Chemical Research in Toxicology

PERSPECTIVE

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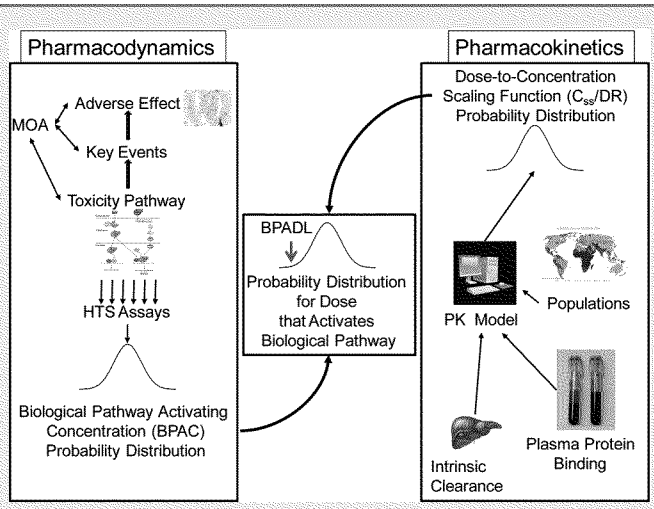
Estimating Toxicity-Related Biological Pathway Altering Doses for High-Throughput Chemical Risk Assessment

Richard S. Judson,*,† Robert J. Kavlock,† R. Woodrow Setzer,† Elaine A. Cohen Hubal,† Matthew T. Martin,† Thomas B. Knudsen,† Keith A. Houck,† Russell S. Thomas,‡ Barbara A. Wetmore,‡ and David J. Dix†

†National Center for Computational Toxicology, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711, United States

‡The Hamner Institutes for Health Sciences, Research Triangle Park, North Carolina 27709, United States

ABSTRACT: We describe a framework for estimating the human dose at which a chemical significantly alters a biological pathway in vivo, making use of in vitro assay data and an in vitro-derived pharmacokinetic model, coupled with estimates of population variability and uncertainty. The quantity we calculate, the biological pathway altering dose (BPAD), is analogous to current risk assessment metrics in that it combines dose - response data with analysis of uncertainty and population variability to arrive at conservative exposure limits. The analogy is closest when perturbation of a pathway is a key event in the mode of action (MOA) leading to a specified adverse outcome. Because BPADs are derived from relatively inexpensive, high-throughput screening (HTS) in vitro data, this approach can be applied to high-throughput risk assessments (HTRA) for thousands of data-poor environmental chemicals. We envisage the first step of HTRA to be an assessment of in vitro concentration - response relationships across biologically important pathways to derive biological pathway altering concentrations (BPAC). Pharmacokinetic (PK) modeling is then used to estimate the in vivo doses required to achieve the BPACs in the blood at steady state. Uncertainty and variability are incorporated in both the BPAC and the PK parameters and then combined to yield a probability distribution for the dose required to perturb the critical pathway. We finally define the BPADL as the lower confidence bound of this pathway-altering dose. This perspective outlines a framework for using HTRA to estimate BPAD values; provides examples of the use of this approach, including a comparison of BPAD values with published dose - response data from in vivo studies; and discusses challenges and alternative formulations.



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INTRODUCTION

Chemical risk assessment and risk management require information on hazard, dose response, use, and exposure to make decisions protective of human health and the environment. One objective of a chemical risk assessment is to identify exposure levels with a reasonable certainty of no harm. Exposures resulting from the use of a chemical that are below these estimated levels are presumed to have a reasonable certainty of no harm[1] or to be without appreciable deleterious effects during a lifetime.[2] Hazard-based limits currently used to inform risk management include quantities such as the reference dose (RfD) for noncancer effects. An RfD is generally derived by estimating the lowest human-relevant point of departure (POD) which may be a NOAEL (no observed adverse effect level) or BMD (benchmark dose) from a set of laboratory animal studies, commonly in rodent and non-rodent species. These are then divided by default factors often in the range of 100 to 1000 to account for uncertainty in cross-species extrapolation, possible database deficiencies which might lead to a failure to identify the most sensitive endpoint, and variability across human populations and life-stages. Where available, human data (e.g., from epidemiological studies) is incorporated into estimates of acceptable exposures.

EPA defines an RfD as representing "... the quantity of a substance which if absorbed on a daily basis over a lifetime, is not expected to pose significant risk of adverse health effects."[3] Alternatives to an RfD, used in certain decision contexts, include allowable daily intake (ADI) and threshold of toxicological concern (TTC).[4] An important component of many risk assessments is the identification of the mode of action (MOA) leading to the critical effect, which is the adverse effect with the lowest NOAEL or BMD.[5-7] Identifying the MOA is important because some MOAs are known to operate in model species but not humans (or vice versa), meaning that the related adverse effect could be discounted (or would have to be accounted for) when determining the human RfD. In addition, some MOA (such as genotoxic carcinogenicity) are assumed to imply no safe threshold dose; therefore, a different risk assessment approach is called for. An important link between MOA and pathway-based analyses is the tenet that it is sometimes possible to relate MOA key events with perturbations of specific pathways.

Current risk assessment approaches for the majority of chemicals face many challenges,[8] including heavy reliance on data from animal studies. In this perspective, we outline one possible alternative formulation for determining permissible exposure levels from in vitro high-throughput screening (HTS) data and informatic analysis. The resulting information could serve as a surrogate for acceptable dose levels derived from animal toxicity studies until such studies are available. Such formulations are desirable because there are thousands of environmental chemicals for which animal data is limited or not available,[9] a situation that is unlikely to change in the near future. Any alternative to the current human health risk assessment approach has to meet several criteria. First, it should be based on an understanding of the modes or mechanisms leading to toxicity, specifically in humans. Second, it should yield relevant dose - response predictions that can be used for setting permissible human exposure levels. Third, it should be at least as health protective as current approaches without imposing unnecessarily strict limits on chemical use.

Over the past decade, in vitro toxicity testing approaches have been widely implemented. In these approaches, chemicals are evaluated using a single or a battery of in vitro assays that probe biological pathways relevant to toxicity. In vitro toxicity testing has been advocated for use in the evaluation of environmental chemicals[10] and is being implemented at the U.S. EPA and NIH through their ToxCast[11,12] and Tox21[13,14] programs. The in vitro toxicity testing approach has several key advantages: (1) the cost is orders of magnitude less than that for animal testing; (2) human molecular targets and cell systems can be directly studied; and (3) hundreds or thousands of chemicals can be analyzed in parallel. Using HTS in vitro methods in hazard assessment screening would address the question, is there a mechanism by which a chemical can lead to a particular adverse effect? Assays are typically run in concentration - response format; therefore, one can estimate the relative potency (i.e., effective concentration) of different chemicals to perturb biological pathways.[12]

Here, we couple the ability of in vitro assays to quantitatively characterize the pharmacodynamics (PD) of a chemical in concentration - response mode with new high-throughput methods for estimating the corresponding pharmacokinetics (PK) of a potential toxicant.[15] By combining these two types of information, we can estimate the external dose that would be required to perturb a biological pathway. In order to complete the analogy with standard risk assessment approaches, we need to incorporate uncertainty and variability into the model. One can then calculate a provisional acceptable exposure level at the low end of the distribution of the pathway-altering dose accounting for uncertainty and variability. We define this value as the biological pathway altering dose or BPAD. The overall process of estimating the BPAD we define as high-throughput risk assessment (HTRA).

The goals of this perspective are to outline a framework for using HTRA to estimate BPAD values; to provide examples of the use of this approach, including comparisons of BPAD values with published lowest effect levels (LELs) and no effect levels (NELs) from animal toxicity studies; and to discuss challenges and alternative formulations. This perspective proposes and evaluates a framework for HTRA and identifies incomplete or unresolved issues, as a first step toward developing an HTRA model for decision-making. The immediate goal of HTRA as described here is not to replace standard risk assessment methods, but instead to provide input into provisional risk assessments for data-poor chemicals. These provisional estimates can then be used to prioritize further study of specific chemicals and could be updated as this new information is collected.

HTRA FRAMEWORK OUTLINE

Our initial goal is to estimate chemical-specific biological pathway-altering doses or BPADs. A BPAD is tied to a particular biological pathway and therefore is analogous to an estimate of a mechanism or MOA-specific LEL or NEL, with the addition of uncertainty and population variability estimates. Although we do not address the equivalent problem of estimating exposure in a high-throughput manner, we recognize that this metric is of comparative importance. Here, we simply outline the key points of the HTRA-BPAD approach, which are illustrated in Figure 1. Implementation details are given in subsequent sections.

1. HTRA is built around biological pathways whose structure is derived from a large body of in vitro and in vivo studies. A number of publicly available biological pathway databases[16-18] exist to guide a selection for use in HTRA.

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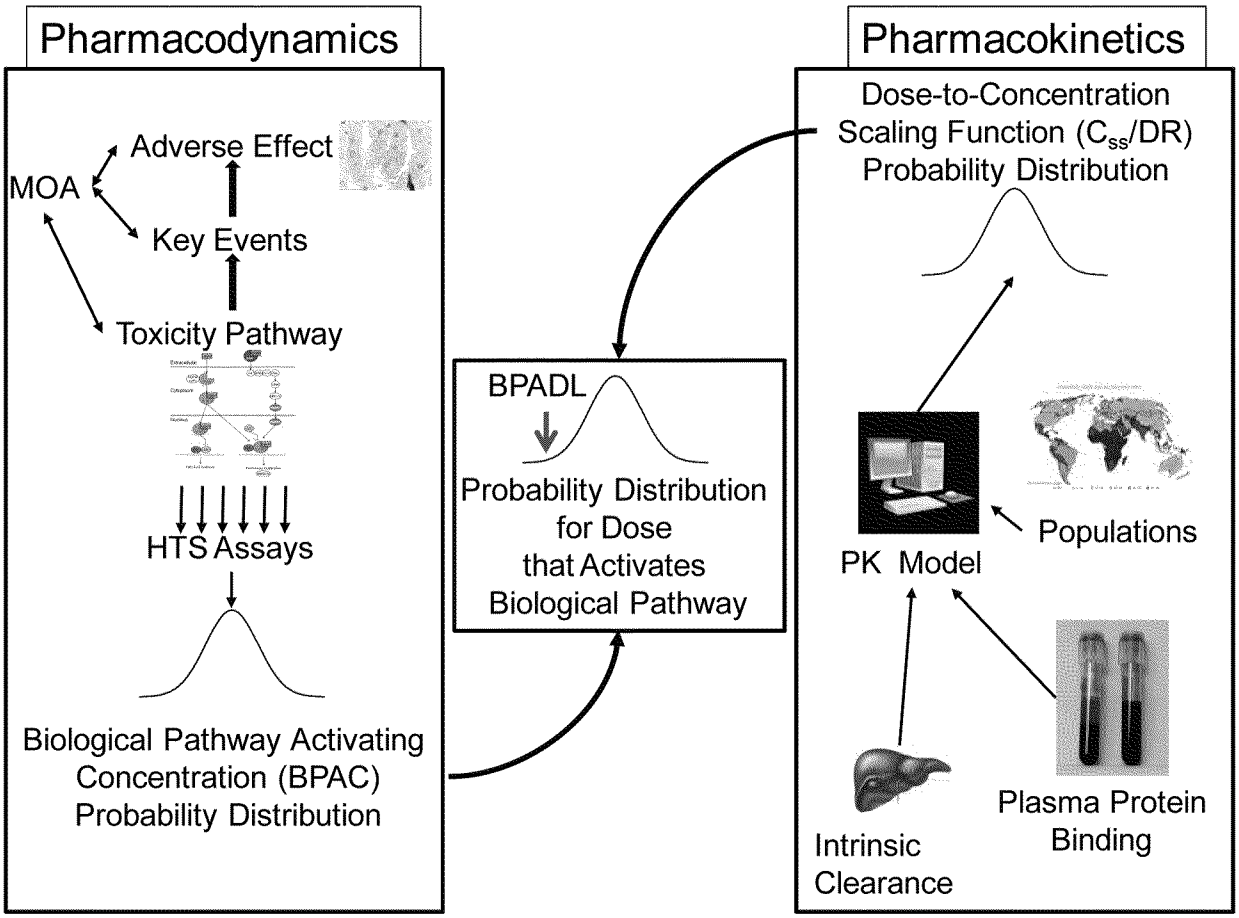

Figure 1. Schematic of the high-throughput risk assessment (HTRA) process, coupling in vitro assay data quantitatively characterizing the pharmacodynamics (PD) of a chemical with high-throughput methods for estimating the corresponding pharmacokinetics (PK) of a potential toxicant. See text for a full description of the HTRA process.

2. For HTRA, we want to focus on toxicity-related biological pathways, i.e., those which, when significantly altered by chemical exposure, are likely to lead to adverse effects in vivo.[10] The distinction between all pathways and toxicity-related pathways is analogous to the distinctions between observations leading to NELs and NOAELs in traditional toxicity testing. An important area of research involves determining linkages between pathways and adverse effects. We purposely avoid the term "toxicity pathway" because there is no real distinction between these and normal biological pathways. Operationally, one way we define "toxicity-related pathways" is by finding associations (either using statistical techniques or from detailed mechanistic analysis) between the perturbation of a pathway or process and the development of adverse outcomes.
3. For each pathway, we select a representative set of targets to probe and develop in vitro assays to measure effects related to those targets. For the examples given here, these targets were selected partly by expert judgment and partly by the availability of off-the-shelf high-throughput assays. Although assays derived from other species that share significant sequence similarity at the specific target gene can also be used, we would primarily focus on human targets and cells for human risk assessment. Assays can be as simple as binding to a single protein or as complex as whole genome microarrays or other genome-scale measurements.
4. All assays must be run in concentration - response format in order to yield values for the BPAC (biological pathway altering concentration).
5. It may be necessary to run several assays associated with a pathway and use a systems-level model to integrate the resulting PD data for estimating BPAC.[19] This model may need to account for some assays being overly sensitive (yielding false positives) and some being under-sensitive (yielding false negatives.) This model should also estimate the PD-related uncertainties and population variability[20] and must characterize the population distribution of the BPAC and its uncertainty using probability distributions.
6. Population-PK modeling is used to estimate the external dose through the relevant route of exposure that would lead to the internal BPAC (dose-to-concentration scaling function). The PK model must also estimate PK-related uncertainties and variability, and then yield a probability distribution for the dose-to-concentration scaling function. In the case of the examples provided here, an oral dose leading to the internal BPAC was used.
7. The PD and PK probability distributions are then combined to yield a probability distribution of the dose at which the chemical would significantly perturb the biological pathway. We calculate a mean value and confidence intervals from this distribution and set the BPAD to be the lower dose boundary of the confidence interval.

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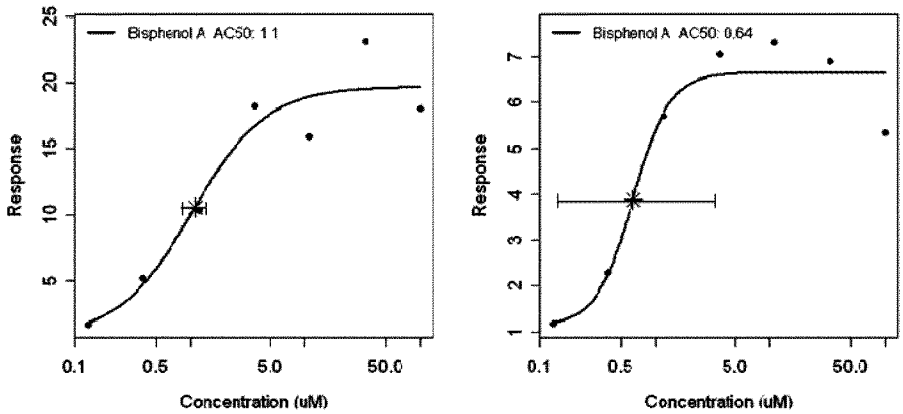

Figure 2. Example concentration - response curves for Bisphenol A in two estrogen receptor assays from the ToxCast program. These assays use multiplexed reporter gene technology in a trans-activating mode (left) and cis-activating mode (right).[22] The y-axis is in units of fold-change. Determination of the AC_{50} (denoted by the horizontal bar with error bands) and associated confidence intervals factor into uncertainties in the estimation of the BPAC.

BIOLOGICAL PATHWAYS AND THEIR LINKAGE TO ADVERSE EFFECTS

Biological pathways are a key connection between MOA-based risk assessment and HTRA when they are mechanistically linked to key events in toxicity modes of action.[21] Pathway-level perturbations can be assessed by testing chemicals in vitro using a suite of assays that measure molecular targets and downstream consequences in the pathway, e.g., binding to key enzymes or receptors, or differential regulation of downstream genes or proteins. By running assays in concentration - response format, one can derive a characteristic concentration (e.g., AC_{50} or concentration at which activity is 50% of its maximum) for each chemical - assay pair. Additionally, in contrast with in vivo studies, it is possible (at least in selected cases) to measure response at arbitrarily closely spaced concentrations and to measure response down to very low concentrations. This eliminates the need to perform low-dose extrapolation using an assumed model: the low dose end of the curve is directly measured. Figure 2 shows examples of concentration - response data, in this case derived from a pair of estrogen receptor assays.[22]

In some instances, there may be a direct link known between an MOA and a corresponding pathway. An example is cholinesterase inhibition. The in vivo key event is measurable cholinesterase inhibition in a blood or tissue sample. The in vitro pathway perturbation is measured by inhibition of cholinesterase activity in a cell-free or cell-based assay. A more complex example is liver hypertrophy driven by peroxisome proliferation.[23-25] in vivo, histopathology can clearly detect peroxisome proliferation. In vitro, numerous assays can measure activity in the underlying peroxisome proliferator-activating receptor (PPAR) pathway.

IN VITRO SCREENING, BIOLOGICAL PATHWAYS, AND THE BIOLOGICAL PATHWAY-ALTERING CONCENTRATION (BPAC)

In the first step of HTRA, we use HTS in vitro assays to measure the extent of chemical-induced perturbation of a biological pathway as a function of concentration. As described above, assays can measure direct binding to key targets, downstream changes of specific biomarkers, or cellular consequences such as cell shape changes or cell death. In some cases, it will be possible to use a single assay to measure pathway perturbation (e.g., cholinesterase inhibition), while in others one may need to integrate over the results of multiple assays. The use of microarrays to estimate pathway perturbation is an alternative or complementary approach for cases where relatively few chemicals need to be examined.[26] HTS-based pathway-based assays offer an alternative and promising technology for screening thousands of chemicals in parallel.[27]

A significant advantage of the current HTS assays is that the majority can be run against human targets or in human cells. This eliminates the need for cross-species extrapolation, but still requires in vitro to in vivo extrapolation. There are many ways to estimate the BPAC, but it remains a challenge to determine which is most appropriate. For the illustrative examples presented below, we used a simple method for estimating the BPAC. This approach takes the collection of assays that map to pathway genes or relevant cellular phenotypes and sets the BPAC for the specified chemical to be the minimum AC_{50} for any of those assays (See Figure 2).

Finally, we need to address uncertainty and variability in our estimate of the BPAC. It is desirable to separately characterize the population variability of the BPAC and its uncertainty because risk assessment uses variability and uncertainty information in different ways. Ideally, population variability information would be available for this analysis, along with a characterization of the uncertainty about that variability. For our examples, and as a suggested default in the absence of an estimate of population variability, we assumed that the population distribution of the BPAC is log-normal and that the ratio of the geometric mean (or, equivalently, the median because of the assumption that the population distribution is log-normal) to the first percentile of the population distribution is $(10)^{1/2}$. This factor is motivated by partitioning the conventional 10-fold uncertainty factor for variability among people into equal-sized portions due to pharmacodynamics and pharmacokinetics. Not all regulatory bodies use the same partition, and this particular factor is intended for illustration, not prescription. Alternative approaches to assessing variability[28] have been based on estimates of appropriate variances from collections of human data.

We do not have a statistically rigorous characterization of the uncertainty about the parameters of this assumed population distribution, but for illustrative purposes, we assume the logarithm of the population median and log-scale standard deviation

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are known to within about a factor of 2 (strictly, that the 1st and 99th percentiles of the uncertainty distribution are 4-fold apart and that the uncertainty distribution is log-normal). Further work needs to be done to better characterize the variability and uncertainty of these parameters.

FROM IN VITRO CONCENTRATION TO IN VIVO DOSE: REVERSE TOXICOKINETICS

This section addresses the task of estimating the in vivo dose that corresponds to the BPAC. A variety of PK models can be used to estimate internal concentration from external dose (or exposure). These models can be reversed to yield the dose (exposure) corresponding to the BPAC. For the present purpose, we require a method that is general enough to be used on a large number of chemicals. The most detailed PK models, usually called physiologically based pharmacokinetic (PBPK) models,[29,30] represent distribution, metabolism, and excretion of a chemical using multicompartment models that account for partitioning between multiple organs and tissues. One challenge to using detailed PBPK models lies in identifying the structure of the model and the values of the corresponding coefficients for each chemical. Generating a complete "validated" model can take years and require the generation of a significant amount of chemical-specific experimental data.

An alternative to detailed PBPK modeling is to use simpler models that make conservative assumptions and require a small number of parameters whose estimates are amenable to high-throughput data generation. One class of models is purely computational, where all parameters are computationally generated, usually through quantitative structure activity relationship (QSAR) models. These models have been used to estimate body burden of chemicals that are not metabolized but which bioaccumulate.[31] Here, we focus on an alternative approach more suitable to chemicals that are largely eliminated through metabolism and renal excretion, which is called reverse toxicokinetics (RTK) or reverse dosimetry.[15,32,33] In this approach, we use a one compartment model and make default assumptions such as chemicals are eliminated wholly through metabolism and renal excretion; renal excretion is a function of the glomerular filtration rate and the fraction of unbound chemical in the blood (i.e., no active transport); and there is 100% oral absorption. Using these assumptions in our published RTK analysis,[15] we only required two experimental chemical-specific parameters to generate an estimate of the plasma concentration of the chemical at steady state per unit dose. These are the rate of disappearance of parent via hepatic metabolism (called intrinsic clearance) and fraction bound (or conversely unbound) to plasma proteins. Both of these parameters can be measured experimentally in a relatively high-throughput manner.

The result of this effort is a chemical-specific ratio of the concentration at steady state (C_{ss}) divided by the dose rate (DR), yielding a concentration-to-dose scaling factor with units of μM/(mg/kg/day). One simply divides the BPAC by the C_{ss}/DR ratio to calculate the steady-state dose required to yield a steady-state BPAC. The estimate of the C_{ss}/DR ratio implicitly contains uncertainties; for instance, the assumption of 100% oral absorption and the assumption that the concentration at the site of action will equal the concentration in plasma. There are also uncertainties in the measurements of experimental values for fraction unbound and intrinsic clearance. Population variability in PK arises from several factors including genetic differences in xenobiotic metabolizing enzymes and heterogeneity of liver mass. The PK software application we use (SimCyp[32,33]) allows us to directly include some sources of population variability, but the current model does not explicitly account for model and parameter uncertainty.

FROM ACTIVITY DOSE ESTIMATES TO THE BIOLOGICAL PATHWAY-ALTERING DOSE (BPAD): INCORPORATING UNCERTAINTY AND VARIABILITY

We have described how to estimate the concentration at which a biological pathway of interest is activated (BPAC), plus the C_{ss}/DR ratio which scales internal concentrations to oral doses. The dose that corresponds to the BPAC is simply BPAC/C_{ss}/DR. While this gives a central estimate, we need to account for uncertainties and variability in each of the estimated values and their resulting ratio.

Our PD estimates are subject to uncertainty. For the in vitro assays, we know that there is statistical noise in the data, which will lead to uncertainties in estimates of the AC_{50}. For some pathways, there may be important biological activity well below the AC_{50}, while for other pathways, relevant in vivo effects will only occur when the assay target is fully activated (or inhibited), well above the AC_{50}. Assays in some cases will yield false positive or false negative results due to a variety of assay artifacts which are not always easy to detect.[34] Further, assays currently in use may not detect the most sensitive signal of pathway activation.

Likewise, the estimates of PK parameters are uncertain. There are uncertainties in the estimates of the experimental parameters such as intrinsic clearance and plasma protein binding. Any PK model will have to make assumptions about the structure of the model used for a given chemical (e.g., in number and types of compartments). For instance, our RTK method assumes that estimated blood concentrations are a good surrogate for the in vitro media/buffer concentrations in the HTS assays.

There is population variability surrounding PD, for example, due to the genetic variation of an enzyme or receptor to which the chemical binds and which then triggers downstream pathway-based processes. As already mentioned, there is significant PK population variability, for instance in xenobiotic metabolism, due to intrinsic genetic variation, and variability due to life stage, health status, and other factors.

Since we expect population variability, and uncertainty about it, in both the BPAC and C_{ss}/DR, the same will be true for their ratio, the BPAD. For purposes of hazard characterization, we propose to set the critical value of the BPAD to correspond to a small percentile, say p%, of the population distribution of the BPAD (designated the $BPAD_{100-p}$ because (100 - p)% of the population would exceed that level, and therefore, in some sense p% would be protected from that level of exposure) and use that level and its lower 95% confidence bound ($BPADL_{100-p}$) to characterize a chronic dose suggested to be of concern. Technically, $BPAD_{100-p}$ is a permissible exposure level that accounts for population variability, and $BPADL_{100-p}$ is the permissible exposure level additionally accounting for uncertainty. For the examples presented in the next section, we assume the population distribution of the C_{ss}/DR is log-normal, and we estimate the population geometric standard deviation from the confidence limits. The ratio BPAC/C_{ss}/DR is then also log-normal. As for the BPAC, we presume to know the parameters of the population distribution of C_{ss}/DR (the geometric mean and standard deviation on the log scale) to within a factor of 2. We focus on the $BPAD_{99}$ and use Monte Carlo sampling

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to generate a confidence interval for the $BPAD_{99}$, allowing us to calculate $BPADL_{99}$.

The center of the BPAD distribution is analogous to an LEL, although it is explicitly the dose at which one would expect to see 50% of maximal perturbation for the pathway. The BPADL for a particular pathway is analogous to a NEL (no effect level) divided by safety factors. The NOAEL from an animal study is the lowest NEL over all effects that are considered by a particular regulatory agency to be adverse and relevant. To make an HTRA analogue to the NOAEL and the NOAEL-related RfD, we need to classify pathway perturbations as adverse or not. Adversity is an important issue requiring more research and eventually policy development to identify the relevant, adverse minimum BPADL for a chemical or stressor. Initially, we foresee the primary utility of HTRA in prioritization of chemicals for targeted testing based on pathway-derived BPADL values and would do this based on our confidence that a particular pathway's perturbation is linked to adversity.

EXAMPLE 1: BISPHENOL A ESTROGENICITY IN VITRO VS IN VIVO REPRODUCTIVE TOXICITY

As a first example, we consider the estrogenicity of Bisphenol A (BPA) relative to the reproductive toxicity of BPA.[35,36] BPA is a high production volume (HPV) chemical widely used in manufacturing polycarbonate plastics and epoxy resins, and humans appear to be exposed primarily through food packaging uses.[37] This is a useful first illustration of the BPAD approach because of the direct link between activity at a single molecular target, the estrogen receptor (ER) ESR1 (formerly known as ERalpha) that can be measured in vitro, and an in vivo effect observed in a rat reproductive model. BPA in vitro pharmacology identifies it as an ER agonist in all six relevant ToxCast assays (listed in Table 1). If we assume that the molecular key event leading to positive findings in female rats from the multigeneration reproduction test is due to BPA estrogenicity, then the ER BPAD should provide an estimate of the corresponding in vivo LEL and NEL. The ToxCast assays provided six ER agonist or binding AC50 values ranging from 0.6 to 1.7 μM.[12] To calculate a conservative BPAD, the lowest ToxCast AC50 is selected (0.64 μM for Attagene Factorial cis ERE assay). We then consider population variability in both the BPAC and the C_{ss}/DR, and the uncertainty about estimates of the population parameters. The assay results used here are not directly amenable to producing estimates of population variability; however, it has become standard practice to quantify variability in the human population with a 10-fold uncertainty factor, comparing the population median to a lower quantile (for our purposes, the 1%ile). This is generally further divided into a PD and PK component.[8] For illustration purposes, we next estimate the PD variability, while the PK variability is explicitly incorporated in the SimCyp confidence intervals. For a log-normally distributed variable, this corresponds to a standard deviation on the log scale of 0.49. The median of the estimated population distribution of C_{ss}/DR is 0.29, with an estimated standard deviation on the log scale of 0.39. The uncertainty of these values is not currently quantified, but for illustrative purposes, we assume the values are relatively uncertain, with the coefficient of variation of the uncertainty distributions at about 36% (corresponding to knowing the value of the parameter to within about a factor of 2). Monte Carlo sampling from log-normal distributions around the estimated population parameters gives a $BPAD_{99}$ of 0.44 mg/kg/day, with lower one-sided confidence limit, $BPADL_{99}$, of 0.16 mg/kg/day.

In vivo, Tyl et al. found diminished female reproductive performance and decreased ovarian weight in the rat reproduction test at 500 mg/kg/day and an NEL of 50 mg/kg/day.[36] The NEL is adjusted for uncertainty/variability (NEL/100) to yield a value of 0.5 mg/kg/day, close to the in vitro ER $BPADL_{99}$ of 0.16 mg/kg/day.

EXAMPLE 2: CONAZOLE CAR/PXR ACTIVITY IN VITRO VS IN VIVO HEPATOTOXICITY

To further illustrate the ideas in the previous sections, we applied HTRA to a set of conazole fungicides. One concern with conazoles is that many of them cause a variety of liver toxicities in rodents, including hypertrophy and tumors.[38,39] One pathway activated by most conazoles, and believed to be involved in these liver pathologies, is the constitutive androstane receptor/pregnane X receptor (CAR/PXR) signaling pathway.[40,41] In the ToxCast project,[12] we evaluated 14 conazoles in a large battery of assays, many of which map to the CAR/PXR pathway. We also converted in vitro AC50 values from the ToxCast assays (concentration response) to equivalent in vivo values for humans using RTK. As described above, we calculated the BPAD distribution corresponding to the lowest AC_{50} across the CAR/PXR-related assays in ToxCast, listed in Table 1. We then compared the BPAD distribution with liver hypertrophy-related LEL, NEL, and NEL/100 values derived from rat and mouse 2-year chronic/cancer studies. Liver hypertrophy alone is not considered an adverse effect that would lead to a LOAEL (lowest observed adverse effect level) and NOAEL; therefore, in this case, we used LEL and NEL (lowest and no effect levels, respectively). Using data from both mouse and rat chronic studies,[42,43] we identified the lowest dose at which either liver hypertrophy or liver weight increase was observed, yielding the liver-hypertrophy LEL. We then set the corresponding NEL to be the dose below the LEL or LEL/10 if the effect was observed at the lowest dose tested.

The results of this comparison are shown in Figure 3. For each chemical, we show a box corresponding to the variability-derived (1% - 99%) confidence interval around the median BPAD and whiskers giving the uncertainty-derived 95% confidence intervals around the ends of the uncertainty range. The $BPADL_{99}$ value is designated with a red circle; the LEL with a blue box; the NEL with a gray triangle; and NEL/100 with a red triangle. We also show the estimated exposure levels based on food residues, all of which are well below the $BPAPL_{99}$ values. Note that for two chemicals (iprodione and imazalil), exposure estimates were not available.

A first observation is that in most cases the $BPADL_{99}$ is within a factor of 10 of the NEL/100, which lends confidence to the use of this approach in more general cases. Using a Kendall rank-correlation test, we see a significant correlation between $BPADL_{99}$ and NEL/100 ($p = 0.025$). This is of particular interest given wide uncertainties going into both estimates. Second, in 9 of 14 cases, the $BPADL_{99}$ is at or below the NEL/100. This suggests that we can potentially use the $BPADL_{99}$ to yield a first order estimate for an upper permissible chronic exposure level in the absence of animal data.

DISCUSSION

Here, we have presented a framework to investigate the application of in vitro pathway-based risk assessment for environmental chemicals. BPADs are in vitro analogues of in vivo point

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Table 1. Description of ToxCast Assays Used in Examples for ER Activity Related to Reproductive Toxicity and CAR/PXR Activity Related to Liver Hypertrophy

assay	gene description	assay description	example
ATG_ERa_TRANS	ESR1 estrogen receptor 1 [human]	transactivation multiplexed reporter gene assay[22]	ER
ATG_ERE_CIS	ESR1 estrogen receptor 1 [human]	transactivation multiplexed reporter gene assay[22]	ER
NCGC_ERalpha_Agonist	ESR1 estrogen receptor 1 [human]	quantitative hts reporter gene assay[27,60]	ER
NVS_NR_bER	ESR1 estrogen receptor 1 [bovine]	cell-free competitive binding assay[61]	ER
NVS_NR_hER	ESR1 estrogen receptor 1 (mutant) [human]	cell-free competitive binding assay[61]	ER
NVS_NR_mERa	ESR1 estrogen receptor 1 [mouse]	cell-free competitive binding assay[61]	ER
ATG_CAR_TRANS	CAR/NR1I3 nuclear receptor subfamily 1, group I, member 3, Constitutive androstane receptor [human]	transactivation multiplexed reporter gene assay[22]	CAR
NVS_NR_hCAR_Antagonist	CAR/NR1I3 nuclear receptor subfamily 1, group I, member 3, Constitutive androstane receptor [human]	cell-free competitive binding assay[61]	CAR
ATG_PXR_TRANS	PXR/NR1I2, nuclear receptor subfamily 1, group I, member 2, Pregnane-X receptor [human]	transactivation multiplexed reporter gene assay[22]	PXR
NCGC_PXR_Agonist_human	PXR/NR1I2, nuclear receptor subfamily 1, group I, member 2, Pregnane-X receptor [human]	quantitative hts reporter gene assay[27,60]	PXR
NVS_NR_hPXR	PXR/NR1I2, nuclear receptor subfamily 1, group I, member 2, Pregnane-X receptor [human]	cell-free competitive binding assay[61]	PXR
ATG_RXRb_TRANS	RXRB - retinoid X receptor, beta [human]	transactivation multiplexed reporter gene assay[22]	CAR/PXR
ATG_RXRa_TRANS	RXRA - retinoid X receptor, alpha [human]	transactivation multiplexed reporter gene assay[22]	CAR/PXR
NCGC_RXRa_Agonist	RXRA - retinoid X receptor, alpha [human]	quantitative hts reporter gene assay[27,60]	CAR/PXR
CLZD_ABCB1	ABCB1 - ATP-binding cassette, subfamily B (MDR/TAP), member 1 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	CAR
CLZD_ABCG2	ABCG2 - ATP-binding cassette, subfamily G (WHITE), member 2 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	CAR
CLZD_CYP2B6	CYP2B6 - cytochrome P450, family 2, subfamily B, polypeptide 6 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	CAR
CLZD_CYP2C9	CYP2C9 - cytochrome P450, family 2, subfamily C, polypeptide 9 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	CAR
CLZD_CYP2C19	CYP2C19 - cytochrome P450, family 2, subfamily C, polypeptide 19 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	CAR
CLZD_CYP3A4	CYP3A4 - cytochrome P450, family 3, subfamily A, polypeptide 4 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	PXR
CLZD_GSTA2	GSTA2 - glutathione S-transferase alpha [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	PXR
CLZD_UGT1A1	UGT1A1 - UDP glucuronosyltransferase 1 family, polypeptide A1 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	CAR/PXR
CLZD_SLCO1B1	SLCO1B1 - solute carrier organic anion transporter family, member 1B1 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	PXR
CLZD_SULT2A1	SULT2A1 - sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1 [human]	gene expression assay in human hepatocytes (24 and 48 h readouts)[62]	CAR/PXR
ATG_HNF4a_TRANS	HNF4A - hepatocyte nuclear factor 4, cofactor for CAR and PXR	quantitative hts reporter gene assay[27,60]	CAR/PXR
ATG_PBREM_CIS	CAR and PXR response element	quantitative hts reporter gene assay[27,60]	CAR/PXR

of departure doses. We posit that BPADs could be used to provide provisional estimates of permissible or acceptable exposure levels for data poor chemicals, upon the basis of discovering which pathways are significantly altered by a chemical and at what concentration those perturbations occur in vitro, and then using a PK model to estimate the external dose that would produce the internal concentration that caused pathway perturbation. Both the PD and PK estimates incorporate uncertainty and variability and when combined yield a probability distribution for the pathway-altering dose. The $BPAD_{99}$ is then calculated as a lower percentile of this distribution, with emphasis on its lower one-sided 95% confidence bound, the $BPADL_{99}$. We have presented examples where BPADs and animal-based LEL and NEL values were compared, and these have yielded interesting insights, including the fact that BPAD values tend to be below or at most a factor of 10 higher than the NEL/100.

HTRA can be compared with the currently used regulatory testing paradigm for food use pesticides and other chemicals for which extensive testing is required. (An alternative, and perhaps more apt comparison is with data requirements for the large

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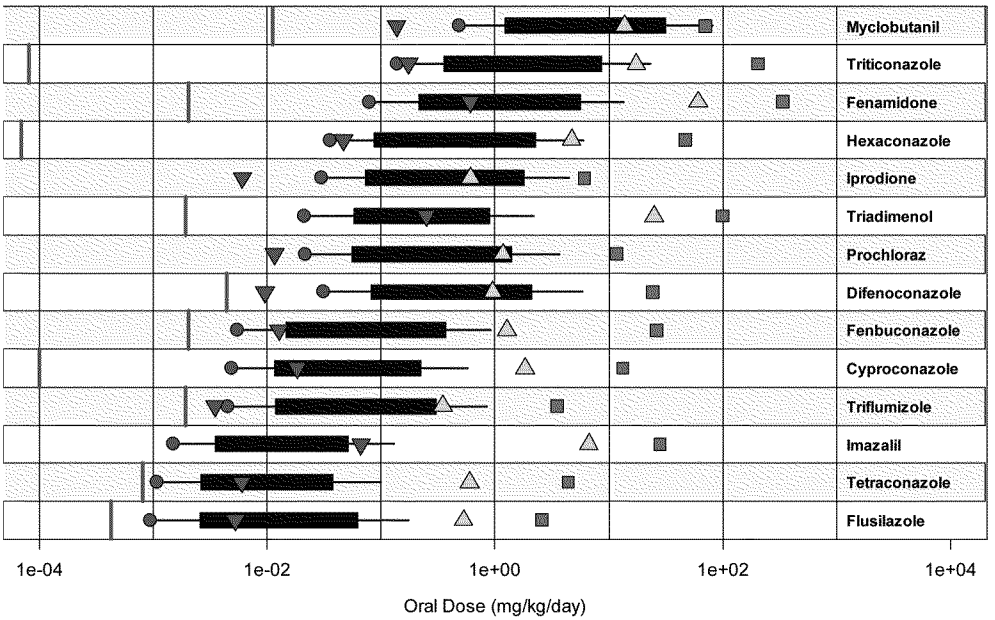

Figure 3. Comparison of HTRA BPAD distributions with LEL and NEL values for liver hypertrophy from animal studies on the 14 conazole fungicides in phase 1 of ToxCast. BPADs are calculated as described in the text. For each chemical, the black box gives the population-variability-derived (1%, 99%) confidence intervals about the median BPAD. The whiskers indicate uncertainty-derived 95% confidence intervals about the extremes of the variability confidence interval. The $BPADL_{99}$ is indicated by a red circle; the LEL by a blue box; the NEL by a gray triangle; and the NEL/100 by a red triangle. Estimated chronic exposure levels from food residues are indicated by vertical red lines. All values are in mg/kg/day.

number of data poor chemicals.) The traditional testing strategy uses relatively high-dose animal tests one chemical at a time to observe what toxic end points occur. These tests provide holistic evidence of toxicity across many organs and over long time scales, and are largely hypothesis free (or hypothesis generating). These tests may then be followed up with more mechanistic studies to understand the underlying basis of toxicity and to provide information needed to better inform extrapolation from animal to human effects and from high doses to typical low doses to which humans will be exposed. With HTRA, we run hundreds to thousands of chemicals in parallel, in human-based assays corresponding to pathways for which there is previous evidence of linkage with toxicity-related end points. Pathways are probed one at a time, and an overall HTRA profile is built up from multiple pathway-based tests.

The basic approach presented here can be extended in a number of ways, some of which are described below. A recent commentary by Crump and colleagues[44] addressed some of these issues and pointed out related challenges, in particular with estimating altering concentrations, performing PK modeling, and treating uncertainty and variability. One particular issue they raise is the danger of making the model too complex in order to better mimic the in vivo situation. We agree with this and emphasize that our goal is not to replace current testing strategies, but instead to develop a new first tier testing approach for data poor chemicals. By keeping the framework relatively simple, the transparency of the approach is facilitated. This is especially important so that all stakeholders can evaluate the model. Extending this transparency, we believe that all data and software used in HTRA should be well documented and made public.

Defining biological pathways and linking them with adverse effects is a key concept in the NRC Toxicity Testing in the 21st Century report.[10] The use of toxicity pathways has been widely discussed over the past few years, yet they remain an ill-defined concept. One issue is that biological pathways themselves are not systematically defined, and a second is that there has not been a concerted effort to organize information linking chemicals, targets (genes, proteins), biological pathways, and their functionally important modular components, key events, MOA, and adverse effects. A database linking all of these types of information together would allow data mining algorithms to find key gene/protein networks whose perturbation would be a risk factor for toxicity. The ToxCast in vitro toxicity testing data, publicly available via ToxCastDB,[43] is an important step in linking chemicals to the perturbation of biological pathways.[11,12] Several other public databases also contain parts of the puzzle, including the Comparative Toxicogenomics Database (CTD)[45,46] and PharmGKB,[47] which link genes and chemicals; OMIM,[48] which links genes and disease; KEGG[17,49] and Pathway Commons,[18] which contain gene-pathway information; and the EPA Aggregated Computational Toxicology Resource (ACToR),[50,51] DSSTox,[52] and ToxRefDB,[42,43,53,54] which link chemicals and adverse effects. An important piece which is lacking is a database of chemicals and their toxicity MOA. EPA's ToxCast program is constructing a database and tools to link all of this data together,[12] but this effort will require help from a much broader community.

One way to approach this problem is to decide when we can equate in vitro activity with in vivo adversity. In some cases (which we call Class 1), the link between in vitro activity and adversity is clear (e.g., cholinesterase activity). There is a single target which, if significantly perturbed, can lead directly to undesirable phenotypic changes. Class 1 could be further subdivided. For instance, Class 1a would be a pathway that is normally off and gets triggered by an exogenous agent (e.g., genotoxicity); whereas a Class 1b pathway would normally be active, but its level is modulated by an exogenous agent, and when that is beyond the realm of homeostasis, damage occurs. Next is an intermediate case (Class 2), where there is an

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association (statistical or otherwise) between perturbations of a pathway and some disease outcome, but the details and causal linkage are not clear (e.g., PPAR pathway perturbations and potential linkage with human disease). Finally, there are many other targets and pathways (Class 3), for which no clear linkage between in vitro activity and adverse In Vivo outcomes is currently known.

Before we can make widespread use of this type of approach, there are a number of challenges that need to be addressed, five of which are discussed below.

Estimating Concentrations at Which Pathways Are Perturbed. We use the results of in vitro assays to determine if a chemical perturbs a pathway and if so, over what concentration range. Because pathways form complex networks that can contain feed-forward and feedback loops, we need to probe the pathway at multiple points because any single assay may miss an important effect. In addition, all assay technologies yield some fraction of false positives and negatives so that it is best to probe pathways using assays from a variety of technologies. These and other factors need to be considered in order to develop robust criteria for determining when a pathway of interest is significantly perturbed, and determining appropriate variability and uncertainty metrics. Our CAR/PXR example is one case where it is possible to refine the estimate of the BPAC by integrating over all of the assays that map to the pathway. A recent publication describes one approach for integrating several nuclear receptor pathways associated with rodent liver tumors.[19] We are investigating a number of other ways to do this, using statistical, Bayesian methods, pathway-level modeling, and agent-based cell simulations in "virtual tissues" in our Virtual Liver and Virtual Embryo projects. Another issue is that some classes of environmental chemicals are not currently amenable to HTS analysis, in particular, volatile chemicals and small molecular weight chemicals that are not expected to directly interact with cellular macromolecules in a pharmacologically relevant fashion.

PK Modeling of in Vitro Concentration to In Vivo Dose. We described one method for estimating the external dose that is required to yield a specified plasma concentration of a chemical, but there are other PK modeling approaches that could be used. Special cases that need to be dealt with include chemicals that bioaccumulate to a significant extent; chemicals that act acutely or through effects at peak concentrations; chemicals that cause toxicity in compartments where there is no full partitioning with plasma (fetus, brain, testis, and milk); analysis of chemicals for which active transport is important; and chemicals for which there is significant nonhepatic metabolism. We incorporate population variability into our current model in an approximate way, but uncertainty is not well captured.

Biotransformation and Other Properties in Vitro Systems Lack. A major criticism of using in vitro assays to predict chemical toxicity is that cells are not tissues, organs or people, i.e. they lack many of the essential interactions that are required to trigger key events in an MOA, or which could prevent key events from being triggered through adaptive responses. Most current HTS assays do not include the possibility for biotransformation, which means that we can make statements about the activity of a parent molecule, but not any potentially more or less toxic metabolites. Technologies are being developed to address this issue[55] but are not currently robust enough to yield relevant results. Most assays do not include multiple cell types and therefore do not incorporate complete paracrine signaling pathways (e.g., those that are needed for the development of an immune response). There are a variety of emergent properties that one will only see with mixtures of cell types, appropriate extracellular matrices, and three-dimensional geometries. Many cell-based assays are carried out in immortalized cell lines that have become highly adapted to growth in vitro and no longer represent their tissue of origin (although use of primary cells for in vitro assays is being increasingly used). Cell-based assays are short-term (hours to days) and cannot be used to directly address the effect of chronic exposures, for instance through accumulation of mutations. All of these factors lead to uncertainty in our BPAD estimates and need to be accounted for in some way.

Relevance of in Vitro Activity to In Vivo Toxicity. There are a variety of issues related to the extrapolation from in vitro to in vivo activity and in particular, to adversity. These include issues related to chronic exposures (months or years) in animal studies as opposed to the hours or days for exposure in the in vitro systems. Another has to do with life-stage sensitivity. A related set of issues have to do with adaptive responses which may occur in the intact animal and over long times, but which are not manifested in a short-time cell assay. We cannot answer all of these here, but restate the basic premise of using in vitro assays in toxicology, namely that for certain disease types, direct perturbation of a target or pathway is a necessary condition for the disease to occur. This is the basis of the notion that there are key molecular initiating events in toxicity modes of action.[7] Because these molecular actions are necessary, but not sufficient, in vitro assays can be overly sensitive in predicting whether a chemical can lead to adversity, but can give specific information on the modes of action that could be driven by chemical exposure. In the ToxCast program, we are using statistical methods to link pathway-level perturbations with adverse outcomes. This is done by using in vitro and in vivo toxicity data on common sets of chemicals in the ToxCast[12] and ToxRefDB[42,53,54] data sets, and finding statistical associations. We then follow-up on strong associations by building a case for biological plausibility by using external validation data (chemicals not used in the initial association analysis) and detailed mechanistic information from the literature. In the case that multiple pathways linked to a given adverse effect are perturbed by a chemical (generating multiple BPADs), we would use the lowest one as the starting point for HTRA, in the same way that the most sensitive adverse end point seen in an animal study is often used in setting a LOAEL.

Dealing with Uncertainties and Variability. We have already mentioned the need to better estimate levels of uncertainty and variability in the modeled PD and PK parameter values. Of note are recent papers that measure the population variability of response to chemicals in vitro using a collection of genetically characterized mouse strains and human cell lines.[20,56] There is additional uncertainty involved in the models themselves that should be considered and a need to optimize the way the separate uncertainty and variability distributions are integrated. The work of Rusyn and co-workers is an example of how one can use in vitro data derived from testing the HapMap cell lines to assess PD population variability. One could use their approach to examine variability, in this case genetic, at the pathway level.[20]

To conclude, our initial goal is to develop a tool for performing rapid evaluations of the potential hazard for data poor chemicals and for setting priorities among those for more detailed testing. One can envision variants of this approach that make use of chemical structure combined with in vitro data to estimate risk across classes of chemicals in the same way that categories are currently evaluated. It should also be possible to formulate methods to make first-order estimates of BPADs for mixtures.

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This HTRA approach lends itself to a tiered testing approach which would not go straight from a finding of high predicted hazard in HTRA to a recommendation of extensive animal testing. Furthermore, this HTRA approach is consistent with a new EPA program advancing the next generation of risk assessment (NexGen) and proposing a tiered approach to risk assessments.[57] In one possible tiered testing approach, a large set of chemicals would be analyzed using some variant of BPAD-HTRA. Those with the lowest BPAD values, and which therefore potentially pose a risk at the lowest exposures, would go into a second, still in vitro tier. The second tier would first include the use of more complex and in depth in vitro analysis, using additional assays in the implicated biological pathways, different cell types, and possibly model organism tests. Any toxicity-related data from structural analogues with of the Tier 1 chemicals should also be included. The second tier should also estimate potential exposures. If the combined, Tier 1 - Tier 2 BPADL approaches exposure levels likely to be encountered in the environment, then the chemical would become a candidate for even more extensive testing and modeling using in silico, in vitro, and in vivo approaches.

An alternative track to be followed for chemicals with high apparent risk in Tier 1,2 is to consider replacements. This would follow the green chemistry/sustainability approaches being developed by the EPA.[58,59] If there is a functionally equivalent chemical (from an end-use standpoint) with a significantly greater BPAD, and no significant sustainability liabilities, then this analysis could help guide a replacement strategy.

AUTHOR INFORMATION

Corresponding Author

*National Center for Computational Toxicology, EPA Office of Research and Development, Research Triangle Park, NC 27711. Phone: 919-541-3085. E-mail: judson.richard@epa.gov.

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DISCLOSURE

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ABBREVIATIONS

AC_{50}, activating concentration (50%); ADI, acceptable daily intake; BMD, benchmark dose; BPAC, biological pathway altering concentration; BPAD, biological pathway altering dose; C_{ss}, concentration at steady state; CAR, constitutive androstane receptor; DR, dose rate; ER, estrogen receptor; HTRA, high-throughput risk assessment; HTS, high-throughput screening; LEL, lowest effect level; LOAEL, lowest adverse effect level; MOA, mode of action; NEL, no effect level; NOAEL, no adverse effect level; PK, pharmacokinetics; POD, point of departure; PD, pharmacodynamics; PXR, pregnane-X-receptor; RfD, reference dose; RTK, reverse toxicokinetics; TTC, threshold of toxicological concern.

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