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DRUG DISCOVERY
TODAY
TECHNOLOGIES

Screening technologies

Preclinical *in vitro* screening assays for drug-like properties

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Besides pharmacological effects, ADMET drug properties, namely, absorption, disposition, metabolism, elimination and toxicity, are important drug properties critical for clinical success. *In vitro* human-based experimental system used in combination with *in vivo* animal systems, using animal species relevant to humans, represent the best approach to assess these important drug-like properties before clinical trials. Selection of drug candidates with the best ADMET properties should enhance the probability of clinical success.

Introduction

The high cost of drug development is partly due to the high failure rate of drug candidates in clinical trials. A successful drug candidate needs to be efficacious without unacceptable adverse effects. During drug development, one needs to select drug candidates with the most appropriate drug-like properties.

The critical drug-like properties can be illuminated by examining the fate of an orally administered drug. Upon ingestion of a drug, the following events would occur: (1) Dissolution: dissociation of the active ingredients of a drug from its matrix. (2) Absorption: passage of the drug through the intestinal epithelium into the systemic circulation. (3) Disposition: entrance of the drug in the system circulation and distribution to various organs and tissues. (4) Metabolism: biotransformation of the drug by the drug-metabolizing enzymes. (5) Biological interaction: interaction of the drug and its metabolites with intended and unintended targets. (6)

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Drug–drug interactions: interaction of the drug with coadministered drugs. (6) Elimination: removal of the drug from the body. The desirable drug properties are therefore high solubility and absorption, ready distribution to intended target tissues, appropriate metabolic stability and minimal formation of toxic metabolites, extensive interactions with intended targets and minimal interactions with unintended targets, minimal interaction with coadministered drugs, minimal toxicity and an appropriate rate of elimination. A drug candidate with these desirable drug-like properties will be probable to be successful in clinical trials. In this review, the state-of-the art technologies for the screening of drug candidates for human ADMET drug properties, such as absorption, distribution, metabolism, elimination and toxicity, will be discussed. The application of these technologies in drug discovery and early drug development should in aid the design and selection of drug candidates with the best probability of success in clinical trials [1,2].

Physical and chemical properties as key determinants of drug-like properties

The physical and chemical properties of a chemical compound can be used as the first determination of drug-like properties. This approach is best exemplified by Lipinski's Rule of Five. Based on a review of the physical and chemical properties of chemical compounds that are drug-like and non-drug-like, Lipinski and colleagues developed the concept that there are physical and chemical properties that appear to

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be critical to solubility and permeability [3]. Lipinski's Rule of Five is stated as follows:

Poor absorption or permeation are more probable for a chemical when there are:

- More than five H-bond donors;
- The molecular weight is over 500;
- The hydrophilicity measurement, $C \log P$ (the logarithm of its partition coefficient between *n*-octanol and water $\log(c_{\text{octanol}}/c_{\text{water}})$), is over 5;
- The sum of Ns and Os is over 10.

Early evaluation of chemical structure and determination of partition coefficient are therefore recommended to allow one to select drug candidates that would be more 'drug-like' as suggested by the Rule of Five.

Screening assays for intestinal permeability

The Rule of Five, however, is not all encompassing. For instance, the rules would not be applicable to compounds that are substrates of efflux transporters (proteins on intestinal epithelium that acts to pump-absorbed xenobiotics back into the lumen, thereby lowering bioavailability) and drug-metabolizing enzymes. Further, drugs can be absorbed via biological pathways, which are not governed by the rules.

Orally administered drugs are absorbed mainly by the intestines. Most of the absorption occurs in the duodenum and the small intestines. The following are the major pathways of absorption [4]:

1. Transcellular absorption: Highly lipophilic drugs (e.g. steroids) can transverse the lipid bilayer cell membrane and thereby can be absorbed through the intestinal mucosal epithelium. This process is driven by passive diffusion via the chemical concentration gradient. Transcellular absorption is the most efficient and therefore the most desirable route of absorption for drugs, except for substrates of efflux transporters (see below).
2. Paracellular absorption: High hydrophilic molecules cannot transverse cell membranes, but they can pass through gaps between cells in the intestinal epithelium. Paracellular absorption is not saturable, driven by concentration gradient, and is regulated by tight junctions (tight junctions prohibit paracellular absorption). Mannitol is mainly uptaken via this pathway.
3. Transporter-mediated absorption: As many nutrients are small, hydrophilic molecules (e.g. sugars, amino acids), specific proteins have been evolved for the uptake of these compounds. Drugs that are substrates of the uptake transporters can enter the systemic circulation after oral administration via this pathway.
4. Transporter-mediated efflux: This is the mechanism of the body to limit the absorption of xenobiotics. The efflux

transporters would pump the xenobiotics that have entered the intestinal epithelium back into the lumen. P-glycoprotein (Pgp) is the most important efflux transporter, both in the intestinal epithelium and the blood-brain barrier. Verapamil and cyclosporin are examples of drugs subjected to Pgp-efflux.

Technologies for the assessment of intestinal absorption include the following:

1. Artificial membranes: These are artificial lipid bilayers via the coating of highly permeable membranes with long-chain fatty acids. Artificial membranes can be used to evaluate the ability of molecules to be absorbed via the transcellular pathway. PAMPA (parallel membrane permeability assay) represents an absorption assay with artificial membranes [5]. Assay with artificial membranes involves the use of multiwell (e.g. 96-well) filtration plates with the hydrophilic filter coated with an artificial membrane solution (0.8% egg lecithin in *n*-dodecane). The test material is applied into the top chamber (donor chamber) and the permeation of the material into the bottom chamber (recipient chamber) containing a buffer solution is quantified. High-performance liquid chromatography (HPLC) coupled with ultraviolet absorbance detection (235 and 254 nm) is routinely used in this permeability assay.
2. *In vitro* cell culture assays: *In vitro* cell culture systems have been developed to model intestinal absorption [6]. Caco-2 cells, a human colon carcinoma cell line known to differentiate in culture to mimic intestinal mucosal epithelium is the most 'complete' experimental model. The Caco-2 cells upon differentiation would develop tight cell-junctions as well as uptake and efflux transporters, thereby modeling the various absorption pathways. Significant progress has been made to develop screening assays for intestinal drug permeability using Caco-2 cells (e.g. [7]). MDCK cells, a canine kidney cell line, also develop tight junctions and can be used to evaluate transcellular absorption. MDCK cells have been transfected to express transporters, especially Pgp, to incorporate the efflux mechanism. The assay involves the culturing of the cells to form an intact cell barrier in a cell culture filter plate similar to those used for PAMPA. The assay is then performed via quantification of the appearance of the test material in the recipient plate.
3. Everted intestinal sac: Intestinal segments can be turned inside out (everted), with both ends tied for intestinal absorption studies. The sacs are placed in a solution containing the drug to be studied, and the amount that enters the lumen of the sac would represent drugs that can be absorbed from the intestinal lumen into the systemic circulation. It has been suggested that everted intestinal sac might provide data closer to human intestinal absorption than the Caco-2 cell model [8]. Maintenance of tissue

viability and the time and labor-intensive procedures represent the most challenging aspects of the use of everted intestinal sacs.

4. *In vivo* animal models: In general, there is a good correlation between intestinal absorption in rodents and humans. However, results with *in vivo* animal models can be complicated by hepatic metabolism and renal excretion. *In situ* intestinal perfusion represents a technique to evaluate drug absorption without hepatic and renal influences. In this procedure, the experimental animal is anesthetized. A portion of the intestine is exposed and a drug solution is perfused into the lumen of the exposed intestine. Drug disappearance from the perfusate is used as a measurement of drug absorption. The intestinal perfusion technique is generally used for specific mechanistic evaluations (e.g. [9]) but not in general screening owing to the need to use live animals and the time- and labor-intensive procedures.

Assays for the evaluation of drug metabolism

An orally administered drug is absorbed via the small intestines into the portal circulation, which enters the liver where drug metabolism occurs. Drug metabolism, the biotransformation of a parent drug by drug-metabolizing enzymes, is an important drug property. Together with bile and renal excretion, drug metabolism determines the duration of a drug in the patients' body. Drug metabolism can determine adverse drug effects – a toxic parent drug can be 'detoxified' or a nontoxic parent drug can be 'activated' to toxic metabolites by biotransformation. Also, pharmacokinetic drug–drug interactions is a result of drug metabolism. Drug metabolism is generally defined as phase 1 oxidation where nonpolar molecules are oxidized via the addition of oxygen atoms, usually in the form of a hydroxyl moiety (–OH group), and phase 2 conjugation where a very water soluble molecular such as glucose (glucuronidation) or sulfate (sulfation) is added to the organic group, especially at the –OH site. Phase 1 oxidation is carried out mainly by P450 mixed function oxygenases. UDP-dependent glucuronosyl transferase (UGT) and phenol sulfotransferase (PST) are examples of phase 2 conjugation enzymes. Early determination of metabolic fate is critical to the design and subsequent selection of the most appropriate chemical structure for further development.

In vitro screening assays with human liver-derived experimental systems represent the most effective approach to estimate human *in vivo* drug metabolic fates [1,2]. These experimental systems include the following:

1. Human liver microsomes: Microsomes are endoplasmic reticulum membrane vesicles prepared by homogenization of the liver, followed by a relative low speed centrifuga-

tion (e.g. $9000 \times g$) to remove whole cells, free nuclei, plasma membranes and mitochondria. The resulting supernatant, conventionally called S-9, which is sometimes used for metabolism studies, is then centrifuged at a higher speed, at $100,000 \times g$, to pellet the endoplasmic reticulum, which contains the P450 isoforms and one of the phase II conjugating enzymes, UGT. Microsomes are mostly used for the evaluation of phase 1 oxidation via the addition of NADP as a cofactor. They can also be used to study glucuronidation via the addition of UDP-glucuronic acid (UDPGA).

2. Human hepatocytes: The parenchymal cells of the liver or hepatocytes contain virtually all the hepatic drug-metabolizing enzymes and cofactors. Although the liver microsomes are used for the evaluation of oxidative metabolism, hepatocytes are used to study both phase 1 and 2 liver drug metabolism pathways. The relatively recent successful cryopreservation of human hepatocytes [10–12] allows this experimental system to be readily used.

Drug metabolism assays include the following [1,2]:

1. Metabolic stability: A successful drug has to stay in the human body for an adequate length of time to interact with the intended pharmacological targets using a reasonable dose regimen. The duration of a drug in the body is mainly determined by hepatic metabolism, hepatic excretion and renal excretion. *In vitro* metabolic assay is used to identify for chemical structures with the most appropriate stability upon hepatic metabolism – metabolic stability. In this assay, the rate of hepatic metabolic clearance of a drug is evaluated. The assays can be performed with liver microsomes or hepatocytes. One approach is to monitor the disappearance of a parent chemical with time. The data are used to estimate the time for 50% disappearance of the parent structure ($T_{1/2}$). Based on $T_{1/2}$, one can estimate *in vivo* hepatic clearance.
2. Metabolite profiling: This assay involves the incubation of a drug candidate with *in vitro* hepatic metabolic systems such as hepatocytes and liver microsomes followed by the identification of the major metabolites, usually by mass spectrometry. Identification of metabolites is important to drug development for the following reasons:
 - a. Structure optimization: Modifying the chemical structure to prevent certain metabolite formation is an effective approach to enhance metabolic stability and to decrease metabolism-related toxicity.
 - b. Selection of relevant animal species: An animal species which produces metabolites identical to that formed in human will more probably yield data representative of human beings – a 'relevant' animal species. Metabolite profiling using *in vitro* hepatic systems from multiple animal species and human will allow one to select logically the most 'relevant' animal species.

Drug–drug interaction potential evaluation

Frequently, patients are given more than one medication. Adverse effects have been observed when one drug interacts with a coadministered drug. Drug–drug interactions are mainly evaluated as ‘pharmacokinetic’ drug–drug interactions, where one drug affects the metabolic fate of another drug [13]. There are two major types of pharmacokinetic drug–drug interactions:

1. Inhibitory drug–drug interactions: One drug might inhibit the metabolism of a coadministered drug. The affected drug thereby might have plasma concentrations, higher than intended, leading to toxicity. A well-known case of inhibitory drug–drug interactions is the inhibition of the metabolism of a nondrowsy antihistamine, terfenadine, by the antifungal drug, ketoconazole. A number of patients developed fatal cardiac arrhythmia after they were administered both terfenadine and ketoconazole owing to the elevated level of terfenadine. Terfenadine has been taken off the market owing to its drug–drug interaction potential. It is now known that ketoconazole is a potent inhibitor of CYP3A4, the P450 isoform responsible for metabolism of terfenadine.
2. Inductive drug–drug interactions: A drug can accelerate the metabolism of a coadministered drug by inducing the corresponding metabolic pathways. In this situation, the major result is the diminished efficacy of the affected drug owing to the lower-than-intended plasma level. Rifampin–birth control pill interaction is an example of this type of drug–drug interactions. Women of child-bearing age on birth control pill experienced pregnancy owing to the induction of the drug-metabolizing enzymes for the active ingredients of the birth control pills. Rifampin is now known to induce two major pathways of the metabolism of birth control pill ingredients: CYP3A4 and estrogen sulfotransferase.

Because of the clear understanding of the mechanisms of pharmacokinetic drug–drug interactions, there is a universally accepted strategy to use *in vitro* human liver-based experimental systems to evaluate drug–drug interaction potential as presented below [1,2]:

1. Pathway identification: Understanding which drug metabolism pathways are involved in the metabolism of a drug will allow one to identify drugs that are known inducers and inhibitors of these enzymes as drugs that would affect the metabolism of the drug. Pathway identification experiments are performed to elucidate which metabolic pathways are important in the metabolism of the drug being investigated. The studies involved firstly identifying the key metabolites, thereby deducing whether the drug is metabolized by phase 1 oxidation or phase 2 conjugation.

If phase 1 oxidation is the major pathway, the following experiments are performed:

- a. P450 isoform-specific inhibitors effects: The drug in question will be incubated with liver microsomes or hepatocytes in the presence of various P450 isoform-specific inhibitors. The inhibitory effects of these inhibitors on the metabolism of the drug would illuminate which P450 isoforms are involved in the metabolism.
 - b. Metabolism by cDNA-expressed microsomes: Microsomes from cells engineered to express only a specific human P450 isoforms are routinely used to identify which P450 isoforms are involved in the metabolism of a drug.
2. Drug-metabolizing enzyme inhibition: These assays are performed to evaluate whether the drug in question would inhibit drug-metabolizing enzymes. The drug to be studied is incubated with liver microsomes or hepatocytes in the presence of substrates of specific drug-metabolizing enzymes. The metabolic rate of these substrates in the presence and absence of the drug would allow one to estimate its inhibitory potential. Results are in general expressed as IC_{50} , a concentration leading to a 50% decrease in activity, or K_i , the inhibitory constant. Low IC_{50} or K_i values as compared with the intended plasma concentration would suggest a high potential of the drug to cause drug interactions with coadministered drugs, which are substrates of the affected enzyme.
 3. Drug-metabolizing enzyme induction: Several drug-metabolizing enzymes, for example CYP1A2, CYP2A6, CYP2B6, CYP2C9 and CYP3A4, can be induced. A drug that can induce one or more of these enzymes will have the potential to cause inductive drug–drug interactions. The assay involves the culturing of human hepatocytes in the presence of the drug in question and measuring enzyme activity, protein or messenger RNA levels of drug-metabolizing enzymes. As this assay requires gene and protein expression, human hepatocyte primary cultures represent the most appropriate experimental system. Several reporter cell lines [14,15] have also been developed as rapid screening assays allowing P450 induction to be evaluated early in drug development. It is generally believed that the results with these screening assays require to be confirmed by that with primary human hepatocytes.

Drug–drug interaction assays are in general focused on P450 isoforms, with CYPs 1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 as the major isoforms studied. The same experimental systems, especially those with intact hepatocytes, are also being applied to evaluate drug–drug interactions involving phase 2 conjugation [16] and transporters [17,18].

Drug excretion

Of the ADMET properties, excretion is probably the least studied. Renal and fecal excretion are in general studied using

whole animals, with no appropriate *in vitro* surrogates. Recently, a technology has been developed using hepatocytes cultured in a sandwich format to allow the development of bile canaliculi *in vitro*. This technology has been applied to evaluate hepatobiliary excretion as well as transporter-mediated drug–drug interactions [19].

Screening assays for drug toxicity

Accurate prediction of drug safety remains the major challenge for the pharmaceutical industry. The routine practice of pre-clinical and clinical drug safety evaluation is apparently inadequate, as evidenced by the yearly withdrawal or severe use-limitation of marketed drugs due to unexpected adverse effects.

Early screening for drug toxicity, especially human-specific toxicity, has become a routine practice in drug discovery and development. It can be argued that because drug candidates will be vigorously tested in laboratory animals, assays to identify human-specific toxicity (which, by definition, will not be detected in nonhuman animals) are critical to drug development.

The use of human cells, which retains organ-specific properties represent important experimental systems for early toxicity screening. The promising primary human cell culture systems include the following:

1. Hepatocytes for liver toxicity;
2. Renal proximal tubule epithelial cells for nephrotoxicity;
3. Vascular endothelial cells for vascular toxicity;
4. Neuronal and glial cells for neurotoxicity;
5. Cardiomyocytes for cardiotoxicity;
6. Skeletal myocytes for rhabdomyolysis.

In general, cytotoxicity endpoints such as membrane integrity, cellular metabolite content, mitochondrial functions and lysosomal functions are used in conjunction with these cells for the screening of organ-specific toxicity. The following endpoints are commonly used in cytotoxicity screening assays:

1. Membrane integrity: This is the measurement of the increase in cytoplasmic enzymes such as lactate dehydrogenase in the culture medium after treatment. This endpoint represents a classical endpoint for cytotoxicity. For some cell types (e.g. hepatocytes), the basal level can be too high and therefore might limit the sensitivity of the endpoint.
2. Cellular metabolite content: Cellular ATP content represents the most commonly measured metabolite content. Dead and damaged cells contain little or no ATP. Bioluminescence assay using the luciferin–luciferase assay represent a sensitive assay for cellular ATP, allowing the use of as little as a few hundred cells per assay.
3. Mitochondrial functions: The chemical, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),

is converted to a blue crystal which can be solubilized and quantified by spectrophotometry. The MTT assay is a common assay for cytotoxicity and usually would yield results similar to the ATP content assay.

4. Lysosomal functions: Neutral red uptake assay is used to measure cell viability as reflected by lysosomal functions. Neutral red is taken up into a cell owing to lysosomal activities. Cell damage would be accompanied by a decreased neutral red uptake. The assay involves incubation of cells with neutral red, followed by washing of extracellular dye, and quantification of uptake by cell solubilization and spectrometry.
5. Apoptosis: Induction of programmed cell death or apoptosis is a known mechanism of drug toxicity. High-throughput screening assays have been developed to quantify the increase in caspase activity as a measurement of apoptosis [20].

There are also specific systems for the evaluation of toxicity of known mechanism. For instance, QT prolongation, a manifestation of cardiac arrhythmia, can be studied in cardiomyocytes [21].

A major drawback of *in vitro* system is that each cell type is studied in isolation, whereas in the human body, there might be multiple organ interactions that are critical to drug toxicity. For instance, a drug can be first metabolized by the liver and the liver metabolites can cause toxicity in a distant organ. To overcome this deficiency of single cell type *in vitro* systems, the independent discrete multiple organ coculture (IdMOC) system [22], has been developed. The IdMOC allows the coculturing of cells from different organs as physically separated cultures that are interconnected by an overlying medium, akin to the blood circulation connecting the multiple organs in the human body. The IdMOC models the multiple organ interaction in human *in vivo*, allowing the evaluation of organ-specific effect of a drug and its metabolites. The IdMOC consists of multiple wells (inner wells) within a larger well (containing chamber). Multiple cell types are firstly individually cultured in the inner wells in media optimized for each cell type (Fig. 1). On the day of experimentation, the individual media are removed and the chamber will be filled with a single medium, flooding all the inner wells, thereby allowing well-to-well communication via the overlying medium. The test materials are added to the overlying medium. After experimentation, the overlying medium can be analyzed for overall metabolism of the test material and the individual cell types can be processed for the quantification of associated test material to evaluate possible organ-specific bioaccumulation, evaluation of cell viability for cytotoxicity, and evaluation of efficacy. The IdMOC can be cultured with primary cells from laboratory animals or human beings to model the respective organisms. The

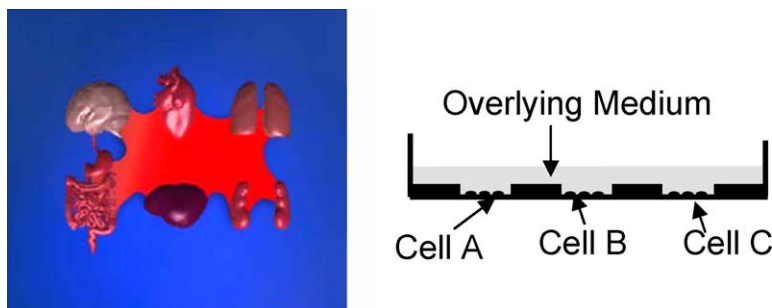


Figure 1. Integrated discrete multiple organ coculture technology. The human body is conceptualized here as multiple organs connected by blood – the principle of the IdMOC system (left panel). A cross-section (right panel) of the IdMOC plate is shown demonstrating the culturing of multiple cell types (cells A–C) as physically separated cultures in different wells but interconnected by a common medium. The IdMOC thereby mimics an animal *in vivo*, with multiple organs as physically separated entities connected by a common fluid (blood *in vivo* and the overlying medium in the IdMOC). From Ref. [21].

IdMOC represents a promising novel *in vitro* experimental system for the routine screening of ADMET drug properties.

Conclusion

ADMET drug properties are critical determinants of the clinical success of a drug candidate. Extensive efforts have been invested to develop scientifically relevant approaches to evaluate these properties. The assays developed in general require only quantities of test materials and can be performed in a relatively high-throughput format. The assays that can be readily applied as early screening assays for drug discovery and development are depicted in Table 1. Applying these screening assays systematically and logically, accompanied by objective data analysis, will aid the efficiency of drug discovery and development. The application of human-based *in vitro* assays, combined with *in vivo* assays with relevant laboratory animal species, should allow one to accurately predict *in vivo* human drug properties, thereby guiding the design and selection of drug candidates with high probability of clinical success.

Table 1. Fate of an orally administered drug and the corresponding *in vitro* screening assays

| Events | Screening assays |
|------------------------|---|
| Dissolution | <i>In vitro</i> dissolution assay |
| Intestinal absorption | Caco-2 permeability; PAMPA |
| Hepatic metabolism | Hepatocyte/liver microsome metabolism (metabolic stability; metabolite profiling) |
| Drug–drug interactions | P450 inhibition; P450 induction |
| Bile excretion | Hepatocyte biliary excretion |
| Organ toxicity | Hepatocyte cytotoxicity (hepatotoxicity); renal proximal tubule cell toxicity (nephrotoxicity); cardiomyocyte QT prolongation (cardiotoxicity); endothelial cell cytotoxicity (vascular toxicity) IdMOC (multiple organ toxicity) |

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