ACUTE TOXICITY PREDICTION IN MULTIPLE SPECIES BY LEVERAGING MECHANISTIC TOXCAST MITOCHONDRIAL INHIBITION DATA AND SIMULATION OF ORAL BIOAVAILABILITY

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ABSTRACT

There is great interest in assessing the in vivo toxicity of chemicals using nonanimal alternatives. However, acute mammalian toxicity is not adequately predicted by current in silico or in vitro approaches. Mechanisms of acute toxicity are likely conserved across invertebrate, aquatic, and mammalian species, suggesting that dose-response concordance would be high and in vitro mechanistic data could predict responses in multiple species under conditions of similar bioavailability. We tested this hypothesis by comparing acute toxicity between rat, daphnia, and fish and by comparing their respective acute data to inhibition of mitochondria membrane potential (MMP) using U.S. Environmental Protection Agency ToxCast in vitro high-throughput screening data. Logarithmic scatter plots of acute toxicity data showed a clear relationship between fish, daphnia, and intravenous rat but not oral rat data. Similar plots versus MMP showed a well-delineated upper boundary for fish, daphnia, and intravenous data but were scattered without an upper boundary for rat oral data. Adjustments of acute oral rat toxicity values by simulating fractional absorption and CYP-based metabolism as well as removing compounds with hydrolyzable linkages or flagged as substrates for glucuronidation delineated an upper boundary for rat oral toxicity versus MMP. Mitochondrial inhibition at low concentrations predicted highly acutely toxic chemicals for fish and daphnia but not the rat where toxicity was often attenuated. This use of a single high-throughput screening assay to predict acute toxicity in multiple species represents a milestone and highlights the promise of such approaches but also the need for refined tools to address systemic bioavailability and the impact of limited absorption and first pass metabolism.

Key words: bioavailability; biotransformation and toxicokinetics; in vitro and alternatives; predictive toxicology; QSAR; mechanisms; systems toxicology, toxicity; acute; safety evaluation

The paradigm for safety testing is shifting from extensive animal testing toward use of computational models and mechanistic in vitro data. However, there are currently no validated or scientifically accepted in silico or in vitro assays that predict acute oral mammalian toxicity. Although updates to older acute testing guidelines minimized the use of animals, determination of acute toxicity for regulatory purposes still requires in vivo testing. Nonanimal alternative approaches could include in silico quantitative structure-activity relationship (QSAR) models (Diaz et al., 2015) or various forms of read-across (European Chemicals Agency, 2012), including mechanistic assays leveraging in vitro data.

Estimates of acute oral toxicity are often needed for compounds before any testing can be done, even in vitro testing. This is especially true under conditions of handling new or investigational R&D chemistries. Assessment of several existing in silico QSAR models for their ability to predict acute oral rat toxicity resulted in poor sensitivity (Diaz et al., 2015), especially for more-highly toxic Globally Harmonized System (GHS) 1 or 2 compounds (manuscript in preparation). Existing QSAR models...
for this endpoint are purely statistical and as such, have limited utility for predicting acute oral toxicity because of the diversity of chemical classes, acute toxicity mechanisms, and impact of bioavailability and metabolism.

Because adequate QSAR models are not available, alternative approaches must rely on read-across, a technique for data-gapping where endpoint information from one chemical is used to predict the same endpoint for another chemical that is considered to be similar for mode-of-action, toxicokinetics, metabolism, etc., in relating to that endpoint (European Chemicals Agency, 2012). Although there is emerging guidance for conducting read-across, there is no widely accepted approach, and most analyses are conducted on a case-by-case basis using nonstandardized approaches requiring sophisticated expertise. Although traditional read-across leverages existing data for the same endpoint for structurally similar compounds, a broad definition includes leveraging data from any combination of other compounds, routes, species, or study types including mechanistic in vitro data. Many compounds will undergo metabolic transformation or hydrolysis, thus an appropriate read-across evaluation should consider both the parent and analogs of metabolic products. A number of reports indicate the potential for read across between species, routes, or in vitro data (Patlewicz et al., 2013a,b; Schüürmann et al., 2011). For example, in vitro basal cytotoxicity has been proposed as a means to predict acute oral toxicity but limitations include a lack of concordance for compounds that are poorly bioavailable or highly metabolized, as well as lack of activity for compounds with those toxicity mechanisms not applicable in vitro (ICCVAM, 2001).

In vitro data potentially amenable for predicting acute in vivo toxicity include high-throughput screening (HTS) data such as those recently generated under the ToxCast program. Starting in 2006, the U.S. Environmental Protection Agency (US-EPA) ToxCast program was developed in several phases to screen thousands of chemicals in high-throughput mode. It contains in vitro biochemical and cell-based platforms as well as small model organisms. In 2008, a collaborative Tox21 program was launched with representation by several federal agencies. Phase II (Dix et al., 2007; Kavlock et al., 2012) of each program has tested approximately 1800 compounds in 900 HTS assays under ToxCast and 8400 compounds in 80 HTS assays under Tox21 (Kavlock et al., 2009; Tice et al., 2013), which includes the 1800 from ToxCast as a subset. A component of these initiatives involved compiling existing animal toxicity data for the compounds and making it publicly available online in a digitized searchable format. Available animal toxicity data currently include repeat-dose mammalian general toxicity and developmental and reproductive toxicity studies. Curated data from short-term study types such as acute oral toxicity and skin sensitization are being compiled by the programs but are not yet publicly available. Data from aquatic species were not collected as part of the ToxCast initiative.

As a proof-of-concept, we examined alternative approaches for the ability to predict acute oral toxicity based on read-across. We hypothesized that mechanisms of acute toxicity are largely conserved across invertebrate, aquatic, and mammalian species and that in vitro mechanistic data could predict responses in multiple species under conditions of similar bioavailability. We examined potential concordance for acute toxicity between routes (oral and intravenous), species (rat, fish, and daphnia), and one in vitro mechanistic data endpoint, inhibition of mitochondrial membrane potential (MMP) (Attené-Ramos et al., 2013, 2015; Sakamuru et al., 2012).

We downloaded MMP inhibition assay data from ToxCast Phase II (Dix et al., 2007; Kavlock et al., 2009, 2012; Tice et al., 2013) and investigated its relationship to acute toxicity. Mitochondria are critical to normal development (St John, 2015) and cellular homeostasis, with key roles in energy production, apoptosis, calcium homeostasis, lipid pathways, steroid and hormone synthesis, and immune responses (Shaughnessy et al., 2014). They are essential to multicellular life and without them an organism would cease to respire aerobically and quickly die. The electron transport chain (ETC) located in the inner mitochondrial membrane generates a trans-membrane proton gradient resulting in an MMP. The MMP drives ATP synthase producing at least 90% of cellular ATP, which equates to approximately $10^8-10^{10}$ ATP molecules per cell per second (Kakkar and Singh, 2007; Newmeyer and Ferguson-Miller, 2003; Scheffler, 2001).

Mitochondrial inhibition is known to be related to acute toxicity for some chemicals but the mechanisms by which compounds can inhibit MMP are varied and complex. Direct targeting of any of the five major complexes involved in ATP generation or indirect uncoupling of the membrane potential by ionophoric mechanisms are all known to occur (Ankley et al., 2010; Jansen and de Boer, 1998). Multiple chemical classes of compounds affect these targets with detailed mechanistic domains still poorly understood. Either direct or indirect induction of apoptosis can also disrupt the MMP, inducing sequelae of diverse mechanisms including general cytotoxicity. The dosimetry related to various mitochondrial sub-organellar targets versus translation to downstream effects is still obscure. Distinguishing the detailed mechanisms of MMP disruption for various classes of chemicals will be important in refinement of adverse outcome pathways (AOPs), potential QSAR models and risk assessment. An AOP is a framework portraying existing knowledge linking molecular initiating events to an adverse outcome, at a level of biological organization relevant to risk assessment (Ankley et al., 2010).

Mitochondrial toxicity is only one mechanism of toxicity; as a result, inhibition of MMP is only expected to predict toxicity for chemicals where this mechanism drives the acute toxicity. In addition, for a chemical active in the MMP inhibition assay at high AC$_{50}$ concentrations, the acute toxicity may be driven by alternative mechanisms. Inhibition of MMP activity should be correlated with the toxicity of a compound suggesting it may be more toxic by another mechanism but not less toxic than the MMP-based prediction, and as expected, the overall toxicity will be rate-limited by factors affecting bioavailability, which vary with route. The chemical may be more toxic but cannot be less toxic than the MMP-based prediction. Thus, plots of acute toxicity (LC$_{50}$ or LD$_{50}$ values) versus MMP AC$_{50}$ should contain considerable scatter but have a sharply defined upper bound that is well predicted by MMP AC$_{50}$ values.

Furthermore, we expected that predictions of oral toxicity using mechanistic assays would often be affected by chemical-specific differences in uptake and metabolism by the oral route that did not occur in the aquatic species and were not well-represented in vitro. We addressed this by examining data concordance to rat intravenous toxicity data and by adjustment of acute oral rat toxicity values by simulating fractional absorption, cytochrome P450 (CYP) - and non-CYP-based metabolism, and hydrolysis.

Our hypothesis on conservation of acute mechanisms across species requires an adequate understanding of bioavailability and metabolism, especially first-pass metabolism. In silico tools that provide a comprehensive validated prediction of bioavailability and metabolism are currently not available. Bioavailability of a compound depends on its physico-chemical properties, diffusion-based parameters, and the extent to which
it is a substrate for transporters or enzymatic phase I or phase II reactions. In mammals, compounds administered via the gastrointestinal (GI) tract are subject to a range of pH values, metabolizing enzymes, and conjugation reactions before reaching the general circulation. Functional groups such as esters, imines, and amides, for example, are prone to hydrolysis. Extensive CYP-based metabolism, oxidative metabolism, and conjugation reactions can occur in the GI lumen and its epithelial wall, the portal blood, and the liver leading to marked loss of parent compound. Initial loss of chemical prior to entering the systemic blood via presystemic metabolism may account for as much as 100% of the administered oral dose. We addressed the potential impact of bioavailability by iterative adjustment of acute oral rat toxicity values. First, we simulated absorption and metabolism using GastroPlus, which predicts the fraction of chemical passively absorbed from the GI tract (F), systemic bioavailability (Fa), and compounds flagged as substrates for glucuronidation. Second, substructure mapping was used to identify compounds with hydrolysable linkages. Potential uridine-5’-diphosphoglucuronosyl transferase (UGT) substrates and hydrolysable compounds were then removed to simulate their loss of bioavailability.

**MATERIALS AND METHODS**

Acute toxicity data sources. Rat oral median lethal dose (LD₅₀) data were collected from several sources including the public literature (Zhu et al., 2009), European Chemicals Agency (ECHA), OECD e-ChemPortal (eChemPortal), and ChemID Plus (ChemIDPlus). Fish 96-h half-maximum lethal concentration (LC₅₀) and daphnia 48-h LC₅₀ data were collected from European Center for Ecotoxicology and Toxicology of Chemicals (ECETOC), Aquasafety, Japan, US-EPA ecotoxicology database (ECOTOX) as available in the OECD QSAR Toolbox v3.2 (http://www.oecd.org/chemicalsafety/risk-assessment/theoecdtqartoolbox.html), and OECD (eChemPortal). Additional data from the public literature were also compiled for fish 96-h LC₅₀ (Lammer et al., 2009; Schirmer et al., 2008; Vittorzi and De Angelis, 1991) and daphnia 48-h LC₅₀ (Cassotti et al., 2014; Guilhermino et al., 2000). Only Klimisch-reliability score 1–2 data (Klimisch et al., 1997) were selected for the data downloaded from ECHA for all three species. Similarly, rat intravenous LD₅₀ data were collected for ToxCast Phase II chemicals from ChemID Plus (ChemIDPlus).

Database development. In many cases, there were multiple data present from various sources for all endpoints including the Chemical Abstract Service (CAS) name and number. Several protocols were written in Pipeline Pilot 8.5 (http://accelrys.com/products/pipeline-pilot/) to select single high-quality endpoint data and to have the correct and single substance identity CAS, name, and Simplified Molecular Input Line Entry System (SMILES) representation for unique compounds. In the case of rat LD₅₀, the lowest value was kept corresponding to the highest toxicity, including the lowest LD₅₀ value of range-finding studies whenever a single LD₅₀ value was not retrieved. In the case of fish 96-h LC₅₀, data were chosen in the order of priority for mortality (LC₅₀) than incipient concentration (IC₅₀).

Because some immobilized organisms may recover. For both fish and daphnia, the flow-through method was given priority over the static method. Significant attention was given to data curation and organization, and finding and maintaining high-quality endpoint data. A master database was thus developed containing more than 18,500 data points belonging to three species and various acute endpoints.

ToxCast phase II chemical library. ToxCast Phase II mitochondrial toxicity data as distributed by the US-EPA were obtained from the ToxCast data download page (version dated December 13, 2013). Data were initially filtered by removing (1) mixtures, (2) compounds with unknown CAS or structure, and (3) all of the inactive compounds corresponding to the “Tox21 Mitochondrial Toxicity Ratio” assay. This resulted in 385 chemicals with numerical AC₅₀ values (ie, the concentration that inhibited the response measured by 50%), known further in this article as in vivo mitochondrial toxicity active chemicals. For these chemicals, acute fish, daphnia, and rat oral and intravenous data were queried from the 18,500 dataset using Pipeline Pilot protocols. Additional protocols were written to query amide and ester functionality using chemical functional group-based SMARTS (SMILES arbitrary target specification) representations (Bhattacharai and Schurer, 2011).

Complex I–V inhibitors of mitochondria. Xenobiotics known to inhibit any of the four complexes (I–IV) of the mitochondrial ETC as well as ATP synthase (complex V) were identified from the literature (Degli Esposti, 1998; Dykens and Will, 2007; Wallace and Starkov, 2000). Their chemical structure SMARTS (Bhattacharai and Schurer, 2011; http://www.daylight.com) representations were defined and used to query the 385 in vivo mitochondrial toxicity active compounds as obtained from the ToxCast Phase II data using structural similarity criteria. The ToxCast-active compounds were loaded as an inventory into the OECD QSAR Toolbox, 3.2, and each of approximately 30 xenobiotics known to inhibit mitochondria were used as targets to search for the ToxCast actives using atom-centered fragment structural analog search criteria with 50% similarity using Tanimoto’s coefficient (Tanimoto, 1957) as well as DICE (Dice, 1945). Similarity calculations were also performed with a Pipeline Pilot component using Tanimoto similarity.

Heat-map. A heat-map was generated using the 627 ToxCast II chemicals for which we have in vivo Fish 96-h data (Y-axis) and ToxCast data (X-axis). The ToxCast “percentage (perc)” assays and assays for which there were less than 10 active chemicals were excluded resulting in 285 assays. Assays from BioSeek were not available from the ToxCast public data download and are not represented in the heat-map. The assays that are mechanistically similar or are targeting the same receptor were collectively organized producing several blocks in the heat-map to highlight potential trends. For example, cytotoxicity-based assays, mitochondrial toxicity-based assays, assays that are functionally similar (such as NRF (nuclear factor), MRE (Metal response element), CRE (CAMP responsive element) assays), CYP based assays, PPAR (peroxisome proliferator-activated receptor) assays, AR (Androgen), ER (Estrogen), and GPCR (G-protein coupled receptor) receptor-based assays were in collective groups (refer ToxCast [Kavlock et al., 2012] for a detailed description of these assays). Assay potency is represented in red, yellow, and green to encode high, medium, and low potency, respectively.

Calculation of bioavailability. Predictions of the fraction of chemically passively absorbed from the GI tract following oral
administration (F), and subsequent systemic bioavailability (Fa), were obtained by using the commercially available software GastroPlus v8.5 (GastroPlus v8.5). A one-compartment pharmacokinetic model was utilized to simulate absorption parameters, systemic bioavailability, and levels of chemical in the blood using a nominal single oral dose of 300 mg/kg in a 250 g rat. The dose formulation type was defined to be a suspension. The oral absorption in GastroPlus utilizes the Advanced Compartmental Absorption and Transit model to predict passive absorption across the gut and accounts for soluble and insoluble portions of the administered dose. Bioavailability predictions were made by including metabolism due to three major CYP enzymes (2C, 2D, 3A) in the rat liver. These QSAR predictions of metabolic clearance (enzyme kinetics -Km and Vmax based on recombinant CYP enzymes) were generated using (ADMET Predictor v7.0) from the SMILES representations of the compounds. As there are no models to account for the effect of hydrolysis on carboxylic acid esters [CC(-O)OC] (aliphatic and aromatic) and amides [NH-C(-O)C] (including lactams and cyclic-diamides), these were tagged using a SMARTS query and flagged by writing protocols in Pipeline Pilot. ADMET predictor gives a qualitative estimation of whether a chemical is a substrate for one phase-2 conjugation enzyme, UGT. Chemicals that were tagged positive for any of the UGTs (1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 1A10, 2B7, or 2B15) were considered to be plausible substrates.

Because of the limitations of ADMET Predictor v7.0, compounds such as organometallics (containing tin, silicone, manganese, mercury), coordination complexes (Ziram, Pyrithione zinc), those with >20 ionizable groups (tannins), and coloring agents (red 3, yellow 5, blue 74) were not predicted. Also compounds with fragmented ions such as sodium, chloride, iodide, citrate, acetate, nitrate, benzoate, and mesylate were predicted by ignoring the ion fragments. ADMET Predictor reduces the formal charge or adds an extra proton, if applicable, for these ionic inputs. For compounds which were dimer-like with similar sub-units (eg, Abamectin), the highest value of Fa/F was used. The existing rat oral LD₅₀ values were iteratively re-derived and re-plotted using a percentile factor of predicted Fa/F to examine the potential impact of bioavailability and first-pass metabolism.

**Scatter plots—millimole regression analysis.** Since toxicological effects represent molecular interactions, it is more meaningful to establish a molecular perspective for toxicity comparisons by converting the LC₅₀ and LD₅₀ values from a mass to a molar basis (Delistraty et al., 1998; Rozman et al., 1996). As the compound collection for the *in vitro* versus *in vivo* comparison is devoid of mixtures and undefined substances, such plots were possible by using the conversion based on molecular weight of the compound as calculated “on the fly” using a Pipeline Pilot component (http://accelrys.com/products/pipeline-pilot/). The millimole regression analysis (Spielmann et al., 1999) was performed by plotting the *in vivo* toxicity data in the logarithmic scale against the *in vitro* assay data. TIBCO SpotFire (SpotFire) and Pipeline Pilot (http://accelrys.com/products/pipeline-pilot/) were used to visualize and generate the distribution of chemicals in the correlation plots. The regression coefficients, slopes, and intercepts of the upper boundary of the plots were calculated by categorizing the *in vivo* data in multiple random bins and finding the corresponding AC₅₀ value for the maximum in *vivo* data per each bin. The distribution of the physicochemical properties of the compounds, for example, molecular weight and hydrophobicity, are presented as histograms (Supplementary Information).

Interspecies correlation. The inter-species (rat, fish, and daphnia) distribution of 1854 ToxCast II chemicals according to their concentration in uM causing 50% lethality (LD₅₀ vs. LC₅₀) was studied using scatter plots. The corrected plot of the rat oral data incorporating bioavailability versus fish as well as daphnia was then derived. In addition, the plot of rat intravenous versus fish and daphnia in uM (LD₅₀ vs. LC₅₀) as well as the plot of rat intravenous versus rat oral and corrected rat oral using bioavailability in uM (LD₅₀ vs. LC₅₀) was derived. The distribution of chemicals according to their toxicity (LD₅₀ or LC₅₀ in uM) for all species (rat, fish, and daphnia) against ToxCast in vitro data (AC₅₀ in uM) was then plotted. In addition, the plot of the rat oral toxicity was re-derived after considering the effect of bioavailability and filtering compounds that could be subject to hydrolysis (esters and amides) and substrates for UGTs.

**RESULTS**

Interspecies correlation. We used the CAS numbers from the 1854 compound ToxCast II library to query the public literature and obtained acute toxicity data for 627 (fish), 539 (daphnia), 1375 (oral rat), and 143 (intravenous rat) compounds. Since comparison of acute toxicity between aquatic species and rat is not straightforward due to the difference in dosimetry units for aquatic (molar) and rat (mass per BW), the rat data were re-derived and expressed in units of molarity/body weight. The potential to predict acute toxicity by using read-across between species was high between fish and daphnia (Fig. 1a) or either aquatic species relative to intravenous rat data (Figs. 1b and 1c). However, the potential to predict acute oral rat toxicity by using read-across to either fish or daphnia was poor (Figs. 1d and 1e). The impact of bioavailability and first-pass metabolism after oral administration in rats was examined iteratively to determine whether the potential to use read-across between aquatic and mammalian species improved. Simulated absorption and metabolism using GastroPlus showed that accounting for the fraction of a compound passively absorbed from the GI tract (F) or after first pass metabolism in the liver (Fa) improved the relationship for either aquatic species versus rat oral data (Figs. 2a and 2b). The potential to predict acute oral toxicity in rats by using read-across to the intravenous route was higher (Fig. 3a) than by using read-across to the aquatic species but was also improved after accounting for the impact of oral bioavailability as described above (Fig. 3b).

Comparison of *in vivo* acute toxicity and ToxCast mitochondrial toxicity. Of the 8400 compounds in Tox21, approximately 1000 were tested in ToxCast II and over 800 others in a suite of assays targeting potential endocrine activity (e1k), totaling approximately 1850. The EPA re-derived the activity calls and AC₅₀ values for these 1850 according to ToxCast dose-response curve-fitting criteria and made the data publicly available.

A representative heat-map of acute fish toxicity data ranked from the most-to-least-toxic compounds plotted versus the AC₅₀’s of more-prominently active ToxCast II assays shows a
clustering of assay activity for several assays, particularly those for cytotoxicity and especially mitochondrial inhibition, suggesting potential predictivity (Fig. 4). There were two in vitro mitochondrial toxicity assays, out of which Tox21 Mitochondrial Toxicity Ratio had 385 chemicals deemed active by the EPA.

In vitro mitochondrial toxicity was measured as the AC50 value of an in vitro measurement of MMP using a fluorescent dye. Logarithmic plots of acute toxicity for fish, daphnia, intravenous rat, and oral rat versus MMP showed clear delineation of an upper boundary of acute toxicity for fish ($r^2 = 0.91$), daphnia ($r^2 = 0.82$), and intravenous rat data ($r^2 = 0.68$) but were scattered without an upper boundary for rat oral data ($r^2 = 0.20$) (Figs. 5a–c). Mitochondrial inhibition at low concentrations predicted high acute toxicity and the ability to identify compounds classified under the GHS as Category 1 for fish and daphnia but not the rat where toxicity was often attenuated due to limited absorption and first pass metabolism.

Iterative adjustments of acute oral rat toxicity values as described under the section Interspecies correlation (Supplementary Figs. 1a–c) by simulating fractional absorption and CYP-based metabolism and removing compounds with hydrolysable linkages or flagged as substrates for glucuronidation showed a trend toward delineation of an upper boundary for rat oral toxicity versus MMP ($r^2 = 0.60$) (Fig. 5d).

Approximately 95% of chemicals with acute fish data also had rat oral data (Supplementary Fig. 2). For the chemicals scattered under the upper boundary in the fish versus MMP plot, their primary mechanism of acute toxicity could be due to mechanisms-of-action such as ion channel blockers or acetyl cholinesterase (AChE) inhibitors (Supplementary Figs. 3a and 3b) rather than mitochondrial inhibition. If we could assign a single toxicity mechanism to every chemical and filter out only those chemicals that are predominantly mitochondrial inhibitors, then we might be able to see a true correlation of in vivo versus in vitro toxicity (such an approach would still need to account for the impact of bioavailability).

Several important chemical classes were observed in the fish versus MMP plot. A set of alkylphenols was among the chemicals that are close to the upper boundary of the plot; the mechanism-of-action for these is thought to be ionophoric uncoupling of the MMP (Supplementary Fig. 4a).

Such an upper boundary was also observed for another ToxCast assay from Apredica that measured mitochondrial inhibition using MMP. Logarithmic plot of acute toxicity for fish versus the APR_MitoMembPot_24h_dn assay also showed clear delineation of an upper boundary ($r^2 = 0.72$) (Supplementary Fig. 4b).

A plot of acute fish toxicity versus the QuikProp bioavailability score provided by EPA in the ToxCast II data download showed a general trend of compounds that were highly bioavailable being more toxic and vice versa (Supplementary Fig. 5a). As well, compounds predicted to be poorly bioavailable by QuikProp showed a clearer trend in the potential to conduct read-across to aquatic species for acute rat oral toxicity (Supplementary Figs. 5b and 5c). Although these are novel findings, the QuikProp tool used to calculate bioavailability also accounts for other parameters such as inherent reactivity.

Correlation with hydrophobicity. We also studied the role of hydrophobicity ($A\log P$) against acute toxicity for the chemicals present in the “upper boundary” as its relationship to fish toxicity has been published (Lipnick, 1999; Veith et al., 2009). A scatter plot of $A\log P$ against acute fish and acute daphnia data for the chemicals along the “upper boundary” shows no clear relationship (Fig. 6). The subset of chemicals which were active for the MMP assay for both fish and daphnia plots are found spread from $A\log P$ scale of –2 to 8 (Supplementary Fig. 6).

**In silico identification of mitochondrial inhibitors.** We used 32 compounds previously identified as mitochondrial complex I-V inhibitors (Canipa and Portinari, 2012) (Supplementary Table 1) from the literature as targets to query the 385 active ToxCast compounds using atom-centered fragment structural similarity
first pass metabolism (Walker, 1978). It is also important to point
based on marked attenuation of acute toxicity in mammals due to
aquatic and mammalian species, the major difference is likely
may be due to differences in categorization criteria between
GHS classification distribution.

EPA. Since this was a proof-of-concept article, we relied almost
with those used by jurisdictions such as some branches of the
exposure. When we plotted acute toxicity data for daphnia, fish,
and rat against their respective GHS classification brackets
and (11) chelants. The final model of oral toxicity would take
protein synthesis inhibitors, (9) alkaloids, (10) anticoagulants,
blockers, (6) GABA blockers, (7) adrenergic receptor blockers, (8)
drial inhibitors, (4) cholinesterase inhibitors, (5) ion channel
ways that usually occur relatively quickly involving at most a
few steps. We have begun cataloging a list of mechanisms for
high acute toxicity, some of which cover diverse chemical
domains, including (1) corrosives, (2) reactives, (3) mitochondrial
inhibitors, (4) cholinesterase inhibitors, (5) ion channel
blockers, (6) GABA blockers, (7) adrenergic receptor blockers, (8)
protein synthesis inhibitors, (9) alkaloids, (10) anticoagulants,
and (11) chelants. The final model of oral toxicity would take
the lowest of all of the mechanism-specific estimates of mini-
mal toxicity. We assume that if a compound becomes systemi-
cally bioavailable, these mechanisms will apply but that many
orally administered compounds will show poor absorption and
undergo marked first pass metabolism. Most of these mecha-
nisms encompass specific targets rather than general physical
targets. Some mechanisms such as corrosivity involve materials
becoming systemically bioavailable based on their intrinsic
physicochemical properties. We chose to study mitochondrial
inhibition because of its precedence in acute toxicity and
because it was represented in ToxCast by many active com-
pounds. We generated a heat-map of the AC50’s for many of the
active ToxCast assays versus acute fish toxicity data that pro-
vided a clear visual relationship to inhibition of MMP.

Mitochondria are complex self-replicating organelles that
evolved to contribute pivotal functions in an integral symbiotic
relationship so it is understandable they are promiscuous tar-
gets with polypharmacologic inhibitors. As shown by the AOP
for mitochondrial toxicity, disruption of MMP occurs by broad
and diverse chemical classes, with each complex in the ETC
known to be inhibited by more than one chemical class (Canipa
and Portinari, 2012). Acidic alcohols and phenols can be

**DISCUSSION**

Some geographies and regulatory jurisdictions require acute
toxicity testing in multiple species and routes with almost no
opportunity for alternative approaches. It is necessary to under-
stand a compound’s acute toxicity to address inadvertent expo-

search criteria using Tanimoto’s coefficient (Tanimoto, 1957)
and DICE coefficient (Dice, 1945). Only two of the ToxCast com-
pounds were identified. Similar calculations with a Pipeline
Pilot component using Tanimoto similarity searches gave the
same results. When we used the Pipeline Pilot similarity proto-
col to query 36 drugs reported as mitochondrial toxicants from
the literature (Supplementary Table 2), six were identified.

GHS classification distribution. There was a nearly equal distrib-
uity across different species using bioa-
availability fraction (f) and removal of esters, amidase assuming hydrolysis (LD0)
will be different than the parent), and uridine-5’-diphosphoglucuronosyl trans-
ferase (UGT) substrates. Log plots of (a) adjusted rat oral LD50 (µM/kg) versus
fish 96 h LC50 (µM/l) and (b) adjusted rat oral LD50 (µM/kg) versus daphnia 48 h
LC50 (µM/l).

![Figure 2](Image)

**FIG. 2.** Scatter plot of adjusted in vivo data between different species using bioa-

vailability fraction (f) and removal of esters, amidase assuming hydrolysis (LD0)
will be different than the parent), and uridine-5’-diphosphoglucuronosyl trans-
ferase (UGT) substrates. Log plots of (a) adjusted rat oral LD50 (µM/kg) versus
fish 96 h LC50 (µM/l) and (b) adjusted rat oral LD50 (µM/kg) versus daphnia 48 h
LC50 (µM/l).
Compounds that chelate iron or copper, metals that help transfer electrons in the ETC, can be acutely toxic when systemically distributed (Horn and Barrientos, 2008). Few notable works on correlation for baseline toxicity was established or because GastroPlus does not currently identify metabolic fate to read-across to either in vitro mechanistic data or between species or routes. It also points out the point that they can be used to train mechanistic QSARs. Currently, there are no AOPs focused on just acute toxicity. As mechanistic AOPs are further developed and refined, these can be incorporated into an overall AOP framework for acute toxicity. Then only the regional chemical structural features domains are needed for use in deriving suitable QSAR models in the future. Such models will likely largely consist of a suite of regional models packaged together under a global heading.

FIG. 3. Scatter plot of in vivo data between rat oral and intravenous. Log plots of (a) rat oral LD₅₀ (µM/kg) versus rat intravenous LD₅₀ (µM/kg) and (b) adjusted rat oral LD₅₀ (µM/kg) using bioavailability fraction (f) and removal of esters, amidines assuming hydrolysis (LD₅₀ will be different than the parent), and uridine-5'-diphosphoglucuronosyl transferase (UGT) substrates versus rat intravenous LD₅₀ (µM/kg).

We showed that potent inhibition of MMP can be used as a stand-alone-assay to predict high acute toxicity; at low test material concentrations high acute GHS toxicity classifications were predicted for fish and daphnia but not the rat. Although there was a nearly equal distribution of studied compounds across GHS classes for the aquatic species very few were in GHS acute mammalian toxicity categories I-III, likely due to marked attenuation of acute toxicity in mammals due to first pass metabolism. This highlights the need for refined approaches to predict route-specific systemic bioavailability that account for a compound’s metabolic fate to read-across to either in vitro mechanistic data or between species or routes. It also points out that read-across to specific mechanistic endpoints will by necessity only apply when the toxicity is driven by that mechanism alone, which will be the case for instances when low doses drives high toxicity.

Bioavailability and metabolism are currently only superficially represented in HTS approaches or in silico models. Bioavailability is influenced by passive and active transport across bio-membranes, protein binding, and metabolic transformation. At least four classes of metabolic reactions are important to acute toxicity: (1) hydrolysis, (2) conjugation, (3) oxidation/reduction, and (4) Cyp-mediated transformations. All can be represented in vitro but only the Cyp-mediated transformations have partially characterized in silico counterparts, which are often only qualitative. Hydrolysis of a test material may occur under facile, acidic, basic, or enzymatic conditions but the ability to model these in silico is poor except at neutral pH. We tried to account for the impact of bioavailability and metabolism in the rat by iteratively adjusting the acute oral toxicity values by simulating fractional absorption and CYP-based metabolism and removing from the dataset any compounds with hydrolyzable linkages or flagged as substrates for glucuronidation. Each adjustment better delineated an upper boundary to the toxicity curve when rat oral toxicity was plotted against MMP. Comparison of in vitro AC₅₀ values that did not include metabolism to the bioavailability-adjusted LD₅₀ values can be rationalized by assuming the in vitro data represent 100% bioavailability to the target (which is an overly simplistic assumption), whereas the acute toxicity would be attenuated proportional to in vivo bioavailability for stable nonhydrolyzed compounds. Using the same rationale, it was necessary to remove from the dataset those compounds with potentially hydrolyzable substructures such as esters and amidines or prone to elimination without systemic bioavailability, such as UGT substrates. Compounds containing other potentially labile substructures (eg, imines, oximes, or hydrazones) or likely substrates for conjugation via sulfation or with glutathione were not removed either because their metabolic fragility is less-well established or because GastroPlus does not currently identify potential substrates for other types of conjugation.

We tested three QSAR models for acute mammalian toxicity and all were poor, especially for GHS category I–III compounds (manuscript in preparation). This is in agreement with a recent publication looking at similar global models (Diaz et al., 2015). The models are global and purely statistical; there are currently very few mechanistic QSAR models for acute mammalian toxicity. Few notable works on correlation for baseline toxicity was published (Lipnick, 1999; Veith et al., 2009). Examining our catalyzed mechanisms for high acute toxicity suggests that a global statistical model would not accommodate predictions that are largely pharmacologic unless each class was highly represented in the training set. The added complexity of marked attenuation of acute toxicity due to hydrolysis and first pass metabolism further emphasizes the need for a mechanistic QSAR model with many regional domains that accounts for pharmacologic and pharmacodynamic nuances of diverse compounds. Until such a model is developed and validated, alternative read-across approaches are needed. Curated acute toxicity data with enough compounds in the mechanistic and structural domains are needed for use in deriving suitable QSAR models in the future. Such models will likely largely consist of a suite of regional models packaged together under a global heading.
FIG. 4. Heat-map showing the correspondence of acute fish toxicity for 627 chemicals (rows) and 285 assays (columns). The assays are clustered based on similar mechanism-of-action or same receptor target producing several blocks in the heat-map for trend analysis. They are sorted against decreasing acute fish toxicity (96 h LC₅₀) data shown by red-pink-blue-green (or by decreasing shades of grey in b/w print) bar on the left-most column which represents Globally Harmonized System (GHS) 1-4 for aquatic species, respectively. Assays with <10 chemicals active were not included. Assay potency is represented in legend on the top-right as red, yellow, and green (or by decreasing shades of grey in b/w print) to encode high, medium, and low potency, respectively. Full color version available online.
FIG. 5. Scatter plots of Tox21 mitochondrial toxicity assay (μM) against different species. Log plots of mitochondrial inhibition (μM) versus (a) fish 96 h LC50 (μM/l) data; (b) daphnia 48 h LC50 (μM/l) data; (c) rat intravenous LD50 (μM/kg); (d) rat oral LD50 (μM/kg); and (e) adjusted rat oral LD50 (μMol/kg). The mitochondrial inhibition assay predicts an upper boundary to fish, daphnia and rat intravenous toxicity but not to the rat oral LD50 (chemicals could cause higher toxicity by another mechanisms but not lower, hence the expected scatter in the data below the boundary). The predictability is improved by the adjustment of rat oral LD50 using the bioavailability fraction (F) and removal of esters and amides assuming hydrolysis (LD50 will be different than the parent) and uridine-5’-diphosphoglucuronosyl transferase (UGT) substrates (see Supplementary Material for upper boundary correlation coefficient calculation).
compounds were identified. When we used a similarity search to query 36 drugs reported as mitochondrial toxicants from the literature, six were identified. This suggests many more compounds than were previously known inhibit mitochondria but current technology allows their identification only in vitro.

There is great enthusiasm for the development of alternative nonanimal approaches in toxicity testing. Our findings represent a milestone and highlight the promise of such approaches but also the need for refined tools that address systemic bioavailability. Various reports in the literature discuss the potential for read-across between species or the use of in vitro data to predict acute toxicity. This is the first instance of laying the framework for broader approaches to read-across that includes interspecies and mechanistic HTS data and also highlights the importance of addressing systemic bioavailability. This study demonstrates the potential for using nontraditional read-across approaches to predict acute toxicity in multiple species.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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