In vitro ADME-Tox characterisation in drug discovery and development

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drug development from bench 2 bedside

CONTENTS

The appropriate ***in vitro*** **ADME-Tox** profiling carried out as early as possible is crucial for the decision-making and selection of the most promising leads.

In vitro ADME-Tox characterisation in drug discovery and development

The research and development (R&D) roadmap is complex, time-consuming, expensive, and unpredictable. In 2021, FDA approved 50 novel drugs while EMA recommended 54 medicines with new active substances for approval, in line with the trends of previous years[1,2]. From the initial idea to the launch, the R&D journey of each one of those drugs took, on average, between 10 to 15 years with an estimated investment of $1.3 to $2.8 billion USD[3–5].

The early-stage drug discovery involves the screening of approximately 5,000 – 20,000 drug candidates, but only five of them will enter the clinical development phase leading to the approval of one drug.

The overall high drug attrition rates have been ascribed to the lack of efficacy (40-50%) mainly due to pharmacokinetic (PK) features, unmanageable toxicity or side effects (30%), and poor drug-like properties (10-15%)[3]. Thus, **the accurate strategic planning with the appropriate *in vitro* absorption, distribution, metabolism, excretion and toxicity** (ADME-Tox) profiling carried out as early as possible in the drug discovery process is crucial for the decision-making and selection of the most promising leads, mitigating the risks of late clinical-stage failures and improving the R&D cost-effectiveness.

The *in vitro ADME-Tox assays*

The *in vitro* assays provide valuable data for deciphering the mechanisms and drug-related determinants underlying the drug candidates'

journey in the body and predicting the *in vivo* pharmacokinetic (PK) and pharmacodynamic (PD) profiles.

Physicochemical properties

Molecular size, **chemical stability, lipophilicity**, aqueous **solubility**, and **ionisation** are interplaying physicochemical properties of utmost importance for predicting the ADME-Tox-related attributes[6–8]. These properties influence the evaluation of compound activity in functional bioassays, plasma protein binding, permeability, tissue distribution, drug-drug interactions, unintended drug promiscuity, and ultimately bioavailability [6–9].

Absorption

Drug passage from the administration site into systemic circulation depends on its physicochemical properties, formulation, and route of administration and modulates drug bioavailability.

Membrane permeability and drug-transporter assays

Permeability refers to the capacity of the drug to cross biological membranes and reach neighbouring extracellular or intracellular compartments being a critical determinant of drug **absorption**, **distribution**, and **excretion**. Different transport mechanisms can be involved in drug permeation across cellular barriers,

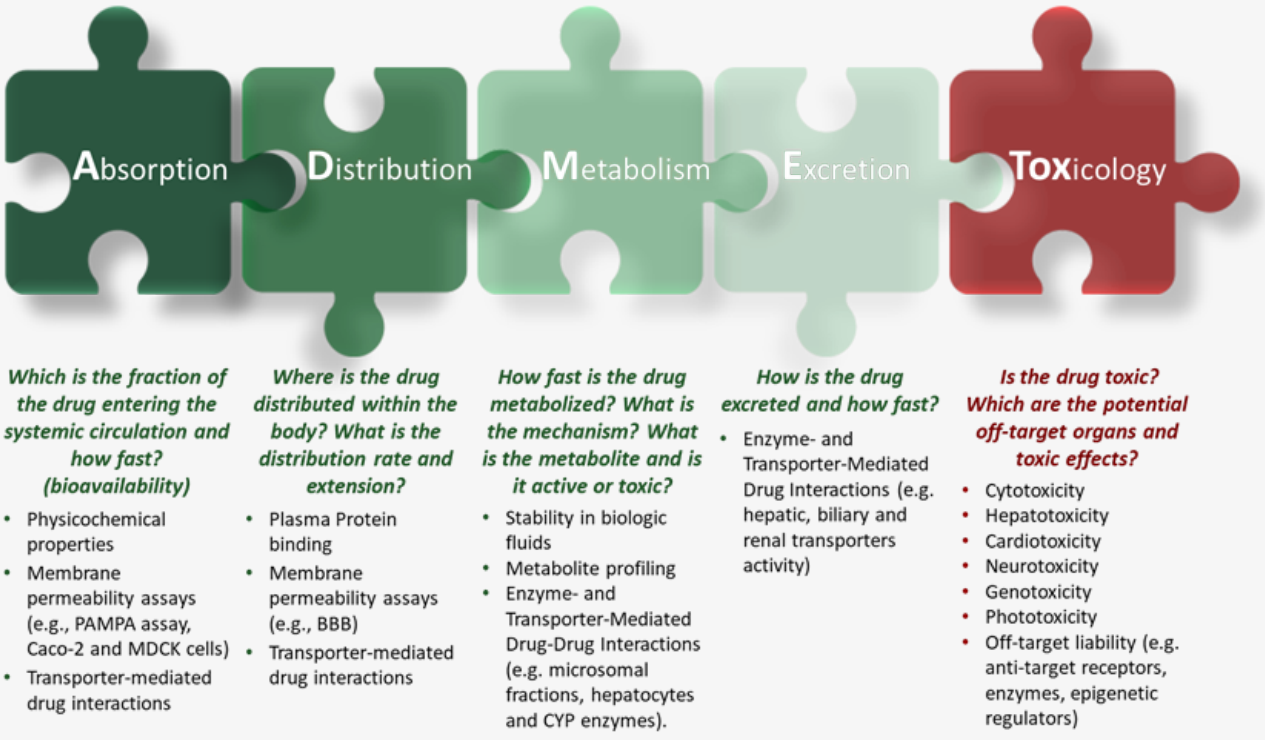

FIGURE 1: *In vitro* ADME-Tox profiling assays and key features to be addressed during drug discovery and development[10,11].

including transcellular passive diffusion, paracellular passive diffusion, carrier-mediated (facilitated or active) transport, receptor-mediated transport or adsorptive-mediated transport[6,9,12].

Two-dimensional (2D) *in vitro* models using synthetic membranes or cell-based cultures are widely adopted to mimic distinct biological barriers and evaluate the apparent permeability by measuring the concentration of test compound in the donor and acceptor compartments[12,13].

The synthetic acellular models (e.g., PAMPA and PVPA assays) reproduce the lipid composition of cellular membranes and are common tools for early-stage ADME screening. Their applicability is restricted to the permeability prediction of lipophilic drugs transported by transcellular passive diffusion[12,14].

Cell-based models using semi-porous membranes better resemble biochemical and structural features of biological barriers, but are labour-intensive techniques with higher costs than synthetic models.

The human colon epithelial cancer cell line (Caco-2) and the Madin-Darby canine kidney cell line (MDCK) of non-human and non-intestinal origin are recognized by regulatory authorities as surrogate models for *in vitro* **permeability assays**, having an acceptable correlation with the *in vivo* passive transcellular transport[10,12,13].

These cell lines have also attracted attention as models for **drug-transporter assays** mediating the influx or efflux of drugs**,** regarding the potential to be transfected with one or more genes and the intrinsic expression of functional active transporter systems (e.g., the ABC transporters P-gp and BCRP)[9,10,12,15,16].

The regulatory authorities encourage the use of *in vitro* data to predict transporter-mediated **drug-drug interactions (DDI)**[10,15,16]. Nevertheless, *in vitro* data should be carefully analysed due to the intrinsic variability of cell-based models, their dependence on culture conditions, and biorelevance for specific applications[10,12,13,17].

Distribution

Physicochemical properties and physiological factors govern the reversible drug distribution between different compartments and tissues within the body.

Plasma protein binding

The **plasma protein binding** reduces the free drug concentration affecting the **distribution**, **metabolism**, and **excretion** by decreasing the fraction of the drug available to permeate cell membranes and exhibit pharmacological effects. The free drug concentration can be determined *in vitro* in the presence of plasma or microsomes by equilibrium dialysis or ultrafiltration. The ratio of the drug amount in whole blood (human and animal) to the amount in plasma can also be determined *in vitro* using the **red blood cell (RBC) partition** assay[6,10,16,18,19].

Blood-brain barrier (BBB) permeability models

Drug delivery across the BBB to treat brain diseases (e.g., Alzheimer's disease, Parkinson's disease, brain tumours) remains one of the unmet clinical challenges.

In vitro transwell models using multiple types of human or non-human brain endothelial cells, including primary and immortalised (e.g., hCMEC/D3, Hbec-5i, bEnd.3) cell lines, and cells generated by differentiation of induced pluripotent or embryonic stem cells, have been exploited to develop *in vitro* models that better recapitulate the BBB cellular components and predict the BBB permeability[20–23].

The conventional static mono-culture models have evolved into static and dynamic co-culture models to mimic the local microenvironment and the functional interactions with other neurovascular unit components (i.e. brain endothelial cells, neurons, astrocytes, pericytes, and microglial cells)[23–25].

Microfluidic devices have been also developed to integrate the effect of the fluid flow in the BBB, albeit their limited capacity for high-throughput screening (HTS)[23].

The expression of **ABC transporters** in the *in vitro* BBB models has been described, allowing the prediction of transporter-mediated DDI and transcellular passive transport[25]. Noteworthy, all these models are not deprived of limitations as they are unable to completely fulfil the BBB complexity[25].

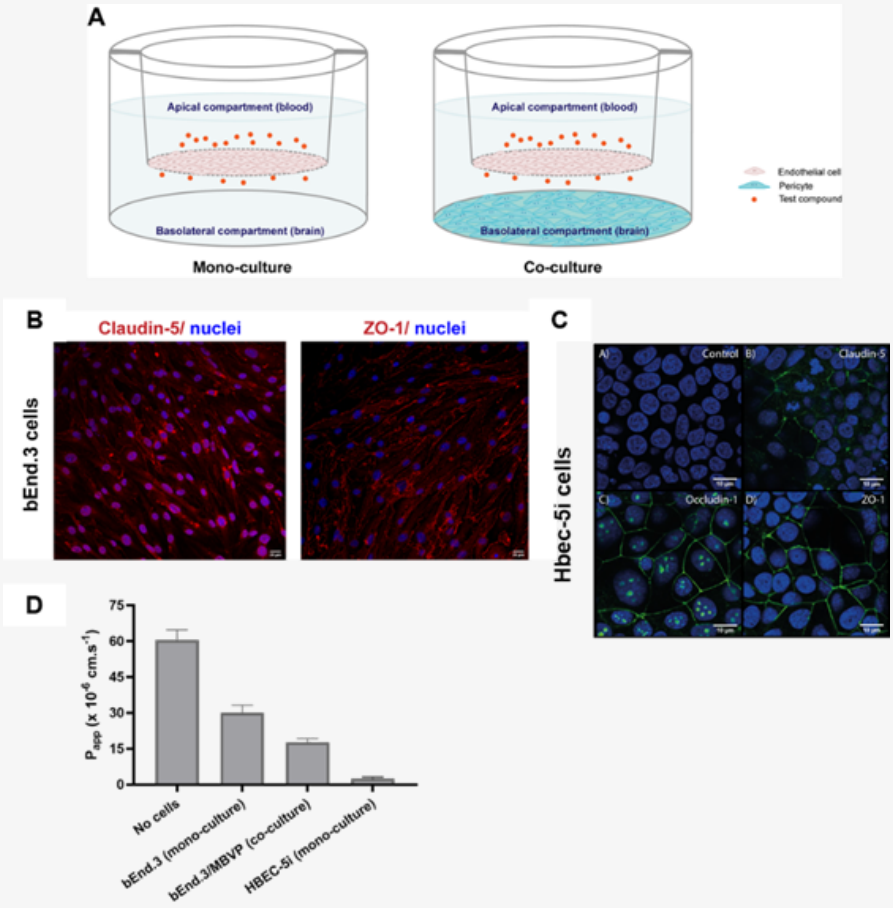

FIGURE 2: /In vitro blood-brain barrier (BBB) permeability models. Schematic representation of monoculture and co-culture models using transwell inserts (A). Confocal images of tight junctions in bEnd.3 (B) and HBEC-5i cells (C) grown in monolayers in transwell inserts. Apparent permeability of Lucifer Yellow in the in vitro BBB models (D)[21].

Metabolism

Identification of the metabolites, pathways, and enzymes involved in the metabolism is required to predict bioactivation (prodrugs), clearance, DDI potential, and toxicity of drug candidates.

Metabolism and transporter-mediated drug-drug interactions (DDI) assays

Metabolism of small-molecule drugs occurs primarily in the liver and intestine.

Drugs are primarily metabolised in the liver through various isoforms of the Phase I cytochrome P450 (CYP) enzymes, but metabolism can also occur through Phase I non-CYP oxidative enzymes and Phase II glucuronosyl- and sulfo-transferases[10,15,16,18].

The metabolic pathways for biologics are more complex with different types of membrane-associated and intracellular proteases, nucleases and peptidases being the preferred players[7,15].

Metabolism studies can use various hepatic *in vitro* systems for **metabolite profiling**, characterisation of **metabolic stability,** identification of metabolic pathways and enzymes (reaction phenotyping studies), and **DDI potential** of a drug candidate[10,15,16,18]. The hepatic *in vitro* systems recognized by regulatory authorities include subcellular liver tissue fractions (microsomes or S9 fractions), liver tissues (freshly prepared or cryopreserved primary hepatocytes or hepatic cell lines) or recombinant human enzymes (CYP and non-CYP enzymes)[10,15,16,18,26,27]. Biological matrices can also be used for metabolic stability evaluation (e.g., plasma, blood)[18].

Human primary hepatocytes are the gold standard *in vitro* model for cytotoxicity and drug metabolism evaluation[10,16,18,26]. Nevertheless, low availability and short-term viability hamper their applicability.

Human hepatic cell lines are also an accepted cell-based model with a poor phenotype and functional correlation to the *in vivo* hepatocytes[10,18,26]. Alternative models have been developed to overcome these limitations, including stem cell-derived hepatocyte-like cells, three-dimensional (3D) liver systems and microfluidic platforms[18,26,28,29].

In vitro **transporter-mediated** assays determining the effect of the drug candidate on substrate uptake or efflux and **DDI** potential should be coupled with *in vitro*-to-*in vivo* extrapolation methods to decide if an *in vivo* drug interaction study is required[10,16,18]. Membrane vesicle systems, cell-based assays for efflux (e.g., Caco-2 cells for ABC transporters), or uptake transporters (e.g., hepatocytes) are accepted as *in vitro* models[10]. The solute carrier (SLC) transporters OATP1B1, OATP1B3, OCT1, and NTCP are some of the uptake transporters that should be studied for drugs with hepatic metabolism and biliary secretion[10,18,30].

Excretion

Excretion is closely related to metabolism regarding the pivotal role of both processes in drug elimination.

Biliary secretion is evaluated *in vitro* using hepatocytes, while primary or immortalized renal epithelial cells (e.g., HEK293, MDCK) cultured in monolayer conditions are the gold standard for characterizing the renal elimination[10,16,31,32].

The regulatory authorities recommend the *in vitro* evaluation of drug transporters inhibition (e.g., P-gp, BCRP, OATP1B1, OATP1B3, OCT2, MATEs, OAT1, and OAT3) when the overall ADME data suggest that active renal secretion is significant to the drug candidate elimination[10,16,32]. The capacity of the *in vitro* models to recapitulate the complexity of the functional kidney and population-related factors is limited, however they provide information to decide whether to conduct an *in vivo* study based on the drug's safety margin, therapeutic index and relevant concomitant medications[10,16,32].

Toxicology

The *in vitro* **toxicology** studies performed early during hit-to-lead and lead optimization support the selection of the most promising compounds and identification of key safety issues at critical points of the development program.

These studies provide information about the toxicity mechanisms of the molecules at the cellular level, guiding the identification of structure-activity relationships and selection of non-toxic concentrations for further *in vitro* and *in vivo* studies.

Cytotoxicity assays

Cytotoxicity of drug compounds is usually evaluated in a range of relevant cell types to predict potential off-target effects. Alterations in cellular morphology, proliferation/growth, metabolism, and impedance, biomarkers of cellular stress or cell death mechanisms, and loss of membrane integrity are within the parameters evaluated to determine the compounds' ability to harmfully interfere with cell proliferation/viability[33,34]. A combination of multiple endpoints should be used to improve the predictive value of the *in vitro* cytotoxicity assays[33,34].

Cardiotoxicity and **hepatotoxicity** as the result of off-target liability are the main causes of life-threatening conditions responsible for drug attrition. Drug-induced modulation of cardiac ion channel proteins (e.g., hERG and non-hERG) and transporters causing QT interval prolongation and delaying ventricular repolarization leads to an increased risk of proarrhythmic and **cardiotoxic** effects.

In vitro electrophysiology studies using non-cardiac cell lines expressing human ion channel proteins, cardiac cell lines, cardiomyocytes isolated from human and non-human relevant species or multicellular preparations are recognized by regulatory authorities as complementary approaches to the *in vivo* cardiotoxicity studies[35,36].

Drug-induced liver injury (DILI) or **hepatotoxicity** has been one of the primary causes of adverse events during clinical trials and drug withdrawal from the market (e.g., troglitazone, nimesulide). DILI risk assessment is a challenge due to the multifactorial nature, poorly understood pathogenesis, and lack of specific biomarkers, remaining a major concern for clinicians, pharmaceutical industry, and regulatory authorities[26,37].

The *in vitro* studies should address a broad range of cell cytotoxicity and stress responses, alterations in gene, protein expression or in other biomarkers of cellular injury, repair and hepatic detoxification pathways to identify toxicity alerts and generate evidence supporting the preclinical *in vivo* studies and the hepatotoxicity risk prediction[26,37].

The physiological and functional complexity of liver has encouraged the development of more efficient and fit-for-purpose *in vitro* models, namely the 3D liver systems and microfluidic organ-on-a-chip platforms[26,38,39].

Genotoxicity assays

During drug development, the evaluation of the compounds ability to induce genetic damage is a regulatory requirement to predict the risk of heritable and carcinogenic effects[40]. Mutations, chromosomal aberrations, and numerical chromosomal changes are manifestations of genotoxicity and their *in vitro* detection suggest that compounds are potential human carcinogens and/or mutagens. A test battery approach, including the *in vitro* and *in vivo* evaluation of mutagenicity and genotoxicity is recommended by regulatory authorities[40]. The mutagenic potential should be assessed *in vitro* using the bacterial reverse gene mutation (Ames) test while the genotoxicity should be evaluated in mammalian cell systems. The metaphase chromosome aberration assay, the micronucleus assay, and the mouse lymphoma L5178Y cell Tk (thymidine kinase) gene mutation assay

(MLA) are recognised as appropriate *in vitro* genotoxicity assays and should be performed in the presence and absence of metabolic activation[40].

When to conduct the in vitro ADME-Tox studies?

At the **hit-to-lead phase**, the hits prioritization is often based in a combined approach of *in silico* and *in vitro* **HTS studies evaluating the molecules ability to interact with the target and assessing the physicochemical properties to predict their drug-likeness and PK features in time- and cost-effective manners**[8,41]. During the **lead optimization**, the main goals of the structural modifications are to preserve the favourable properties while mitigating the undesirable attributes of the selected leads. Likewise, a more comprehensive and physiologically relevant *in vitro* **ADME-Tox analysis is required to guide the preclinical** *in vivo* safety and toxicity studies towards the selection of candidates with a better ADME-Tox profile[8]. A tiered approach is applied to perform the *in vitro* ADME-Tox studies as early as feasible in the drug development program. **The selected assay panel and their timing should be established case-by-case since they will depend on the type of drugs (small-molecules or biologics) and the de-risking strategy for advancing to the next stage of development.** For example, *in vitro* metabolism and specific DDI studies may be required during lead optimization, *in vivo* preclinical studies after candidate selection or deferred until the early clinical development phase.

Opportunities and challenges for *in vitro* ADME-Tox assays

The *in vitro* ADME-Tox assays are indispensable tools for reducing and refining the *in vivo* testing and progressing compounds in the R&D value chain with an improved cost-effectiveness. Significant efforts have been made in the past decades to develop innovative *in vitro* 3D organotypic/organoid cultures and microfluidic (organ-on-a-chip) platforms providing more realistic organ-like models to assess compounds efficacy and safety while adding the inter-individual population variability[26,39].

Advanced body-on-a-chip systems developed as multi-organ systems have the potential to predict the efficacy and toxicity in different organs while simultaneously incorporate various PK processes[38,42,43]. The application of machine learning and artificial intelligence to data analysis and ADME-Tox modelling is also an opportunity to improve the in vitro-to-in vivo extrapolation and the decision-making process during drug development[44]. The development of models for accurately predicting the efficacy and safety of biologics and the standardization and reproducibility of the protocols for the advanced cell-based models are still the major challenges for the *in vitro* ADME-Tox assays.

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