# Probe molecule (PrM) approach in adverse outcome pathway (AOP) based high throughput screening (HTS): *in vivo* discovery for developing *in vitro* target methods

Michelle M. Angrish,[†] Michael C. Madden,[‡] and Joachim D. Pleil[§,*]

[†]ORISE Participant, US EPA, Research Triangle Park, NC

[‡]Environmental Public Health Division, NHEERL/ORD, US EPA, Chapel Hill, NC

[§]Human Exposure and Atmospheric Sciences Division, NERL/ORD, US EPA, Research Triangle Park, NC

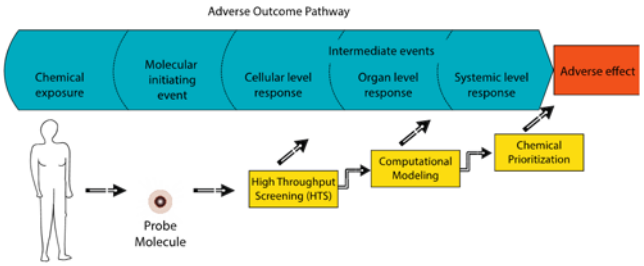
*Author to whom correspondence may be addressed:

Dr. Joachim Pleil

E-mail: pleil@email.unc.edu

Phone: 919-541-4680

# Table of Contents Graphic



## Abstract

Efficient and accurate adverse outcome pathway (AOP) based high-throughput screening (HTS) methods use a systems biology based approach to computationally model *in vitro* cellular and molecular data for rapid chemical prioritization; however, not all HTS assays are grounded by relevant *in vivo* exposure data. The challenge is to develop HTS assays with unambiguous quantitative links between *in vitro* responses and corresponding *in vivo* effects, which is complicated by metabolically insufficient systems, *in vitro* to *in vivo* (IVIVE) extrapolation, cross-species comparisons, and other inherent issues correlating IVIVE findings. This article introduces the concept of ultrasensitive gas phase probe molecules (PrM) to help bridge the current HTS assay IVIVE gap. The PrM concept assesses metabolic pathways that have already been well defined from intact human or mammalian models. Specifically, the idea is to introduce a gas phase probe molecule into a system, observe normal steady state, add chemicals of interest, and quantitatively measure (from headspace gas) effects on PrM metabolism that can be directly linked back to a well-defined and corresponding *in vivo* effect. As an example, we developed the pharmacokinetic (PK) parameters and differential equations to estimate methyl-tertiary butyl ether (MTBE) metabolism to tertiary butyl alcohol (TBA) via cytochrome (CYP) 2A6 in the liver from human empirical data. Because MTBE metabolic pathways are well characterized from *in vivo* data, we can use it as a PrM to explore direct and indirect chemical effects on CYP pathways. The PrM concept could be easily applied to *in vitro* and alternative models of disease and phenotype, and even test for volatile chemicals while avoiding liquid handling robotics. Furthermore, a PrM

can be designed for any chemical with known empirical human exposure data, and used to assess chemicals for which no information exists. Herein, we propose an elegant gas phase probe molecule-based approach to *in vitro* toxicity testing.

**Key Words:** high throughput screening (HTS), adverse outcome pathway (AOP), probe molecules (PrM), *in vitro* to *in vivo* extrapolation (IVIVE)

## Introduction

The U.S. Environmental Protection Agency is tasked with evaluating the human health, environmental, and wildlife effects of over 80,000 chemicals registered for use in the environment and commerce. The challenge is that limited chemical data exists; traditional toxicity testing methods are slow, costly, involve animal studies, and are not able to keep up with a chemical registry that grows by at least 1000 chemicals every year. The emerging paradigm is to use *in vitro* cell based and cell free assays or *in vivo* alternative species such as zebra fish embryos in high-throughput screening (HTS) assays to generate data that can be computationally linked to biological pathways.[1-4] The underlying premise is that HTS assays may target one or more molecular initiating events (MIEs) or key events (KEs) in an adverse outcome pathway (AOP) that uses a systems biology approach to mechanistically anchor chemical exposure with adverse outcomes and expedite risk assessment.[5-7] Therefore, the US EPA has launched a "Toxicity Forecaster" program termed ToxCast (http://www.epa.gov/ncct/toxcast/) that systematically evaluates chemicals with a series of HTS assays; the ultimate goal is to limit the number of laboratory animal-based toxicity tests while quickly and efficiently screening large numbers of chemicals. To date, chemicals listed in ToxCast 1 include pesticides and other compounds of public interest such as Aldicarb, Bisphenol-A, Carbaryl, Cyfluthrin, Fipronil, and Permethrin that already have extensive *in vivo* data and would be a good choice for initial testing for the evaluations proposed in the ensuing discussions.

AOPs (as described by the Organization for Economic Co-operation and Development program guidelines) are linear pathways where a molecular initiating or key event is expected (but not necessarily demonstrated) to reflect an *in vivo* adverse outcome to chemical exposure (Figure 1).[8, 9] The underlying idea is that an adverse outcome pathway provides a framework for linking a key event with an adverse outcome by extending across multiple layers of biological organization.[2] Therefore, if a chemical elicits a key event, risk assessors can use the AOP as a rapid predictive approach in risk assessment to infer that the adverse outcome will occur. The fundamental basis for high throughout screening data to defensibly inform key events in an AOP is that the *in vitro* and *in vivo* alternative assays rapidly and accurately describes an *in vivo* chemical exposure scenario.[10] However, HTS assays often lack relevant human *in vivo* dosimetry,[11, 12] biological response (i.e. exposure level, absorption, distribution, metabolism, elimination (ADME), and inter-individual genetic variability [11] information.

To sufficiently support an AOP, the underlying data derived from *in vitro* toxicity testing should come from *in vivo* observations and discovery analyses.[13] Certainly, the overall goal of HTS cannot be achieved with such detailed approaches; however, some subset of the applied *in vitro* methods should have direct empirical *in vivo* analogs either from human or animal research. The high throughput screening community has recognized the need for such complementary approaches. The earliest articles regarding AOP-based toxicity testing describe the intrinsic difficulties with *in vitro* testing; cells isolated from tissue require immortalization for continued renewal, which essentially changes innate cellular and molecular function.[14] An option is to use primary cells that better represent *in*

*vivo* activity, but they do not propagate and have a limited life cycle. Furthermore, cells removed from their natural environment no longer represent toxicological responses related to systems biology.[14, 15]

A second issue is the determination of potency and dosimetry among *in vitro* assays and its use for high throughput risk predictions without considering the influence of systems level ADME. The high-throughput risk assessment-biological pathway altering dose model developed by Judson, et al. 2011 takes into account chemical pharmacokinetics, but assumes 100% oral absorption and elimination through metabolism and renal excretion.[16] Furthermore, the PK parameters are estimated by reverse dosimetry from *in vitro* data and may misrepresent *in vivo* systems biology effects due to over-modeling and over-simplification of bioavailability and clearance parameters.[17]

We propose that a HTS component should be the implementation of *in vivo* empirical research to help structure the *in vitro* assays. For example, controlled human studies of methyl tertiary butyl ether (MTBE) exposure have provided the kinetic parameters of metabolism to tertiary butyl alcohol (TBA) by the CYP2A6 pathway in the liver.[18-21] Similarly, the study of clinical administration of the anesthetic sevoflurane has provided the kinetic parameters for metabolism to hexafluoropropanol by the CYP2E1 pathway in the liver.[22] Because the MTBE and sevoflurane metabolic pathways are well characterized from *in vivo* data, we can use them as probe molecules to explore the effects of chemicals of interest on their respective CYP pathways. Specifically, human pharmacokinetic models from controlled human exposure studies exist; therefore the *in*

*vitro* test results can forward modeled and linked directly back to the human pharmacokinetic response.

Such probe molecule tests can be designed for any chemical/metabolite pair that have been studied and pharmacokinetically modeled from environmental exposure studies. Furthermore, a suite of PrMs can be selected based on their linkage to key events in AOPs relevant to toxicity testing. The elegance of this approach for designing high throughput screening assays is that probe molecules provide a single quantitative real time measurement from air of an *in vitro* response to chemical exposure that encapsulates all cellular and molecular responses affecting a key event. Because corresponding probe molecule ADME and PK parameters, their human metabolites, and their metabolic pathways are well defined, they directly bridge the *in vitro* to *in vivo* uncertainty gap. In essence, chemicals with empirical human exposure assessments can be used as PrMs for chemicals for which we have no information. Herein, a proof of principle approach to PrM testing of liver function is described in an *in vitro* model.

## Probe Molecule Conceptual Framework

Our initial goal was to develop a conceptual framework for implementing gas phase probe molecules to screen chemicals of interest for effects on liver function in an *in vitro* cell model. This objective requires an innovative approach that applies a direct quantitative link between an *in vitro* response and a corresponding *in vivo* effect for improved AOP-based toxicity testing and chemical prioritization. Here we outline the conceptual framework for implementing the probe molecule approach diagrammed in Figure 2 and described in detail below.

### *Data from literature*

The probe molecules pair MTBE/TBA will be used as a model compound, but any chemical with known human PK values can be used as a PrM and applied to controlled *in vitro* gas-phase assays that are designed to directly measure the effects of chemical exposure on organ (in this example, liver) function. Essentially, liver cells exposed to an MTBE gas phase input will be held at equilibrium with the gas phase output metabolic conversion product TBA. The steady state model will simulate "normal" liver function under conditions that are based on human MTBE PK parameters that were adapted from human studies.[23, 24] Chemicals of interest will be added to the steady state system and evaluated for their effects on MTBE metabolism by direct measurement of the gas from air (Figure 2).

#### *Conceptual Pharmacokinetic Model*

A one-compartment model (Figure 3) was simplified from a two-compartment model for human inhaled breath data that defined the PK parameters for the metabolic conversion of MTBE to

TBA. Briefly, MTBE and TBA breath data were mathematically modeled and validated from blood and breath data.[23] MTBE pharmacokinetic parameters (Table 1) were fit to our one-compartment conceptual model (Figure 3). The central compartment represents the *in vitro* liver system at steady state, i.e. the liver cells in equilibrium with the gas flow. The model assumes that the probe molecule MTBE is absorbed by and distributed to the central compartment and that the central compartment characterizes blood MTBE concentrations directly proportional to breath concentrations (via blood/breath coefficients). The model also assumes the central compartment is the primary site of MTBE metabolism and that TBA is the primary metabolite. The rate constants $K_0$ (liver uptake), $K_{MT}$ (MTBE loss to TBA), $K_{MB}$ for (MTBE loss to air), $K_{TL}$ (TBA loss to air) are first order and in units concentration/time. MTBE metabolism to TBA is adjusted by the change from $K_{MT}$ to $K_{TT}$ where $K_{TT} = K_{MT} \times k_d$. The lower case $k_{d\text{-}e}$ are rate adjustments for reducing the two compartment human model to one compartment and correct for MTBE metabolism by tissues other than the liver in the human model. It is important to note that the proposed one-compartment model provides the conceptual framework for linking a known human PK model (that is based on external exposure and measured internal dose parameters) with an *in vitro* outcome. It is not within the scope of this work to outline physiologically based pharmacokinetic (PBPK) modeling and *in vitro* to *in vivo* extrapolation (IVIVE), but rather to propose a framework for evaluating the proposed probe molecule concept. We anticipate that future empirical *in vitro* data will be appropriately compared to *in vivo* data and used to calibrate the proposed model simulations for extrapolating *in vitro* assays to human exposure outcomes as previously described.[25]

*Calculation Model: Differential Equations*

Based on the concepts of first order kinetics and the one-compartment model outlined in Figure 3 we wrote differential equations to approximate the concentration of probe molecule ($C_{mc1}$) and primary metabolite ($C_{TC1}$) as an incremental function of time. We assumed a constant input in units μg/L, an initial uptake rate ($K_0$) in units of concentration/time, and a closed system at steady state that is given by equations 1–2:

$$dC_{MC1}(t)/dt = K_0 C_I(t) - (K_{MT} + K_{MB}) C_{MC1}(t) \quad (1)$$

$$dC_{TC1}(t)/dt = K_{TT} C_{MC1}(t) - K_{TL} C_{TC1}(t) + K_0 k_e \quad (2)$$

such that $C_{MC1}$ and $C_{TC1}$ represent the cellular concentrations of probe molecule and metabolite, respectively. The rates $K_{MT}$ and $K_{TL}$ represent loss to air whereas the rate for MTBE metabolism ($K_{MT}$) is adjusted to $K_{TT}$ for the conversion to TBA.[23] The one compartment calculation model assumes the same compartment: air MTBE and TBA proportions as the blood: breath model[23] such that the concentration of MTBE ($C_{MB}$) and TBA ($C_{TB}$) are given by equations 3 and 4.

$$C_{MB}(t) = [A_{pMB0} + A_{pMB1} C_i(t)] C_{MC1}(t) \quad (3)$$

$$C_{TB}(t) = [A_{pTB0} + A_{pTB1} C_i(t)] C_{TC1}(t) \quad (4)$$

*Example: An MTBE/TBA Probe Molecule Simulation Model*

The MTBE two-compartment PK parameters were adapted to our conceptual *in vitro* liver system (Figure 2), mathematically modeled in MATLAB® R2014a (Natick, MA, USA), and graphed in GraphPad Prism V5.0b (La Jolla, CA, USA). The code is available in supporting information. Initial estimates for steady state conditions were fit to our one compartment model

described by equations 1-4. The *in vitro* model approximated the MTBE time to steady state in compartment one ($_{C1}$) and in air ($MTBE_{C1}$ and $MTBE_{air}$, respectively) at 2.4 h, whereas TBA in air required ~60 h (Figure 4A). Although we do not expect *in vitro* cellular kinetics to perfectly align with human PK parameters, we do expect rapid PrM and metabolite diffusion across cellular space to reduce the *in vitro* time to steady state to a fraction of model estimates. Using the steady state parameters from Figure 4A, the expected levels of MTBE and TBA in the air were modeled in a time-dependent manner after adding the Cyp2A6 and Cyp2E1 selective inhibitor diethyldithiocarbamate (DETC)[26] to the *in vitro* system at 60 h. The model assumed a five min lag (calculated by spline interpolation) until 90% CYP2A6 inhibition ($K_{TT}$) based on previous CYP450 inhibition studies.[26, 27] Figure 4B shows the expected MTBE and TBA curves in response to CYP450 inhibition by DETC. Note that steady state MTBE concentrations are not appreciably altered by changes in TBA (Figure 4B and equation 1 and 3); therefore, in this case, the only measurement required is for TBA. The system can conceptually be returned to steady state, reinstituted for another round of toxicity testing, and implemented longitudinally.

### *In vitro cell model for probe molecule metabolism*

Probe molecules can be quantified from a variety of *in vitro* cell culture models, but because chemical biotransformation and detoxification primarily occurs in the liver, our initial focus was on hepatocytes. A variety of *in vitro* liver cell culture methods exist with distinct advantages and disadvantages. Immortalized cell lines are one of the most common and easy to use cell lines; however, they have little to no phase I and II enzyme activity.[28] Compared to other immortalized human cell lines, HepaRG™ cells have high CYP450 activity, including CYP2A6 and CYP2E1, and express various nuclear receptors.[29, 30] However, they are clonal and fail to illustrate

phenotypic and genetic diversity. Human primary hepatocytes isolated from various donors reflect genetic heterogeneity and maintain high phase I and II enzyme activity for up to 72 h[15], but require low density monolayer growth between a collagen or Matrigel® layer (likely slowing probe molecule diffusion to intracellular targets) for the maintenance of liver-specific functions. Primary cells (and in particular anchorage-dependent cells such as hepatocytes) grown in suspension without a solid support lose activity within a few hours. Three-dimensional cell cultures systems have the potential to solve all of the issues described above including (1) the need for a solid support and suspension culture, (2) a microenvironment favorable for "normal" *in vitro* hepatocyte phenotype, and (3) cell densities amenable to a quantitative signal that is HTS scalable. Specifically "in microcarrier culture, cells grow as monolayers on the surface of small spheres or as multilayers in the pores of macroporous structures that are suspended in culture medium…. (with) yields of up to 200 million cells per milliliter".[31] However, nutrient deliver is often limited or insufficient to the nutrient core. Alginate cell encapsulation bioreactors reportedly avoid carrier cell core by immobilizing cells in an alginate gel network that enables nutrient circulation and delivery.[32] In either case, 3D cell culture suspensions can be grown with a $CO_2$/air/PrM mixture, including a test volatile of human health concern, sparged directly into the culture media or headspace (Figure 2). Advantageously, probe molecules are amenable to any *in vitro* cell culture model, but particularly with metabolically competent systems that more accurately represent an *in vivo* exposure scenario.

## *Probe Molecule Chemical Analysis*

*Chemical selection and measurement criteria*

The ingenuity of the probe molecule approach is that it also allows for screening of volatiles for which there is no current ToxCast assay. Furthermore, it is desirable to compare PrM empirical data with ToxCast assay data for chemicals that have known *in vivo* exposure data. Therefore, a small subset of chemicals previously characterized in ToxCast (http://www.epa.gov/ncct/toxcast/chemicals.html) and *in vivo* assays should be chosen for of the probe molecule system.[4, 17, 33] Chemical selection should be biased towards known hepatotoxins targeting (1) Phase I (CYP450) metabolism, (2) Phase II conjugation activities, and (3) other (un)characterized mechanisms. The assay's strength is that probe molecules provide both a *cis* (direct) and *trans* (indirect) assay, so that chemical effects on a PrM biological network would activate the key event and indicate an adverse outcome. All tested chemicals quality control (QC) standards should be consistent with the U.S. EPA ToxCast program. Ideally, a concentration response curve would be constructed to envelop a chemical's lowest point of departure (POD), potency (AC50, concentration at 50% activity), and efficacy (Emax or maximum response). Chemicals could even be indexed against a well-known hepatotoxicant such as acetaminophen (APAP) so that the APAP Emax = 1, AC50 = 0.5, and so on. The broad mechanisms underlying APAP hepatotoxicity[34] cover likely *cis* and *trans* chemical effects that may alter probe molecule metabolism, making APAP an ideal PrM indexing compound for hepatotoxicity.

To rule out cytotoxicity effects, cells can be assayed for viability by dye exclusion[35, 36] where a concentrated cell suspension will be mixed with one drop of a 0.05% Trypan Blue and loaded

into the counting chamber of a hemocytometer, incubated for 1-2 minute, and counted. Living cells exclude the dye whereas dead cells appear blue.

### *Gas sampling and Quantitation*

Measurement of probe molecules and their associated metabolites can be performed with a variety of existing and emerging technologies that can be divided into offline (snapshot) or online (real-time continuous) monitoring.

#### *Off-line analysis*

The standard analytical methods for gas-phase organic compounds are generally off-line, that is, aliquots of the headspace are collected via passive headspace sampling using a gas-lock syringe, and then directly injected into a gas chromatograph with appropriate detector giving a periodic snapshot in time of the concentrations (e.g. http://www.restek.com/pdfs/59895B.pdf). Given a flow-through PrM system, the effluent gas could be concentrated by collection onto an adsorbent trap tube that is periodically swapped out. Samples can be analyzed with automated analytical systems that thermally desorb the tube, further concentrate analytes, and inject directly into bench top gas chromatography-mass spectrometry (GC-MS) instrumentation. The underlying analytical technology is based on EPA Method TO-17[37] and has been applied to various projects including those for breath analysis that are closely aligned with the requirements for probe molecules.[38-40] A recent innovation in offline gas analysis is the implementation of a hybrid syringe/adsorbent technology termed "needle trap" collection.[41, 42] Needle trap allows efficient collection/concentration at low volumes in one step and is likely a simple solution for short term probe molecule evaluation.

*On-line analysis*

In the long term, more sophisticated analytical approaches can be applied that use online real-time monitoring. This provides advantage for higher throughput applications because a single detector can multi-task flows from different reactors and the manual sample collection and transfer procedures can be avoided. Specifically, there are two types of gas-phase analytical schemes that could be implemented: real-time MS or mid-infrared tunable diode laser (TDL) spectroscopy.

*Mass Spectrometry*

Commercially available MS systems could be implemented for analysis of PrM metabolites. Real-time analysis of volatiles (that eliminate the need for sample collection) can be achieved with proton transfer reaction mass spectrometry (PTR-MS) or selected ion flow tube mass spectrometry (SIFT-MS).[43, 44] Both analytical techniques deliver sufficient specificity and sensitivity to analyze "cellular breath", but with distinct differences between (1) production of precursor ions, (2) reaction conditions, and (3) sample detection. Specifically, with SIFT-MS a quadrupole simultaneously produces precursor ions that are diluted with and carried by helium to the quadrupole mass spectrometer detector. In contrast, PTR-MS precursor ions generated by switching to other gaseous proton donors are drawn by an electric field down a drift tube to a quadrupole MS, and, for the option of higher mass resolution and associated increased specificity, time of flight (ToF) MS.[45] The advantages of PTR-MS over SIFT for the detection of PrM metabolites are that precursor ions are not selected and samples are not diluted by a carrier gas. Recent commercial products developed for exhaled breath analysis are approaching

low part per billion by volume (ppbv) sensitivities and can provide real-time results from small gas flows (<1 ml/min) with a time resolution of ~50 ms.[46]

As such, PTR-MS and SIFT-MS are well suited for reactor cell gas analysis as the outflowing matrix will be saturated with water vapor, contain carbon dioxide and, in general, can be considered cellular breath.[40] Therefore, because the real-time application for cellular off gassing is a developing field, any MS implementation for PrM will be developed in conjunction with the International Association of Breath Analysis (IABR, http://iabr.voc-research.at) which has a number of participating experts in instrument development.

*Tunable Diode Laser (TDL) Spectroscopy*

A different option involves recently developed optical sensors that are extremely specific and sensitive for individual compounds. The technique is broadly termed "laser absorption spectroscopy" (LAS) and has various implementations including multi-pass White cell, cavity ring-down cell, integrated cavity output, photo-acoustic cell, and external cavity.[47-49] Briefly, the technique relies on an optical path containing the gas sample illuminated by a specific modulated solid-state laser light-source that is specifically manufactured for short scans in the mid-infrared (3–30 μm) optical range. Early TDL systems were only capable of scanning across one or two vibrational absorption bands and had to be specially constructed for individual compounds. New technologies are now capable of "hop-free" tuning and cover much broader ranges making them universally applicable.[50] One potential advantage for TDL measurement is that the analyses could be performed without the perturbation of extracting an aliquot of gas by using IR transparent reaction cells.

TDL is a rapidly evolving field and any configuration chosen for the analytical finish will implement state of the art technology developed under the guidance of the Mid-Infrared Technologies for Health and the Environment (MIRTHE), an Engineering Research Center (ERC) funded by the National Science Foundation (NSF).

## DISCUSSION

The conceptual framework outlined above offers probe molecules for assaying chemical effects *in vitro* to infer *in vivo* effects. Importantly, the probe molecule approach is poised to (1) measure toxicity profiles of volatile and liquid chemicals in a metabolically competent system, (2) measure any or all *cis* and *trans* biological pathways that might activate a key event in a single assay, and (3) is founded on the premise that chemicals are evaluated for their ability to alter probe molecule metabolism that has been well characterized *in vivo*. The underlying assumption is that cell-based and cell-free *in vitro* assays of molecular initiating or key events suggest (or can be used as model inputs for) adverse outcome at the systems level.[1, 51-56] We agree with the need for a modern approach to risk assessment that addresses the ethical, throughput, and cost concerns of animal testing. However, current high throughput screening assays using cell lines and proteins often lack corresponding *in vivo* exposure data and, therefore, pose challenges when predicting *in vivo* effects to therapeutics, consumer products, pesticides, etc. [57] We suggest complementing the current AOP-based HTS assay paradigm with a PrM approach for improved *in vitro* to *in vivo* risk prediction.

The probe moelcule concept provides a novel quantitative "black box" measure of all KE and, unlike other *in vitro* assays, is based on focused human exposure studies. Specifically, probe molecules directly bridge the *in* vitro to *in vivo* gap by applying established systems level exposure parameters, including absorption, distribution, metabolism, and elimination along with empirical PK and PBPK data to an adverse effect.[18-21, 23, 24] Furthermore, the probe molecule approach is a *cis* and *trans* effect sensor because the system funnels all molecular and cellular responses to chemical exposure (xenobiotic metabolism and biotransformation, receptor-ligand interactions, DNA binding, protein production and degradation, gene activation, signaling, etc.) into a common biological target (e.g., MTBE metabolism) that is quantifiable and measurable. One needs only select an appropriate probe molecule to target an MIE or KE(s) of biological and toxicological interest. We note that the PrM model and simulations outlined above will require at minimum PBPK and IVIVE "tweaking" to extrapolate an *in vitro* dose to human exposure.[25] What we propose is that, with a probe molecule, we already have a validated model for the expected *in vivo* metabolic response that we can apply *in vitro* to assess metabolic perturbations from test chemicals.

The probe molecule approach described above introduces the idea of gas phase sampling and measurements that eliminate the need for expensive, complicated, and bulky liquid handling robotics systems. A major advantage of gas-phase over liquid is that samples require no preparation or chromatographic separation for mass spectrometry. Instead, the analytical signal is measured by direct infusion mass spectrometry or spectroscopy, i.e. directly from headspace (air), in real time, and within single-digit part per trillion by volume (pptv)-range detection limits.[58] A second major advantage is that the gas phase system can be used to examine the

effects of air toxics including volatiles for which there is no current HTS assay system. Furthermore, in the case of volatile assays, the system can be returned to steady state for longitudinal and high-throughput assays of chemicals of interest. As such, the gas-phase probe is much more amenable to routine and rapid analysis than molecular or chemical analytes from cellular or liquid fractions.

The probe molecule concept is broadly applicable not only to liver toxicity, but many cell (human primary, pulmonary, skin, neural, steriodogenic, embryonic, etc.), disease (asthma, metabolic syndrome, diabetes, chronic obstructive pulmonary disease, etc.) or metabolic (i.e. lipid metabolism[59-61]) models. Furthermore, implementation of primary cells from various donors allows PrM measurements of stochastic responses and the examination of genotypes that confer sensitivity. The system can even be "primed" with bacteria and viruses to reflect various ambient environmental conditions. In fact, genetic and "exposome" parameters of a PrM-themed assay can be engineered to provide a systems relevant *in vitro* input for *in silico* tissue reconstruction tools (i.e. v-Embryo, www.epa.gov/ncct/virtual_liver; and v-Liver, www.epa.gov/ncct/virtual_liver).[62, 63] Therefore, quantitative cellular and molecular effects to chemical exposure can be measured with a single quantitative probe molecule assay that is grounded by real human exposure data.

## CONCLUSION

21st Century HTS assays and computational toxicology research programs represent an elaborate approach to assess and prioritize the ~1000 new chemicals introduced into the U.S. every year

and >80,000 chemicals already registered in the Toxic Substances Control Act inventory (TOSCA 15USC2601). However, risk assessments purely based on computational models of "model" *in vitro* systems run the risk of decision making without relevant *in vivo* data[64] and under- or over-estimating chemical risk. The AOP-based HTS approach to chemical prioritization addresses this concern by using mechanistic *in vitro* data to link an MIE and/or a KE within biological pathways to an adverse outcome[2, 51, 65]; the primary challenge, however, is the limited relevant human and *in vivo* empirical systems biology data anchoring *in vitro* HTS assays. We agree a high-throughput approach to chemical prioritization and risk assessment is required to address the rapidly growing chemical list, but put forward cautious consideration when inferring an adverse outcome from modeled *in vitro* data with no relevant *in vivo* link. As such, we propose an elegant probe molecule-based approach to *in vitro* toxicity testing that may bridge the *in vitro* to *in vivo* information gap.

## DISCLOSURE

This research was supported in part by an appointment to the Research Participation Program for the U.S. Environmental Protection Agency, Office of Research and Development, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. EPA. The United States Environmental Protection Agency through its Office of Research and Development has subjected this article to Agency administrative review and approved it for publication. Mention of trade names or commercial products does not constitute endorsement for use. The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.

## ACKNOWLEDGEMENT

The authors are grateful for the expert advice and guidance from Robert Kavlock, Lyle Burgoon, Mark Higuchi, Kathleen Holm, and Rusty Thomas of US EPA, Jose Zavala-Mendez of EPA/ORISE, Jens Herbig of Ionicon Analytik, Innsbruck, Austria, and Terence Risby of Johns Hopkins University, MD USA.

## ABBREVIATIONS

$AC_{50}$, activating concentration (50%); ADME, absorption, distribution, metabolism, and elimination; AOP, adverse outcome pathway; CSS, chemical safety for sustainability; EPA, Environmental Protection Agency; EDC, endocrine disrupting chemical; HFIP, hexafluoropropanol; HTS, high throughput screening; KE, Key event; MTBE, methyl tertiary butyl ether; MIE, molecular initiating event; MS, mass spectrometry; NRC, national research council; OECD, Organisation for Economic Cooperation and Development; POD, point of departure; PrM, probe molecule; PTR, protein resonance transfer; QC, quality control; SEV, sevoflurance; SIFT, selected ion flow tube; TBA, tertiary butyl ether; TOF, time of flight; TOSCA, Toxic Substances Control Act.

## Supporting Information

The supporting information contains published MATLAB code and comments describing the MTBE one-compartment model. This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES


(1) Andersen, M. E., Al-Zoughool, M., Croteau, M., Westphal, M., and Krewski, D. (2010) The future of toxicity testing. *J. Toxicol. Environ. Health. B Crit. Rev. 13*, 163-196.

(2) Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., Mount, D. R., Nichols, J. W., Russom, C. L., Schmieder, P. K., Serrrano, J. A., Tietge, J. E., and Villeneuve, D. L. (2010) Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem. 29*, 730-741.

(3) Judson, R., Richard, A., Dix, D. J., Houck, K., Martin, M., Kavlock, R., Dellarco, V., Henry, T., Holderman, T., Sayre, P., Tan, S., Carpenter, T., and Smith, E. (2009) The toxicity data landscape for environmental chemicals. *Environ. Health Perspect. 117*, 685-695.

(4) Judson, R. S., Houck, K. A., Kavlock, R. J., Knudsen, T. B., Martin, M. T., Mortensen, H. M., Reif, D. M., Rotroff, D. M., Shah, I., Richard, A. M., and Dix, D. J. (2010) In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. *Environ. Health Perspect. 118*, 485-492.

(5) Edwards, S. W., and Preston, R. J. (2008) Systems biology and mode of action based risk assessment. *Toxicol. Sci. 106*, 312-318.

(6) Adeleye, Y., Andersen, M., Clewell, R., Davies, M., Dent, M., Edwards, S., Fowler, P., Malcomber, S., Nicol, B., Scott, A., Scott, S., Sun, B., Westmoreland, C., White, A., Zhang, Q., and Carmichael, P. L. (2014) Implementing Toxicity Testing in the 21st Century (TT21C): Making safety decisions using toxicity pathways, and progress in a prototype risk assessment. *Toxicology*, (epub doi:10.1016/j.tox.2014.02.007).

(7) Perkins, E. J., Chipman, J. K., Edwards, S., Habib, T., Falciani, F., Taylor, R., Van Aggelen, G., Vulpe, C., Antczak, P., and Loguinov, A. (2011) Reverse engineering adverse outcome pathways. *Environ. Toxicol. Chem. 30*, 22-38.

(8) OECD. (2013) Guidance Document on Developing and Assessing Adverse Outcome Pathways., ENV/JM/MONO(2013)6.

(9) Allen, T. E., Goodman, J. M., Gutsell, S., and Russell, P. J. (2014) Defining Molecular Initiating Events in the Adverse Outcome Pathway Framework for Risk Assessment. *Chem. Res. Toxicol.*, In Press.

(10) Pleil, J. D., Williams, M. A., and Sobus, J. R. (2012) Chemical Safety for Sustainability (CSS): human in vivo biomonitoring data for complementing results from in vitro toxicology--a commentary. *Toxicol. Lett. 215*, 201-207.

(11) Wetmore, B. A., Allen, B., Clewell, H. J., 3rd, Parker, T., Wambaugh, J. F., Almond, L. M., Sochaski, M. A., and Thomas, R. S. (2014) Incorporating Population Variability and Susceptible Subpopulations into Dosimetry for High-Throughput Toxicity Testing. *Toxicol. Sci. 142*, 210-224.

(12) Wetmore, B. A., Wambaugh, J. F., Ferguson, S. S., Li, L., Clewell, H. J., 3rd, Judson, R. S., Freeman, K., Bao, W., Sochaski, M. A., Chu, T. M., Black, M. B., Healy, E., Allen, B., Andersen, M. E., Wolfinger, R. D., and Thomas, R. S. (2013) Relative impact of incorporating pharmacokinetics on predicting in vivo hazard and mode of action from high-throughput in vitro toxicity assays. *Toxicol. Sci. 132*, 327-346.

(13) Pleil, J. D., and Sheldon, L. S. (2011) Adapting concepts from systems biology to develop systems exposure event networks for exposure science research. *Biomarkers 16*, 99-105.
(14) Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B. J., Boobis, A., Carere, A., Kevekordes, S., Lhuguenot, J. C., Pieters, R., and Kleiner, J. (2002) Methods of in vitro toxicology. *Food Chem. Toxicol. 40*, 193-236.
(15) Soldatow, V. Y., Lecluyse, E. L., Griffith, L. G., and Rusyn, I. (2013) models for liver toxicity testing. *Toxicol Res (Camb) 2*, 23-39.
(16) Judson, R. S., Kavlock, R. J., Setzer, R. W., Hubal, E. A., Martin, M. T., Knudsen, T. B., Houck, K. A., Thomas, R. S., Wetmore, B. A., and Dix, D. J. (2011) Estimating toxicity-related biological pathway altering doses for high-throughput chemical risk assessment. *Chem. Res. Toxicol. 24*, 451-462.
(17) Rotroff, D. M., Wetmore, B. A., Dix, D. J., Ferguson, S. S., Clewell, H. J., Houck, K. A., Lecluyse, E. L., Andersen, M. E., Judson, R. S., Smith, C. M., Sochaski, M. A., Kavlock, R. J., Boellmann, F., Martin, M. T., Reif, D. M., Wambaugh, J. F., and Thomas, R. S. (2010) Incorporating human dosimetry and exposure into high-throughput in vitro toxicity screening. *Toxicol. Sci. 117*, 348-358.
(18) Buckley, T. J., Pleil, J. D., Bowyer, J. R., and Davis, J. M. (2001) Evaluation of methyl tert-butyl ether (MTBE) as an interference on commercial breath-alcohol analyzers. *Forensic Sci. Int. 123*, 111-118.
(19) Kim, D., Andersen, M. E., Pleil, J. D., Nylander-French, L. A., and Prah, J. D. (2007) Refined PBPK model of aggregate exposure to methyl tertiary-butyl ether. *Toxicol. Lett. 169*, 222-235.
(20) Lindstrom, A. B., and Pleil, J. D. (1996) A methodological approach for exposure assessment studies in residences using volatile organic compound-contaminated water. *J. Air Waste Manag. Assoc. 46*, 1058-1066.
(21) Lindstrom, A. B., and Pleil, J. D. (1996) Alveolar breath sampling and analysis to assess exposures to methyl tertiary butyl ether (MTBE) during motor vehicle refueling. *J. Air Waste Manag. Assoc. 46*, 676-682.
(22) Ghimenti, S., Di Francesco, F., Onor, M., Stiegel, M. A., Trivella, M. G., Comite, C., Catania, N., Fuoco, R., and Pleil, J. D. (2013) Post-operative elimination of sevoflurane anesthetic and hexafluoroisopropanol metabolite in exhaled breath: pharmacokinetic models for assessing liver function. *J Breath Res 7*, 036001.
(23) Pleil, J. D., Kim, D., Prah, J. D., and Rappaport, S. M. (2007) Exposure reconstruction for reducing uncertainty in risk assessment: example using MTBE biomarkers and a simple pharmacokinetic model. *Biomarkers 12*, 331-348.
(24) Prah, J., Ashley, D., Blount, B., Case, M., Leavens, T., Pleil, J., and Cardinali, F. (2004) Dermal, oral, and inhalation pharmacokinetics of methyl tertiary butyl ether (MTBE) in human volunteers. *Toxicol. Sci. 77*, 195-205.
(25) Croom, E. L., Shafer, T. J., Evans, M. V., Mundy, W. R., Eklund, C. R., Johnstone, A. F., Mack, C. M., and Pegram, R. A. (2014) Improving in vitro to in vivo extrapolation by incorporating toxicokinetic measurements: A case study of lindane-induced neurotoxicity. *Toxicol. Appl. Pharmacol.* In Press.
(26) Ono, S., Hatanaka, T., Hotta, H., Satoh, T., Gonzalez, F. J., and Tsutsui, M. (1996) Specificity of substrate and inhibitor probes for cytochrome P450s: evaluation of in

vitro metabolism using cDNA-expressed human P450s and human liver microsomes. *Xenobiotica 26*, 681-693.
(27) Yuan, R., Madani, S., Wei, X. X., Reynolds, K., and Huang, S. M. (2002) Evaluation of cytochrome P450 probe substrates commonly used by the pharmaceutical industry to study in vitro drug interactions. *Drug Metab. Dispos. 30*, 1311-1319.
(28) Rodriguez-Antona, C., Donato, M. T., Boobis, A., Edwards, R. J., Watts, P. S., Castell, J. V., and Gomez-Lechon, M. J. (2002) Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. *Xenobiotica 32*, 505-520.
(29) Aninat, C., Piton, A., Glaise, D., Le Charpentier, T., Langouet, S., Morel, F., Guguen-Guillouzo, C., and Guillouzo, A. (2006) Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab. Dispos. 34*, 75-83.
(30) Guillouzo, A., Corlu, A., Aninat, C., Glaise, D., Morel, F., and Guguen-Guillouzo, C. (2007) The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-biological interactions 168*, 66-73.
(31) Science, G. H. a. L. (2014) Microcarrier Cell Culture Principles and Methods.
(32) Orive G., H. R. M., Gascon A.R., Pedraz J.L. (2006) *Immobilization of Enzymes and Cells*. Vol. 22, 2 ed., Humana Press.
(33) Houck, K. A., Dix, D. J., Judson, R. S., Kavlock, R. J., Yang, J., and Berg, E. L. (2009) Profiling bioactivity of the ToxCast chemical library using BioMAP primary human cell systems. *J Biomol Screen 14*, 1054-1066.
(34) Hinson, J. A., Roberts, D. W., and James, L. P. (2010) Mechanisms of acetaminophen-induced liver necrosis. *Handb. Exp. Pharmacol.*, 369-405.
(35) Darzynkiewicz, Z., Li, X., and Gong, J. (1994) Assays of cell viability: discrimination of cells dying by apoptosis. *Methods Cell Biol. 41*, 15-38.
(36) Kaltenbach, J. P., Kaltenbach, M. H., and Lyons, W. B. (1958) Nigrosin as a dye for differentiating live and dead ascites cells. *Exp. Cell Res. 15*, 112-117.
(37) EPA. (1999) Compendium of Methods for the determination of toxic organic compounds in ambient air: Compendium Method TO-17, US Environmental Protection Agency.
(38) Pleil, J. D., Hubbard, H. F., Sobus, J. R., Sawyer, K., and Madden, M. C. (2008) Volatile polar metabolites in exhaled breath condensate (EBC): collection and analysis. *J Breath Res 2*, 026001.
(39) Pleil, J. D., Miekisch, W., Stiegel, M. A., and Beauchamp, J. (2014) Extending breath analysis to the cellular level: current thoughts on the human microbiome and the expression of organic compounds in the human exposome. *J Breath Res 8*, 029001.
(40) Pleil, J. D., Stiegel, M. A., and Fent, K. W. (2014) Exploratory breath analyses for assessing toxic dermal exposures of firefighters during suppression of structural burns. *J Breath Res 8*, 037107.
(41) Lord, H. L., Zhan, W., and Pawliszyn, J. (2010) Fundamentals and applications of needle trap devices: a critical review. *Anal. Chim. Acta 677*, 3-18.
(42) Trefz, P., Kischkel, S., Hein, D., James, E. S., Schubert, J. K., and Miekisch, W. (2012) Needle trap micro-extraction for VOC analysis: effects of packing materials and desorption parameters. *J. Chromatogr. A 1219*, 29-38.

(43) Herbig, J., and Beauchamp, J. (2014) Towards standardization in the analysis of breath gas volatiles. *J Breath Res 8*, 037101.
(44) Herbig, J., Muller, M., Schallhart, S., Titzmann, T., Graus, M., and Hansel, A. (2009) On-line breath analysis with PTR-TOF. *J Breath Res 3*, 027004.
(45) Blake, R. S., Monks, P. S., and Ellis, A. M. (2009) Proton-transfer reaction mass spectrometry. *Chem. Rev. 109*, 861-896.
(46) Winkler, K., Herbig, J., and Kohl, I. (2013) Real-time metabolic monitoring with proton transfer reaction mass spectrometry. *J Breath Res 7*, 036006.
(47) McCurdy, M. R., Bakhirkin, Y., Wysocki, G., Lewicki, R., and Tittel, F. K. (2007) Recent advances of laser-spectroscopy-based techniques for applications in breath analysis. *J Breath Res 1*, 014001.
(48) Wysocki, G. C., R.F.; Tittel, F.K.; Maulini, R.; Bulliard, J.M.; Faist, J. (2005) Widely tunable mode-hop free external cavity quantum cascade laser for high resolution spectroscopic applications. *Applied Physics B 81*, 9.
(49) Mürtz, M. a. H., P. (2008) *Online Monitoring of Exhaled Breath Using Mid-Infrared Laser Spectroscopy*. Vol. 3, Springer Netherlands.
(50) Nasim, H. a. J., Y. (2013) Recent advancements in spectroscopy using tunable diode lasers. *Laser Phys. Lett. 10*, 043001.
(51) Boekelheide, K., and Campion, S. N. (2010) Toxicity testing in the 21st century: using the new toxicity testing paradigm to create a taxonomy of adverse effects. *Toxicol. Sci. 114*, 20-24.
(52) Dix, D. J., Houck, K. A., Martin, M. T., Richard, A. M., Setzer, R. W., and Kavlock, R. J. (2007) The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicol. Sci. 95*, 5-12.
(53) Collins, F. S., Gray, G. M., and Bucher, J. R. (2008) Toxicology. Transforming environmental health protection. *Science 319*, 906-907.
(54) Hubal, E. A. (2009) Biologically relevant exposure science for 21st century toxicity testing. *Toxicol. Sci. 111*, 226-232.
(55) Raunio, H. (2011) In silico toxicology - non-testing methods. *Front. Pharmacol. 2*, 33.
(56) Schmidt, C. W. (2009) TOX 21: new dimensions of toxicity testing. *Environ. Health Perspect. 117*, A348-353.
(57) Shah, F., and Greene, N. (2014) Analysis of Pfizer compounds in EPA's ToxCast chemicals-assay space. *Chem. Res. Toxicol. 27*, 86-98.
(58) Sulzer, P. K., T.; Juerschik, S.; Lanza, M.; Hartungen, A.; Jordan, A.; Edtbauer, S.; Feil, S.; Hanel, G.; Maerk, J1. L., et al. (2013) Detection of Toxic Industrial Compounds (TIC) with Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) for a real-life monitoring scenario, In *6th International Conference on Protin Transfer Reaction Mass Spectrometry and its Applications* (Hansel A., D. J., Ed.) p 196, innsbruck university press, Obergurgl, Austria.
(59) Angrish, M. M., Dominici, C. Y., and Zacharewski, T. R. (2013) TCDD-elicited effects on liver, serum, and adipose lipid composition in C57BL/6 mice. *Toxicol. Sci. 131*, 108-115.
(60) Angrish, M. M., Jones, A. D., Harkema, J. R., and Zacharewski, T. R. (2011) Aryl Hydrocarbon Receptor-Mediated Induction of Stearoyl-CoA Desaturase 1 Alters Hepatic Fatty Acid Composition in TCDD-Elicited Steatosis. *Toxicol Sci 124*, 299-310.

(61) Angrish, M. M., Mets, B. D., Jones, A. D., and Zacharewski, T. R. (2012) Dietary fat is a lipid source in 2,3,7,8-tetrachlorodibenzo-rho-dioxin (TCDD)-elicited hepatic steatosis in C57BL/6 mice. *Toxicol. Sci. 128*, 377-386.
(62) Kavlock, R., and Dix, D. (2010) Computational toxicology as implemented by the U.S. EPA: providing high throughput decision support tools for screening and assessing chemical exposure, hazard and risk. *J. Toxicol. Environ. Health. B Crit. Rev. 13*, 197-217.
(63) Shah, I., and Wambaugh, J. (2010) Virtual tissues in toxicology. *J. Toxicol. Environ. Health. B Crit. Rev. 13*, 314-328.
(64) Glaze, W. H. (1998) Too little data, too many models. *Environ. Sci. Technol. 32*, 207A.
(65) NRC. (2007) Toxicity testing in the 21st century: a vision and a strategy. The National Academies Press, Washington, DC.

**Tables**

**Table 1.** Pharmacokinetic parameters for MTBE and TBA: calculated from data and PK models derived from human inhalation studies conducted at EPA. [23]

| Compound | Parameter | Description | Value |
|---|---|---|---|
| MTBE | $K_0$ | MTBE uptake | 6.85 L $min^{-1}$ |
| | $K_{MT}$ | MTBE loss to TBA | 0.820 L $min^{-1}$ |
| | $K_{MB}$ | MTBE loss to air | 0.380 L $min^{-1}$ |
| TBA | $K_{TT}$ | TBA production | 0.165 L $min^{-1}$ |
| | $K_{TL}$ | TBA loss to air | 0.597 L $min^{-1}$ |

**Figure Legends**

**Figure 1.** Schematic illustrating a linear adverse outcome pathway that also includes input from a computational, high-throughput screening approach to chemical prioritization.

**Figure 2.** Schematic diagram illustrating an *in vitro* liver function model for gas phase PrMs. $CO_2$, air, and PrMs are bubbled into the micro-reactor through a sterilizing membrane. Cell suspension cultures are exposed to chemicals of interest through the exposure inlet and the system is held at steady state concentration. Chemicals of interest are added to the steady state system and effects on PrM metabolites are measured from air by quantitative mass spectrometry or similar methods.

**Figure 3.** Conceptual model for probe molecule absorption, distribution, metabolism, and elimination. The model assumes first order kinetics and proportional elimination into the air. Arrows represent rate constants, $MTBE_I$ and $TBA_C$ represents the concentrations of parent and metabolite, respectively, and subscripts $_C$ or $_{air}$ represent distribution to the *in vitro* liver (central compartment) or air.

**Figure 4**. Methyl tertiary butyl either (MTBE) pharmacokinetic (PK) model: (A) steady state conditions with constant MTBE exposure and (B) steady state with 30 μM diethyldithiocarbamate (DETC) co-exposure at 60 hour (h). TBA, tertiary butyl alcohol; $_{CC}$, central compartment; $_{air}$, chemical concentration in the air.

## Figures

### Figure 1

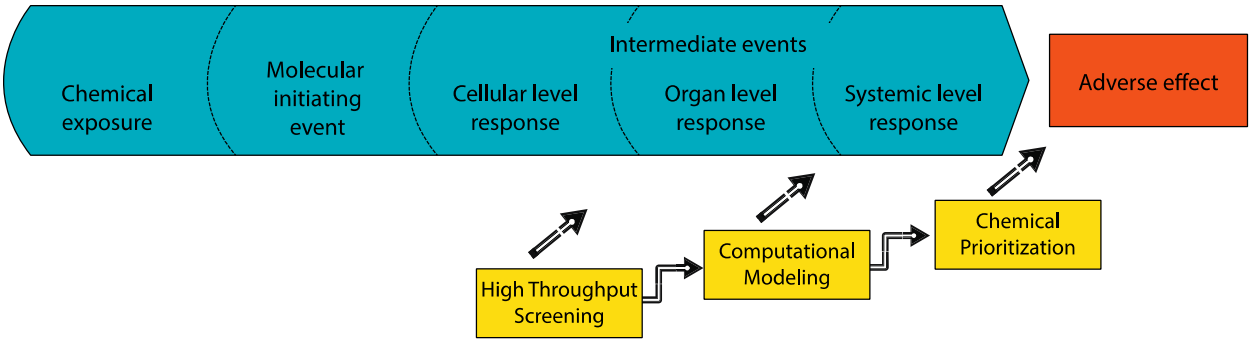


**Figure 2**

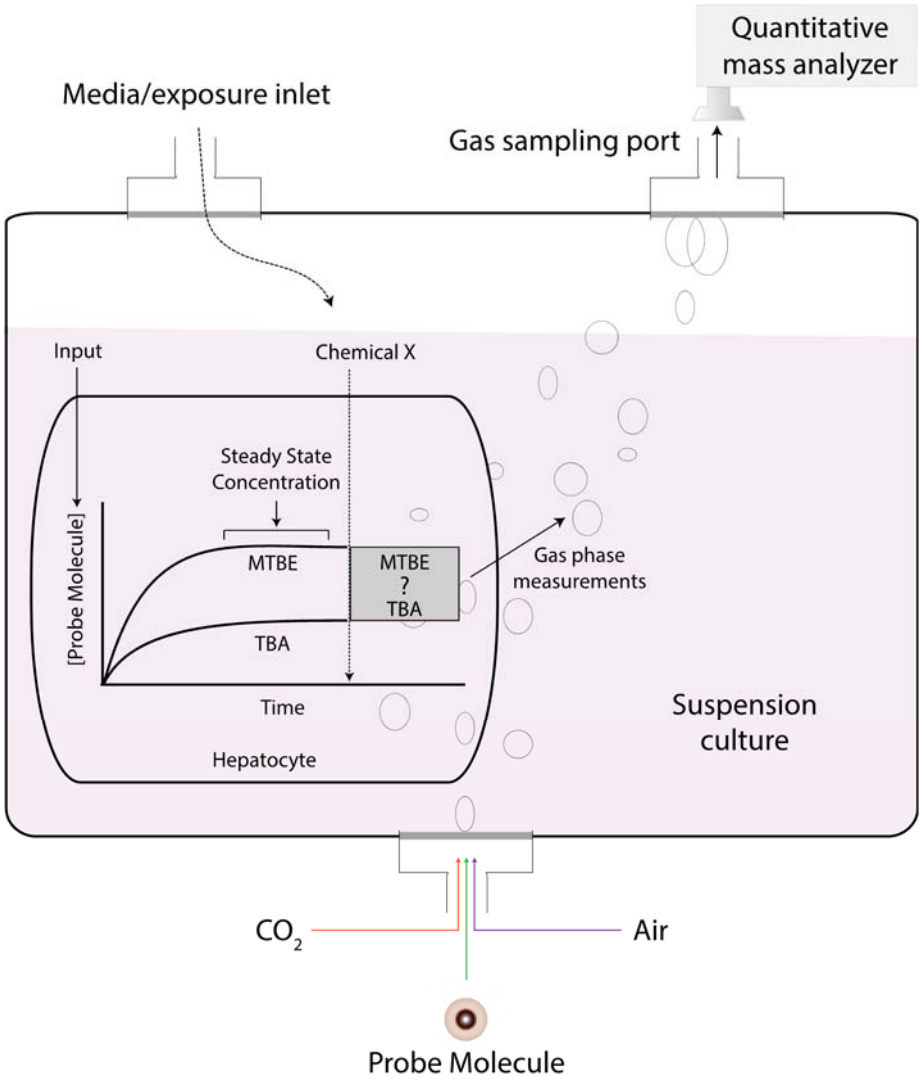

Quantitative mass analyzer
Media/exposure inlet
Gas sampling port
Input
Chemical X
Steady State Concentration
[Probe Molecule]
MTBE
MTBE ? TBA
TBA
Time
Hepatocyte
Gas phase measurements
Suspension culture
$CO_2$
Air
Probe Molecule

**Figure 3**

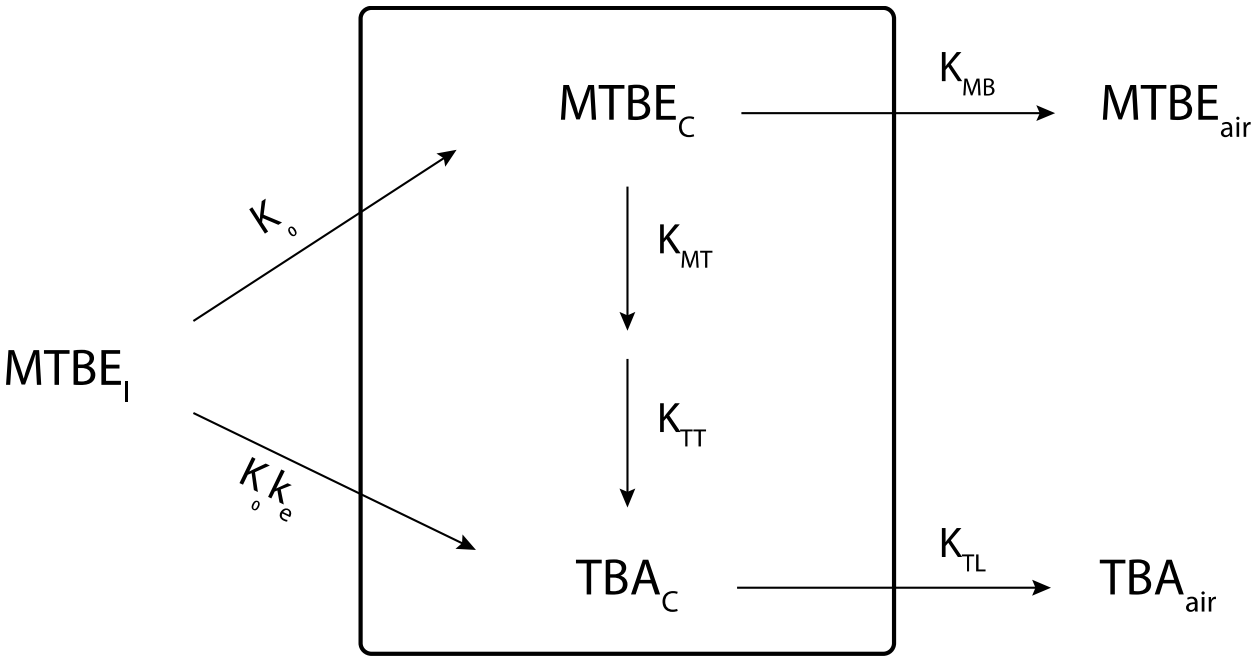
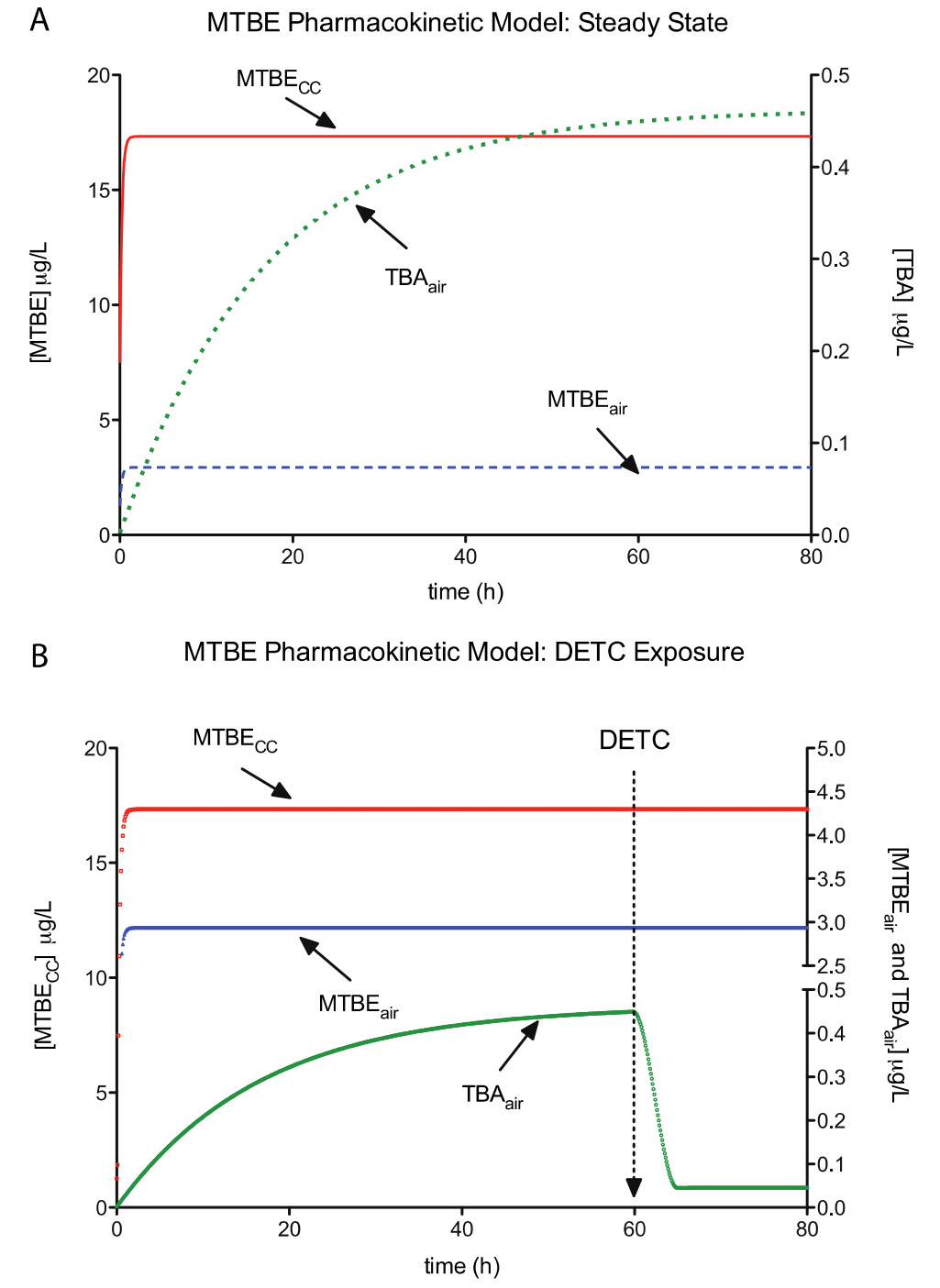


**Figure 4**


A
MTBE Pharmacokinetic Model: Steady State
MTBE$_{CC}$
TBA$_{air}$
MTBE$_{air}$
[MTBE] µg/L
[TBA] µg/L
time (h)
B
MTBE Pharmacokinetic Model: DETC Exposure
MTBE$_{CC}$
DETC
MTBE$_{air}$
TBA$_{air}$
[MTBE$_{CC}$] µg/L
[MTBE$_{air}$ and TBA$_{air}$] µg/L
time (h)