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Drug Discovery and ADMET process: A Review

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Abstract

Pharmaceutical drug discovery is an expensive and time-consuming process. In order to have one effective and safe drug, millions of molecules are screened. A successful IND application requires that the sponsor demonstrate reasonable evidence concerning safety of the candidate molecule. This evidence is derived from a battery of *in vitro* assays as well as *in vivo* studies using animals. Animal studies often fail to predict clinical results due to inter-species differences in enzymes, transporters and biochemical pathways. Although many *in vitro* assays use human cells, they lack the interplay of body systems and biochemical pathways. Due to these shortcomings, both *in vitro* and *in vivo* studies often fail to predict clinical outcomes. Nearly 30% of molecules entering clinical trials fail because of insufficient safety. This inadequacy opens opportunities for new technologies that can address these issues, thereby leading to a more predictive human model. In addition, due to time-consuming and expensive nature of *in vivo* studies, improved *in vitro* assays that lead to reduction in animal usage present interesting opportunities. With these ideas in mind we framed the objective to summarize the *in vitro* ADME/T tests in order to better predict human clinical outcomes.

Keywords: ADME, drug discovery, metabolism, toxicity, 3D culture.

1. Introduction

Drug Discovery Process

The drug discovery process begins with first choosing a specific disease to treat. Understanding something about the biochemical pathway(s) or genetics of the disease allows researchers to focus on a specific class of enzymes or receptors potentially responsible for the disorder. After identifying and validating the target enzyme/receptor, a high-throughput screen of potentially millions of small molecules is conducted. The results of this screening allow medicinal chemists to focus only on those molecules containing certain functional groups, core structures and 3D shapes. These leads are then subjected to further biochemical and *in vitro* assays to eliminate molecules with potential side effects, drug-drug interactions or toxicity.

A select few candidates are pursued further during the lead optimization phase. Herein, medicinal chemists fine tune the reactivity and structure of these ligands. These leads are again tested using both *in vitro* and *in vivo* methods. This cycle of chemical fine tuning and biological testing is repeated several times until the most likely molecule to succeed in clinical trials has been synthesized and characterized as best as possible. The optimized molecule is then chosen to be subjected to more extensive studies for IND filing; meanwhile, the scale-up process synthesis is executed. This assessment involves bioanalytical quantification of metabolites, plasma concentration measurements, regular vet assessments, and ultimately necropsy of several organs. These more extensive *in vivo* ADME/T studies are then used to estimate the PK/PD parameters in humans and also provide guidance for physicians in clinical trials. A schematic flowchart of this process is shown in Figure 1.

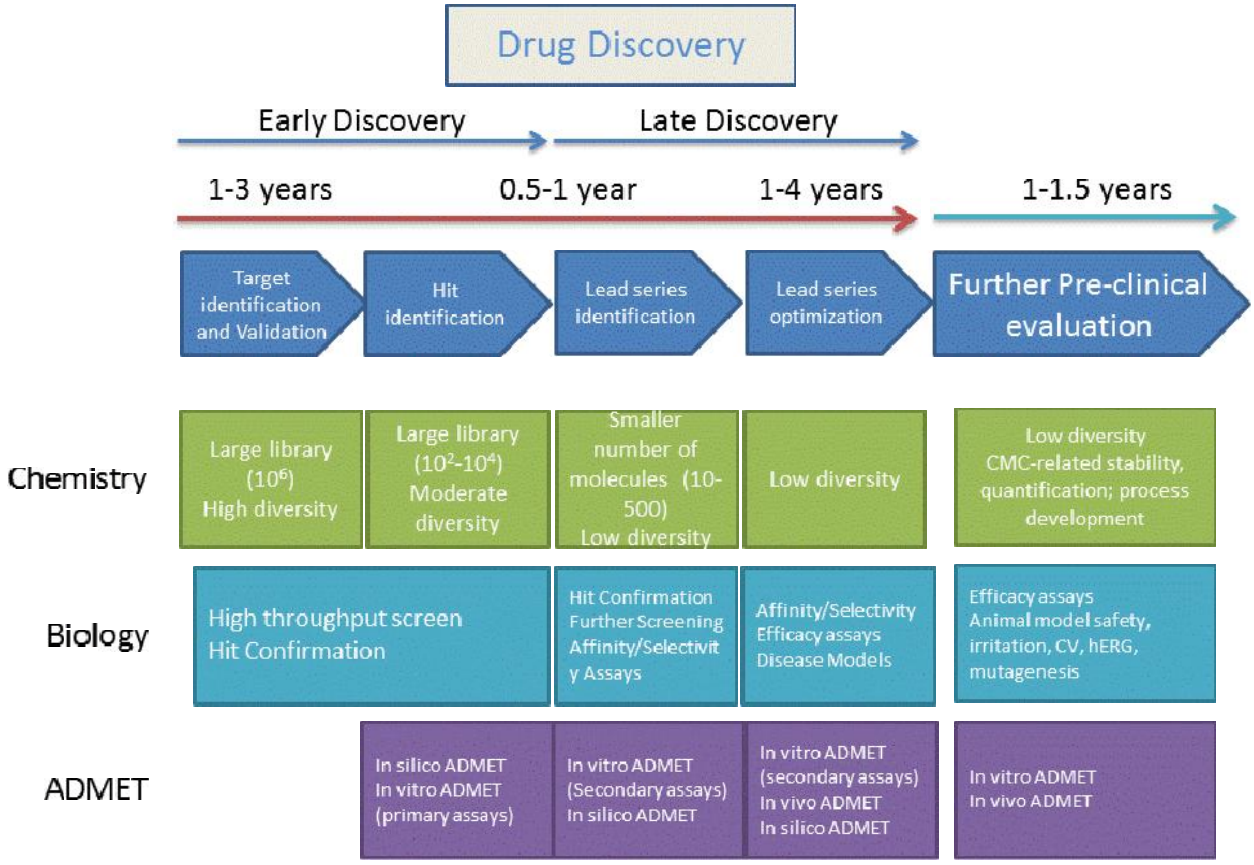

Figure 1. Schematic flowchart of preclinical drug discovery process showing relative contributions of different branches (Biology, chemistry and preclinical pharmacology). Compounds in a chemical library are screened to identify those molecules that interact with the intended target. Molecules that are positive in this assay as "Hits" begin the process of lead identification (Hit-to-Lead) and Lead optimization followed by further preclinical tests. Along the way different ADME/T assays are carried out with varying complexity.

2. Drug Failure: Fail Early, Fail Cheap

The discovery and development of a new pharmaceutical drug requires more than a billion dollars and can take 12 years of research effort (Adams and Brantner 2010; DiMasi, Hansen, and Grabowski 2003). The drug discovery and development process can be divided into four distinct phases: early discovery, late discovery, preclinical, (Fig.1) and finally the clinical trials.

Although there are several factors that contribute to attrition, two major reasons are efficacy and toxicity. It has been estimated that only 1 of 10,000 chemicals that enter the discovery process ever reaches the market. This is not hard to imagine when one considers that 40% of NCEs that begin preclinical safety studies in animals will fail due to toxicity, and that 89% of NCEs that enter clinical trials will fail. The greatest cost in terms of time occurs in early and late discovery, which can require 6 to 8 years of research effort. In comparison, the highest cost in terms of dollars occurs in the preclinical and clinical studies (DiMasi, Hansen, and Grabowski 2003; Kletter et al. 2013; McCarter et al. 2013). The ability to identify and reduce risk early can significantly improve the process of drug development by improving efficiency and improving the probability of success. The new paradigm in drug discovery should include a robust means of identifying issues related to toxicity early in the discovery process where the cost of dropping a molecule is less than in later phases.

3. Preclinical ADME/T Testing

As discussed above, every potential drug molecule needs to be tested for ADME/T properties. In this section, we briefly describe the commonly performed *in vitro* and *in vivo* ADME/T studies.

3.1. *In vitro* Assays

ADME/T studies are done throughout the drug discovery and development process (Guttendorf) . They can be done in preclinical studies leading up to an IND or be assessed in humans during clinical trials to file an NDA (Guttendorf). *In vitro* ADME studies are typically conducted at the same stage as *in vivo* PK studies to facilitate selection of drug candidates with the best safety and pharmacological profile while understanding the mechanisms behind their activity (Steinmetz and Spack 2009).

A. Absorption & Distribution

Most orally bioavailable drugs are absorbed via the intestine. To understand pathways behind intestinal permeability (Li 2005) , the following assays are usually conducted:

Artificial membranes: These are artificial lipid bilayers used to measure the absorption of drugs across lipid membranes by passive diffusion. One example of such a membrane is PAMPA (Parallel Membrane Permeability Assay) which can be done on a high throughput basis. The drug is filtered through a hydrophilic membrane in the donor chamber into the recipient chamber. This ability to permeate is measured by UV analysis of the drug present in the recipient chamber (Li 2005).

In vitro cell culture assays: As PAMPA measures only passive diffusion and does not consider active uptake and transport, cell culture systems which can mimic *in vivo* intestinal epithelium are required to provide insights into mechanisms involved in drug absorption ('Note for Guidance on Toxicokinetics'). These assays can be performed using a similar technique as PAMPA, by culturing on a plate ('Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals'). The following cell culture systems are used:

- Caco-2 : Human colon carcinoma cells are cultured to measure active efflux ('Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals') of drugs that are pumped back into the lumen after being absorbed in the intestine (Li 2005). An important transporter involved in this mechanism is P-gp(Li 2005), which is assayed by measuring active efflux of drugs like verapamil or cylcosporin('Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals').
- MDCK (Madine Darby Canine Kidney): This cell line is also used to measure drug efflux ('Testing of Carcinogenecity of Pharmaceuticals'). They are used in conjunction with Caco-2 because they can become confluent quickly ('Testing of Carcinogenecity of Pharmaceuticals'). This assay can be performed by either using wild-type MDCK cells or cells which have been transfected to express the MDR1 gene which expresses the P-gp transporter ('Testing of Carcinogenecity of Pharmaceuticals').The MDR1-MDCK cell line is used so that the involvement of only the P-gp transporter can be measured as compared to Caco-2 which expresses different transporters as it is derived from human intestinal epithelium ('Testing of Carcinogenecity of Pharmaceuticals'). Both wild type MDCK and Mdr1-MDCK assays are compared to eliminate any canine transporter activity.

Other transporters: Apart from P-gp, transporters such as organic anion transporter (OAT), organic anion transporting polypeptide (OATP), organic cation transporter (OCT), multi-drug resistance proteins (MDRP) and breast cancer resistant protein (BCRP) have been recommended to be studied by the FDA ('Need for Carcinogenecity Studies of Pharmaceuticals').

B. Metabolism

It is important to understand how the body will metabolize a drug, and how quickly those changes will occur. Metabolites of a drug can be toxic, can have new efficacy properties, and/or can interact alter the metabolism of other co-administered drugs. The adverse effects of a drug may arise from its breakdown into toxic metabolites or can be associated to its interactions with other drugs already being administered to the patient (Li 2005). To assess its safety profile and pharmacokinetics, *in vitro* metabolism studies are required (Li 2005). Drug metabolism is facilitated by phase I oxidation by cytochrome P450 mono-oxygenases and phase II conjugation by UDP-dependent glucoronosyl transferase (UGT) and phenol sulfotransferase (PST) (Li 2005).

To study drug metabolism, *in vitro* systems are constructed using tissue, cells and enzymes from human liver (Li 2005) . Inter- species differences can also be determined by using other animal sources like rats, mice, guinea pigs or rabbits .

Liver microsomes: These sub- cellular fractions consisting of endoplasmic reticulum of the liver are utilized to study the activity of phase I enzymes (Li 2005).

Hepatocytes: Primary hepatocytes from rat, dog, monkey and human have been used extensively to evaluate chemical and drug toxicity, metabolism, bioactivation, transporter interaction, intrinsic clearance, and other biochemical processes (Li, Qu, and May 2001; Brown et al. 2007; Hewitt et al. 2007; Wilkening, Stahl, and Bader 2003; Naritomi et al. 2003). An important advantage of primary hepatocytes is that NCEs can be evaluated *in vitro* with cells prepared from normal tissue. Because primary hepatocytes have both phase I and phase II drug metabolizing systems they have been used extensively to study drug metabolism, and toxicity (McKim 2010), (Li 2005). Primary hepatocytes can be used to identify compounds that undergo metabolic activation and to determine how these metabolites affect cellular toxicity (Maggs et al. 2004; Kaminski and Stevens 1992). Optimal growth of hepatocytes is usually achieved with culture plates that have been coated with extracellular matrix proteins, such as matrigel or collagen. Culture time is limited in most laboratories to a maximum of 72 h without significant loss of metabolic activity. Cytochrome P450 activity is typically inducible and present at constitutive levels with good metabolic capacity, provided the cells are of high quality and are cultured under conditions that optimize cell viability and maintain CYP activities.

The following assays are performed:

- ***Metabolic stability***(Li 2005): This assay can be performed on microsomes, supernatant fraction containing microsomes and cytosol (S9) (Dash et al.) and hepatocytes (Li 2005) . Since a drug has to stay in the body for a long period of time, its stability can be measured by its rate of clearance (Li 2005).
- ***Microsomal stability***(Prestwich): Large numbers of microsomes pooled from various donors can be screened to measure clearance involving phase I and some phase II enzymes in a high throughput fashion (Prestwich).
- ***S9 stability***(Dash et al.) :S9 fractions contain both phase I and phase II enzymes as they are supernatants obtained during the process of extraction of microsomes, consisting of both microsomes and cytosol (Dash et al.). They are assayed by methods similar to microsomal stability assays (Dash et al.).
- ***Hepatocyte stability*** (Gomez-Lechon et al.): Hepatocytes are used as a secondary screen to measure intrinsic clearance of compounds which performed well in the primary screens on microsomes and S9 fractions (Gomez-Lechon et al.). Cells from at least 3 donors are used in each well to reduce variability and are screened for both phase I and phase II enzymes(Gomez-Lechon et al.). Both fresh and cryopreserved hepatocytes can be used (Gomez-Lechon et al.).
- ***Metabolite identification and profiling*** (Knop et al.): When a drug is incubated with microsomes, hepatocytes or plasma samples, metabolites formed *in vitro* can be identified using Liquid Chromatography/ Mass Spectrometry (LC/MS)(Knop et al.), (Li 2005). Early identification of these metabolites is important to detect any harmful forms which can lengthen or halt the drug development process altogether (Bissell et al.). If such metabolites are found, then the structure of the compound can be modified to prevent their creation. Alternatively, favorable metabolites can lead to selection of the best *in vivo* animal model for further downstream studies (Li 2005).

C. Drug-drug Interactions:

The administration of one drug can significantly alter the plasma concentration, half-life, or toxicity of another drug. This phenomenon is called a drug-drug interaction or DDI. Drugs that inhibit cytochrome P450 (CYP) enzymes, such as CYP3A4, or that can induce the production of CYP enzymes have a high probability of producing a DDI (Wienkers and Heath 2005; Ito, Brown, and Houston 2004; Ito et al. 2005). In the case of permeability, bioavailability, metabolic stability, and potential DDIs, it makes sense to build *in vitro* screening models focused on predicting human outcomes. The Food and Drug Administration (FDA) requires the submission of an Investigational New Drug (IND) package prior to manufacturing, transport across state lines, and testing in humans. A large part of the IND submission consists of animal safety studies. Typically, these studies are performed in a rodent (rat) and non-rodent species (dog) (Kola and Landis 2004).

According to FDA's guidance document on *in vitro* testing, "*in vitro* studies can frequently serve as an adequate screening mechanism to rule out the importance of a metabolic pathway and drug-drug interactions that occur via this pathway so that subsequent *in vivo* testing is unnecessary"

(Liu, LeCluyse, et al.). A drug might increase or decrease the metabolic activity of a co-administered drug by inhibiting the metabolic enzymes involved (Li 2005). The most commonly studied enzyme isoforms are CYPs 1A2, 2C8,2C6, 2C9, 2C19,2D6,2E1 and 3A4 (Li 2005) . The phase II enzyme UGT1A1 is also studied as it can be inhibited by drugs that are cleared by glucoronidation(Liu, Chism, et al.). Drug-drug interactions (DDIs) are evaluated by performing the following assays:

- ***Enzyme inhibition***: Inhibition of Cytochrome P450 enzymes can be analyzed by incubating the drug in question with liver microsomes(Li 2005) . Since liver microsomes express the whole panel of CyP450 enzymes as in vivo, they are ideal for this assay (Marion, Leslie, and Brouwer). A decrease in the metabolites produced at different concentrations of the drug can be monitored to calculate an IC_{50} value (Marion, Leslie, and Brouwer) . The K_i can also be measured for a specific enzyme to further determine the type of inhibition and any further potential *in vivo* effects (Marion, Leslie, and Brouwer). If the IC_{50} or K_i values are low, that can indicate the drug can lead to DDIs (Li 2005).
- **Enzyme induction**: In this assay, primary hepatocytes are incubated with the drug being evaluated and CYP450 enzyme activity, protein or mRNA levels are measured (Li 2005) . If enzyme induction is significantly greater than that of controls, then the drug can lead to potential DDIs with other drugs (Mathijs et al.). Primary hepatocytes are used because mRNA level is also monitored to provide insights into the mechanism behind induction (Li 2005).

D. Excretion

This is an area where there are no appropriate *in vitro* alternatives to *in vivo* models for excretion (Li 2005). Hence excretion has not been studied in detail in an *in vitro* setting.

E. Toxicity

There is no specific FDA guidance document for *in vitro* toxicity testing, therefore small customized safety panels are created for each drug and used for early screening for toxicity Different cell lines created from hepatocytes, cardiomyocytes and skeletal myocytes can be studied for organ-specific toxicity (Li 2005). Enzyme based and receptor binding assays can be part of the testing panel too. Even though assays can vary, the most commonly measured endpoints are mentioned below.

- ***Membrane integrity***(Li 2005): Usually, lactate dehyrogenase levels are measured to evaluate membrane integrity (Li 2005) as they might be increased due to a damaged membrane (Ng et al.). Hemolysis assays are used to screen for any degradation of red blood cells by the drug. An alternative endpoint is “cellular metabolite content” where bioluminiscence assays using luciferin can be employed to measure intracellular ATP which can be decreased if the cell is damaged or dead (Li 2005).
- ***Mitochondrial function***(Li 2005): The MTT assay is usually used to measure cytotoxicity; as normal cells will reduce MTT to purple crystals (Li 2005). Absorbance can be measured and correlated to cell number and cell death (Zhang et al.).
- ***Lysosomal functions*** (Li 2005): Neutral red dye is taken up by live cells through lysosomes but damaged cells will show decreased uptake (Li 2005).
- ***Mutagenic potential***: The Ames test is used to screen for carcinogens by investigating the potential of the drug to reverse mutations(Meng). Bacteria containing a defective mutant gene which renders them unable to grow in media without histidine are plated with the drug (Sakai et al.). If this mutation is reversed, then these bacteria will be able to grow in the histidine-free media (Sakai et al.). This assay would be usually performed late in the lead optimization phase, unless there were reasons to believe that there might be mutagenic potential (e.g., for nucleoside analogs, probably incorporated earlier than normal).
- ***hERG assay:*** The hERG (human Ether a go-go Related Gene) is essential for creating potassium channels in the heart which in turn, are necessary for its proper functioning. Since abnormal heart rhythm or arrhythmias can be caused by various drugs due to blockages of these potassium channels, it is important to check whether they inhibit the current produced by these channels ('hERG Safety Assay'). Chinese Hamster Ovary (CHO) cells transfected with hERG are used. In a single well of a 384 well plate, such a cell is used to block the gap between two fluid-containing chambers. A pore forming agent is added to create a pore i.e. a channel in this cell. The test compound is then added to screen for inhibition. Electric current before and after addition of the compound is measured and translated to an IC_{50} value.

3.2. *In vivo* ADME/T assays:

A. ADME

Although most of the useful data for IND filing obtained from *in vivo* models relates to toxicity, the ADME analysis of these animals is useful in predicting the PK parameters for phase I clinical trials as well. These animal studies are generally conducted on two species, one rodent and one larger animal (usually rabbit, dog, pig or primate). These two points are used to extrapolate (or interpolate) to determine parameters for clinical administration. Absorption is easily determined by monitoring the concentration of the drug in the blood plasma after dosing and at regular time intervals thereafter. Distribution to different tissues can be observed by administering a radiolabeled agent and tracking this through the various systems and organs using body scans. Metabolite formation is determined using LC-MS to analyze blood samples. The use of an isotopic agent (such as a radiolabel) makes it easy to identify metabolites, as there will be dual peaks corresponding to the labeled and naturally abundant agents in the mass spectrum. Chemists then use this MS data to hypothesize how a drug might be metabolized in humans. Similarly, this same procedure can be used to determine excretion products, with these specimens coming from feces or urine instead of blood. In this fashion, the ADME data derived from animal studies allows biologists and medical doctors to estimate the appropriate does in humans. This also provides analytical chemists with an idea of what metabolites they might observe in the clinic.

B. Toxicology

Preclinical *in vivo* toxicology studies need to be conducted in two species of animals for both acute and chronic exposure. Rats and mice are the preferred first subjects used *in vivo* due to their body mass and extensive knowledge of these biological systems. Dogs, pigs and primates are the other preferred species for *in vivo* studies. Acute toxicity studies generally last about 2-4 weeks whereas chronic studies are anywhere between one month and one year in duration. Chronic studies need not be conducted in preparation for Phase I clinical trials. Furthermore, the length of these studies will depend up on the intended length of treatment in patients which varies between indications. The ICH recommends 6 month studies using rodents and 9 month studies when using other species for most chronic toxicology studies ('Duration of Chronic Toxicity Testing in Animals' 1998).

Toxic effects resulting from acute exposure is determined by monitoring drug levels and metabolites in the blood during exposure ('Note for Guidance on Toxicokinetics' 1994; Birendra, Afzal, Sochaki, et al. 2015; Birendra, Afzal, Wentland, et al. 2015) as well as necropsy of the test subjects when necessary. For these tests, only one sex may be used if there is no reason to believe there is a difference between genders or if the drug is only to be administered clinically to one gender ('Guidance on Genotoxicity Testing and Data Interpretation' 2008). If the test subject(s) die during the acute toxicology testing, the organs are harvested and examined by a certified veterinarian pathologist to determine details about injury to specific organs. If the test subjects do not display any toxicity as a result of the study, their lives may be spared and potentially used again for other toxicology studies.

In addition to organ-specific toxicology, immunotoxicity may also be monitored. This is of minimal importance for most small molecules; however, this is a much more pronounced concern for biologics as they often appear as ‘invaders’ from the immune system’s perspective. Immunotoxicity could be either a suppression or enhancement of the immune system and may be monitored for both acute and chronic exposure. These studies are generally conducted over 28 days with consecutive daily dosing. These implications of these tests might involve hematological changes such as leukocytopenia/leukocytosis, alterations in immune organ (i.e. spleen and thymus) size/mass, changes in serum globulin levels and/or increased incidence of infections or tumors. If immunotoxicity is suspected, assays such as the T-cell dependent antibody response (TDAR) or other targeted cell type assays are recommended. Immunophenotyping of leukocytes maybe performed to identify specific cell populations affected that could potentially provide useful clinical biomarkers. Immunophenotyping is conducted using either flow cytometric analysis or immunohistochemistry. Additional immunogenicity tests required for biologic candidates. Immunogenicity tests examine the production of antibodies in response to a biologic, and are infrequently predictive of the human immune response when attempting to translate animal experiments to clinical trials ('Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals' 2011).

Beyond acute- and immuno-toxicity, effects resulting from chronic exposure can be divided into 3 categories: genotoxicity, carcinogenicity and

reproductive toxicity. As such, these effects are investigated for treatments intended for use beyond a month or two. Genotoxicity is assessed by an array of *in vitro* assays required for IND filing. Positive results from genotoxicity experiments warrant the investigation of carcinogenicity and reproductive toxicology experiments, which are usually conducted concurrent with Phase III clinical trials.

For genotoxicity, usually two experiments need to be conducted. First, bacterial mutation *in vitro* assays (Ames) are performed ('Guidance on Genotoxicity Testing and Data Interpretation' 2008), which are predictive of rodent carcinogenicity. Additional assays to assess genotoxicity include the DNA double-strand break assays (including "Comet"), the alkaline elution assay, DNA covalent-binding assays and unscheduled DNA synthesis assay from liver ('Testing of Carcinogenecity of Pharmaceuticals' 1997). This battery test approach is designed to reduce the risk of false negative results for compounds with genotoxic potential; however, a positive result in any assay for genotoxicity does not necessarily mean that the test compound poses a genotoxic or carcinogenic hazard to humans ('Guidance on Genotoxicity Testing and Data Interpretation' 2008). As such, this approach uses genotoxicity results to determine if carcinogenicity studies need to be conducted

To examine chromosomal damage in animals, treated rodents are assessed using erythrocytes in the blood or aberrations during metaphase within bone marrow. Other cells, such as hepatocytes are also acceptable targets for *in vivo* studies when specific organs toxicity is suspected. Micronucleus assays are also used to assess carcinogenicity. If *in vivo* results suggested toxicity, either further negative *in vitro* assays or additional *in vivo* assays using other tissues with equal exposure would suffice to demonstrate lower safety risk.

Since carcinogenicity studies are time consuming and resource intensive they are only being performed when human exposure warrants the need for information from life-time studies in animals in order to assess carcinogenic potential ('Need for Carcinogenecity Studies of Pharmaceuticals' 1995). Carcinogenicity studies are also conducted in two species by administering the drug via the same anticipated route of that to be used in humans. In the absence of clear evidence favoring one species, it is recommended that the rat be selected as one species ('Testing of Carcinogenecity of Pharmaceuticals' 1997). Most filings with the FDA conduct carcinogenicity studies for treatments which are expected to be administered routinely in excess of 3-month dosages in humans. Carcinogenicity testing is nearly universal for drugs administered 6-months or longer. Even if the anticipated therapeutic dosing does not meet or exceed these lengths, carcinogenicity studies are recommended for drugs that fall within certain categories of compounds known to be carcinogenic, when SARS suggests potential carcinogenicity or if pre-neoplastic lesions are observed in repeated dose studies. Experiments generally include noting both cellular changes and biochemical measurements ('Testing of Carcinogenecity of Pharmaceuticals' 1997). Cellular changes may include observations involving the morphological, histochemical or functional attributes of the relevant tissues. Biochemical measurements may include hormone levels, growth factors and enzyme activity. Unequivocally genotoxic compounds, in the absence of other data, are presumed to be trans-species carcinogens, thus implying a hazard to humans ('Need for Carcinogenecity Studies of Pharmaceuticals' 1995).

Reproductive toxicology is tricky as the PK parameters for pregnant or lactating animals will be different and is unlikely that this data will be available ('Note for Guidance on Toxicokinetics' 1994). For male reproductive toxicity, fertility is the focus of such studies. Given the complexity of these tests and the scope of this report, further discussion involving reproductive toxicology will be omitted.

3.3. 3D Tissue Culture for ADME/T Studies

A. Need of 3D culture for Drug discovery

In the pharmaceutical industry, late ailure of developmental candidates is a cause of concern. Such failure translates into loss of millions of dollars and several years of wasted time and efforts. This is primarily the reason why they prefer to identify "failure" drug molecule in early developmental stages. To accomplish this goal, researcher must develop novel and state of the art efficient assays and methodologies to better characterize these prospective drug molecules.

Traditional state of art *in vitro* methodologies to characterize molecules in ADME/T processes have been 2D cell cultures. The 2D monolayer culture of hepatocyte and other cell type such as HEK 293T cells are relatively cheap and convenient to use for high throughput screening to identify hepatotoxicity and

other ADME/T properties of drugs(Singh, Simpson, and Bennett 2015; Singh and Bennett 2009). In *in vitro* ADME/T studies using hepatocyte monolayers on hard plastic or glass surface, it is assumed that the monolayer reflects the essential physiology of liver. In fact, liver-specific architecture, mechanical and biochemical characteristics and cell-cell communication are lost under simplified *in vitro* conditions. More than 50% of drugs tested *in vitro* in 2D hepatocytes fails to show their *in vivo* effects because of the lack of entire physiological system. For example, consider lack of idiosyncratic hepatotoxicity (IH) in in vitro, a result of lack of immune-mediated reactions (Gomez-Lechon et al. 2010). An improved *in vitro* method of liver that could mimic *in vivo* liver functions and could predict drug pharmacokinetics and pharmacodynamics in an *in vitro* environment is highly desirable.

It is hypothesized that 3D cell culture that closely reflects *in vivo* environment will better predict to characterize pharmacokinetics and pharmacodynamics of different chemical entities. This early characterization of ADME/T properties will help drug researchers to exclude unpromising molecules early enough to save time and money.

B. Advantages of 3D Tissue models over 2D models

A reliable ex vivo information of physiological relevance can be offered by 3D cell cultures because 3D scaffolds reduce the gap between hepatocyte cell culture and resembling close to physiological liver. Liver consists on many types of cells including hepatocytes (main metabolic cells with *in vivo* value 60%), endothelial cells (secrete cytokines with *in vivo* value 20%), kupffer cells (macrophages, inflammatory response *in vivo* value 15%), and stellate cells (fibroblasts, store fat and secrete ECM *in vivo* value 5%) (Dash et al. 2009). These different cell types interact with neighboring cells and with ECM through biochemical and mechanical cues to establish a 3D communication network that maintains specificity and homeostasis of the tissue. Key events of cellular uptake of bio molecules and drugs, their metabolism, regulation of gene expression such as CYP and other metabolism related enzymes, signaling through ECM all together compiles into a complex network of information exchange to derive a process including drug metabolism into many inter-mediatory bioactive products as well as their later excretion.

C. Hepatocyte 3D culture

Primary hepatocytes within hydrogel of extracel are used for 3D culture, to evaluate hepatotoxicity *in vitro (Prestwich 2008)*. These primary hepatocytes using collagen or matrigel(Knop et al. 1995; Bissell et al. 1987) 3D culture sandwich technique retain their biochemistry and have enhanced longevity (Prestwich 2008). The sandwich model provides cell-cell interaction, cell-extracellular matrix interaction, maintain polarity and morphology (Liu, LeCluyse, et al. 1999; Liu, Chism, et al. 1999; Marion, Leslie, and Brouwer 2007). These 3D hepatocytes maintain their metabolic competence but show some degree of reduction of phase I activity and gene expression(Mathijs et al. 2009). Later, an overlay sandwich culture has been developed that reduces shear stress and preserve mass transport consistency using laminar-flow perfusion(Ng et al. 2006). Another sandwich model is based on ultra-thin micro fabricated porous silicon nitride membrane. These models have improved mass transport features with decreased stress markers and enhanced liver like functions (Zhang et al. 2008; Bennett et al. 2014). Sandwich-cultured hepatocytes are limited to mechanistic studies of drug toxicity in association to transportation and partial identification of major intermediate metabolites(Meng 2010). This is because of gradual loss of metabolic enzyme CYP 2E1.

***Spheroid Cultures*:** Another 3D version is spheroid cultures with microvilli projections expressing plasma proteins such as albumin and transferrin (Sakai et al. 1996; Tong et al. 1992). These spheroids have liver like histology but have limited transport of oxygen and other nutrition. These spheroids show higher sensitivity to drug hepatotoxicity than regular 2D cultures (Du et al. 2007), but the tightly associated spheroids showed different endpoints for drug hepatotoxicity as compared to 2D. It was suggested that lack of compound penetration into these tight spheroids may limit their use in chronic *in vitro* toxicity assays (Meng 2010; Walker, Rhodes, and Westmoreland 2000).

***Gel Entrapment Culture*:** is a semi-permeable hollow fiber cartridge with separate cells from the perfusion compartment and is a scaffold for hepatocyte entrapment(Tzanakakis et al. 2000). In gel entrapment culture medium circulation system provides nutrition and oxygen. Gel entrapped hepatocytes are more sensitive than 2D culture. The gel entrapment detects 5-10 fold less toxic drug levels as compared to monolayer and shows high sustained levels of CYP

expression (Shen et al. 2006; Meng et al. 2007). This model is sensitive to increased oxygen species production and mitochondrial dysfunction to account for hepatotoxicity induced by drug (Shen et al. 2009).

Hollow Fiber Bioreactors (HFB): is an alternative to conventional cell culture methods and is being used for many PK studies. Cells grow on and around the large surface area provided by the network of hollow fibers. Hollow fiber cell and tissue culturing maximize surface area to minimize the volume of culture medium required. The porous hollow fibers form a 3 dimensional network of capillaries that continually delivers a supply of fresh media and nutrients to the cultured cells that remain isolated in the Extra-Capillary Space. The fiber membrane provides a selectively permeable barrier that permits passage of metabolic by-products away from the cells while culture-dependent nutrients permeate into the culture. When perfused with culture media, the hollow fibers allow oxygen and nutrients to be supplied to the cells while metabolic waste products are eliminated. The process increases the accumulation of the cell-secreted growth factors required for optimal growth. Due to the system's efficient delivery of media and removal of waste, the process uses less culture media than other methods. Cultures can be sustained for 6 months or more while maintaining high production yields and titers. Some of the salient features of HFB are its support to many human cell types, promoting high cell density, supporting cell-cell interaction, low sheer, fluid mechanics imitating *in vivo* interstitial flow, controllable media flow & exchange, and improved pharmacodynamics potential (Whitford and Cadwell 2009)

D. Precision Cut Tissue Slices

Precision cut tissue slice (PCTS) is a process whereby cores of excised tissue, such as liver can be sliced into discs of uniform thickness. Both phase I and phase II metabolizing systems are intact (Heinonen et al. 1996) and inducible in PCTS (Lupp et al. 2002). Some advantages to this system include an intact organotypic architecture that is similar in composition to the original tissue. This allows evaluation of cell-to-cell interactions in vitro. Thus, PCTS is an excellent model for evaluating drugs of chemically-induced hepatobiliary toxicity (Amin et al. 2006). The system can be applied to problems that require the ability to discern toxicity, including target organ toxicity, and species-specific toxicity (Vickers and Fisher 2005; Parrish, Gandolfi, and Brendel 1995; Lerche-Langrand and Toutain 2000; Rodrigues et al. 1994).

One disadvantage of PCTS has been the relatively small number of slices that could be obtained from a single animal and tissue type making high volume screening less practical. Increasing the number of small slice, CHIP, can improve the technique. Another disadvantage that has limited the use of PCTS as an early screening tool has been the inability to cryopreserve slices, and to recover them with a high degree of viability. Issues of viability related to time in culture, regional changes in viability due to the thickness of the slice, diffusion of nutrients, and the type of incubator employed for culture (Toutain et al. 1998) must be tested and optimized within each laboratory.

4. Summary

Development of small molecules is full of challenges but pioneering ADMET investigations on new approaches could advance our knowledge in drug discovery. A better understanding of ADME processes, by pharmacologists in early discovery through late development, could be crucial to enhance success rate and reduce the cost of drug molecules. In particular, predicting toxic effects of preclinical molecules have been the major challenge in early stage of drug cycle. Though, predictive *in silico* or rule-based ADME tools could boost the discovery and reduce time as well as expense in early identification stages, yet better pre-clinical assays are in need to be developed. Recently, scientists have shown a lot of interest in 3D cell culture models as compared to 2D cell cultures because they could better replicate *in vivo* conditions.

Conflict of Interest: None

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