used in subsequent POD level analyses. Although using data across study type may allow for comparisons made with a larger number of chemicals, data taken across study types and pathologies may have a higher variance.

**In vitro bioactivity data**

The Toxicity Forecaster (“ToxCast”) project consists of a suite of *in vitro* high throughput screening assays conducted using a variety of different technologies and preparations [46]. ToxCast assays include both biochemical and cell-based assays. These assays are primarily focused on human toxicity and are generally derived from human proteins and tissues. Assays are conducted in concentration-response format and are analyzed to determine if a concentration-dependent response is observed. These responses are modeled by either a Hill function or a Gain-Loss model, which can be summarized by the concentration required for 50% activation (AC$_{50}$). *In vitro* data were accessed from the ToxCast summary files from October 2015 [47]. The AC$_{50}$ value (μM) for each assay component endpoint was used for the evaluations in this study. We restricted our analysis in this work to *in vitro* bioactivity data with positive results (i.e., positive hit-calls) and filtered by disregarding positive hit-calls with any curve-fitting flags. *In vitro* bioactivity data are available in the S1 File.

**Analysis**

An overview of the analysis workflow we used is shown in Fig 1. This workflow applies to both the endpoint level data and POD level data. Scripts to reproduce the analysis are available in the S1 File. As a first step, data for *in vitro* bioactivity and *in vivo* toxicity were merged by chemical. In the endpoint level analysis (for which doses correspond to observed effects for specific pathologies in rat), the number of unique chemicals with values for both dose and AC$_{50}$ were counted for each combination of *in vivo* endpoint and *in vitro* assay. Only those *in vitro-in vivo* comparisons with at least 5 chemicals were included in the subsequent analyses.

In the POD level analysis (for which doses correspond to minimum values by chemical from combined data for LOEL and LOAEL taken across pathologies and study types), *in vitro* assays in the merged data set having AC$_{50}$ values and corresponding *in vivo* POD doses for at least 5 chemicals were included in subsequent analyses.

**Randomization test**

The resultant merged data sets each contained a vector of chemicals having a particular distribution. In addition to comparing the results of IVIVE using the PBTK model relative to untransformed values of dose and AC$_{50}$, we also compared performance of the PBTK model relative to randomized results. If the performance of the random results were similar to the PBTK result, it would suggest that differences between the performance of the PBTK result and untransformed values were due to the mechanics of the model or analysis, rather than chemical specificity. In each of the two separate analyses of *in vivo* data (endpoint and POD level data), the vector of chemicals from the combined data set was permuted ten times with resampling (Fig 1, step 2). Subsequently, these ten vectors of randomly permuted chemicals were used to produce ten sets of “randomized” results. Effectively, each randomized result was determined by parameterizing the PBTK model and accessory calculations based on the corresponding random chemical.
Fig 1. Diagram for the analysis workflow. $\kappa$ is the ratio of internal concentration (\(\mu M\)) to dose (mg/kg/day) determined using the PBTK model for an external dose of 1 mg/kg/day. ORMSE is the orthogonal root mean square error. Example chemicals are denoted by Ex1, etc. to enable demonstration of data mergers.

https://doi.org/10.1371/journal.pone.0217564.g001
PBTK model

A diagram of the PBTK model used in this work is shown in Fig 2. The model allows for doses to be absorbed through the gut or injected intravenously, although analysis was restricted to oral doses in this work. The fraction absorbed was assumed to be 100%. We accounted for organ and tissue partitioning in the PBTK model following the methods described by Pearce et al. [24]. When experimental values for log P (EPI Suite, [48]) were unavailable, predicted values from the OPERA model [49] were used. pKa values were from the work by Strope, et al. [50] or predictions from ChemAxon (Budapest, Hungary). The time course internal concentration of the PBTK model was evaluated using the EPA’s hHtk R package.

For a given set of assumptions, a dose of 1 mg/kg/day administered once a day was used to determine in vivo concentration-to-dose ratios ($\kappa$) by the application of the PBTK model and any accessory calculations with respect to the dose and study length. $\kappa$, effectively the ratio of the concentration (μM) at 1 mg/kg/day to the dose of 1 mg/kg/day, is determined from the PBTK model and any additional calculations (e.g., concentration selection, in vitro disposition). As such, $\kappa$ is a function (generically denoted by $f$) of the chemical, time, assumptions, and internal concentration selection (Eq 1). Concentrations ($C$) produced by the PBTK model are proportional to dose (Eq 2); this holds for concentrations at a given point in time, linear means, maxima, and steady state concentration. This characteristic allows for simple application of reverse dosimetry, where in vitro concentrations (i.e., $AC_{50}$) are converted to administered equivalent doses (AED) by dividing the concentration by the corresponding concentration determined for a dose of 1 mg/kg/day (Eq 3) [12, 17, 19–21, 27]. In previous work, this type of conversion was applied for steady state concentrations [20]. The same method is applicable to the transient solution, but with the requirement that the dosing regimen (study length and doses per day) are model inputs [23, 26].

\[
\kappa = f(1 \text{ mg/kg/day}, \text{ chemical, time, assumptions, dose selection})
\]

\[
C = \text{dose} \times \kappa
\]

\[
\text{AED} = \frac{AC_{50}}{\kappa}
\]

In the analysis workflow (Fig 1, step 4), values for $\kappa$ determined for different study lengths and chemicals were then merged with the data set by matching chemical and study length to produce the vector of $\kappa_{PBTK}$. Similarly, ten separate vectors of random results ($\kappa_{rand,i} = 1:10$) were produced by assigning $\kappa$ values corresponding to the permuted chemicals and study lengths (Fig 1, table iii). It is important to note that only the TK and physicochemical parameters are randomized in this analysis, the initial inputs (dose and study length; $AC_{50}$) remain specific to the original vector of chemicals. Predicted values for AED and internal concentration were then determined following Eqs 2 and 3. Various combinations of the assumptions in the application of the PBTK model for IVIVE were evaluated, each producing a unique vector of $\kappa_{PBTK}$ and 10 unique vectors of $\kappa_{rand,i}$.

Forward and reverse dosimetry

The models employed in this work are phenomenological in that they are structured to represent the physical processes that have been observed experimentally. As such, if internal concentration (determined from external dose) were associated with $AC_{50}$, then AED (determined from $AC_{50}$) may be expected to be similarly associated to external dose. To evaluate this, we performed two separate comparisons (Fig 1, step 5a-b):
a. Forward dosimetry: Evaluate the strength of the association between in vitro AC$_{50}$ and predicted in vivo concentration (C$_{PBTK}$) corresponding to dose as determined following Eq 2. The association between C$_{PBTK}$ and the in vitro AC$_{50}$ was compared with the association between in vitro AC$_{50}$ and the non-PBTK adjusted external dose values. To evaluate performance relative to chance, the randomized results (C$_{rand, i = 1:10}$) were predicted ten times using TK for permuted (random) chemical identifiers and the associations between in vitro AC$_{50}$ and each C$_{rand, i = 1:10}$ were determined.

b. Reverse dosimetry: Evaluate the strength of the association between in vivo dose and predicted external dose (AED$_{PBTK}$) determined following Eq 3. The association between these doses was compared to the strength of the association between the non-PBTK adjusted in vitro AC$_{50}$ and dose. Again, to evaluate performance relative to chance randomized TK result (AED$_{rand, i = 1:10}$) were calculated using the permuted chemical identities.
Model selection

For each comparison of in vitro assay and in vivo endpoint, a series of univariate, orthogonal regressions were performed for the standardized log_{10} transforms of the variables (Fig 1, step 6). First, all values (dose, $AC_{50}$, $C_{PBTK}$, $C_{rand,i}$, AED$_{PBTK}$, AED$_{rand,i}$) were log_{10} transformed. Values were then standardized by subtraction of the mean and division of the standard deviation for the respective variable following Eqs 4 and 5. In this subsection, generic variables ($y$, $x$, $\varphi$, and $\theta$) are used to describe calculations. For forward dosimetry, the $y$-variable is log$_{10}$ in vitro $AC_{50}$ and the $x$-variables are log$_{10}$ transforms of $C_{PBTK}$, $C_{rand,i}$ and in vivo external dose. For reverse dosimetry, the $y$-variable is log$_{10}$ in vivo external dose and the $x$-variables are log$_{10}$ transforms of AED$_{PBTK}$, AED$_{rand,i}$ and in vitro $AC_{50}$.

$$\phi_i = \frac{y_i - \bar{y}}{\sigma_y}$$

$$\theta_i = \frac{x_i - \bar{x}}{\sigma_x}$$

The statistic used as the basis for the comparisons made in this work is the root mean square error based on the orthogonal distance between a point and the regression line. Both standardized variables are centered on zero and have a variance of 1, subsequently the orthogonal regression line is either $\varphi = \theta$ or $\varphi = -\theta$. In either case, we can define a root mean square error based on the orthogonal distance between a point and the $\varphi = \theta$ line, which we refer to as the orthogonal root mean square error (ORMSE):

$$ORMSE = \sqrt{\frac{1}{n} \sum_{i=1}^{n} ((\phi_i - \theta_i)/\sqrt{2})^2}$$

Effectively, the ORMSE serves as a representation of the association between the two variables (i.e., a lower ORMSE indicates a stronger, more quantifiably definable relationship). For forward dosimetry, ORMSEs were determined between in vitro $AC_{50}$ and $C_{PBTK}$, between $AC_{50}$ and each of the ten sets of $C_{rand,i}$ and between in vitro $AC_{50}$ and external dose. For reverse dosimetry, ORMSEs were determined between in vivo external dose and AED$_{PBTK}$ between external dose and each of the ten sets of AED$_{rand,i}$ and between external dose and in vitro $AC_{50}$. As noted previously, $C_{rand,i}$ and AED$_{rand,i}$ represent single sets of the ten random results determined by parameterizing the PBTK model and other calculations using random sampling from a distribution of chemicals.

Resulting ORMSE can be organized as in Fig 1, table iv. The ten separate sets of random results allow for ten comparisons to be made for each in vitro assay and in vivo endpoint comparison. Note that the ORMSE between $y =$ external dose and $x =$ $AC_{50}$ is equivalent to the ORMSE between $y =$ $AC_{50}$ and $x =$ external dose. The only values that change with evaluation subset for a given in vitro-in vivo endpoint comparison are the ORMSE for the randomized result. The forward and reverse dosimetry results were treated separately. For a given in vitro-in vivo endpoint comparison, ORMSE were compared for each evaluation subset, and “wins” were allocated to the predictor (PBTK, Rand., or Dose/$AC_{50}$) with the lowest ORMSE (Fig 1, step 7). The number of times a predictor had the smallest ORMSE were then counted (Fig 1, step 8) and summarized per evaluation subset (Fig 1, table v). The medians and standard deviations of the counts allocated to each predictor were then determined across the ten evaluation subsets, again treating forward and reverse dosimetry separately (Fig 1, step 9). This process (Fig 1, steps 3–9) was then repeated for every combination of assumption and concentration.
selection (internal concentration metric and in vitro distribution) of interest. An analogous methodology was carried out for the POD level analysis, noting that doses in that case were determined across in vivo response types.

Assumptions evaluated

In addition to understanding the overall impact of applying our high-throughput PBTK model for IVIVE, we also evaluated the influence of some of the main assumptions in the application of the high-throughput PBTK model. These include restrictive vs. nonrestrictive hepatic clearance, selection of concentration from the PBTK model, and application of the Armitage model [51] for distribution of chemicals in vitro. A list of the evaluated assumptions is included in S3 File.

Restrictive vs nonrestrictive hepatic clearance

Hepatic clearance is influenced by the effective rate of desorption of a chemical from the plasma protein to which it is bound [52]. When this rate is relatively slow, the clearance is dependent on the fraction of unbound chemical in the plasma \( f_{up} \) and therefore considered restrictive. When the desorption rate is fast, the clearance is independent of \( f_{up} \) and termed nonrestrictive. In the PBTK model, restrictive clearance may be accounted for by multiplying the intrinsic hepatic clearance, \( Cl_{int} \), by \( f_{up} \). For limited sets of chemicals with known in vivo clearance, assuming nonrestrictive clearance may potentially provide more accurate results, although certain chemicals (e.g., warfarin) are known to be metabolized under restrictive clearance [19, 52].

Selection of concentration from the PBTK model

The PBTK model produces concentration vs. time profiles for each tissue/compartment in the model. Different concentrations taken at different times may either be more or less indicative of the conditions that lead to an observed in vivo response [28] and either more or less relatable to an in vitro \( AC_{50} \) value. Mean and maximum concentrations were extracted from the solution of the PBTK model for the venous plasma concentration and the tissue plasma concentration from each tissue compartment in the model. The mean concentration is equivalent to the area under the concentration vs. time curve divided by the time-length of the dosing. When comparing predicted in vivo concentration (e.g., in plasma) with in vitro \( AC_{50} \), it may also be preferable to compare concentrations from similar compartments. \( AC_{50} \) may either be 1) assumed to be a nominal concentration directly comparable to \( C_{PBTK} \), or 2) assumed to be similar to a free concentration so that \( C_{PBTK} \) should be converted to a free concentration by multiplying \( C_{PBTK} \) by \( f_{up} \). Furthermore, in cases where a model of the distribution of chemical in vitro is incorporated in the interpretation of in vitro results, it may be that the in vitro free concentration should be compared to the predicted in vivo unbound concentration, \( f_{up} \cdot C_{PBTK} \).

Distribution in in vitro cell-based assays

The distribution of a chemical in an in vitro environment results in different concentrations for different assay compartments (cells, water, lipids, proteins). The concentration in a particular assay compartment may be better associated with bioactivity in vivo than the nominal concentration (total amount per total well volume). This is particularly true for cell-based assays where there is more material (cells, serum) available for chemical binding. For in vitro cell-based assays, we evaluated an updated version of the in vitro distribution model by Armitage et al. 2014 [51]. Updates include absorption to the walls, distinction between storage and
membrane lipids in the cells, and changes in methods to estimate membrane concentrations. Assay well geometry, media volume, and cell volume were taken from values reported by Corning [53] that correspond to the assay footprint (i.e., well number). Other descriptors of assay components, including fetal bovine serum (FBS) and cell content (lipid, protein, and water fractions), are taken as averages of those values reported by Armitage. The values for water solubility and Henry’s law constant used were from experimental values in EPI Suite [48] if available or predictions from OPERA [49]. The aqueous phase concentration estimated from the Armitage model was used for subsequent comparisons. For a given in vitro-in vivo comparison, corresponding aqueous phase concentrations were calculated for each in vitro result. A factor was then defined by the aqueous concentration divided by the nominal AC₅₀. The result for including the in vitro distribution model was then given by the concentration from the PBTK model divided by the factor, thereby producing a unique k_PBTK for the given set of assumptions.

Results

Since the propensity for bioactivity is proportional to chemical concentration, TK frames the dose-response relationship and associated hazard characterization by linking external exposures (theoretical or relevant, depending on the scenario) to resultant internal concentrations. This work evaluates varying sets of IVIVE assumptions by examining the impact of a PBTK model on the association between in vitro bioactivity and in vivo toxicity data. In vitro HTTK parameters of f_{up} and C_{int} were measured for 65 new chemicals and analyzed jointly with data from the literature for 97 chemicals. For the data considered, TK parameters, in vitro AC₅₀, and in vivo doses were simultaneously available for 84 chemicals. Forward and reverse dosimetry results were determined using the PBTK model for each set of assumptions, and comparisons were made using a regression analysis. Two different in vivo data sets were used for the basis of this analysis: the endpoint level data containing 80 chemicals with doses corresponding to observed responses for 106 specific endpoints (68 pathological responses and 3 study types), and the POD level data containing 84 chemicals where effects had been aggregated into a single point of departure per chemical (the minimum LOEL-LOAEL taken across all available pathologies, studies, and study types). For the endpoint level data, different in vitro assay-in vivo endpoint combinations had different numbers of chemicals that were both active in vitro and that had a particular in vivo endpoint observed; 2787 endpoint combinations had at least 5 chemicals, while 48 combinations had at least 20. For the comparisons made between the POD level data and in vitro assay endpoints, there were 69 comparisons with at least 5 chemicals that were active in vitro and in vivo, while 17 comparisons had at least 20 chemicals.

For each chemical, time dependent mean and maxima plasma and tissue concentrations were determined by the PBTK model for a dose of 1 mg/kg/day for various study lengths. Model linearity of the ratio of internal concentration to external dose for a given time-point allowed for extrapolation to various dosing scenarios and evaluation of forward and reverse dosimetry. Comparisons were made based on the ORMSE of the standardized log₁₀ transforms of the variables; depending on the direction of the dosimetry, AC₅₀ (forward) or dose (reverse) was compared vs the PBTK result (internal concentration or AED) relative to a randomized result (internal concentration or AED) and the untransformed value (dose or AC₅₀). While other parameters could have been selected, ORMSE was deemed appropriate as a basis for the broad evaluation in this work. Results were compiled by the number of times a variable (PBTK, random, or untransformed) had the lowest ORMSE from each in vitro assay and in vivo endpoint comparison.
Example regression

To illustrate features of the regressions, an example analysis is demonstrated assuming nonrestrictive hepatic clearance in the PBTK model, as well as using the total mean venous plasma concentration and the nominal AC_{50}. The results from the Attagene assay endpoint, ATG_PX_RE_CIS_up, characterizing the regulation of the pregnane X receptor transcription factor response element in HepG2 cells [54], were compared with the endpoint level in vivo data from chronic studies for systemic, non-neoplastic, liver pathology. Outside of this example, each in vivo endpoint was compared with every in vitro assay endpoint for the complete analysis; more than 750,000 regressions were made in total across all the analyses. For the example case, regressions of the standardized, log_{10} transformed variables are shown in Fig 3 for forward dosimetry (a-c) and reverse dosimetry (d-f). A lower ORMSE indicates a stronger association. The lowest ORMSE in the forward solution comparison is achieved using the PBTK model (Fig 3A). However, for the AED comparison, the lowest ORMSE is for random (Fig 3E). As expected, the ORMSE is the same for AC_{50} vs dose in the forward dosimetry comparison and dose vs AC_{50} in the reverse dosimetry comparison (Fig 3C and 3F); in these latter two comparisons, the points are mirrored around the y = x line, so the orthogonal distance to y = x does not change.

For the PBTK and random results, the difference between the forward dosimetry and reverse dosimetry results is not a simple switching of the axis. Rather, the comparisons of AC_{50} vs concentration are distinct from those for dose vs AED. In Fig 3A (AC_{50} vs C_{PBTK}), there is a possible outlier near x = -4 and y = -2 (all variables were standardized and log_{10} transformed, as described in Methods); in the corresponding reverse dosimetry plot (Fig 3D), this is the point at y = -4 and x = 2. If this point were omitted, the trends with respect to ORMSE would be similar for the forward and reverse comparisons. However, this would be a subjective alteration; instead our interest is in evaluating the effect of TK in general across comparisons of a large range of assay endpoints and in vivo effects. A broad comparison among various in vitro and in vivo data should mitigate the influence of any outliers; the example regression shows results for only one of the comparisons that were made, and only one of the ten sets of random results.

Fig 4 shows the distribution of ORMSE for the endpoint level analysis with the assumptions of nonrestrictive clearance and mean total plasma concentration across all comparisons of in vitro assay and in vivo endpoint. For demonstrative purposes, the random results in this case are median values from the 10 sets of results. Results are restricted to those comparisons having at least 5 different chemicals. The distributions for the forward and reverse solutions are identical for dose (forward solution comparison) or AC_{50} (reverse solution comparison). Results are also similar between the forward and reverse solutions for the random result, but differences are apparent between forward and reverse dosing for the PBTK result and are particularly noticeable for those comparisons with greater than 20 chemicals. In that case, the reverse dosimetry result (median ORMSE: 0.87) is shifted towards slightly higher values of ORMSE than the forward dosimetry result (median ORMSE: 0.80). Relative to the random result (median ORMSE: 0.91 for forward and reverse, ≥ 20 chemicals), the PBTK result is slightly lower in either case. The PBTK result is also slightly lower than the comparison of untransformed dose and AC_{50} (median ORMSE: 0.90, ≥ 20 chemicals). Similar trends are observed for the corresponding POD level analysis across all the assays in Fig 5. For comparison based on at least 20 chemicals, the reverse result again appears shifted towards higher values of ORMSE (median: 0.94) than the forward solution (median ORMSE: 0.88). The example analysis for the assumption set of nonrestrictive clearance with mean total plasma concentration suggests differences in the results for forward dosimetry and reverse dosimetry. To
Evaluating results across all assumption sets

For the endpoint level data, ORMSE values were determined for the forward and reverse dosimetry comparisons for each *in vitro* assay endpoint and *in vivo* endpoint. For the POD level data, ORMSE were determined for each assay endpoint. The number of times a variable yields the lowest ORMSE were counted for the forward and reverse dosimetry results using both the endpoint level *in vivo* data and POD level *in vivo* data following the methods outlined in Fig 1, step 7–8. Following those methods for the example results in Fig 3, in the forward dosimetry comparison a win would be counted for the PBTK result while, in a separate set of counts for reverse dosimetry, a win would be counted for AC$_{50}$. Fig 3 included values for 1 of the 10 sets of random results, wherein the TK were randomized. In the following results, comparisons were made for each separate set of random results, so that a total of 10 sets of comparisons were made. Medians and standard deviations were then determined from these counts.

The results of applying the analyses across all combinations of assumptions and concentration selection are shown in Figs 6 and 7 for comparisons based on at least 20 different chemicals. Figs 6 and 7 represent the results of more than 15,600 comparisons across assumptions, *in...
The assumptions evaluated include clearance (restrictive and nonrestrictive), internal concentration selection (mean or max; total venous plasma, free venous plasma, or tissue), and estimation of the free concentration in the in vitro bioactivity assay using the model by Armitage et al. [51]. Results for comparisons based on at least 5 different chemicals are available in S1 Fig and S2 Fig. In the endpoint level analysis, study types were evaluated separately and then the counts were compiled in Figs 6 and 7. A breakdown of the results for the endpoint level analysis by study type is available in S3 Fig, S4 Fig, S5 Fig, and S6 Fig, and these results exhibit similar trends. Across all comparisons, minimal differences were observed between selection of the mean or maximum in vivo concentration. As such, the results for use of the Armitage in vitro disposition model are only shown in combination with the mean venous plasma concentration.

For the forward dosimetry comparison (Fig 6), the endpoint level analysis suggests that in general, using the PBTK model for IVIVE better clarifies the association between in vitro bioactivity and in vivo toxicity data than either the random result and untransformed dose. Improvements in performance appear greater when nonrestrictive clearance is assumed, or when the free plasma concentration is used with restrictive clearance. Utilizing the Armitage model to estimate the free in vitro concentration performs well when combined with the selection of the in vivo mean free venous plasma concentration (res.-free-vein-mean-Armitage). Similar trends are observed in the POD level analysis, although the differences between counts are smaller. For the reverse dosimetry comparisons shown in Fig 7, the trends in the endpoint
level analysis differ from those observed for forward dosimetry. The counts for PBTK with restrictive clearance are slightly higher relative to those with nonrestrictive clearance, while the highest counts are achieved when comparisons are made using the in vivo mean free venous plasma concentration in combination with the in vitro free concentration predicted by the Armitage model (res.-free-vein-mean -Armitage). For the reverse dosimetry POD level analysis, the random result produced higher counts for certain assumptions, but these exhibit large deviations.

In general, the PBTK results performed better for the endpoint level analyses than the POD level analyses. For the endpoint level analysis comparisons versus assay results were made for specific in vivo endpoints and study types, whereas for the POD level analysis doses were taken as the minimum across study types and pathology for a given chemical. It would be expected that trends may be stronger across a specific endpoint as opposed to comparisons across different pathologies and different study types, as in the POD level analyses. Separately from the determination of ORMSE, variances for log$_{10}$ transformed AC$_{50}$ as well as variances for log$_{10}$ transformed dose were determined for each comparison made in the two separate analyses for an arbitrary assumption set. Table 1 shows the medians of these variances determined for all comparisons of in vitro assay endpoint and in vivo endpoint (endpoint level analysis) and comparisons of in vitro assay endpoints to POD data (POD level analysis). While the variance in AC$_{50}$ was similar in either analysis, the variance in dose was larger for the POD level.

PBTK performs better than the randomized result and the untransformed result in general for the endpoint level analysis. However, given the limitations of the available chemical-
specific data, an optimal set of assumptions for IVIVE among those evaluated in Figs 6 and 7 cannot be clearly identified. The assumptions that appear to perform best include restrictive clearance with free mean or free max venous plasma concentration, nonrestrictive clearance with total mean or max tissue concentration, and restrictive free mean venous plasma concentration in vivo combined with estimation of the free concentration in vitro by the Armitage model. Prior work [27] has been based on only one set (httk v1.8) of these assumptions (restrictive clearance; total steady-state concentration which is analogous to the total mean

Fig 6. Allocated counts from the forward dosimetry method. Counts compare in vitro AC₅₀ with predicted in vivo concentration for the endpoint level analysis (top row) and POD level analysis (bottom row) as a function of the assumptions used in application of the PBTK model. Counts are from assay-effect pairs with at least 20 unique chemicals and are median values from the 10 sets of comparisons. The error bars are plus or minus two standard deviations from the 10 comparisons. Labels on the x-axis indicate assumption set: for clearance (res.–restrictive, nres.–nonrestrictive), in vivo concentration selection (tot.–total, free, vein, tis.–tissue, mean, max), and use of the Armitage disposition model to estimate the free concentration in vitro.

https://doi.org/10.1371/journal.pone.0217564.g006
Fig 7. Allocated counts from the reverse dosimetry method. Counts compare in vivo dose with predicted AED from in vitro toxicity assay results for the endpoint level analysis (top row) and POD level analysis (bottom row) as a function of the assumptions used in application of the PBTK model. Counts are from assay-effect pairs with at least 20 unique chemicals and are median values from the 10 sets of comparisons. The error bars are plus or minus two standard deviations from the 10 comparisons. Labels on the x-axis indicate assumption set: for clearance (res.–restrictive, nres.–nonrestrictive), in vivo concentration selection (tot.–total, free, vein, tis.–tissue, mean, max), and use of the Armitage disposition model to estimate the free concentration in vitro.

https://doi.org/10.1371/journal.pone.0217564.g007

Table 1. Median of variances for log_{10} transforms of AC_{50} and dose by analysis level.

| Analysis Level | AC_{50} | Ddose |
|----------------|---------|-------|
| Endpoint Level | 0.021   | 0.046 |
| POD Level      | 0.017   | 0.017 |

https://doi.org/10.1371/journal.pone.0217564.t001
concentration used here) which has been shown to be slightly inferior to other assumptions for forward-dosimetry in Fig 6 but more equivocal for reverse-dosimetry (Fig 7) and remains a plausible option. Using those set of conditions, Wetmore, et al demonstrated the potential to use TK for IVIVE to compare lowest in vivo doses with the lowest AED estimated from in vitro bioactivity data [27]. Both httk v1.8 [23] and Wetmore et al. [20] include a correction by Kilford et al. [37] for the fraction of chemical bound in the in vitro clearance assays [55]. Analogous comparisons produced in our current work are shown in Fig 8 for four of the assumption sets. The log<sub>10</sub> transforms of the lower 10<sup>th</sup> percentile dose from POD level in vivo data vs the 10<sup>th</sup> percentile AED using the PBTK model, determined with a time scale of the corresponding lowest dose. Each point corresponds to a particular chemical. Results are for the assumption set of total mean concentration, and are otherwise indicated by the panel labels: a) restrictive clearance with in vivo mean total venous plasma concentration, b) restrictive clearance with in vivo mean free venous plasma concentration, c) restrictive clearance with in vivo mean free venous plasma and free concentration in vitro predicted by the Armitage model, d) nonrestrictive clearance with mean tissue concentration. The dashed lines are y = x lines. Corresponding RMSE and ORMSE (the latter defined for the standardized variables) are also reported.

![Fig 8. Plots of the log<sub>10</sub> transforms of the 10<sup>th</sup> percentile dose from POD level in vivo data vs the 10<sup>th</sup> percentile AED using the PBTK model, determined with a time scale of the corresponding lowest dose. Each point corresponds to a particular chemical. Results are for the assumption set of total mean concentration, and are otherwise indicated by the panel labels: a) restrictive clearance with in vivo mean total venous plasma concentration, b) restrictive clearance with in vivo mean free venous plasma concentration, c) restrictive clearance with in vivo mean free venous plasma and free concentration in vitro predicted by the Armitage model, d) nonrestrictive clearance with mean tissue concentration. The dashed lines are y = x lines. Corresponding RMSE and ORMSE (the latter defined for the standardized variables) are also reported.](https://doi.org/10.1371/journal.pone.0217564.g008)

correspondence used here) which has been shown to be slightly inferior to other assumptions for forward-dosimetry in Fig 6 but more equivocal for reverse-dosimetry (Fig 7) and remains a plausible option. Using those set of conditions, Wetmore, et al demonstrated the potential to use TK for IVIVE to compare lowest in vivo doses with the lowest AED estimated from in vitro bioactivity data [27]. Both httk v1.8 [23] and Wetmore et al. [20] include a correction by Kilford et al. [37] for the fraction of chemical bound in the in vitro clearance assays [55]. Analogous comparisons produced in our current work are shown in Fig 8 for four of the assumption sets. The log<sub>10</sub> transforms of the lower 10<sup>th</sup> percentile dose from POD level in vivo data vs the 10<sup>th</sup> percentile AED using the PBTK model, determined with a time scale of the corresponding lowest dose. Each point corresponds to a particular chemical. Results are for the assumption set of total mean concentration, and are otherwise indicated by the panel labels: a) restrictive clearance with in vivo mean total venous plasma concentration, b) restrictive clearance with in vivo mean free venous plasma concentration, c) restrictive clearance with in vivo mean free venous plasma and free concentration in vitro predicted by the Armitage model, d) nonrestrictive clearance with mean tissue concentration. The dashed lines are y = x lines. Corresponding RMSE and ORMSE (the latter defined for the standardized variables) are also reported.

Additional figures for the other assumption sets and mean concentration are available in S7 Fig. In this exercise, it would be desirable for dose and AED to be highly associated and for AED to be less than the corresponding dose, the argument being that the in vitro assays should be more sensitive to the initial perturbation that may cause an adverse effect. Using the in vivo mean free venous plasma concentration with the free in vitro concentration from the Armitage
model (Fig 8D) has a significantly lower RMSE. While this might be preferable in terms of predicting the mean, the restrictive clearance results using mean total plasma concentration (8a) are more conservative (i.e., they rarely underestimate the POD_10). Additionally, in this exercise Fig 8A has a slightly lower ORMSE, indicating a potentially stronger association despite a greater RMSE. Lower RMSE alone suggests improved accuracy of the mean, but in this case does not correspond with improvements in association.

Lastly, it is useful to understand if model performance depends on the type of chemical. As a simple evaluation, we can examine the association between physical chemical properties and residual error. Importantly, this also serves as an evaluation of model bias. The models in this work are based on input parameters including \( f_{up} \), the octanol-water partition coefficient (logP), the dissociation constant (logD), and molecular weight (MW). As a final test, we evaluate the association between these parameters and the residuals from the comparison of the log10 transforms of POD_10 vs AED_10 for each model. For this test, we use the absolute value of the Pearson’s correlation coefficient, |COR|, separately determined between the residuals and each parameter. Results are represented by the heatmap shown in Fig 9, where brighter colors correspond to higher values for |COR|. Under ideal circumstances, the model should accurately account for the relationship between the chemical, the parameter, and the outcome (AED_10) such that |COR| should be low. However, Fig 9 shows that |COR| is high for certain combinations of model and input parameter. Comparisons relative to the 10th percentile log10 AC50 values are included for reference. For assumptions that performed poorly in previous tests, we generally see little correlation with the different input parameters. This is true for the base set of assumptions of restrictive clearance with mean total venous plasma concentration. However, the residuals for those models that performed well appear to exhibit some dependence on the input parameters. For the mean or max free in vivo plasma concentration with restrictive clearance, the residuals appear to be highly correlated with \( f_{up} \), logP, and logD. This suggests that the model using these assumptions may not fully account for the differences in binding between the in vitro and in vivo systems. However, using the Armitage model to estimate the free concentration in vitro has lower correlation between the residuals and the parameters of \( f_{up} \), logP, and logD. Using nonrestrictive clearance exhibits higher values for |COR|, particularly for \( Cl_{int} \) across the different combinations of assumptions. This holds for nonrestrictive clearance with mean or max tissue concentration.

**Discussion**

Understanding our ability to use in vitro and in silico methods to quantitatively predict known doses exhibiting pathological effects in vivo is a prerequisite to estimating toxic doses for chemicals without in vivo toxicological data. In this work, the application of a PBTK model to clarify the association between in vitro bioactivity and in vivo toxicity data [11, 13, 27] was evaluated across a broad range of chemicals, in vitro assays, in vivo endpoints, and modeling assumptions. Evaluations were carried out for two analysis levels: 1) each specific in vivo endpoint was compared with each in vitro high-throughput screening assay component endpoint and 2) POD determined across pathologies and study types were compared with each in vitro high-throughput screening assay component endpoint. For both analysis levels (endpoint level and POD level, respectively), results were compared for forward dosimetry and reverse dosimetry. In the endpoint level analysis, both the forward and reverse dosimetry results demonstrated an improved performance when using the PBTK model. This strongly suggests that applying toxicokinetic models elucidates the association between in vitro bioactivity and in vivo toxicity data, particularly when study type and specific effect are considered.
The analyses presented here were designed to clarify the association between in vitro bioactivity and in vivo toxicity data. Results are not meant to suggest prediction of a particular in vivo endpoint by a specific in vitro assay component endpoint. Rather, improved performance in general indicates that incorporating TK would be beneficial when extrapolating quantitative results from models that rely on an ensemble of assay results, such as the estrogen receptor pathway model of Judson et al. that used 18 different ToxCast assays [8]. Furthermore, in vitro bioactivity and in vivo toxicity data in this work were restricted to positive only results. While TK may suggest that an AED associates with an in vivo dose, TK will not convert a negative assay hit to a positive one or vice-versa. Although there was a general observation of improved performance for the PBTK model in the endpoint level analysis, there was dissimilarity in the trends observed with respect to the different assumption sets. There were also differences in the results between the endpoint level and POD level analyses. The ambiguity of the trends with respect to association (i.e., Figs 6 and 7) when examining various assumptions and dose metrics suggested the potential of a range of possible results in different applications of TK. In
In some cases, untransformed \textit{in vivo} dose was associated with \textit{in vitro} $AC_{50}$ (nonzero counts for dose and $AC_{50}$ in Figs 6 and 7). This may be a reflection of the fact that doses are often selected to reflect the amount of chemical that can be tolerated by the test animal [56]. Additionally, the relatively higher counts for random in the POD level analysis may be due to the selection of minimum AED. We did observe, however, that using TK yielded a stronger association more often than it did not. Finally, subsequent analysis comparing residuals with input parameters suggested that several of the model assumptions had certain pronounced biases. Collectively, utilizing restrictive clearance with the mean free venous plasma concentration and estimation of free concentration \textit{in vitro} using the Armitage model appeared to perform slightly better in terms of 1) the ability to clarify the association between \textit{in vitro} bioactivity and \textit{in vivo} data (Figs 6 and 7), 2) accuracy (i.e., RMSE) of AED$_{10}$ extrapolation relative to POD$_{10}$ (Fig 8), and 3) relatively lower dependence on input parameters (Fig 9).

**The metrics evaluated**

The ORMSE of the standardized log$_{10}$ transforms was selected as the statistical parameter for the basis of the comparisons made in this work to 1) enable comparison between forward and reverse dosimetry, 2) allow comparison across data with different units, and 3) elucidate the association between the \textit{in vitro} bioactivity and \textit{in vivo} toxicity data. As in the example regression in Fig 3, the ORMSE may be sensitive to outliers. However, as we carried out the analysis across a range of assay endpoints, \textit{in vivo} effects, and chemicals, we expect this effect was moderated, as evidenced by the general observation of improved performance with the PBTK result for the endpoint level analysis. Furthermore, we also used ten sets of randomized results as additional references for comparison of the performance of the application of the PBTK model. If the performance across all \textit{in vitro} and \textit{in vivo} comparisons were due to small differences in ORMSE, then the randomized result would perform similarly to the PBTK result. However, this was not observed to be the case for the endpoint level analysis. Standard parameters such as $R^2$ and RMSE are y-oriented and would be less comparable between forward and reverse dosimetry. Evaluating the total sum of squares of the residuals would be dimensional and would not necessarily be relatable to the strength of an association. Another alternative to using RMSE would be to represent the uncertainty in the measured \textit{in vitro} bioactivity and \textit{in vivo} toxicity data via bootstrapping or another methodology, but this would require an \textit{a priori} understanding of the uncertainty at different levels of the data.

As the example regression in Fig 3 showed, the comparison of $AC_{50}$ and $C_{PBTK}$ in the forward dosimetry result is separate from the comparison of \textit{in vivo} dose and AED$_{PBTK}$ in the reverse dosimetry result. From Eq 1, we see that the values of $C_{PBTK}$ and AED$_{PBTK}$ are inversely related, that $C_{PBTK}$ is a function of the dose and the study length (i.e. dosing regimen), and that AED$_{PBTK}$ is a function of $AC_{50}$ and the corresponding study length of interest. The residuals of the log$_{10}$ transforms are therefore equal and opposite (Eqs 7 and 8).

$$\log_{10}AC_{50,j} - \log_{10}C_{PBTK,j} = \text{residual}_j \quad \text{(7)}$$

$$\log_{10}\text{dose}_j - \log_{10}\text{AED}_{PBTK,j} = -\text{residual}_j \quad \text{(8)}$$

As such, the total sum of squares would be equivalent for both comparisons. However, any statistic that accounts for the distribution of the variables (e.g., the variance, ORMSE of standardized variables) will likely differ. In this work, we are more concerned with the latter as our focus is on association. While related, $C_{PBTK}$ and AED$_{PBTK}$ will produce separate sets of results in their comparisons, unless the residuals between variables approach zero. Importantly, the basic relationship between dose and concentration suggests the need to compare results for
both forward and reverse dosimetry when evaluating any model applied for quantitative IVIVE.

The influence of the evaluated assumptions

Although this work demonstrates the potential of TK to elucidate the association between in vitro bioactivity and in vivo toxicity data, it was unclear which specific set of assumptions in the application of TK for IVIVE yielded an optimal result in Figs 6 and 7. For ease of reference, the results in Figs 6 and 7 are summarized in Fig 10 by the fraction that the PBTK result was selected over the random result and untransformed values (i.e., ratio of counts for PBTK in Figs 6 and 7 to the corresponding total count per dosimetry, analysis level, and assumption set). Of the assumptions evaluated, selection of either the mean or maximum in vivo concentration seemed to have little influence on performance. While there were clear differences between the results for the other layers of the assumptions, trends in the forward dosimetry results were not always corroborated by the reverse dosimetry results. Those sets of assumptions that consistently performed well include restrictive clearance with free venous plasma concentration, restrictive clearance with free venous plasma in vivo concentration combined with free in vitro concentration predicted by the Armitage model, and nonrestrictive clearance with tissue concentration.

Free concentration in vivo is generally assumed to be the relevant concentration for effecting a biological response; however, this should be specific to the local active site and as such may be intracellular and subject to active transport or other processes [57]. In this work, we have only evaluated the free concentration in venous plasma, in part due to the perfusion limited assumption of our model. Furthermore, we have only evaluated the free (aqueous phase) in vitro concentration as many of the evaluated assays are not cell-based. Even for comparison with a given assay endpoint, the relative prevalence of various types of binding sites in vivo and in vitro may differ and result in differences in the fraction of free chemical. Despite these qualifications, using the free in vivo concentration with restrictive clearance and the nominal in vitro concentration performed reasonably well, likely because many of the in vitro assays evaluated are not cell-based and have limited material available for binding. A more accurate result, particularly for cell-based assays, may be expected if the in vitro concentration were corrected for nonspecific binding by using an in vitro disposition model. For restrictive clearance, this expectation was corroborated by our results which demonstrated improved performance when using free in vivo concentration in combination with the Armitage model to estimate free in vitro concentration [51]. Furthermore, the analysis of residuals (Fig 9) showed that using the free in vivo concentration alone exhibited bias in the results with respect to binding related parameters of \( f_{\text{up}} \), logP, and logD. However, this bias appeared to be reduced when using free in vivo concentration and free in vitro concentration.

This differs from the conclusions proposed in the work by Casey et al. [58], which evaluated the use of TK to extrapolate the ER pathway model [8] and compared results relative to OECD guideline uterotrophic in vivo data. Based on direct comparison of log_{10} RMSE between models, the authors concluded that using either a steady state free plasma concentration (in vivo) or the in vitro intracellular concentration predicted by the Armitage model provided a more accurate result for this specific pathway. However, lower RMSE indicates improved accuracy of the mean, but does not necessarily imply a stronger association or that the model is more informative. Our results instead suggest the possibility that using the mean free plasma concentration with restrictive clearance is one of several options exhibiting mild improvements in association over other model assumptions/selections when applied across a broad range of in vitro and in vivo data. Furthermore, we observed additional improvement when using free in...
vivo concentration in combination with free in vitro concentration, which might be considered a theoretically more justifiable comparison.

Nonrestrictive clearance with tissue concentration also appeared to perform well in terms of clarifying the association between in vitro bioactivity and in vivo toxicity data (Fig 10). However, high values for \(|\text{COR}|\) were observed for several input parameters (Fig 9), particularly with respect to \(C_{\text{int}}\). This trend was observed for other combinations of assumptions with non-restrictive clearance. As such, the results call into question the suitability of using nonrestrictive clearance as a broad assumption. It should be noted that restrictive and nonrestrictive clearance are the two limits of an equilibrium assumption made to simplify dynamic binding, mass transport, and reaction processes that occur during hepatic clearance. Further understanding of the dependence of the rates of those processes on the chemical may provide more representative results.

Implications for future work

The results of this work demonstrate that TK improves our ability to elucidate the association between in vitro bioactivity and in vivo toxicity data when evaluations are made per specific in vivo endpoints and study types. Therefore, in vitro bioactivity results should be translated...
using TK for IVIVE when developing machine learning and other statistical models for predicting *in vivo* toxicity. This potential suggests that improvements to the understanding of TK for environmental chemicals would be beneficial to enabling new approach methods. In this work, the assumptions were evaluated based on application across all the data; there was no selection made based on the particular assay or *in vivo* effect. In general, it appeared that using restrictive clearance with free *in vivo* venous plasma concentration and free *in vitro* concentration performed well. Although this was somewhat less conservative than the base set of assumptions (restrictive clearance with mean total plasma concentration, Fig 8A), if uncertainty were extrapolated it may be reasonable to select a lower bound (e.g., the 5th percentile) as a more conservative estimate of POD. Additionally, for certain *in vitro* assays or *in vivo* endpoints, some assumptions may work better than others, particularly with respect to concentration selection and use of an *in vitro* disposition model. Variation of those assumptions based on relevancy to the assay in question may be reasonable. Similarly, it may be possible to vary certain assumptions based on chemical. This may particularly be applicable to nonrestrictive and restrictive clearance, which should vary by chemical.

We have shown that PBTK clarifies the association between *in vitro* screening assays and *in vivo* toxicity. Using restrictive clearance with free *in vivo* venous plasma concentration and free *in vitro* concentration appeared to perform as well as or better than other model assumptions. While in that case the residual error was somewhat less dependent on the input parameters, there remained some bias—this suggests the potential for model improvement. Based on the performance afforded by PBTK, other methods to further improve the accuracy and understanding of TK should be pursued [59], particularly with respect to chemical-specific TK parameters. For example, this work assumed 100% fraction absorbed in the gut and no gut metabolism—a better understanding of oral bioavailability would be beneficial. Differences were observed in the performance of restrictive and nonrestrictive clearance, although they were not consistent between forward and reverse dosimetry. Restrictive and nonrestrictive clearance are two limits of a potential range of off-binding rates of an absorbed chemical from protein. Where available, *in vitro* methods could be used to measure these and other toxicokinetic parameters. Further evaluation of both forward and reverse dosimetry is important to fully understand the relationship between *in vitro* bioactivity and *in vivo* toxicity data.

This work evaluated applying toxicokinetics for rat to predict doses for which *in vivo* effects may be observed in rat. The PBTK model used here can be scaled to human by using human physiological parameters and human specific *in vitro* or *in silico* determined toxicokinetic parameters (i.e., $\text{Cl}_{\text{int}}$ and $f_{\text{up}}$). Human specific data have already been collected for hundreds of chemicals [12, 19]. Thus, the IVIVE principles evaluated here may be applied to humans. Ultimately, AED for humans can then be compared with predicted rates of exposure to provide an estimate of chemical risk [2, 5, 17–21].

**Supporting information**

S1 Fig. Allocated counts from forward dosimetry for comparisons with at least 5 chemicals. Counts compare *in vitro* AC$_{50}$ with predicted *in vivo* concentration for the endpoint level analysis (top row) and POD level analysis (bottom row) as a function of the assumptions used in application of the PBTK model. Counts are from *in vivo-in vitro* pairs with at least 5 unique chemicals and are median values from the 10 sets of comparisons. The error bars are plus or minus two standard deviations from the 10 comparisons. Labels on the x-axis indicate assumption set: for clearance (res.–restrictive, nres.–nonrestrictive), concentration selection (tot.–total, free, vein, tis.–tissue, mean, max), and use of the Armitage disposition model to estimate
the free concentration in vitro.

S2 Fig. Allocated counts from reverse dosimetry for comparisons with at least 5 chemicals. Counts compare in vivo dose with predicted AED from in vitro toxicity assay results for the endpoint level analysis (top row) and POD level analysis (bottom row) as a function of the assumptions used in application of the PBTK model. Counts are from in vivo-in vitro pairs with at least 5 unique chemicals and are median values from the 10 sets of comparisons. The error bars are plus or minus two standard deviations from the 10 comparisons. Labels on the x-axis indicate assumption set: for clearance (res.–restrictive, nres.–nonrestrictive), concentration selection (tot.–total, free, vein, tis.–tissue, mean, max), and use of the Armitage disposition model to estimate the free concentration in vitro.

S3 Fig. Allocated counts from forward dosimetry for comparisons with at least 5 chemicals by study type. Counts compare in vitro AC50 with predicted in vivo concentration for the endpoint level analysis (top row) and POD level analysis (bottom row) as a function of the assumptions used in application of the PBTK model. Counts are from in vivo-in vitro pairs with at least 5 unique chemicals and are median values from the 10 sets of comparisons. The error bars are plus or minus two standard deviations from the 10 comparisons. Labels on the x-axis indicate assumption set: for clearance (res.–restrictive, nres.–nonrestrictive), concentration selection (tot.–total, free, vein, tis.–tissue, mean, max), and use of the Armitage disposition model to estimate the free concentration in vitro. Results are separated by study type: chronic (CHR), subchronic (SUB), and developmental (DEV).

S4 Fig. Allocated counts from reverse dosimetry for comparisons with at least 5 chemicals by study type. Counts compare in vivo dose with predicted AED from in vitro toxicity assay results for the endpoint level analysis (top row) and POD level analysis (bottom row) as a function of the assumptions used in application of the PBTK model. Counts are from in vivo-in vitro pairs with at least 5 unique chemicals and are median values from the 10 sets of comparisons. The error bars are plus or minus two standard deviations from the 10 comparisons. Labels on the x-axis indicate assumption set: for clearance (res.–restrictive, nres.–nonrestrictive), concentration selection (tot.–total, free, vein, tis.–tissue, mean, max), and use of the Armitage disposition model to estimate the free concentration in vitro. Results are separated by study type: chronic (CHR), subchronic (SUB), and developmental (DEV).

S5 Fig. Allocated counts from forward dosimetry for comparisons with at least 20 chemicals by study type. Counts compare in vitro AC50 with predicted in vivo concentration for the endpoint level analysis (top row) and POD level analysis (bottom row) as a function of the assumptions used in application of the PBTK model. Counts are from in vivo-in vitro pairs with at least 20 unique chemicals and are median values from the 10 sets of comparisons. The error bars are plus or minus two standard deviations from the 10 comparisons. Labels on the x-axis indicate assumption set: for clearance (res.–restrictive, nres.–nonrestrictive), concentration selection (tot.–total, free, vein, tis.–tissue, mean, max), and use of the Armitage disposition model to estimate the free concentration in vitro. Results are separated by study type: chronic (CHR), subchronic (SUB), and developmental (DEV).
S6 Fig. Allocated counts from reverse dosimetry for comparisons with at least 20 chemicals by study type. Counts compare in vivo dose with predicted AED from in vitro toxicity assay results for the endpoint level analysis (top row) and POD level analysis (bottom row) as a function of the assumptions used in application of the PBTK model. Counts are from in vivo-in vitro pairs with at least 20 unique chemicals and are median values from the 10 sets of comparisons. The error bars are plus or minus two standard deviations from the 10 comparisons. Labels on the x-axis indicate assumption set: for clearance (res.–restrictive, nres.–nonrestrictive), concentration selection (tot.–total, free, vein, tis.–tissue, mean, max), and use of the Armitage disposition model to estimate the free concentration in vitro. Results are separated by study type: chronic (CHR), subchronic (SUB), and developmental (DEV).

(TIFF)

S7 Fig. Plots of the log10 transforms of the 10th percentile dose from POD level in vivo data vs the 10th percentile AED using the PBTK model, determined with a time scale of the corresponding lowest dose. Each point corresponds to a particular chemical. Results are for the assumption set of total mean concentration, and are otherwise indicated by the panel labels: a) restrictive clearance with in vivo mean free total venous plasma concentration, b) restrictive clearance with in vivo mean venous plasma concentration and free concentration in vitro predicted by the Armitage model, c) nonrestrictive clearance with in vivo mean total plasma concentration, d) nonrestrictive clearance with in vivo mean free venous plasma concentration, e) nonrestrictive clearance with in vivo mean total venous plasma concentration and free concentration in vitro predicted by the Armitage model, f) nonrestrictive clearance with in vivo mean free venous plasma concentration and free concentration in vitro predicted by the Armitage model. The dashed lines are y = x lines. Corresponding RMSE and ORMSE (the latter defined for the standardized variables) are also reported.

(TIFF)

S1 File. Analysis scripts and input data.

(ZIP)

S2 File. List of abbreviations.

(DOCX)

S3 File. List of assumptions evaluated.

(DOCX)

S1 Table. Measured Clint and fup for rat.

(CSV)

S2 Table. GC-MS methods.

(XLSX)

S3 Table. LC-MS methods.

(XLSX)

S4 Table. Endpoint level in vivo toxicity data for rat.

(CSV)

S5 Table. POD level in vivo toxicity data for rat.

(CSV)

S6 Table. In vivo endpoints evaluated in this work.

(CSV)
Acknowledgments

The views expressed in this publication are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Reference to commercial products or services does not constitute endorsement. We thank James Armitage for providing a revised version of his model. We thank Dr.s Dustin Kapraun and Katie Paul Friedman for providing internal technical review of this manuscript. We thank Dr. Maria Bacolod for technical assistance in the conduct and documentation of the in vitro experiments. We thank Dr. Ann Richard for chemical library management support. We thank Dr. Antony Williams for technical assistance in the calculation of pKa values. We appreciate government contracting support from David Murphy, Sandra Roberts, Stephen Little, and Dr. Matthew Martin.

Author Contributions

Conceptualization: Gregory S. Honda, R. W. Setzer, Barbara A. Wetmore, Nisha S. Sipes, Russell S. Thomas, John F. Wambaugh.

Data curation: Gregory S. Honda, Jon Gilbert, Briana Franz, John F. Wambaugh.

Formal analysis: Gregory S. Honda.

Investigation: Gregory S. Honda, Jon Gilbert, Briana Franz.

Methodology: Gregory S. Honda, Ly L. Pham, R. W. Setzer, Barbara A. Wetmore, Nisha S. Sipes, John F. Wambaugh.

Software: Gregory S. Honda, Robert G. Pearce, John F. Wambaugh.

Supervision: Russell S. Thomas, John F. Wambaugh.

Validation: Gregory S. Honda.

Visualization: Gregory S. Honda.

Writing – original draft: Gregory S. Honda, John F. Wambaugh.

Writing – review & editing: Robert G. Pearce, Ly L. Pham, R. W. Setzer, Barbara A. Wetmore, Nisha S. Sipes, Russell S. Thomas, John F. Wambaugh.

References

1. Judson R, Richard A, Dix DJ, Houck K, Martin M, Kavlock R, et al. The toxicity data landscape for environmental chemicals. Environ Health Perspect. 2009; 117(5):685–95. https://doi.org/10.1289/ehp.0800168 PMID: 19479008

2. NASEM. Using 21st Century Science to Improve Risk-Related Evaluations. Washington, DC: The National Academies Press; 2017. 200 p.

3. USGAO. Options for Enhancing the Effectiveness of the Toxic Substances Control Act. In: USGAO, editor. 2009.

4. USGAO. Toxic Substances: EPA Has Increased Efforts to Assess and Aonctrol Chemicals but Could Strengthen Its Approach. In: USGAO, editor. 2013.

5. Thomas RS, Philbert MA, Auerbach SS, Wetmore BA, Devito MJ, Cote I, et al. Incorporating new technologies into toxicity testing and risk assessment: moving from 21st century vision to a data-driven framework. Toxicological Sciences. 2013; 136(1):4–18. https://doi.org/10.1093/toxsci/kft178 PMID: 23958734

6. Judson R, Houck K, Martin M, Knudsen T, Thomas RS, Sipes N, et al. In vitro and modelling approaches to risk assessment from the U.S. Environmental Protection Agency ToxCast programme. Basic Clin Pharmacol Toxicol. 2014; 115(1):69–76. https://doi.org/10.1111/bcpl.12239 PMID: 24684691
7. Tice RR, Austin CP, Kavlock RJ, Bucher JR. Improving the human hazard characterization of chemicals: a Tox21 update. Environ Health Perspect. 2013; 121(7):756–65. https://doi.org/10.1289/ehp.1205784 PMID: 23603828

8. Judson RS, Magpantay FM, Chickarmane V, Haskell C, Tania N, Taylor J, et al. Integrated Model of Chemical Perturbations of a Biological Pathway Using In vitro High-Throughput Screening Assays for the Estrogen Receptor. Toxicol Sci. 2015; 148(1):137–54. https://doi.org/10.1093/toxsci/kfv168 PMID: 26272952

9. Liu J, Mansouri K, Judson RS, Martin MT, Hong H, Chen M, et al. Predicting hepatotoxicity using ToxCast in vitro bioactivity and chemical structure. Chem Res Toxicol. 2015; 28(4):738–51. https://doi.org/10.1021/acs.chemresox.5b00501h PMID: 2567799

10. Kleinstreuer NC, Ceger P, Watt ED, Martin M, Houck KA, Browne P, et al. Development and Validation of a Computational Model of Androgen Receptor Activity. Chem Res Toxicol. 2017; 30(4):946–64. https://doi.org/10.1021/acs.chemrestox.6b00347 PMID: 27933809

11. Blaauwboer BJ. Biokinetic modeling and in vitro–in vivo extrapolations. Journal of Toxicology and Environmental Health, Part B. 2010; 13(2–4):242–52.

12. Wetmore BA. Quantitative in vitro-to-in vivo extrapolation in a high-throughput environment. Toxicology. 2015; 332:94–101. https://doi.org/10.1016/j.tox.2014.05.012 PMID: 24907440

13. Coecke S, Pelkonen O, Leite SB, Bernauer U, Bessem JG, Bois FY, et al. Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. Toxicology in vitro. 2013; 27(5):1570–7. https://doi.org/10.1016/j.tiv.2012.06.012 PMID: 22771339

14. Kramers HA. Brownian motion in a field of force and the diffusion model of chemical reactions. Physica. 1940; 7(4):284–304.

15. Yung-Chi C, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_50) of an enzymatic reaction. Biochemical pharmacology. 1973; 22(23):3099–108. PMID: 4202581

16. National Research Council. Toxicity testing in the 21st century: A vision and a strategy: National Academy Press; 2007.

17. Rotroff DM, Wetmore BA, Dix DJ, Ferguson SS, Clewell HJ, Houck KA, et al. Incorporating human dosimetry and exposure into high-throughput in vitro toxicity screening. Toxicol Sci. 2010; 117(2):348–58. https://doi.org/10.1093/toxsci/kfq220 PMID: 20639261

18. Kavlock RJ, Bahadori T, Barton-Maclaren TS, Gwinn MR, Rasenberg M, Thomas RS. Accelerating the pace of chemical risk assessment. Chemical research in toxicology. 2018; 31(5):287–90. https://doi.org/10.1021/acs.chemresox.7b00339 PMID: 29600706

19. Wetmore BA, Wambaugh JF, Ferguson SS, Sochaski MA, Rotroff DM, Freeman K, et al. Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment. Toxicol Sci. 2012; 125(1):157–74. https://doi.org/10.1093/toxsci/kfr254 PMID: 21948869

20. Wetmore BA, Wambaugh JF, Allen B, Ferguson SS, Sochaski MA, Setzer RW, et al. Incorporating High-Throughput Exposure Predictions With Dosimetry-Adjusted In vitro Bioactivity to Inform Chemical Toxicity Testing. Toxicol Sci. 2015; 148(1):121–36. https://doi.org/10.1093/toxsci/kfv171 PMID: 26251325

21. Ring CL, Pearce RG, Setzer RW, Wetmore BA, Wambaugh JF. Identifying populations sensitive to environmental chemicals by simulating toxicokinetic variability. Environment International. 2017; 106:105–18. https://doi.org/10.1016/j.envint.2017.06.004 PMID: 28628784

22. Arnot JA, Brown TN, Wania F. Estimating screening-level organic chemical half-lives in humans. Environ Sci Technol. 2014; 48(1):723–30. https://doi.org/10.1021/es4029414 PMID: 24298879

23. Pearce RG, Setzer RW, Strope CL, Sipes NS, Wambaugh JF. httk: R Package for High-Throughput Toxicokinetics. J Stat Softw. 2017; 79(4). https://doi.org/10.18637/jss.v079.i04 PMID: 30220889

24. Pearce RG, Setzer RW, Davis JL, Wambaugh JF. Evaluation and calibration of high-throughput predictions of chemical distribution to tissues. Journal of Pharmacokinetics and Pharmacodynamics. 2017; 44(6):549–65. https://doi.org/10.1007/s10928-017-9548-7 PMID: 29032447

25. Sipes NS, Wambaugh JF, Pearce R, Auerbach SS, Wetmore BA, Hsieh JH, et al. An Intuitive Approach for Predicting Potential Human Health Risk with the Tox21 10k Library. Environmental Science & Technology. 2017; 51(18):10786–96.

26. Wambaugh JF, Wetmore BA, Pearce R, Strope C, Goldsmith R, Sliuka JP, et al. Toxicokinetic Triage for Environmental Chemicals. Toxicological Sciences. 2015; 147(1):55–67. https://doi.org/10.1093/toxsci/kfv118 PMID: 26085347

27. Wetmore BA, Wambaugh JF, Ferguson SS, Li L, Clewell HJ 3rd, Judson RS, et al. Relative impact of incorporating pharmacokinetics on predicting in vivo hazard and mode of action from high-throughput in vitro...
vitro toxicity assays. Toxicol Sci. 2013; 132(2):327–46. https://doi.org/10.1093/toxsci/kft012 PMID: 23358191

28. Toutain P-L, Bouquet-Melou A. Free drug fraction vs. free drug concentration: a matter of frequent confusion. Journal of veterinary pharmacology and therapeutics. 2002; 25(6):460–3. PMID: 12485352

29. Groothuis FA, Heringa MB, Nicol B, Hermens JLM, Blaauwboer BJ, Kramer NJ. Dose metric considerations in in vitro assays to improve quantitative in vitro–in vivo dose extrapolations. Toxicology. 2015; 332:30–40. https://doi.org/10.1016/j.tox.2013.08.012 PMID: 23978460

30. Williams AJ, Grulke CM, Edwards J, McEachran AD, Mansouri K, Baker NC, et al. The CompTox Chemistry Dashboard: a community data resource for environmental chemistry. Journal of Cheminformatics. 2017; 9(1):61. https://doi.org/10.1186/s13321-017-0247-6 PMID: 29185060

31. Tan Y-M, Liao KH, Conolly RB, Blount BC, Mason AM, Clewell HJ. Use of a physiologically based pharmacokinetic model to identify exposures consistent with human biomonitoring data for chloroform. Journal of Toxicology and Environmental Health, Part A. 2006; 69(18):1727–56.

32. Tan Y-M, Liao KH, Clewell HJ. Reverse dosimetry: interpreting trihalomethanes biomonitoring data using physiologically based pharmacokinetic modeling. Journal of Exposure Science and Environmental Epidemiology. 2007; 17(7):591–603. https://doi.org/10.1038/sj.jes.7500540 PMID: 17108893

33. Sohlenius-Sternbeck A-K, Jones C, Ferguson D, Middleton BJ, Projean D, Floby E, et al. Practical use of the regression offset approach for the prediction of in vivo intrinsic clearance from hepatocytes. Xenobiotica. 2012; 42(9):841–53. https://doi.org/10.3109/00498254.2012.669080 PMID: 22509824

34. Sohlenius-Sternbeck A-K, Fagerhol M, Bylund J. The volume of distribution is an indicator of poor in vitro–in vivo extrapolation of clearance for acidic drugs in the rat. Xenobiotica. 2013; 43(8):671–8. https://doi.org/10.1080/00498254.2012.755578 PMID: 23323549

35. Hertzberg RP, Pope AJ. High-throughput screening: new technology for the 21st century. Curr Opin Chem Biol. 2000; 4(4):445–51. PMID: 10959774

36. Waters NJ, Jones R, Williams G, Sohal B. Validation of a rapid equilibrium dialysis approach for the measurement of plasma protein binding. J Pharm Sci. 2008; 97(10):4586–95. https://doi.org/10.1002/jps.21317 PMID: 18300299

37. Kilford PJ, Gertz M, Houston JB, Galetin A. Hepatocellular binding of drugs: correction for unbound fraction in hepatocyte incubations using microsomal binding or drug lipophilicity data. Drug Metabolism and Disposition. 2008.

38. Houston B, Carlile D. Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. Drug metabolism reviews. 1997; 29(4):891–922. https://doi.org/10.3109/03602539709002237 PMID: 9421679

39. Wood FL, Houston JB, Hallifax D. Clearance Prediction Methodology Needs Fundamenta1 Improvement: Trends Common to Rat and Human. Drug Metabolism and Disposition. 2017; 45:1178–88. https://doi.org/10.1124/dmd.117.077040 PMID: 28887366

40. Naritomi Y, Terashita S, Kagaya A, Sugiyama Y. Utility of hepatocytes in predicting drug metabolism: comparison of hepatic intrinsic clearance in rats and humans in vivo and in vitro. Drug Metabolism and Disposition. 2003; 31(5):580–8. PMID: 12695346

41. Knudsen TB, Martin MT, Kavlock RJ, Judson RS, Dix DJ, Singh AV. Profiling the activity of environmental chemicals in prenatal developmental toxicity studies using the U.S. EPA’s ToxRefDB. Reprod Toxicol. 2009; 28(2):209–19. https://doi.org/10.1016/j.reprotox.2009.03.016 PMID: 19446433

42. Martin MT, Judson RS, Reif DM, Kavlock RJ, Dix DJ. Profiling chemicals based on chronic toxicity results from the U.S. EPA ToxRef Database. Environ Health Perspect. 2009; 117(3):392–9. https://doi.org/10.1289/ehp.0800074 PMID: 19337514

43. Martin MT, Mendez E, Corum DG, Judson RS, Kavlock RJ, Rotroff DM, et al. Profiling the reproductive toxicity of chemicals from multigeneration studies in the toxicity reference database. Toxicol Sci. 2009; 110(1):181–90. https://doi.org/10.1093/toxsci/kfp080 PMID: 19363143

44. EPA U. ToxRefDB Washington, DC: United States Environmental Protection Agency; [updated 10/07/2015; cited 2018]. Available from: ftp://newftp.epa.gov/comptox/High_Throughput_Screening_Data/Animal_Tox_Data.

45. EPA U. Series 870—Health Effects Test Guidelines Washington, DC: United States Environmental Protection Agency; [cited 2018]. Available from: https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-870-health-effects-test-guidelines.

46. Kavlock R, Chandler K, Houck K, Hunter S, Judson R, Kleinstreuer N, et al. Update on EPA’s ToxCast program: providing high throughput decision support tools for chemical risk management. Chem Res Toxicol. 2012; 25(7):1287–302. https://doi.org/10.1021/tx3000939 PMID: 22519603
47. EPA U. In Vitro DB Washington, DC: United States Environmental Protection Agency; [updated 10/28/2015; cited 2018]. Available from: ftp://newftp.epa.gov/comptox/High_Throughput_Screening_Data/Previous_Data/ToxCast_Data_Release_Oct_2015/.

48. EPA U. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.11 Washington, DC, USA: United States Environmental Protection Agency; [updated 09/13/20167/27/2018]. Available from: https://www.epa.gov/tsca-screening-tools/epi-suitem-estimation-program-interface.

49. Mansouri K, Grulke CM, Judson RS, Williams AJ. OPERA models for predicting physicochemical properties and environmental fate endpoints. Journal of Cheminformatics. 2018; 10(1):10. https://doi.org/10.1186/s13321-018-0263-1 PMID: 29520515

50. Strope CL, Mansouri K, Clewell HJ, Rabinowitz JR, Stevens C, Wambaugh JF. High-throughput in-silico prediction of ionization equilibria for pharmacochemical modeling. Science of The Total Environment. 2018; 615:150–60. https://doi.org/10.1016/j.scitotenv.2017.09.033 PMID: 28964990

51. Armitage JM, Wania F, Amol JA. Application of mass balance models and the chemical activity concept to facilitate the use of in vitro toxicity data for risk assessment. Environ Sci Technol. 2014; 48(16):9770–9. https://doi.org/10.1021/es501955g PMID: 25014875

52. Yoon M, Efremenko A, Blaauboer BJ, Clewell HJ. Evaluation of simple in vitro to in vivo extrapolation approaches for environmental compounds. Toxicology in vitro. 2014; 28(2):164–70. https://doi.org/10.1016/j.tiv.2013.10.023 PMID: 24216301

53. Corning. [05/29/2017]. Available from: https://www.corning.com/worldwide/en/products/life-sciences/support-main/drawings-specs.html.

54. Martin MT, Dix DJ, Judson RS, Kavlock RJ, Reif DM, Richard AM, et al. Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA’s ToxCast program. Chem Res Toxicol. 2010; 23(3):578–90. https://doi.org/10.1021/tr100195f PMID: 20143881

55. Pelkonen O, Turpeinen M. In vitro–in vivo extrapolation of hepatic clearance: biological tools, scaling factors, model assumptions and correct concentrations. Xenobiotica. 2007; 37(10–11):1066–89. https://doi.org/10.1080/00498250701620726 PMID: 17968737

56. Bucher JR, Portier CJ, Goodman JL, Faustman EM, Lucier GW. National Toxicology Program Studies: Principles of Dose Selection and Applications to Mechanistic Based Risk Assessment. Toxicological Sciences. 1996; 31(1):1–8.

57. Smith DA, Di L, Kerns EH. The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. Nat Rev Drug Discov. 2010; 9(12):929–39. https://doi.org/10.1038/nrd3287 PMID: 21119731

58. Casey WM, Chang X, Allen DG, Ceger PC, Choksi NY, Hsieh JH, et al. Evaluation and Optimization of Pharmacochemical Models for in vitro to in vivo Extrapolation of Estrogenic Activity for Environmental Chemicals. Environ Health Perspect. 2018; 126(9):97001. https://doi.org/10.1289/EHP1655 PMID: 30192161

59. Basketter DA, Clewell H, Kimber I, Rossi A, Blaauboer B, Burrier R, et al. A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing-t4 report. ALTEX-Alternatives to animal experimentation. 2012; 29(1):3–91.