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Physiologically Based Pharmacokinetic Models: Integration of In Silico Approaches with Micro Cell Culture Analogues

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Abstract

There is a large emphasis within the pharmaceutical industry to provide tools that will allow early research and development groups to better predict dose ranges for and metabolic responses of candidate molecules in a high throughput manner, prior to entering clinical trials. These tools incorporate approaches ranging from PBPK, QSAR, and molecular dynamics simulations in the *in silico* realm, to micro cell culture analogue (CCAs) in the *in vitro* realm. This paper will serve to review these areas of high throughput predictive research, and highlight hurdles and potential solutions. In particular we will focus on CCAs, as their incorporation with PBPK modeling has the potential to replace animal testing, with a more predictive assay that can combine multiple organ analogs on one microfluidic platform in physiologically correct volume ratios. While several advantages arise from the current embodiments of CCAs in a microfluidic format that can be exploited for realistic simulations of drug absorption, metabolism and action, we explore some of the concerns with these systems, and provide a potential path forward to realizing animal-free solutions. Furthermore we envision that, together with theoretical modeling, CCAs may produce reliable predictions of the efficacy of newly developed drugs.

Keywords

Physiological based pharmacokinetic models; microfluidic; cell culture analogue; QSAR; molecular dynamic simulation

1.0. Introduction and Background

1.1. Industrial Significance

Whole animal testing has traditionally taken the central role in evaluating the toxicological and pharmacological profiles of drugs. However, there are a number of substantial problems with this approach. According to the PhRMA (Pharmaceutical Research and Manufacturers of America), the cost to bring a drug to market has risen from \$138 million in 1975 to over \$1 billion in 2010 [1-8]. In fact, U.S. drug companies spent \$67.4 billion on research and development in 2010 [1, 3, 8]. 50% of candidate drugs fail to pass Phase II clinical testing, and 90% fail to pass the final stages of development, due to human toxicity and bioavailability issues. Very often, the sheer difference in size scale between rodents and humans often leads to poor translation from benchtop experimentation to human application

[7, 9, 10]. The complexity and variability within the organism itself makes it difficult for researchers to definitively isolate the molecular mechanisms of action for a drug candidate using animal-based studies – too many additional processes are occurring simultaneously. This inability to determine the exact mechanism of action prevents reliable extrapolation across species and dosage levels [10]. These problems, combined with the exorbitant costs, cumbersome timelines, and potential questions of ethicality, create a substantial need for practical and reliable alternatives to animal studies.

To reduce the tremendous cost and attrition rate of the drug development process, much recent research [11-34] has been dedicated to identifying *in vitro* screening systems i.e., approaches that can be used in preclinical phases of discovery and development to predict *in vivo* subcellular and cellular physiological responses. This work has also led to a federal consortium of groups with a similar goal known as the Tox21 group; a collaboration between EPA, the National Institutes of Environmental Health Sciences, the National Toxicology program, NIH, National Human Genome Research Institute, the NCGC, and the FDA. *In silico* techniques such as physiologically based pharmacokinetic (PBPK) modeling, quantitative structure-activity relationships (QSAR), and molecular dynamic simulations (MDS) have been the focus of much recent research because of their potential to improve the predictive capabilities of drug screening at the onset of development [13, 19, 20, 24-26]. PBPK models mathematically simulate animal metabolism by predicting the absorption, distribution, metabolism, and excretion (ADME) of pharmaceutical compounds within a system of interconnected tissue compartments [10, 26, 27, 35-42], while QSAR and MDS augment the PBPK model by predicting the *in vivo* effects of chemical structural changes on key chemical properties [25, 43-55].

Meanwhile, cell culture analog (CCA) systems have garnered interest in the field for their potential as an alternative to animal testing when driven by PBPK predictions. CCAs physically replicate the PBPK using mammalian cells cultured in compartments that represent organs, which are then connected by a circulating medium that represents blood [10, 40, 56-63]. In this way, an *in vitro* screening strategy that combines CCAs with *in silico* PBPK modeling may produce reliable projections of drug efficacy while avoiding the difficulties of animal testing.

1.2. Historical Perspective

PBPK modeling can be traced back to 1937 to an article by Teorell, in which the first pharmacokinetic model was described in scientific literature [35]. While the article postulated the basics of the technique, the model remained unsolvable due to the computational limitations of the time. The focus therefore shifted to simpler models for which analytical solutions could be obtained. The seminal papers on the liver PBPK model by Rowland *et al.* [60] - which describe the influence of blood flow, intrinsic clearance, and binding on hepatic clearance - inspired further development of liver, kidney, intestine, and whole-body PBPK modeling. The availability of computers and numerical integration algorithms marked a renewed interest in physiological models in the early 1970s [34]. By the late 2000s, with the advent of high throughput screening creating a need for high throughput pharmacokinetic assessment in the pharmaceutical industry, hundreds of scientific publications have adopted the PBPK strategy and at least two private companies are basing their business on their expertise in this area.

1.3. Overview of Paper

Within this review paper we will review current research efforts for the development of *in vitro* and *in silico* approaches that can be used to overcome limitations in conventional cell-based assays that are utilized to determine key pharmacokinetic properties of new chemical

entities. First we will summarize PBPK models and the *in vitro* assays needed to provide key model parameters. Additionally we will evaluate the limitations of the current systems. We will then review developments in microelectromechanical systems (MEMS) that can be used as microscale cell culture analogs (CCA) to overcome the limitations of conventional cell-based assays. We will conclude with an analysis of other *in silico* approaches that can be used to augment PBPK systems through the integration of chemical level analysis.

2.0. In Silico Models

2.1. PBPK

Physiologically-based pharmacokinetic (PBPK) modeling is a mathematical modeling technique used in pharmaceutical research and development to predict the absorption, distribution, metabolism and excretion (ADME) of a compound in humans and other animal species [60, 62].

The concept of physiologically-based PK/PD modeling has been described in papers within the past decade [26, 27, 39-42, 61, 62, 64-68]. In the most complete sense, PBPK models attempt to recapitulate the entire body as a closed circulatory system with interconnected compartments, each of which represents a specific organ or tissue (Fig. 1). The transport between the various tissue compartments is modeled using mass balance equations that describe organ and tissue-specific blood flow into and out of each compartment. In the tissue specific compartments, it is paramount to distinguish between blood, interstitial space, and intracellular space because these compartments are separated from plasma by membranes that can form physiological barriers.

There are a variety of key parameters necessary in establishing a PBPK model. The first sets of inputs are physical (binding and distribution) and biochemical (Michaelis–Menten parameters, V_{max}/K_m) data. Other requisite data sets include enzymatic constants for metabolism, passive diffusion clearance and transport clearances for influx or efflux at the basolateral membrane. Additionally transport is needed at the apical membrane for secretion of the eliminating organ. These, taken together with estimates of tissue to plasma or blood partitioning coefficients for weak bases and acids provide fairly accurate ADME predictions [37, 38]. Furthermore, as the researchers gain knowledge about new chemical entities, subsections of the whole body PBPK can be refined to model the drug environment in more detail.

It should be noted that, in practice, a PBPK model is distinct from compartmental modeling. The compartmental model usually combines all of the metabolite formation and elimination organs into a single compartment, whereas the PBPK model treats each organ and tissue as a separate entity. Unlike the compartmental model, the PBPK is able to account for the amount of metabolite formed, as well as the amount that does not reach systemic circulation; the sink terms in each tissue compartment (metabolism and excretion) reduce the rate of metabolite appearance into systemic circulation. The PBPK model also addresses the difference in transporters among tissues and describes how the transport occurs; it can reveal the dynamic process by which passive diffusion and/or active transporters facilitate entry of the parent drug or its metabolites into eliminating organs. These added functionalities allow PBPK modeling to provide more accurate predictions of first-pass removal during drug absorption.

2.2. QSAR

Human exposure to exogenous chemicals, drugs and factors creates a complex response both locally and systemically; there is not a singular approach in predictive toxicology that can fully simulate this complexity. Nevertheless, in order to regulate the use of chemicals,

regulatory agencies and authorities, such as the EPA in the US and EEC for the European Union, require reliable information pertaining to human health effects [69, 70]. To date, the “goto” tools for toxicology testing has been predominantly limited to *in vivo* animal testing and *in vitro* assays. Recently, however, much debate has arisen over the suitability of animal models, especially rodent models, in predicting human physiological response.

Computational approaches then represent a potential alternative to animal testing. One approach is based on the relationship between a compound's molecular structure and its chemical activity. Shortly termed as QSAR (Qualitative/Quantitative Structure Activity Relationships), these approaches attempt to find consistent mathematical relationships between variations in the molecular properties and biological activities of a series of compounds [71, 72]. These rules can then be applied to new chemical entities. This body of work operates on the assumption that a molecule's physical, chemical, and biological effect is implicit from its geometric and electronic properties; based on this assumption, QSARs deduce toxicity and other biological endpoints of a new compound by comparing an array of physicochemical descriptors that characterize the compound to a database of existing “similar” compounds. These physicochemical descriptors, which include parameters to account for hydrophobicity, topology, electronic properties, and steric effects, can range from simple atom counts to two dimensional structural information and even three/four dimensional shape signatures [71, 73].

QSARs can be classified based on structural representation. 1D-QSAR correlates activity with global molecular properties like pKa. 2D-QSAR correlates activity with structural patterns like connectivity indices and 2D pharmacophores. 3D-QSAR expands to the correlation of activity with non-covalent interaction fields surrounding the molecular. 4D-QSAR additionally includes the ensemble of ligand configurations. 5D-QSAR represents different induced-fit models in the 4D-QSAR, and 6D-QSAR further incorporates different solvation models in 5D-QSAR [74]. Classical QSAR methods utilize a 2D scheme and are simpler and faster than the 3D approach. However, they often suffer from a lack of parameters describing drug-receptor interactions, such as steric effects, and do not account for the stereochemistry or 3D geometry of the molecule. As such, the extension to 3D has become very important in structural *in silico* drug screening; the increasing body of information regarding structural biology has added to the assumptions that form the basis of 3D-QSAR methods, and many forms of 3D-QSAR have been developed in the past decade.

In general QSARs focus on several endpoint outcomes which are relevant to environmental and regulatory agencies as well as the pharmaceutical industry. These endpoints are acute, chronic reproductive and developmental toxicity; immunological toxicity; neurotoxicity; hepatotoxicity; carcinogenicity, phospholipidosis; mutagenicity, skin absorption, irritation and sensitization [69, 75]. We have to note that QSARs are generally empirical and statistical approaches; however, there are approaches to enable numerical representation based on descriptors/substructure information [73]. One such approach for computing descriptors is a freeware by the FDA National Center for Toxicological Research; there are also a plethora of confidential industrial and commercial approaches [71].

However, while QSAR has the potential to enable rapid computational assessment of drug toxicity, it is limited by the dearth of biological data available for developing and validating predictive models. The data pool is very narrow in terms of chemical and biological space (i.e. mechanisms of action) for nearly all toxicity end-points. The data for *in vitro* toxicity is limited to a few thousand chemicals, and the data for *in vivo* toxicity is further restricted to a few hundred chemicals. This is compounded by the general unevenness of data quality within the field and the lack of understanding regarding the impact of poor-quality data on the models [70, 76]. Another obstacle for QSAR is the difficulty of modeling the nonlinear

relations that characterize multiple modes of action and multiple drug interactions [25]. An even more compounding hurdle is the current inability to quantify the majority of endpoints, such as carcinogenicity, neural toxicity, and behavioral toxicity. In a quest to predict relevant toxicity for millions of chemicals, these issues become fundamental impediments for the QSAR approach [25, 76].

2.3. Molecular Dynamic Simulations

The second major approach for in silico toxicology prediction utilizes molecular dynamics (MD) simulations to investigate the physical movements of atoms within the NCE. In a similar vein to the QSAR approach, the premise for MD is that a drug molecule's biological activity depends directly on the three-dimensional arrangement of its functional groups, i.e. its pharmacophore [47, 51-53, 77, 78]. MD simulations model molecular structures in three dimensions and predict their behavior in the biological environment using either Newtonian deterministic dynamics or Langevin-type stochastic dynamics [79, 80]. MD simulations operate by taking energy calculations and integrating Newton's equations of motion over small time steps ($\sim 10^{-15}$ sec-1 fsec) [47, 54, 79]. Once the velocities are computed, new atom locations can be calculated. These values are then used to calculate trajectories, or time dependent locations for each atom. Trajectory analysis can yield important information regarding the formulation solubility enhancement, drug binding, or drug diffusion in lipid membranes, as well as the macroscopic behavior of such systems.

MD methods originated within the theoretical physics community during the 1950s. The earliest MD simulations, conducted by Alder and Wainright in 1957 [81], analyzed only those atomic interactions that occurred through perfect collisions. As computational power improved in the 1970s, the first MD simulations for protein interactions were developed through the use of physics-based first-principle empirical energy functions [47, 54]. While the earliest protein MD simulations covered only several picoseconds of simulation (or equilibration) time, theoretical, methodological, and computational advancements have allowed modern implementations to routinely perform simulations at the nano- and microsecond time scale. Furthermore, while the original MD simulations used only 500 atoms, modern techniques are able to account for 10^4 - 10^7 atoms [53, 54].

In the context of drug discovery and testing, MD allows for a detailed investigation of the enzyme-substrate interactions that drive key biological processes. Such processes include signal transduction, metabolic regulation, enzyme cooperativity, and physiological response, all of which depend on noncovalent binding at the molecular level [54]. MD allows for estimations of the binding modes and binding free energies for protein-ligand and protein-protein complexes. Specifically, the prediction of protein-ligand binding is known as ligand docking. Older ligand-docking methods predominantly used molecular mechanics-based scoring functions; however, the present discussion will be limited to the newer methods that apply molecular dynamics to the analysis.

The AutoDock 4.0 simulation software, which is one of the most cited docking programs in the research community, provides a good example of how ligand docking is employed. AutoDock 4.0 uses molecular dynamics to predict the docking of a flexible ligand to a binding site of a non-flexible enzyme [51, 82-90]. The program consists of three subcomponents: AutoTors, AutoGrid, and Auto-Dock. AutoTors, the simplest of the three, defines the rotatable bonds within the ligand. Each rotatable bond adds an extra degree of freedom (DOF) to the ligand and thus increases the complexity of the simulation. As such, large molecules containing many rotatable torsional angles can quickly cause computational overload. AutoGrid pre-calculates a three-dimensional grid of interaction energies based on the targeted macromolecule. With the rigid receptor protein's structure defined, interaction energies between the target and its surroundings can be calculated, and hydrogen bonding,

dispersion/repulsion, and electrostatic effects can be assessed. After the full grid is defined, AutoDock performs the simulation itself by moving the ligand randomly in the available degrees of freedom and calculating the energy of the new ligand “state”. Ultimately, the objective is to find, within a reasonable computational timeframe, the global minimum in the interaction energy between the substrate and the target protein [82, 85].

One of the major concerns for these types of ligand-docking simulations is the balance between accuracy and computational cost [53-55]. The tremendous complexity of the molecular environment requires one to account for hundreds of thousands of degrees of freedom, as well as the multitude of energetic forces, when analyzing only one receptor-ligand combination. In particular, the ligand binding process can lead to conformational changes in the receptor protein that may be necessary for the receptor to accommodate the bound ligand. While it is important to explore the conformational space available to the receptor protein, it is often difficult to represent the plasticity of the binding site without overloading the computation [54]. This issue becomes especially important when multiple allosterically connected binding sites are present. As such, modifications are needed to allow further exploration of receptor conformations so that these MD simulations are more practical [91]. One such solution involves varying the temperatures that specifically affect the flexibility of the ligand being simulated [92]. In doing so, sampling for the docking simulations can be enhanced. A second major concern that arises from computational limitations is the duration length [54, 55]. In fact, short MD procedures have been shown in certain cases to improve the performance of docking simulations compared to long, static structures.

Very recently, hybrid methods that combine 3D-QSAR, MD, docking, and molecular mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) have arisen to investigate the binding interactions of early-stage drug candidates [93]. 3D-QSAR using comparative molecular field analysis (CoMFA) allowed for the exploration of the specific contributions of electrostatic, steric, and hydrophobic effects of dihydroquinoline analogues binding to glucocorticoid receptor – a promising pharmaceutical target to treat inflammatory diseases. Here, 3D-QSAR was not used model the activities of new ligand analogs, but instead to assess the structure–activity relationships between the different molecular entities. Molecular dynamics simulations were then performed to assess binding modes while accounting for receptor flexibility. Molecular docking studies were also performed wherein receptor flexibility was not a factor of consideration. Here, the position, conformation, and ligand orientation relative to the receptor were investigated. Finally MM/PBSA analyses confirmed the binding modes from the previous MD simulations by computing their exact binding free energies based on the MD trajectories. Combined in silico studies [94-101] use an integrated approach, are very new. However, their rapid adoption into numerous studies within the past year signifies the potential for this paradigm to improve the accuracy and reliability of in silico drug screening.

3.0. Determining Parameters PBPK Model Parameters

3.1. Metabolism and Clearance

Metabolic modeling, used to describe the clearance parameter of PBPK models, is most often characterized by one of three processes: 1) first order kinetics; 2) zero order kinetics; or 3) Michaelis–Menten kinetics . First-order metabolism is classified as a linear dependency with respect to substrate concentration, i.e. a constant fraction of compound is metabolized per unit time. Zero order kinetics is less common at physiologically relevant concentrations; it is classified as a constant amount of compound metabolized per unit time. Michaelis–Menten kinetics is the approach used most often. Here, metabolism at lower concentrations (below the Michaelis–Menten constant, K_m) is first order; meanwhile at

higher concentrations the metabolic capacity of the system is exceeded and metabolism becomes zero order. Michaelis–Menten kinetics describes enzymatic reaction rate by relating the rate of reaction (V) to the substrate concentration (S) as follows:

$$V = \frac{V_{\max}[S]}{K_m + [S]}$$

The key variable here is $V_{\max}$, which represents the maximum rate achieved by the system, at maximum (saturating) substrate concentrations. The Michaelis constant K_m is the substrate concentration at which the reaction rate is half of $V_{\max}$. If saturation does not occur, a zero- or first-order metabolic process is described; however, Michaelis–Menten kinetics is usually assumed for single substrate biochemical reactions. Once the mechanism (zero-order, first-order, or Michaelis–Menten) is described, one can then determine the *in vitro* rate constants.

The gold standard for ascertaining the clearance/metabolic parameters of the Michaelis–Menten model *in vitro* culture systems uses cultured primary hepatocytes, either in suspension or in a plated configuration. Hepatocytes contain all of the enzymes (cytochrome P450s, transporters, etc.) in the cytosol and in microsomes.

It is generally accepted that, in a suspension-based culture, hepatocyte enzymatic activity rapidly diminishes three hours after the cells are thawed. Plating paradigms have thus been developed to extend the functional competency of hepatocytes. These approaches include cocultures, collagen sandwich configurations, and microfabricated placement using protein patterning or microstencils [102–110]. While plating may extend the practical life of hepatocytes, it can also affect the concentrations of important drug metabolizing enzymes [102, 111]. For this reason, quantitative extrapolation of metabolic rate constants derived from plated cells, when extrapolated based on cell count, should be carefully evaluated for multiple donor hepatocyte populations. This is important in determining both the temporal competency and the population variation of hepatocyte functionality.

It should also be noted that Michaelis–Menten is a simplistic reduction of clearance though, and more sophisticated models are currently utilized. For example, a well stirred model, and parallel tube model are implemented to calculate hepatic clearance for new chemical entities, either using suspension culture hepatocytes, or microfluidics systems. In these models the *in vitro* intrinsic clearance obtained from the static culture conditions to the estimated hepatic clearance (CL_H), assuming the drug is totally unbound in the serum-free culture medium:

$$CL_H = Q_H \times \frac{CL_{\text{int}} \times SF}{Q_H + CL_{\text{int}} \times SF} \quad \text{well-stirred model}$$

$$CL_H = Q_H \left(1 - e^{-\frac{CL_{\text{int}} \times SF}{Q_H}} \right) \quad \text{parallel tube model}$$

where Q_H is the human hepatic blood flow (20.7 mL/min/kg) and SF is the scaling factor (2.3) from *in vitro* to *in vivo* based on the consideration of the hepatocyte content in the liver

(108×10^6 hepatocytes per g of liver) and the liver weight (20 gram liver weight per kg of body weight). The *in vivo* hepatic clearance (CL_H) has a unit of mL/min/kg.

3.2. Permeability

Along with solubility, the permeability of a drug candidate is a key factor in the Biopharmaceutics Classification System (BCS) [112, 113]; together these two factors determine the entry of cells into an organ system. It is therefore imperative to assess both the active and passive permeability of new chemical entities (NCEs) through the gastrointestinal tract and into specific cells. In simplest terms, permeability is the rate of transport of substances in and out of cells. Specifically, it represents the sum of passive absorption and active (transporter protein and enzyme-based) transport. Passive absorption is governed by the partition coefficient (log P), distribution coefficient (log D), molecular weight (MW), ionization state and the hydrogen bonding capacity [114].

Active transport modeling however is more difficult and in its infancy as it requires the incorporation of kinetic modeling of specialized trans-membrane proteins, or transporters, that will recognize the substance and allow it access (or, in the case of secondary transport, expend energy on forcing it) to cross the membrane when it otherwise would not; either because it is one to which the phospholipid bilayer of the membrane is impermeable or because it is moved in the direction of the concentration gradient. Including active transport within PBPK models is now generally regarded as a necessary improvement over prior models, as it will increase the accuracy of bioavailability calculations. However, kinetic modeling of ligand-receptor kinetics is typically limited by difficulties in specifying model topology and parameter values. Additionally, incorporating entities across different biological scales ranging from molecular to organismal in the same model is not trivial. Thus if active transport were to be incorporated into a model multiple kinetic parameters would need to be determined with respect to the specific transporter utilized to allow for cellular uptake of the compound of interest. Due to the inherent time costs and complexity of this endeavor, most models are thus simplified in only considering passive transport.

With the advancement of the pharmaceutical industry in the 1990s drastically increasing the rate at which drugs pass through the development pipeline, the industry now demands assessment of solubility and permeability in a robust, mechanistic and high throughput fashion. Strategies in this regard fall into two broad categories: cell monolayer cultures and artificial membrane systems. Cell monolayer culture systems usually rely on human colon adenocarcinoma (Caco-2) cells and are recognized by the Food and Drug Administration (FDA) for classifying the absorption characteristics of new drugs and compounds; the system was first developed by Ronald T. Borchardt [115]. The model consists of a monolayer of Caco-2 cells grown on a collagen-coated porous polycarbonate membrane. The system is designed to allow easy access to both the apical and basolateral sides of the monolayers, providing an ideal environment to examine passive and active mechanisms of absorption, excretion, and metabolism [115-117]. Permeability measured using the Caco-2 system has shown strong correlation with fractional absorption in humans. However, there are two significant problems with the Caco-2 monolayer model. First, the preparation and measuring process is cumbersome, expensive and labor intensive; the assay does not answer the industry's demand for high throughput screening across thousands of compounds [118]. Second, results attained from the Caco-2 model have demonstrated poor inter-laboratory reproducibility; the different cell growth environments in different labs have led to large variances in enzyme and transporter protein expression [119]. To alleviate this problem, Caco-2 cells need to be genetically modified to remain consistent under a range of growth conditions. However they have been shown to resist such modification. As such, strategies using the Caco-2 model have reached a roadblock.

The second major approach toward assessing drug permeability is the artificial membrane system. These systems only examine passive transport and do not address active (enzyme or transporter based) mechanisms. The earliest artificial membrane systems measured partition coefficients in an immiscible two phase octanol/water mixture; this method proved labor intensive [120]. More recent approaches use parallel artificial membrane permeability assays (PAMPAs). The PAMPA represents the first flux based assay in this field and the first true high-throughput screening assay.

Made possible by the discovery of black lipid membranes in the 1960s, the PAMPA is based on the formation of many artificial single-layer lipid membranes on a microporous filter [121]. A recent definition by Ruell and Ardeef defines the model as follows: "PAMPA is a permeability assay that uses a microporous filter infused with a lipid or a mixture of lipids to separate two aqueous, pH buffered solutions in a multiwell microplate sandwich." The assay provides a simple way to predict transcellular drug absorption by measuring the flux of compounds of interest between the two aqueous "donor" and "acceptor" compartments. In the seminal work by Kansy [118], the PAMPA consisted of an egg lecithin/dodecane membrane, which has since demonstrated much better correlation with human fractional absorption than the octanol/water mixture - egg lecithin is known to have a similar lipid composition to that of mammalian cell membranes [118, 121, 122]. Recent evidence has shown that PAMPAs can exceed cell based assays in speed, versatility, reproducibility and cost while presenting biologically relevant models of transport [123, 124].

3.3. Volume of Distribution

The volume of distribution (V_d), also known as apparent volume of distribution, is a proportionality factor that relates the amount of drug in the body to the concentration of drug measured in a biological fluid (plasma, in this case). It is defined as the theoretical volume of uniformly distributed drug needed to produce a desired blood concentration. V_d at steady state is therefore one of the parameters that strongly determines the disposition of a compound. In effect, V_d describes tissue binding, which greatly affects drug bioavailability over time and often determines that drug's half-life [6, 125].

At present, the methods used to predict V_d include: the extrapolation of animal data [126, 127], PBPK modeling, and *in silico* approaches that employ quantitative structure-pharmacokinetic relationships [128]. Animal models are time consuming and costly, and cannot be used for high throughput screening. Furthermore, data extrapolated from animal models may not always translate into accurate human V_d values. *In silico* approaches such as PBPK and QSAR do offer high throughput screening power. However they ultimately are theoretical models that do not account for all the variables that affect V_d *in vivo*.

To date, *in vitro* models have not been established for assessing V_d . Tissue engineering has only recently provided a means of culturing adult human cells effectively. As such, the amount of throughput demanded by today's pharmaceutical markets will make current *in vitro* methods very costly.

In this light, there is a strong need for robust, high throughput *in vitro* screening systems. It has recently been demonstrated that *in vivo* assessment of plasma partition coefficient can be predicted by using *in vitro* data on drug lipophilicity and plasma protein binding [129-132]. However, predictive capability is limited by the range of compounds that can be modeled. An example of this restriction is the underestimation of V_d in cationic amphiphilic compounds bases. Thus a multi-tissue analogue system is needed for more effective V_d prediction.

4.0. CCAS as *In Vitro* Surrogates For Animal Models

Although *in vivo* animal models are still a vital part of both preclinical testing, the cost, efficiency, and ethicality issues associated with animal studies have pushed the field to find viable alternatives. Based on the original work of Russel and Burch [133], the goal here is to progressively achieve reduction, refinement and replacement of animal testing. This “3R Plan” does not strive simply to eliminate animal testing. Instead, it seeks to find technologies with strong predictive capabilities that simultaneously reduce the cost, time, and variability associated with animal testing [134].

Approaches that use isolated primary cells or cell lines have shown great potential in meeting the requirements of the 3R Plan. These cell culture analogs (CCAs) are physical representations of PBPK models; they aim to predict the ADME-TOX of drugs in the human body, as demonstrated in (Fig. 2). With the advancement of stem cell technologies that enable differentiated cells with primarylike synthetic and metabolic functions [135], CCAs will have even more of an impact in reducing and replacing animal testing. Here we briefly review the strategies that use live cell cultures to improve drug testing. We then assess their advantages and shortcomings.

Functionally, CCAs are surrogates for human physiology. They consist of live cells plated on a variety of platforms that range from static well cultures to microfluidic perfusion systems. Observations made on the platform scale can be subsequently correlated to human physiological events. Such observations include common viability and ADME-TOX studies; binding affinity studies; catalytic effects of chemical compounds on enzymatic reactions; and gene expression analyses [133, 134, 136].

With the advancement of aseptic techniques, commercially available sterile merchandise, and assay equipment that support high throughput formats (such as the 96 well plate [137] and the corresponding multi-well automated assay reader [138, 139]), static well culture techniques have made important contributions to both toxicology and PBPK studies. One concern with these cell cultures has been the loss of phenotype and cell viability over extended culture times. Some three-dimensional cell culture studies claim improved viability and phenotypic retention compared to their two-dimensional counterparts. Likewise, multi-cell cocultures have also demonstrated improvements over single cell type cocultures. Nevertheless, static well cultures have been the subjects of a more fundamental debate over their suitability [140]: the static nature of the experiments as opposed to the physiological reality, in which the system is continually perfused within the visceral system. Static well cultures have had problems providing continuous removal of metabolic waste and continuous supply of fresh nutrients. Instead, these functions only performed periodically at long intervals. A second important difference between the static and physiological environments concerns their relative scales. In contrast to the microscopic length and time scales characteristic of the physiological environment, the multi-well format operates on the macroscopic level [57, 140].

Most of these studies are utilize rodent cells and use IC₅₀ correlation testing to predict human response [141]. However, recent concerns have been raised regarding the usefulness of rodent cells in predicting human outcomes, even when the study uses deleted genes [142, 143].

In recent years, with the advance of microfabrication technology, it has become possible to design precise, well-defined microscale cell culture systems. The premise of such microscale cell culture analogs (mCCAs) is to mitigate the time and length scale problems that arise in traditional static systems due to the lack of perfusion [18, 59, 63, 144, 145]. The small

dimensions of the microfluidic structures allow cells cultured within mCCAs to exist in realistic physiological conditions; liquid-to-cell ratios, physiological shear stresses, and fluid resistance forces can all be kept at relevant levels. With the aid of computer controlled pumping systems, mCCAs can achieve precise flow conditions at these small scales. Earlier work in this area focused on two dimensions, single cell-type microperfusion cultures, and simple endpoints such as clearance, viability and secretion. In contrast, newer designs have begun to operate in three-dimensional space, utilize multiple cell types, and assess a wider set of endpoints [18, 145-148]. High throughput techniques that have been developed for other microfluidic platforms readily carry over to the mCCAs and enable massively parallel dynamic experiments. For example, integrated gradient generators in microfluidic devices provide a rapid and convenient way to study dose responses for toxicity, clearance, gene expression, and a multitude of other endpoints and even temporal responses via the incorporation of fluorescent proteins [149-151].

Similar to their macroscale counterparts, microscale cell culture analogs have also made forays into three dimensional culture models and cocultures [12, 102, 145-148, 152, 153]. In this context, studies by Bhatia *et al.* showcase the importance of microscale patterning on cell function and viability [154-156]. Microscale 3D perfusion systems have likewise proven useful in toxicity studies. Toh *et al.* have fabricated and tested a microfluidic 3D hepatocyte chip to predict drug hepatotoxicity [149]. The 3D HepaTox Chip uses multiplexed microchannels to maintain a microenvironment conducive to hepatocyte metabolism and synthesis, and is able to administer simultaneous drug doses to primary hepatocytes in conjunction with a concentration gradient generator. Going one step beyond the macroscale analogs, however, microscale systems offer physiological analogs via miniature artificial visceral systems, such as those designed by Shuler *et al.* and Hurel Corporation [57, 145]. These systems separate parenchymal cells from different organs into microculture chambers that are perfused via connected microchannels. The premise here is that the ADME TOX studies for these systems would better reflect the systemic and nonlinear relationships of the human body than the ADME TOX studies for single-cell microscale cell culture systems [18].

In the following sections, we take a closer look at the design principles, advantages, and shortcomings of mCCAs using our own computational efforts. We then discuss the prevailing scaling approaches in the context of PBPK modeling and ADME TOX testing.

5.0. Limitations of CCAs

While there is large inherent value in developing CCAs for *in vitro* drug screening, current embodiments are limited in several ways. As a first point of comparison, we refer to liver-based CCAs as we assess the physiological relevance of current *in vitro* animal surrogates. CCAs in the past have tried to replicate the *in vivo* environment by scaling based on tissue volume ratios or residence time. However, these approaches tend to lead to devices that miss other *in vivo* parameters [18]. To illustrate this point we compiled key physiologic parameters from the human liver and devised a fluidics device that could incorporate all of these features (Table 1), described as the “Proposed *In vivo* Match”. We also compiled relevant parameters from a conventional CCA. Then through the use of computational fluid dynamics, aided with the software package COMSOL, and utilizing methods described in [157], we assessed the clearance of carbamazepine and the consumption of oxygen in the proposed CCA as well as conventional CCAs that exist. As shown in (Fig. 3), there are two limitations to a near replica of a PBPK system: 1) the oxygen content in the system at such high cell-to-volume ratios seen *in vivo* quickly leads to depletion; 2) the extremely fast, non-*in vivo* relevant clearance profile. To address this, we tried a device that increased the cell to volume ratio one fold greater than the conventional CCAs (Table 2). However, the oxygen

content in the system was still too low to achieve a theoretical seeding density of 3,000 cells/uL, even assuming an oxygen permeable membrane along the top of the fluidics chamber. To truly adopt a PBPK-relevant system, one would need to include an oxygen carrier analogous to hemoglobin to ensure sufficient oxygenation, as well as a higher plasma protein concentration to ensure relevant clearance predictions. Regarding the quick compound depletion, a multi-layer, multi-cellular analogue would need to be established to ensure the multiple types of mass transfer that exist *in vivo*.

Aside from these limitations in an ideal *in vivo* replica device, current devices are primarily designed based on residence times. The problem arises because one can find large variations in the clearance parameters while holding the residence time constant. To illustrate this point, we again performed CFD simulations (utilizing COMSOL and methods previously described [157]) for a variety of geometries (G1 through G4) at a wide range of flow rates (Fig. 4). (Fig. 4A) shows multiple geometries constructed with the same residence time. Running the various geometries at the same flow rate (5uL/min) yielded vastly different clearance profiles for the same compound. There are two reasons for this. First, as the cell-seeding surface area decreases, the cell count increases. As such, the maximum number of reaction sites (i.e. cells) is reduced. Second, the surface area decreases, the overall depth of the device must be increased to maintain the same residence time. In this case, the transport distance between the chemical of interest and the cell surface is increased. For further evidence, one can obtain the same clearance profile for different geometries by changing the flow rate (Fig. 4B) and in turn the residence time.

Another issue with modern CCAs is the inclusion of barrier tissues. For example, CaCO₂ cells are often added to ascertain permeability, and fluidics systems are often used to ascertain clearance [68]. The challenge is to design a culture system containing membranes that are sturdy enough to provide mechanical support for the barrier tissue, yet porous enough to allow metabolites to pass through. Mechanically, it is important to match the properties of the support structure to the properties of *in vivo* ECM. Meanwhile, even with a porous membrane, a diffusional transport limitation still exists that must be initially analyzed with the compound of interest alone. Furthermore, certain systems – especially those using skin-based analogs – need to maintain an air-liquid interface. This not only complicates the overall CCA, but requires special attention to maintain the viability of the barrier membrane [158, 159]. Additionally, as aforementioned, permeability calculations require the inclusion of active transport, where applicable, a feature which is not currently embodied in CCAs.

Because most CCAs are created using polydimethylsiloxane (PDMS), non-specific binding arises as another major concern. In systems that do not study compound clearance, this problem is trivial. However, the problem is paramount in systems that specialize in PBPK-based parameter estimation. For certain systems, researchers have moved from PDMS to plastics-based models, whose smaller pore sizes marginalize compound sequestration [68, 160, 161]. Another approach, should one need the flexibility and ability to derive complicated fluidics architectures, inerts the PDMS by depositing parylene on its surface, thereby closing its pores and preventing sequestration [162-165].

Duration of operation represents the final concern for CCAs today. Currently, CCA devices are capable of operating 24 to 72 hours without losing its function [160, 161]. However, unless the system is connected to a reservoir with frequent media changes, metabolic waste products begin to accumulate after 72 hours, and cell viability drops drastically. Efforts to increase the lifetime of CCAs will require both the removal of metabolic waste products and the replenishment of consumed nutrients in the blood surrogate. For on-chip devices, metabolic waste products might be removed from the blood surrogate stream using a

microfluidic dialysis procedure [166-169]. Meanwhile, depleted medium needs to be removed and replaced with fresh medium containing nutrients. Both processes would require additional microfluidic loops, again emphasizing the need for precise pressure and fluid flow controls. Additionally, air bubble accumulation represents a major obstacle for the long term viability of cells cultured within CCAs. While bubbles are purged at the setup of the device, they become trapped within the system over time, either due to the dissolution of oxygen from the media or the intrusion of air into the media as media transfers are made at the reservoir within the recirculation loop. Recent advances have been made to alleviate these problems [170, 171], but for now are applicable only to PDMS-based microsystems.

6.0. Future Directions For *In Vitro* Development

6.1. New Multi-tier CCAs and mCCAs

With the advent of microfabricated devices, we now have the control needed to develop *in vitro* platforms for assessing drug volume of distribution. Future devices use multi-tissue compartmental designs to represent plasma distribution into different tissues. The amount of cells will be determined based on their percentage exposure to plasma *in vivo*. This compartment can be implemented in microfluidic chips to measure the volume of distribution for a given drug. The precise control over volume, flow and cell ratio can be established in a compartment to mimic the volume distributions in the body. In this manner, a compartment will be created to represent the tissues that contribute to volume of distribution *in vivo*. The overall goal of the proposed chip is to facilitate high throughput screening of multiple drug characteristics.

The proposed device (Fig. 5) will contain three compartments and two flow systems. The first flow system represents the absorption pathway *in vivo*, and will contain a diffusion layer into the second flow system. The amount of drug in the second flow system will represent the drug's bioavailability. The drug will circulate throughout the two compartments, representing *in vivo* circulation through the liver and other tissues/organs. Using this design, several DMPK parameters can be assessed simultaneously and with greater accuracy. The size of the micro-device allows high throughput systems that can evaluate DMPK characteristics for several NCEs. As such, the device will signify the first multi-compartmental microsystem for simultaneously evaluating multiple DMPK properties.

6.2. Integration of mCCAs and *In Silico* Modeling

In a similar vein to the recent push toward combining multiple modeling techniques for *in silico* drug assessment, the future direction for comprehensive *in vitro* screening will likely combine the aforementioned *in silico* techniques with CCAs [10, 56-58, 145]. In one potential embodiment, mammalian cells are cultured in a CCA reactor that physiologically embodies the corresponding PBPK model. Where the PBPK specifies organ/tissue compartments, the CCA bioreactor contains physical compartments with the corresponding cell types. By coupling a CCA bioreactor to the PBPK, one can assess the plausibility of the PBPK's representation of *in vivo* kinetics, thermodynamics, molecular mechanisms, and anatomical characteristics. In the PBPK, kinetic and thermodynamic relationships can be written for each compartment that predicts the activity of a drug in the corresponding organ [56]. Meanwhile, the CCA replaces the theoretical compartments with cell systems that contain the chemical reactions described by the PBPK kinetics. As discussed before, inter-organ reactions are enabled by perfusing the entire CCA with cell culture medium.

In a study conducted in 2000, Ghanem and Shuler designed early-generation PBPK-CCA systems to assess naphthalene metabolism [56, 57]. The researchers tested two CCA reactors. In the first prototype, based on previous work by Sweeney [58], cells were cultured

as a monolayer on the surface of two glass dilution bottles to represent lung and liver compartments. A dosage of naphthalene was administered to the system, which resulted in the circulation of metabolite from the liver compartment that caused glutathione depletion and apoptosis in the lung compartments. A second, improved CCA reactor consisted of perfused organ compartments containing cells cultured on packed beds of microcarrier beads [172]. This approach offered lower residence times, higher cell density, and a more realistic lung-to-liver cell ratio. These studies demonstrated two important observations regarding the effectiveness of the CCA-PBPK concept. First, it can be seen that the concept can be applied to toxicity studies. The researchers were able to use the combined approach to discover the exact toxic mechanism for naphthalene. Based on earlier studies and literature, it was assumed that the binding of naphthalene epoxides to proteins caused the toxic response; yet the differences between the two CCAs brought this hypothesis into question. Upon adjusting naphthalene metabolism in the PBPK model by incorporating quinone formation and binding, they attained model results that were more consistent with their experimental results. In doing this, they uncovered the possible importance of naphthoquinones as metabolites involved in naphthalene toxicity. Ultimately, however, their system uses a single cell type for each compartment and does not incorporate an expansive range of kinetic parameters, e.g. the rates of formation of multiple chemicals and conjugates for the cells they used. Thus it remains a simplified approximation of human metabolism.

Other studies have integrated physiologically based pharmacokinetic/pharmacodynamics (PBPK/PD) and QSAR modeling are combined with mechanistically-based experimental toxicology; PBPK/PD, QSAR, and molecular mechanics/dynamics (MM/MD) modeling have been combined with time-course medium-term liver foci assays [173], molecular biology studies [174], and evaluation of cell proliferation [175, 176]. There has also been work using *in silico* and *in vitro* approaches to quantitatively evaluate cellular and molecular biomarkers critical to carcinogenesis between different chemicals and/or chemical mixtures proliferation [173, 174, 177-184]. One such study examined chlorobenzene isomer carcinogenicity by combining *in silico* modeling of cancer, *in vitro* experimentation, and infrared spectroscopic analyses in the Fourier domain [173, 183]. Similar approaches have been devised to test chemical mixtures using Syrian hamster embryo cell transformation assays with human keratinocytes [185-188].

6.3. More Realistic (3D) CCAs and mCCAs

While most CCA studies for pharmaceutical testing have been conducted on two-dimensional surfaces, they are often inadequate for new challenges presented in the field. Most obviously, 2D systems are fundamentally different from the complex 3D mesh that makes up the *in vivo* extracellular matrix. Furthermore, the 3D geometrical environment of the many cells and tissues in the body strongly affects their functionality; 2D culture systems cannot simulate this effectively. Many recent studies have shown that cells behave very differently in two-dimensional flat layers than as three-dimensional constructs. For example, 3D cell cultures have been shown to enhance the hematopoietic efficacy of embryonic stem cells, while 2D cultures showed no such effect [189]. Recent studies also indicate that, when cultured in 3D, breast cancer cells revert to their original non-cancerous state when treated with B1-integrin surface receptors; however this was not observed for breast cancer cells grown in 2D monolayers [190]. These discrepancies suggest that mCCAs consisting of 3D cell culture constructs will behave more authentically than those consisting of 2D monolayers. For these reasons, many recent studies have focused on creating 3D scaffolds that closely simulate the biochemical and mechanical properties and signaling cues found in native tissues [191, 192].

3D scaffolds are porous matrices designed to support cell system. Based on the specific application, scaffolds can be tailored to the desired porosity, permeability, mechanics, surface properties, and physical appearance. Many fabrication techniques have been developed, and the specific process can greatly affect the resulting architecture. Common fabrication approaches include electro-spinning, particulate leaching, and solid free-form manufacturing. The overall motivation behind moving from 2D monolayers to 3D scaffolds is to mimic the properties of natural ECM so that cells can function as they would *in vivo*. Natural ECM consists of a highly porous hydrogel-like polysaccharide material (such as glycoproteins, glycosaminoglycans, and proteoglycans) containing a complex meshwork of collagen and elastic fibers. But beyond simply serving as a physical scaffold for cells, natural ECM also performs dynamic modulation of cellular activity. The ECM can direct cellular organization, mediate proliferation and differentiation, induce morphological changes. Cells can in turn respond to the environment that the ECM creates, and often synthesize or destroy specific ECM elements.

The majority of the 3D scaffolds today are made from either natural or synthetic hydrogels [193-197]. The major advantage of natural hydrogels such as Matrigel [198] (derived from mouse tumor cells) and collagen gels [199] (the most prevalent protein in ECM) is their ability to promote cellular interactivity by having a large portion of the native tissue's biochemical signals already in place. However, natural hydrogels tend to have larger batch-to-batch variability compared to their synthetic counterparts. The relative unpredictability and complexity of natural products can hinder the accuracy with which they characterize cellular behavior. Furthermore, because there are more cellular interactions going on, it can be difficult to pinpoint and isolate the exact mechanisms of interest. Compared to natural hydrogels, synthetic hydrogels are more consistent, more easily characterized, and more conducive toward manipulation of their mechanical properties [200]. The most common forms of synthetic materials are: polyethylene glycol, polyacrylamide, polymethacrylamide, and polyvinyl alcohol [197]. Polyethylene glycol (PEG) in particular is often selected as the main structural component of the hydrogel due to its hydrophilicity and resistance to protein adsorption [201]. Synthetic hydrogels, however, do not provide functional sites for cellular interactions, though it is possible improve their ability to interact with cells by linking their backbones with biomimetic cues such as RGD peptides [63].

As discussed earlier, the integration of CCAs with the small size and time scales offered by microfluidic devices enables increased spatial and temporal control of molecules. These micro-CCAs (mCCA) can provide more physiologically relevant environments for simulating drug action and toxicity. For example, recent studies have used Matrigel scaffolds for colon tumor and liver cells in conjunction with alginate microcapsules to encapsulate myeloblasts within a microfluidic environment [63]. Similarly, mCCAs with 3D hydrogel cultures of multiple cell lines have been utilized to examine the metabolism-dependent toxicity of anti-cancer drugs [146]. Studies on the ability of the microscale environment to elicit authentic cell function showed enhanced biochemical activity and gene/protein expression in 3D mCCA systems compared to traditional *in vitro* environments [202]. More systematic studies are currently underway to further improve the design of 3D mCCA systems. One study used *in silico* modeling to evaluate the effects of surface tension, spatial geometry, and hydrophobic interactions on 3D mCCAs [203]. Collagen polymerization within the microfluidic environment has also been studied, and cell viability in these microfluidics-embedded collagen matrices were compared with viability in conventional systems.

The recent development of microfluidics based drug discovery systems such as Caliper's *LabChip3000* serve as examples of where this technology is currently positioned. Such systems use fluorescence excitation and detection wavelengths together with plate-handling

automation for selective screening, enzyme kinetic optimization, and IC₅₀ determinations. Chip-based enzymatic assay protocols are available for the major target classes, including kinases, phosphatases and proteases. Most assays are performed based on mobility shift electrophoresis, in which product and substrate peaks are separated and detected independently. The idea here is that the sensitivity of the chip-based assays can lead to improved data quality for detection weak inhibitors and lower false positive and false negative readings [204, 205].

6.4. On Chip Detection

The relative inexpensiveness of fabricating mCCAs presents an exciting proposition for pharmaceutical drug testing. Their small size allows for such studies to be conducted with miniscule amounts of reagents and cells. The advancement in this field has been enabled by the development of novel cell lines and reporter gene techniques, as well as new microfabrication, microfluidics and optical and electrochemical sensor technologies. Non-invasive detection methods using reporter genes and label-free techniques allow for real-time dynamic monitoring of viable cell number and cellular activities. Microbioreactors with continuous perfusion allow for long-term culturing to study chronic toxicity effects. Systemic toxicity and interactions between different cell types can also be studied on a biochip [206]. New microfabrication techniques, optical and electrochemical sensor technologies have enabled significant advancement in this field. MCCAs with continuous perfusion can now allow for dynamic studies on the effects of systemic and chronic toxicity through long-term cell culturing within the biochip [203]. In conjunction with multi cell type scaffolds, cellular interactions between different cell and tissue types can also be assessed [207]. High-density microfluidic arrays have the potential to dramatically influence future approaches toward rapid screening [68, 206]. The gold standard of high throughput analysis will be to completely alleviate the need for off-chip detection and quantification.

One important area for future development is the incorporation of micro-biosensors for assessing cell viability, reporting cell stress, quantifying the amount of enzymes released, measuring parent compound concentrations in the media over time, and monitoring toxicity changes dynamically. Using such sensors, cell stress and the presence of particular metabolites could be used as indicators of the actions of drugs in the mCCA [206, 208-213]. For example, an oxygen sensor such as that utilized by Sin *et al.* (2004) could be used to measure the rate of oxygen consumption by liver cells on the chip as a measure of their metabolic activity. Meanwhile, on chip immunoassays and ELISAs have the potential to be established in a multi-plex format, thereby providing a monitoring capacity of a variety of secreted products by the cultured cells, giving a readout of the functional state of the cells. Another potential area of integrated function is the development of on chip LCMS which will provide a means for the assessment of parent compound clearance, and the formation of metabolites [214-220]. For example, Gonzolez *et al.* have used on-chip sensors in mCCAs to measure toxicity and clearance of harmful contaminants in drinking water [221]. Similarly, Stangegaard *et al.* have designed biocompatible MCCAs to culture and monitor eukaryotic cells for on-chip optical recording of cellular events [222].

The field of micro-biosensors has also grown immensely during the past few years, and a large number of sensors are now available for the monitoring mCCAs, thereby providing an additional level of analytic capacity. Among these are sensors that record optical, electrical, and electrochemical signals [223-226]. Most assays use optical methods of detection [227-234], wherein optical waveguides direct light waves through the chip, across the microfluidic channels, and back out of the chip where it is detected. Frequently, optical interferometers are used to detect small changes in the refractive index of samples flowing through the microchannels; the liquid flowing through the channel induces tiny changes in

the path length of the light, which the interferometer can perceive [227]. In this way, optical detection is integrated with the microdevice to form a micro-opto-electromechanical-system (MOEMS). Elman *et al.* have recently developed a full lab-on-chip system for inexpensive and efficient toxicity detection that integrates whole-cell sensors, a MOEMS modulator, and solid-state photodetectors [235]. The whole-cell sensors were genetically engineered for lux-lac reporter-promoter expression, while the MOEMS modulator was designed to overcome the thermal noise, switching noise, and dark current that traditionally inhibit low intensity bioluminescence detection.

For applications that require portability, optical methods have been limited because of the difficulty of connecting to on-chip waveguides outside the benchtop environment. Klob *et al.* have developed an electrode-based array to monitor the electrical parameters of anti-cancer drug cellular response in real time [236]. 3D multi-cell type models for microtumors/metastases were cultured and transferred to the microarray. Impedance spectra were then measured over time as an indication of cellular response to a large number of drugs. Gonzolez *et al.* have been working on the development of on-chip electrochemical sensors to detect the presence of liver cell stress markers. In doing so, they have been able to fabricate photo-patternable carbon electrodes using conventional photolithography techniques. Using this technique, they were able to install carbon electrode arrays within the mCCA platform that could covalently bind to biological recognition agents through salinization chemistry. This holds as a potential method for immobilizing antibodies within the microfluidic device.

As discussed, the performance of current mCCA systems would be enhanced and their operation in routine diagnostic laboratories would be easier if a variety of micro-biosensors were installed on the platforms. At the same time, computerized control of medium flow and pressure within the microfluidic circuit and built-in pumps would add to the simplicity of use of mCCA systems [171]. As an alternative, interfacing with currently available robotic liquid handlers is also a potential work flow [63, 171]. Automated positioning in the microfluidic chambers and automated pipetting systems alongside multiplexed read-outs can further improve experimental efficiency [237-239]. Compartmentalized multiwall systems can enable parallel testing of difference substances on one array while reducing requisite media volumes [236, 240]. Though these remain as areas for improvement, the high throughout potential of mCCAs may soon be realized.

7.0. Summary

The field of *in vivo* pharmacokinetic prediction has seen a rapid expansion over the past few decades. The most notable *in silico* advancement has been the development of PBPK modeling, which, in conjunction with the assays that provide inputs for the PBPKs, have yielded fairly robust *in vitro* – *in vivo* prediction. More recently, there has been a movement to produce high throughput, animal-free devices for drug testing. This push has led to the development of cell culture analogs as physiological embodiments of PBPK models. While CCAs are currently limited, future advancements in duration time, physiological scaling, and on-chip detection will mark CCAs as a prominent tool for the pharmacokinetic prediction of new chemical entities.

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Fig. (1). Whole Body PBPK Model

Whole body PBPK model depicting the liver as the only tissue for metabolite formation. Metabolism of drug to other metabolites also occurs in the intestine, and both drug and metabolite are secreted back to the intestinal lumen.

Fig. (2). Overview of a PBPK cell culture analogue

In this illustration, cell and tissue compartments are established on a microfluidics chip, with key physiologic parameters matched to the *in vivo* counterparts.

Fig. (3). Analysis of oxygen consumption and Carbamazepine clearance in various device configurations

Clearance of carbamazepine, and consumption of oxygen for both the static and flow conditions were calculated using COMSOL Multiphysics. An experimentally determined intrinsic clearance rate was used as a consumption rate boundary condition (flux) at the modeled cell surface for each of the geometries. An overview of the methods is presented in [157].

Fig. (4). CFD Assessment of Clearance within a microdevice at different flow rates

Simulations were run for four different geometries at 4 different flow rates, and were computed using COMSOL. Geometry G1 has a reactive, cell-containing surface area of $1.36 \times 10^{-5} \text{ m}^2$. Geometry G2 has a reactive surface area of $6.8 \times 10^{-6} \text{ m}^2$. Geometry G3 has a reactive surface area of $3.4 \times 10^{-6} \text{ m}^2$. Geometry G4 has a reactive surface area of $1.7 \times 10^{-6} \text{ m}^2$. The residence time across all four geometries is held constant. In the second panel, a study of inlet velocity flow rate was conducted, with V set to 5 $\mu\text{L}/\text{min}$, V/2 set to 2.5 $\mu\text{L}/\text{min}$ and so on. An overview of the methods is presented in [157].

Fig. (5). Microcell Culture Analogue

Top: Diagram of a pharmacokinetic microfluidic device comprises of an absorption compartment, a metabolism compartment, a biodistribution compartment and a reservoir. The fluid (culture medium or buffer) from the reservoir splits in certain proportion and flows through the absorption compartment or the biodistribution compartment. The fraction flows through the absorption compartment then enter the metabolism compartment simulating the liver first-pass metabolism. The fraction flows through the biodistribution compartment provides tissue binding effect. Both fractions merge before entering the reservoir. Bottom: the side view of the absorption and the metabolism compartments.

Table 1
Theoretical PBPK Relevant System

	In vivo	Conventional	Proposed In vivo Match
Length (mm)	-----	4.0	40
Width (mm)	-----	3.4	10
Depth (mm)	-----	0.1	0.1
Percent of fluid in liver (%)	40	2	40
Percent of fluid in other tissue (%)	60	98	60
VOD	0.07	3.5	0.175
Total number of hepatocytes	1.6×10^{11}	30,000	1,000,000
Total blood / liquid volume (uL)	3.5×10^6	100	100
Cell to volume ratio (cell / uL)	47,000	300	10,000
Flow rate	1.4 (L/min/person)	5 (uL/min/chip)	150 (uL/min/chip)
Single pass (min)	2.5	20	0.67
Transit time (sec)	16	16	16

Table 2
Toward a PBPK Relevant System

	In vivo	Proposed Geometry	Proposed Geometry with Oxygen Membrane
Length (mm)	-----	10.0	10.0
Width (mm)	-----	8.0	8.0
Depth (mm)	-----	0.5	0.5
Percent of fluid in liver (%)	40	40	40
Percent of fluid in other tissue (%)	60	60	60
VOD	0.07	0.175	0.175
Total number of hepatocytes	1.6×10^{11}	100,000	300,000
Total blood / liquid volume (uL)	3.5×10^6	100	100
Cell to volume ratio (cell / uL)	47,000	1,000	3,000
Flow rate	1.4 (L/min/person)	150 (uL/min/chip)	150 (uL/min/chip)
Single pass (min)	2.5	0.67	0.67
Transit time (sec)	16	16	16