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# REVIEW ARTICLE

# USE OF CELL CULTURE IN DRUG DISCOVERY

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**ABSTRACT**

The first step in drug discovery is, in many cases is to test compounds in cell culture to find out the activity in terms of pharmacological actions. *In vitro* cell culturebasedstudies in the non-clinical laboratory serve various functions along the path from the discovery of new molecular entities to approval and marketing of a therapeutic. Various techniques have been developed to study many aspects of drug disposition including absorption, metabolic stability, elucidation of elimination pathways, potential for inhibition and induction of CYP450 enzymes, metabolite profiling in various model species and humans. Typically, this means that the drug product must undergo a series of robust tests and experiments using *in vitro*, *in vivo*, *ex vivo*, and in silico models as per the needs of the focused indication and regulatory guidelines. A thorough understanding of the metabolic profile in various species and man is crucial in successful evaluation of potential of new therapeutics. This is also particularly important as it will assist in minimizing dosing levels in toxicology studies which are chosen on the basis multiples of the pharmacologically effective doses. For pharmacokinetic *in vitro* absorption, distribution, metabolism and excretion (ADME) studies, various models of cell lines grown in 2D are generally used. Human colon carcinoma cells (Caco-2) are used for absorption analyses, and canine kidney cells (MDCKII-MDR1) are generally employed in distribution studies, while hepatocytes are utilized in metabolism and excretion investigation. The pharmaceutical industry presently relies on several widely used *in vitro* models, including two-dimensional and three-dimensional cell culture models.

## INTRODUCTION

Cell culture is an indispensable *in vitro* tool used to improve our perception and understanding of cell biology, development of tissue engineering, tissue morphology, mechanisms of diseases and drug action. Efficient cell culturing techniques allow researchers to design and develop new drugs in preclinical studies. In clinical context, cell culture is most commonly linked to creating model systems that study basic cell biology, replicate disease mechanisms and to investigate the toxicity of novel drug compounds. The aim of the modern pharmacology is to first identify active compounds from natural elements that can constitute a starting point to develop therapeutic drugs.

### DRUG DISCOVERY

Drug discovery is a process which aims at identifying a compound therapeutically useful in curing and treating disease. The history of drug discovery and development is as old as some of the oldest human civilizations. The practice of Ayurveda in India and traditional Chinese medicine are over 5000-year-old therapeutic traditions that are still in practice at large. Papyrus Ebers is evidence of medicinal practice in Egypt about 3000 years ago. The Greek and Roman medicines became popular in Europe and western Asia between 700 BC and 200 BC (Dias *et al*., 2012). The ancient Arab medicines were in practice to a great extent until 1500 AD and are still in use in the Mediterranean gulf. The beginning of modern era in medicine can be considered from the time when Edward Jenner discovered immunization for smallpox. The development in the field was gradual until Sir Alexander Fleming discovered Penicillin in 1928; since then, the field of medicinal chemistry and drug discovery has flourished and by the end of the twentieth century, it became a complex interdisciplinary platform primarily based on synthetic organic chemistry expanding into various biological specificities. At the beginning of the twenty-first century, drug discovery research faced new challenges transforming the classical concept of drug development that was in practice for half a century.

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With advances in science and technology, the pharmaceutical, health care and IT industry, accompanied by high-pace shifts in the global economy, bolstered the process of modern-day drug discovery and development to a large significance. Novel interdisciplinary research involving metal and polymer nanoparticles, liposomes, antibodies, and neo-antibiotics in both academia and industries have opened venues for precision diagnosis, targeted drug delivery, and innovative immunotherapy (Karmakar *et al*., 2020).The classical steps in drug discovery involving target validation, lead molecule design, chemical synthesis, pre-clinical evaluation, ADME(absorption, distribution, metabolism, excretion), clinical trials and development for market of the pharmaceutical agentsare followed till date, the distribution of funding at each stage have changed due to the changing global market and healthcare policies (Gilliland *et al.,* 2016).The development of a new medicine, from target identification through approval for marketing, takes over 12 years and often much longer. The cost to develop a New Molecular Entity (NME; a small molecule compound) or New Biological Entity (NBE; an antibody, protein, gene therapy or other biological medicine) is certainly over $1 billion and on averagehas been estimated to be about $2.6 billion. The drug development process is set up, particularly at the stage of clinical development, to "fail fast, fail early" in a strategy to eliminate key risks before making a expensive late-stage investment (Mohs and Greig, 2017).

**Phases of drug discovery:** The Drug Discovery Process involves many different stages and series of actions. Typically, it can be divided into four main stages: Early Drug Discovery, Pre-Clinical Phase, Clinical Phases, and Regulatory Approval.

**Early Drug Discovery:** A drug discovery programme is initiated because there is a disease or clinical condition without suitable medical products available and it is this unmet clinical need which is the underlying driving motivation for the project. The initial research, often occurring in academia, generates data to develop a hypothesis that the inhibition or activation of a protein or pathway will result in a therapeutic effect in a disease state. The outcome of this activity is the selection of a target which may require further validation prior to progression into the lead discovery phase in order to justify a drug discovery effort. During lead discovery, an intensive search ensues to find a drug-like small molecule or biological therapeutic, typically termed a development candidate, that will progress into preclinical, and if successful, into clinical development and ultimately be a marketed medicine (Hughes *et al.*, 2011).

**Target identification and validation:** One of the most important steps in developing a new drug is target identification and validation. A target is a broad term which can be applied to a range of biological entities which may include for example proteins, genes and nucleic acids etc. A good target needs to be efficacious, safe, meet clinical and commercial needs and above all be 'druggable'. A 'druggable' target is accessible to the putative drug molecule, be that a small molecule or larger biologicals and upon binding, elicit a biological response which may be measured both *in vitro* and *in vivo*. Certain target classes are more amenable to small molecule drug discovery, for example, G-protein-coupled receptors (GPCRs), whereas antibodies are good at blocking protein/protein interactions.

Good target identification and validation enables increased confidence in the relationship between target and disease and allows us to explore whether target modulation will lead to mechanism-based side effects. Data mining of available biomedical data has led to a significant increase in target identification. In this context, data mining refers to the use of a bioinformatics approach to not only help in identifying but also selecting and prioritizing potential disease targets (Yang **et al**., 2012). The data which are available come from a variety of sources but include publications and patent information, gene expression data, proteomics data, transgenic phenotyping and compound profiling data. Identification approaches also include examining mRNA/protein levels to determine whether they are expressed in disease and if they are correlated with disease exacerbation or progression (Bertram and Tanzi, 2008).

**The hit discovery process:** Following the process of target validation, it is during the hit identification and lead discovery phase of the drug discovery process that compound screening assays are developed. A 'hit' molecule defined as a compound which has the desired activity whose activity is confirmed upon retesting. A variety of screening paradigms exist to identify hit molecules. High throughput screening (HTS) involves the screening of the entire compound library directly against the drug target or in a more complex assay system, such as a cell-based assay, whose activity is dependent upon the target but which would then also require secondary assays to confirm the site of action of compounds (Fox *et al*., 2006). This screening paradigm involves the use of complex laboratory automation without prior knowledge of the nature of the chemo type likely to have activity at the target protein. Knowledge-based screening involves selecting from the chemical library smaller subsets of molecules that are likely to have activity at the target protein based on knowledge of the target protein and literature or patent precedents for the chemical classes likely to have activity at the drug target. This type of knowledge has given rise, more recently, to early discovery paradigms using pharmacophores and molecular modelling to conduct virtual screens of compound databases (McInnes, 2007).

A typical programme critical path within the lead discovery phase consists of a number of activities and begins with the development of biological assays to be used for the identification of molecules with activity at the drug target. Once developed, such assays are used to screen compound libraries to identify molecules of interest. The output of a compound screen is typically termed a hit molecule, which has been demonstrated to have specific activity at the target protein. A plethora of assay formats have been enabled to support compound screening. The choice of assay format is dependent upon the biology of the drug target protein, the equipment infrastructure in the host laboratory, the experience of the scientists in that laboratory, whether an inhibitor or activator molecule is sought and the scale of the compound screen. For example compound screening assays at GPCRs have been configured to measure the binding affinity of a radio- or fluorescently labelled ligand to the receptor, to measure guanine nucleotide exchange at the level of the G-protein, to measure compound-mediated changes in one of a number of second messenger metabolites including calcium, cAMP or inositiol phosphates or to measure the activation of downstream reporter genes (Steinmetz and Spack, 2009).

**Hit-to-lead phase**: The aim of this stage of the work is to refine each hit series to try to produce more potent and selective compounds which possess pharmacokinetic properties adequate to examine their efficacy in any in vivo models that are available. It comprises of structural activity relationship investigations around each core compound structure, with measurements being made to establish the magnitude of activity and selectivity of each compound (Peet, 2003). This needs to be carried out systematically and where structural information about the target is known, structure-based drug design techniques using molecular modelling and methodologies such as X-ray crystallography and Nuclear magnetic resonance can be applied to develop the SAR faster and in a more focused way. This type of activity will also often give rise to the discovery of new binding sites on the target proteins. As animal models are used to validate the activity of compounds in in vivo disease models and in preclinical toxicity studies, it is important to have data on activity in vitro on orthologues. Microsomal stability is a useful measure of the ability of in vivo metabolizing enzymes to modify and then remove a compound. Hepatocytes are sometimes used in this sort of study instead and these will give more extensive results. CYP450 inhibition is examined as, among other things, it is an important predictor of whether a new compound might have an influence on the metabolism of an existing drug with which it may be co-administered (Hughes *et al*., 2011).

**Lead optimization phase:** The objective of lead optimization phase is to maintain favorable properties in lead compounds while improving on deficiencies in the lead structure. For example the aim of the this phase is now is to modify the structure to improve the absorption of the compound. Thus, more regular checks of hERG affinity and Human colon carcinoma cells (Caco-2) permeation were undertaken and compounds were soon available which maintained their potency and selectivity at the principal target but which had a muchreducedh ERG affinity and a better apparent permeation than initial lead compounds. Compounds at this stage may be deemed to have met the initial goals of the lead optimization phase and are ready for final characterization before being declared as preclinical candidates. The team has to continue to explore synthetically in order to produce potential back up molecules, in case the compound undergoing further preclinical or clinical characterization fails and more strategically, to look for follow-up series(Peet, 2003). The stage at which the various elements that constitute further characterization are carried out will vary from company to company and parts of this process may be incorporated into the lead optimization phase. However, in general, molecules need to be examined in models of genotoxicity such as the Ames test and in in vivo models of general behaviour such as the Irwin's test. High-dose pharmacology, PK/PD studies, dose linearity and repeat dosing PK looking for drug-induced metabolism and metabolic profiling all need to be carried out by the end of this stage considering chemical stability issues and salt selection for the putative drug substance. Typically, within industry for each project 200 000 to $>10^6$ compounds might be screened initially and during the following hit-to-lead and lead optimization programmes 100's of compounds are screened to hone down to one or two candidate molecules, usually from different chemical series (Jorgensen, 2009).

**Pre-Clinical Phase:** Preclinical studies are performed in *in vitro*, in vivo, ex vivo and in silico models to obtain information about the safety and biological efficacy of a drug candidate before testing it in a final target population, i.e., humans. Preclinical studies or tests are mainly performed in compliance with GLP/GSP guidelines (good laboratory practice and good scientific practices) to ensure reliability and reproducibility of results. The FDA **(**The Food and Drug Administration) require supporting basic preclinical data to investigational new drug application especially on toxic effects, safety profile, pharmacokinetics and pharmacodynamics. The data from preclinical trials must be accurate, reliable and based on the best suitable and comparable model available to the target population. Typically, this means that the IND or drug product must undergo a series of robust tests and experiments using *in vitro*, *in vivo*, ex vivo, and in silico models as per the needs of the focused indication and regulatory guidelines. Preclinical studies are conducted according to good laboratory practice (GLP) guidelines, which regulate how laboratory studies are performed. Clinical trials are conducted according to good clinical practice (GCP) guidelines, which are internationally required quality and safety standards for designing, conducting and reporting clinical trials (Honek, 2017).

**Clinical Phase**: The goal of clinical transition studies is to demonstrate the safety of the drug before it is first dosed in humans. From this step forward, every experiment and study that is carried out will be reviewed by the Food and Drug Administration [FDA]. Phase 1 Clinical investigations of a new drug candidate start with a group of studies commonly called Phase 1 testing. A series of ethical considerations are involved in the design of all clinical studies since human subjects could potentially be put at risk. It involves healthy volunteers, not patients, with the primary aim to assess the safety of the new drug and these volunteers are financially compensated for their participation. Clinical trials are conducted according to good clinical practice (GCP) guidelines, which are internationally required quality and safety standards for designing, conducting and reporting clinical trials (Tamimi and Ellis, 2009). The Phase1 study will also produce data on the pharmacokinetics of the drug (absorption, distribution, metabolism, and elimination) and its pharmacodynamic properties (biochemical and physiological effects on the body). The aim of Phase 2 clinical trials is to test the safety and in a preliminary fashion, the effectiveness, of a therapeutic candidate compound in patients with the targeted disease. Patients are carefully randomized into the control and drug groups to ensure that average disease severity is the same in the two groups. Phase 2 trials have dual and sequential goals, they are commonly divided into two subtrials: Phase 2a and Phase 2b. Phase 2a concentrates on safety and dosing while Phase 2b is an extension of 2a with an increased focus on efficacy. The goal of the Phase 3 trial is to show whether or not a new compound is effective in treating the target disease and are pivotal in the industry because they will make or break the success of the drug and combines scientific and financial considerations. While from a scientific perspective it is critical to ensure that the Phase 3 study is adequately powered, financial considerations will influence the design of the trial toward the minimum size that effectively meets the desired goal. Phase 4 clinical trial also known as post-marketing surveillance trials involving safety surveillance (pharmacovigilance) and ongoing technical support after approval. There are multiple observational designs and evaluation schemes that can be used to assess the effectiveness, cost effectiveness and safety of intervention in real-world settings.

The safety surveillance is designed to detect any rare or long-term adverse effects over a much larger patient population and longer time period (Mahan, 2014).

**Regulatory Submission/Approval:** After the clinical studies have completed and delivered a positive outcome, then compilation of the data submission to the regulatory agencies. This usually takes several months and can be done one by one region at a time, e.g. in the United States or could be done globally, targeting major regions simultaneously. Classically, the major markets include the United States, the European Union and Japan. However, recently more attention is given to the 'emerging markets' such as Latin America, India and China, amongst others. As for the United States, a routine New Drug Application 'NDA' can take up to 15 months for review. However, in cases of particularly high medical need or in areas lacking treatments (e.g. oncology and human immunodeficiency virus), an expedited review can be granted. If the new drug is a biologic, then a biologic license application 'BLA' rather than a 'NDA', is submitted (Tamimi and Ellis, 2009).

**Cell culture in drug discovery:** *In vitro* studies in the non-clinical laboratory serve various functions along the path from the discovery of new molecular entities to approval and marketing of a therapeutic. *In vitro* techniques have been developed to study many aspects of drug disposition including absorption, metabolic stability, elucidation of elimination pathways, potential for inhibition and induction of CYP enzyme and metabolite profiling in various model species and humans and are essential for submission of IND and post IND filing from both the preclinical and clinical arena. Although, many types of *in vitro* assays are conducted during drug development, use of cell cultures is the most reliable one. The first step in drug discovery is, in many cases, to test compounds in cell culture to find out activity in terms of pharmacological actions. Selecting the cell line and defining the optimum conditions for both the cell culture media and the drug solvent, in addition to the other reagents is an important issue since the compound activity may be specific(Ghanemi, 2015). Another important element is the choice of the positive control, which is in many cases a commonly used drug that is well studied and well known for the activity we are about to test. Importantly, the use of negative controls assures a better interpretation of the results since it allows us to distinguish the effects of the tested drugs from those due to other elements such as the reagents or the cell culture medium ingredients. For instance, many pharmacological discoveries related to one of the most important pharmacological targets, G protein coupled receptors have been made. The use of *in vitro* models, mainly cell cultures, is an important and long-lasting component of preclinical drug tests. Production of a new medical product takes approximately 12 years, half of which are devoted to *in vitro* studies. For pharmacokinetic *in vitro* absorption, distribution, metabolism and excretion (ADME) studies, various models of cell lines grown in 2D are generally used. Human colon carcinoma cells (Caco-2) are used for absorption analyses, and canine kidney cells (MDCKII-MDR1) are generally employed in distribution studies, while hepatocytes are utilized in metabolism and excretion investigation (Jaroch *et al.,* 2018).

**Cell cultures:** Cell culture refers to laboratory methods that enable the growth of eukaryotic or prokaryotic cells in physiological conditions.

Different variants of cell culture found application in modelling diseases, IVF technology, stem cell and cancer research, monoclonal antibody production, regenerative medicine and therapeutic protein production. Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favourable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been already established. The advantage of using cell lines in scientific research is their homogeneity and associated reproducibility in data generated (Segeritz and Vallier, 2017). These different scientific approaches would not be possible without some crucial discoveries that had been made over the centuries. From the ancient Romans, through the Middle Ages, to the late of the nineteenth century, the Aristotelian doctrine of spontaneous generation was one of the most basic laws. In the eighteenth century, the spontaneous generation doctrine was laid by Louis Pasteur. In the first decade of the eighteenth century, nucleus was observed in plant and animal tissues and Virchow and other scientists presented the view that cells are formed via scission of pre-existing cells. In the first decade of the twentieth century, Ross Harrison developed the first techniques of cell culture *in vitro*, and Burrows and Carrel improved Harrison's cell cultures. In mid-twentieth century, the basic principles for plant and animal cell cultures *in vitro* were developed, and human diploid cell lines were established. On the basis of knowledge about the cell cycle and gene expression regulation, the first therapeutic proteins were produced using mammalian cell cultures.

The end of twentieth century and early twenty-first century brought the progress in 3-D cell culture technology and created the possibility of the tissue engineering and the regenerative medicine development (Rodriguez *et al.,*2014*).* Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). At this stage, the cells have to be subcultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.After the first subculture, the primary culture known as a cell line or subclone. Cell lines derived from primary cultures have a limited life span, and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line. Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as senescence; these cell lines are known as finite. However, some cell lines become immortal through a process called transformation, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line (Chaudhary and Singh, 2017).

**Morphology of Cells in Culture:** Cells in culture can be divided in to three basic categories based on their shape and appearance. Fibroblastic (or fibroblast-like) cells are bipolar or multipolar, have elongated shapes, and grow attached to a

substrate. Epithelial-like cells are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches. Lymphoblast-like cells are spherical in shape and usually grown in suspension without attaching to a surface.There are two basic systems for growing cells in culture, as monolayers on an artificial substrate (i.e., adherent culture) or free-floating in the culture medium (suspension culture). The majority of the cells derived from vertebrates, with the exception of hematopoietic cell lines and a few others, are anchorage-dependent and have to be cultured on a suitable substrate that is specifically treated to allow cell adhesion and spreading (i.e., tissue-culture treated).Cells that are cultured in suspension can be maintained in culture flasks that are not tissue-culture treated, but as the culture volume to surface area is increased beyond which adequate gas exchange is hindered (usually 0.2–0.5 mL/cm$^2$), the medium requires agitation. This agitation is usually achieved with a magnetic stirrer or rotating spinner flask (Danielsson *et al*., 2010; Park *et al*., 2018).

**The Physical and Chemical Environment:** The aim of cell culture is to provide an environment that mimics, to the greatest extent possible, the *in vivo* environment of that specific cell type. The cell culture incubator, the culture dish or apparatus, and the medium together create this environment *in vitro*. They provide an appropriate temperature, pH, oxygen, and CO2 supply, surface for cell attachment, nutrient and vitamin supply, protection from toxic agentsand the hormones and growth factors that control the cell's state of growth and differentiation. The surface for cell adhesion, growth, proliferation and also determines the cellular secretion activity of cells. Earlier the glass surface was widely used, now in most of laboratories use plastic (usually polystyrene) labware is used for typical monolayer cultures. The surface of that cell culture vessels can be enhanced by coating with proteins, such as collagen, gelatin, laminin, fibronectin that are components of extracellular matrix. For that purpose also Polymers such as poly-L-lysine or other commercial matrices can be used for that purpose (Rodriguez *et al.,*2014*).*Media used for the cells usually composed of basal nutrient medium and supplements. The balanced salt solution, for example, DPBS, HBSS, EBSS form basis of complex media. The supplements complete media with nutrients, proteins, amino acids, buffering system and vitamins. The most popular media are Dulbecco's Modified Eagle's Medium (DMEM), Eagle's Minimal Essential Medium (EMEM), Medium 199 (M199), Roswell Park Memorial Institute (RPMI–1640). The amino acids essential for growth and cell proliferation, for example, cysteine, L-glutamine and tyrosine. For proper metabolism, cells require B vitamins (especially presence of B12 is essential), choline, folic acid, inositol, biotin. Ions and trace elements: The major ions such as $Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $Cl^-$, $PO_4^{3-}$, $SO_4^{2-}$, $HCO_3^-$ affect osmolarity of culture media. Trace elements such as zinc, copper, selenium and tricarboxylic acids intermediates are used in cultures media. Carbohydrates and organic supplements are usually provided. Glucose is mainly used as an energy source, but in some cell types galactose, mannose, fructose or maltose can be used. The culture media can be also supplemented with pyruvate, lipids (cholesterol, steroids, fatty acids), citric acids intermediates (Priyabrat *et al.,* 2014). Serum is a complex mixture of proteins, source of minerals, lipids, hormones, and growth and adhesion factors. Fetal bovine serum (FBS) and newborn calf serum (NCS) are most common. For more specific cultures human, horse or rabbit sera are used.Antibiotics and antifungal with laminar flow hoods reduced the frequency of contamination. In cell cultures most often penicillin streptomycin solutions are used. As the antimycotic agents the kanamycin or amphotericin B are applied. Hormones and growth factors are used especially in serum-free media. Those factors ensure cellular growth, division, and differentiation. The most popular are fibroblastic growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) or platelet derived growth factor (PDGF). In the group of hormones the most common are hydrocortisol and insulin ((Pilgrim*et al*., 2022).

**Physico-chemical properties of cultures *in vitro*:** For animal and human cells a pH was determined in the range of 7.0 -7.4. Some cells require higher pH levels, for example, normal fibroblasts (7.4–7.7). The pH level can be checked by presence of phenol red in culture medium. The buffering system is essential to maintain proper pH. The bicarbonate buffers not only show low toxicity, but also help in glucose metabolism. The other buffering system include use of HEPES buffer.Most of cell lines are maintained at 37°C but temperature is determined by origin of tissue, for example, lower temperature is usually used for skin and testicles cell cultures(Rodriguez *et al.,*2014*).*

**CELL-BASED STUDIES OF ABSORPTION AND DISTRIBUTION IN *IN VITRO* CONDITIONS:** The goal of producing an orally active therapeutic has made the investigation of gut penetration an importantart of drug development. For this purpose, the human colon carcinoma cell line Caco-2 has been well characterized and establish structurally differentiated and polarized monolayers with characteristics typical of enterocytes of the intestinal epithelium. Permeability studies with Caco-2 monolayers cultured on polycarbonate membranes correlate well with oral human absorption. Recently expression of cytochrome P450 3A4 has been enhanced in Caco-2 cell by addition of 1α,25-Dihydroxyvitamin $D_3$ to culture media and increase in the levels of this enzymeimproves correlation with metabolism seen in intestinal mucosa. The permeability assay is conducted in transwell microplates - i.e., 24-well or 96-well plates and therefore it fits the HTS (Sevin *et al*., 2013).Alterations of absorption assays speed up throughput to meet the need for rapid feedback to medicinal chemists earlier in the discovery process such as the application of an automated system for liquid handling. Screening of mixtures increase overall throughput whereas pooling of samples from individual absorption assays has proven to speed up the analytical portion of a screen. Other approaches have attempted to address the labor and time intensive 21day Caco-2 model by either substituting proprietary culture environment to form a differentiated monolayer in only 3 days or by replacing the Caco-2 cell line with faster growing MDCK cells. P-glycoprotein (P-gp) mediated efflux is another important potential barrier to drug absorption. Mechanistic studies of such transporters can be investigated in the Caco-2 model. Other procedures incorporate inhibition of efflux of known fluorescent P-gp substrates in cytometric assay formats. A competitive binding assay using [$^3$H]-verapamil is also potentially more amenable to increased throughput and purports to directly quantify affinity for the ligand (Peng *et al*., 2014). A rapid screening program allow structure transport relationships to be cataloged aid in improving the absorption characteristics of a potential therapeutic which has shown efficacy and such screening is linked not only to the validity of the data but also for feedback to the chemists involved in synthesis.

In contrast, when seeking to describe P-gp modulators, a more formal study may be prudent. The impact of this point is illustrated by the case where a P-gp modulator results in increased morphine exposure to the brain. In such cases, a detailed and specific study of the interaction of a drug with P-gp may have implications relevant to regulatory submission. If data from such studies will support an application, the absorption assays should be performed in a manner that ensures data integrity. Therefore conduct of such studies necessitates increased attention to the particulars of GLP (Bajpai and Esmay, 2002).Another, quite commonly used cell line for absorption assessment, is Madin-Darby canine kidney cell line named MDCK. *In vitro* model, MDCK-MDR1 cell line seems to be better than Caco-2 for drugs, which transport is known to be mediated by P-glycoprotein for screening for compounds that could be potentially transported by this protein. Drugs that are not transported by P-glycoprotein do not differentiate significantly between these two tested models. Both Caco-2 and MDCK cell lines are appropriate for blood-brain barrier (BBB) transportation studies. By testing 22 compounds with different BBB transport degree, results obtained with MDCK-MDR1 cell line were in good concordance to *in vivo*. Absorption and distribution cell-based assays have multiple applications in pharmaceutical industry as they allow screening during lead optimization because HTS provides testing many synthesized drug candidates or prodrugs simultaneously. Due to this, structure-property relationship can be assessed. Permeability assays are conducted for the establishment of blood-brain barrier penetration, thus distribution studies are applicable. Prediction of gut wall metabolism is possible by inducing these cell lines with new drugs candidates (Garberg *et al*.,2005).

## CELL - BASED STUDIES OF LIVER METABOLISM END EXCRETION IN *IN VITRO* CONDITIONS:

Metabolic stability assays have been developed which employ many different liver tissue systems including microsomes, slices and hepatocyte culture to address the barriers such as oral bioavailability. These assays are early screening tools and for rank ordering based on estimation of *in vitro* half-life ($t_{½}$) or the apparent clearance ($Cl_{app}$). Design of the experiment should be considered aiming to make reliable clinical predictions (Bajpai and Esmay, 2002). The establishment of structure-activity relationships is essential in discovery research. Iterative feedback from stability screens help target chemical modifications aimed at improving the ADME profile. Throughout this process, laboratory reports are more likely to be communicated to the medicinal chemist than to regulatory agencies. The FDA also recognizes the value and challenges of *in vitro* systems in elucidating the role of liver metabolism on clearance as it relates to clinical outcome. For example, the guidance for use of the microsomal test system states that "Microsomes from several donors should be used to avoid reliance on microsomes deficient in one pathway". A simple metabolic stability assay establishes initial rate conditions which would then support design of later studies in an exploratory fashion, the relevance to validity of other studies with attention to details such as adequate test article characterization, instrument calibration and documentation so that the data can support further studies. Alternatively, these experiments may need to be repeated preliminary to a study that is planned for submission in order to satisfy a particular GLP parameter. A thorough understanding of the metabolic profile in various species and man is crucial in successful evaluation of potential new therapeutics. Simultaneously, the liver is responsible for the first pass effect, which does not always have a positive effect on the bioavailability of drugs. For IVIVE (*In vitro* to *In vivo* extrapolation) of hepatic clearance, human or animal microsomal enzymes, isolated hepatocytes, hepatocyte cell lines and cuttings of the liver are commonly employed (Schaeffner *et al*., 2005). Cryopreserved primary hepatocytes (retain activity of phase I and phase II metabolism enzymes) and freshly isolated hepatocytes are the firstchoice model for studies of *in vitro* metabolism. Immortalized hepatocytes cell lines such as HepG2 (Human hepatic cell line), HepaRG (Human hepatocellular carcinoma cell line),Huh7(Human hepatic cell line), Sk-Hep-1(Human hepatoadeno carcinoma cell line),Fa2N-4 (human hepatic cell line) are also used for the studies and they exhibit incomplete expression of metabolic enzymes compared to freshly isolated hepatocytes. On the other hand, liver cell lines are easily available, relatively cheap and are easy to cultivate. A variety of *in vitro* systems have been designed for toxicity studies of liver that utilize 3D cultures, such as bioreactors, hanging drops and fluid flow systems. These systems are suitable for studies of liver toxicity for tested compounds (Jaroch, *et al*., 2018).

**Cyp450 pathway elucidation**: *In vitro* assays designed to identify the particular CYP450 enzyme(s) mediating the major pathways of elimination are likely to be carried out later in the development of promising drug candidates. Assays designed around the selectivity's of chemical inhibitors for the most common and important CYP's 450 (i.e. 1A2, 2C9, 2C19, 2D6 and 3A4) have been thoroughly evaluated and are well established. Other strategies which clarify the major CYP enzymes include correlation with substrates known to be metabolized by specific pathways and incubation with cDNA expressed enzymes. These assays can provide valuable information on the potential for variability in drug exposure due to elimination by polymorphically expressed enzymes, the comparative metabolic fate in preclinical species to that in man and as a predictor of clinical interactions due to inhibition or induction. For these reasons, the FDA has addressed concerns over study design and, more specifically, "careful consideration of both inhibitor and substrate concentration to maintain a selective approach". Preliminary experiments to show physiological relevance of substrate and inhibitor concentrations, lack of depletionand initial rate conditions would provide important supporting data in order to satisfy this regulatory guidance and documentation of the individual or pooled microsomal phenotypes may be necessary to support clinical prediction. (In other words one must show that the test system is not deficient in one CYP450 isozyme)**.**Inhibition of a drug's metabolic pathway may result in increased drug concentrations. A recent case study revealed an adverse reaction to concurrent therapy with methadone and ciprofloxacin revealed that inhibition of CYP450 1A2 and 3A4 by ciprofloxacin have increased methadone levels and profound sedation, confusion and respiratory depression. Such drug-drug interactions have been established using well characterized isoform specific substrates, in order to make these predictions earlier in the drug discovery process these assays have been adapted to more high throughput formats. Inhibition studies guide clinical development programs by providing information on the potential for drug-drug interactions. These *in vitro* inhibition studies can obviate the need for *in vivo* drug interaction studies as is addressed in FDA guidance which also indicates that this "opportunity should be based on appropriately validated experimental method"

(Kocarek*et al*.,1995). Another facet of drug-drug interactions is the potential for induction of metabolic enzymes. For example,phenytoin enhanced acetaminophen hepatotoxicity brings to light the previously underappreciated role of CYP3A4 induction in producing the toxic metabolite N-acetyl-p-benxoquinone (NAPQI). In addition to CYP3A, CYP1A, CYP2C9 and CYP2E1 are also known to be inducible in man. Though the potential for increased pharmacological and/or toxic effect remains due to the formation of metabolites, induction usually results in reduced pharmacological effect due to increased clearance of parent drug. The study of induction potential *in vitro* has taken on additional importance as species differences in CYP expression have been realized. Consequently, primary human hepatocyte cultures have become important in the investigation of new drug candidates because of more reliable predictive power and established *in vitro* methods for the analysis of total enzyme content of liver tissues and or biopsies associated with the toxicology or *in vivo* induction studies could be expected to follow the GLP requirements (Bajpai and Esmay, 2002).

**CELL CULTURE BASED *IN VITRO* TEST SYSTEMS FOR ANTICANCER DRUG SCREENING:** The number of patients diagnosed with cancer is increasing worldwide and one of the most important challenges remains the development of effective, safe and economically viable antitumor drugs. Clinical approval for drugs tested in preclinical studies enabling them to enter phase I clinical trials is essential. Currently, potential anticancer drugs have a very low rate of gaining clinical approval than drugs for other diseases. Despite the high cost and duration of anticancer drug clinical development it is necessary to develop new, more effective preclinical platforms for screening antitumor compounds (Imamura *et al*., 2015). *In vitro* tumor models are a necessary tool in not only the search for new substances showing antitumor activity but additionally for assessing their effectiveness. The behavior of the tumor in the body is determined by cells within the tumor and stromal tumor microenvironment (TME) and the extracellular matrix (ECM), which provides structural support for cells in the extracellular space. The TME is characterized by a low extracellular pH and a high level of hypoxia, both factors moderate dormant phenotypes of tumor cells. As a result, these factors are associated with development of therapy resistance and poor prognosis of tumor-bearing patients. The tumor biological characteristics are similar to the chronically unhealed wound with constant inflammation, which contributes toward tumorigenesis, tumor progression and metastasis. Attracted by the tumor stromal microenvironment, other cell types also play a key role in not only tumor progression and metastasis, but also in the formation of resistance to therapies (Wu and Dai, 2017). Within the TME many other cellular components reside including immune cells [T-lymphocytes, B-lymphocytes, neutrophils, natural killer cells (NK-cells) and macrophages], endothelial cells associated with the tumor, fibroblasts, myofibroblasts, adipocytes, pericytes and mesenchymal stem/stromal cells (MSCs) (Vincenza*et al*., 2015).

**Two-Dimensional Cultures:** Until the 1980s, the National Cancer Institute (NCI) used *in vivo* mouse models of P388 or L1210V leukemia for systematic screening of drugs. These models possessed high levels of productivity and stability, were convenient for data interpretation, and were relatively inexpensive. Despite these qualities, a significant drawback to these models was the inability to identify potential antitumor substances aimed at treating solid tumors. This drawback was taken into account, and by the end of the 80s, an *in vitro* panel for drug screening was developed, consisting of 60 different human cell lines originating from tumors (leukemia, melanoma, tumors of the central nervous system, cancer of the lungs, colon, ovaries, breast, kidney, and prostate), which was called NCI60 (Mingaleeva *et al*., 2013).Testing a drug of interest using the NCI60 panel involves the application of two-dimensional (2D) tumor cell cultures, grown in a monolayer on a flat surface. During the first stage of screening, testing is carried out on the three cell lines that are frequently the most sensitive to drug therapy, MCF7 (breast adenocarcinoma), NCI-H460 (lung carcinoma) and SF-268 (glioma). The cytotoxicity of the test substance is determined using the pink anionic dye sulforodamine B. If the test substance inhibits the growth of at least one cell line, testing proceeds to the next stage comprising of the full 60 cell line panel. In 2017, the NCI ALMANAC database was created based on screening results using the NCI60 panel. The database helped identify new effective combinations of existing antitumor drugs and new clinical trials were launched. By analogy with the NCI60 panel, the Japanese Foundation for Cancer Research (JFCR) developed a panel in the 1990s consisting of 30 tumor lines from the NCI60 panel, plus nine tumor cells lines specific to the Japanese population, specifically gastric cancer cells) and breast cancer cells. Thus, the panel included 39 cell lines and was therefore called JFCR39. However, during clinical trials, it became apparent that drugs that have shown high efficacy in 2D *in vitro* models do not always work or can have a low efficacy in oncology patients. This phenomenon is partially explained by the fact that cells grown in 2D cultures do not have a complex three-dimensional tissue architecture and do not exactly reflect the complex interactions between TME or ECM and cells which exist in the body(Rizvanov*et al*., 2010).

**Boyden Chamber:** The Boyden chamber is a chamber consisting of two compartments filled with medium and separated by a microporous membrane and a convenient tool for the study of chemotaxis, assessing cell motility and invasion. Boyden chamber was used to assess cell motility in a study on the effect of free paclitaxel and paclitaxel-loaded pyromellitic nanorods on reducing the growth and invasiveness of melanoma cells (Kitaeva*et al*., 2020). However, despite the ease of use of the Boyden chamber, researchers are increasingly turning to more advanced systems that take into account a greater number of TME conditions, in particular, microfluidic systems (Clemente *et al.,* 2019).

**Three-Dimensional Cultures:** It is known that 2D cultures do not fully reflect the pathophysiology of tumor cells and the actual level of resistance to radiotherapy or chemotherapy in the tumor niche in the *in vivo* system . Studies have shown that gene expression profiles as well as treatment responses in multicellular spheroid 3D models are more similar to the *in vivo* situation. For example, liver tumor cells in 3D culture have high resistance to drug treatment, similar to the resistance of solid tumors *in vivo*. Thus, the BT-549, BT-474, and T-47D breast cancer cell lines cultured as spheroids showed greater resistance to paclitaxel and doxorubicin compared to cells in a 2D culture (Imamura *et al.,* 2015). Cells of squamous cell carcinoma originating from the head and neck (lines LK0902, LK0917, and LK1108) cultured as spheroids were shown to be less sensitivity to cisplatin when compared with 2D cultures. It is known that the TME may significantly change the susceptibility of tumor cells to drugs.

New methods were developed for culturing cells using the ECM to model spatial organization, as well as adding various types of cells included in the TME to the culture. 3D co-cultures of non-small cell lung cancer (NSCLC) and fibroblasts embedded in a Matrigel or encapsulated in alginate are used as models in drug discovery for analysis of immune cell infiltration. Also, described is a high-potential tumor spheroid model drug screening, which consists of pancreatic ductal adenocarcinoma (PDAC) cell lines (Panc-1 and BxPC-3) and cancer-associated fibroblasts (CAFs) surrounding by oligomeric type I collagen (Oligomer) for creation of the interstitial ECM supports definition (Puls *et al.*, 2018).An alternative way to create a novel 3D tumor-tissue model is organoid manner. The novel *in vitro* system allowed the propagation of mammary stem and progenitor cells into functional ductal/acinar structures. Organoids can be received by two main types of stem cells: pluripotent embryonic stem cells and their synthetic induced pluripotent stem cell counterparts and organ-restricted adult stem cells (Clevers, 2016). Also, organoids received by cultivation of small tissue fragments and explants on matrixes or from cultured or sorted cells assembled to organoids *in vitro*. Organoids from primary lung cancer tissues demonstrated the high reproduction levels of histological and genetic characteristics of *in situ* tissue and their high ability for using them in patient-specific drug trials. Organoid manner was used for modeling PDAC from patient derived xenografts (PDX) tumors and organoids derived from patient prostate cancer bone metastasis. Organoids derived from patients with bladder cancer were tested with epirubicin, mitomycin C, gemcitabine, vincristine, doxorubicin, and cisplatin, this model was presented as a prospective model of human bladder cancer (Mullender*et al*., 2019).

**Microfluidic Systems**: Microfluidic systems are prospective models for reconstructing the migration, microenvironment, and microcirculation of cells in tumor tissue. Microfluidic systems are small devices that can reproduce a specific fluid flow, constant temperature, fresh medium, flow pressure and chemical gradients characteristic of *in vivo* systems. The microfluidic system using collagen-matrigel hydrogel matrices made it possible to reproduce the microenvironment and experimental conditions for studying the migration and invasion of H1299 lung adenocarcinoma cells. At the same time, matrigel in low concentrations facilitated the migration of H1299 cells, however, at a high concentration matrigel slowed the migration of cells, possibly due to their excessive attachment. It has also been shown that the use of antibody-based integrin blockers significantly modulated the mechanisms of H1299 cell migration. A microfluidic system with an incessant supply of nutrient medium through a syringe pump has also been described. It is used to study the effect of the matrix metalloproteinase inhibitor (GM6001) on the formation of invadopodia in A549 lung cancer cells, which is characteristic of cells during invasion. Microfluidic systems also make it possible to obtain a metastatic model of a tumor, such as breast cancer, which allows the study of antitumor drugs effects on the inhibition of tumor cell migration (Mi *et al.*, 2016). To simulate the extravasation process, a microfluidic system was constructed containing two microfluidic channels and a porous membrane sandwiched between them. The first channel represents the vascular equivalent and contains primary endothelial cells isolated from the pulmonary artery. The second channel acts as a reservoir for collecting migratory tumor cells. In this case, endothelial cells showed *in vivo*-like behavior under flow conditions. The introduced GFP-labeled tumor cells of epithelial or mesenchymal origin were detected using vital imaging, which showed tightly attached tumor cells to the endothelial membrane (Kuhlbach*et al*., 2018).

## CONCLUSION

The process of developing a novel drug is time consuming and costly. To increase the chances of successfully completing a clinical trial leading to the approval of a new drug, the choice of appropriate preclinical models is of utmost importance. Identifying a safe, potent, and efficacious drug requires thorough preclinical testing, which evaluates aspects of pharmacodynamics, pharmacokinetics, and toxicology in *in vitro* and in vivo settings. For ethical and cost-related reasons, use of animals for the assessment of mode of action, metabolism and toxicity of new drug candidates has been increasingly scrutinized in research and industrial applications. Although, many types of *in vitro* assays are conducted during drug development, use of cell cultures is the most reliable one. Two-dimensional (2D) cell cultures have been a part of drug development for many years. Two-dimensional (2D) cell cultures have been used since 1900s and are still a dominant method in many biological studies. Thus, cell culture continues to be not just a tool but also a window into the *in vivo* environments of each cell type studied *in vitro*. Improvement in the number of drug candidates that succeed in clinical trials, thus reducing costs involved in developing a drug as well as the time necessary to introduce a new therapeutic agent into clinical practice. *In vitro* absorption, distribution, metabolism, and excretion assessment, as well as drug-drug interaction (DDI), can be studied with the use of various cell culturebased assay. There is no one “correct” way to discover a new drug. Instead, drug developers have a “tool kit” of strategies and methods they can choose from among which, when properly deployed and exploited, will on occasion lead to the discovery of a new medicine. In view of the growing incidence of oncology, increasing the pace of the creation, development and testing of new antitumor agents, the improvement and expansion of new high-tech systems for preclinical *in vitro* screening is becoming very important. Studies with cancer cell lines give an opportunity to understand tumor biology and allow high-throughput screening for drug development. The choice of the correct tumor model at the stage of *in vitro* testing provides reduction in both financial and time costs during later stages due to the timely screening of ineffective agents. The availability of these tissue-, cellular- or molecular-based assays would then allow the pharmaceutical scientist in collaboration with the medicinal chemist to use an iterative process to refine the “pharmaceutical properties’’ of a drug candidate. Using a similar strategy, medicinal chemists, working in collaboration with biochemists, cell biologists, immunologists and molecular pharmacologists, have been very successful in optimizing the “pharmacological properties” of drug candidate.

**FUTURE PERSPECTIVES:** Yet, an active compound does necessary mean a future drug since toxicological studies, chemical investigation, clinical trials and legal issues may exclude a compound from further development processes towards a recognized drug. However, cell culture-based assays cannot provide the full pharmacological profile since the data they provided are limited to some molecular and cellular aspects such as pharmacodynamic, biochemical pathways and genetic variations.

The model must be complex enough to take into account most of the microenvironment factors, but at the same time be reproducible, with the ability to correctly interpret the screening results. Existing trends in science, particularly in the field of preclinical screening, are heading precisely toward complicating the models need to be developed. Recently three-dimensional (3D) cell cultures have received remarkable attention in studies such as drug discovery and development. Optimization of cell culture conditions is very critical in ensuring powerful experimental reproducibility, which may help to find new therapies for cancer and other diseases. Although many important investigations were performed using cancer cell lines, the results give limited information and present low clinical correlation. The genetic aberrations of cancer cell lines that are related with increasing passage numbers are one of the reasons why this type of study does not fully represent clinical situation. Poor correlation between preclinical *in vitro* and *in vivo* data with clinical trials remains a major concern. While having the potential to provide mechanistic insights, *in vitro* models are constrained by the fact that isolated cells may not behave in a petri dish as they would within the body where they partake in crosstalk and interaction with millions of other cells. Consequently, more sophisticated preclinical models are required to establish the investigational compound's safety profile before transitioning to a clinicalsettings. Importantly, some pharmacokinetic parameters cannot be studied by cell cultures, and animal experiments remain required to achieve this purpose. Focusing on the challenges facing the application of cell culture techniques in drug discovery and overcoming them would further exploit this important method for drug discovery and drug development research. Since cell-culture studies focus on isolated cells apart from tissues or organisms, the influence of some elements that can interfere with the pharmacological receptors for example, including some chemical environments (hormones and fluid pressure cannot be studied. Many potentially useful drug candidates were never developed clinically because these molecules lacked the structural features needed to circumvent the epithelial (e.g., intestinal mucosa), endothelial (e.g., BBB) and/or elimination (e.g., liver) barriers that limit the access of the drug to its site of action To date, challenges remain in the creation of an *in vitro* system in which all ADME analyses can be simultaneously performed within one experiment.

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