Preclinical In Vivo Drug Development Studies: Limitations, Model Organisms, and Techniques

Seema Negi, Sanjay Kumar, and Ajeet Singh

Abstract The process of drug discovery and development encompasses target identification, validation, assay development, identification of hits, lead optimization, preclinical evaluation, and finally human clinical trials. Once a new chemical entity (NCE) is discovered, it progresses toward the development process that includes preclinical and clinical pharmacology. Preclinical research includes in silico, in vitro, ex vivo, and in vivo studies using cell lines, tissues, and animal models for predicting pharmacokinetic and pharmacodynamic properties of lead candidates. In vivo studies are critical in the drug development process because these investigations are useful to assess the properties of drugs and physiological and biochemical processes like adverse drug reactions and drug-drug interactions, which are difficult to examine in vitro. This chapter provides the detailed insight on in vivo studies that includes animal models and toxicology testing methodologies to identify a safe, potent, and efficacious drug. This chapter also highlights the importance of predictive and validated animal models for absorption, distribution, metabolism, and excretion (ADME) studies, along with the disease-based animal models for understanding disease pathophysiology that ultimately helps in making decisions that lead to human clinical trials for a drug candidate.

Keywords Drug discovery · Drug development · In vivo studies · Animal models · Pharmacokinetic · Toxicology · Techniques

S. Negi (✉)
Central Research Station, Subharti Medical College, Swami Vivekanand Subharti University, Meerut, U.P., India

S. Kumar
Glocal College of Paramedical Science and Research Centre, The Glocal University, Mirzapur Pole, Saharanpur, U.P., India

School of Life and Allied Health Sciences, The Glocal University, Mirzapur Pole, Saharanpur, U.P., India

A. Singh
Department of Pharmaceutical Sciences, J. S. University, Shikohabad, Firozabad, U.P., India

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1 Introduction

The process of discovering possible new medicines is known as drug discovery and development. It involves a broad range of scientific disciplines, including biology to molecular biology, chemistry to computational chemistry, and pharmacology to molecular pharmacology and takes an average of 10 to 15 years to bring a single drug into market (Csermely et al. 2013; Hughes et al. 2011). The first steps in this process are carried out largely by basic studies, and their findings facilitate the identification of potential new targets for drug discovery. The whole procedure of drug discovery and development follows a defined process and is guided by regulatory requirements, with the goal of avoiding excessive costs by eliminating unlikely drug candidates early on (Haber and Spaventi 2017). A schematic diagram of the overall drug discovery and development process is depicted in Fig. 1. The process is divided into the following five primary steps: drug discovery, preclinical research, clinical research/trial, Food and Drug Administration (FDA) review, and FDA post-market safety monitoring with three subdivisions under clinical research (Nys and Fillet 2018). Thousands of compounds are assessed before moving on to the preclinical step of the drug development process, which takes an average of 6 years. Target identification and lead discovery are the first steps in drug development, which can then advance to the preclinical stage for determining the drug's efficacy and safety. The new drug is biologically evaluated in preclinical studies for

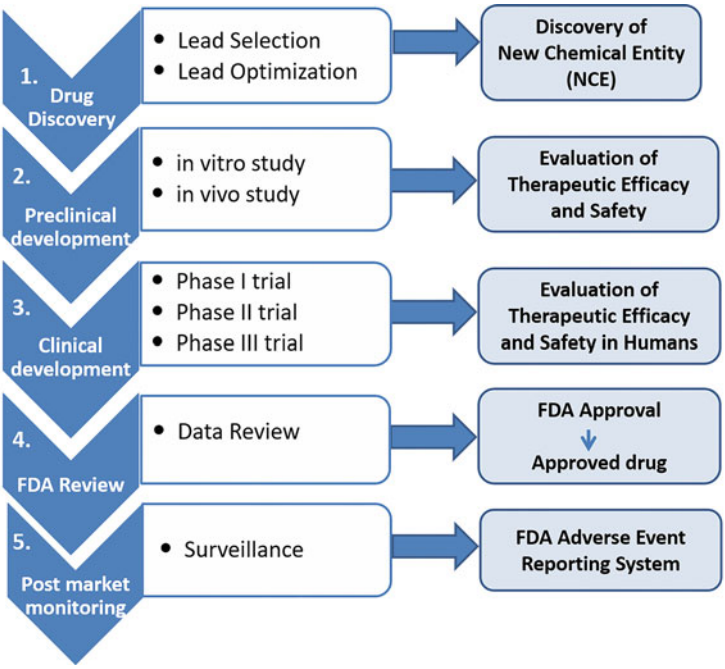

Fig. 1 An illustration of the stages involved in the drug discovery and development process with major strategies and aims at different phases

pharmacological and toxicological effects, as well as potential therapeutic applications. In vitro and in vivo studies are used in the preclinical stage to develop a safe and effective drug that can then be assessed in clinical trials. For assessing the safety, efficacy, and pharmacology of a drug in humans, clinical trials are further divided into three phases (Bjorklund et al. 2002; Lipsky and Sharp 2001; Martini et al. 2001). The procedure then moves from clinical trials to FDA approval, with the FDA either approving or rejecting the drug following its evaluation. If the application is denied, the applicant is given an explanation for the rejection of the application as well as the information to enhance the claim (Lipsky and Sharp 2001).

Validation procedures used in preclinical investigations range from in vitro (studies performed in cell lines and tissues separated from living organisms) to in vivo (studies performed on laboratory animals). In vivo studies are critical to determine the safety, bioequivalence, dosing regimen, adverse drug reactions, and drug-drug interactions in a living system, as well as to monitor and observe the drug's long-term effects. In vitro-in vivo correlation (IVIVC) data is utilized to choose suitable excipients and optimize the formulation process for quality control, leading in lower total costs (Nainar et al. 2012; Segovia-Zafra et al. 2021). Fig. 2

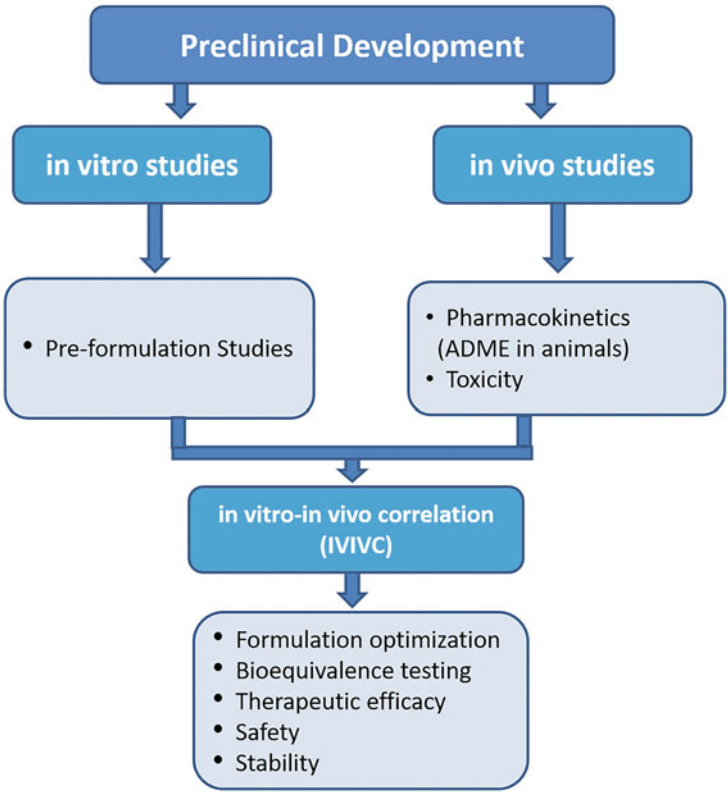

Fig. 2 A model representing preclinical development including in vitro and in vivo studies to predict in vitro-in vivo correlation (IVIVC)

depicts the role of in vitro and in vivo studies for predicting IVIVC at the preclinical phase of drug discovery and development. In vitro studies not only reduce the cost of a drug testing but also reduce ethical conflicts and experimental restriction. In vivo studies are important for drug development because these studies are useful for assessing a drug's characteristics such as therapeutic effects, side effects, drug metabolism, and drug-drug interactions that are difficult to detect in vitro. This chapter's aim is to highpoint the importance of in vivo studies in drug development, discussing in detail various diseases including cancer and metabolic, genetic, cardiovascular, and neurodegenerative diseases based on in vivo animal models.

2 Preclinical In Vivo Studies of ADME in Drug Development

Preclinical studies are used for establishing a starting, safe dose for first-in-human studies and to analyze the molecule's potential toxicity, which usually includes prescription drugs as well as diagnostics and new medical devices.

2.1 Importance of In Vivo ADME Studies

Routine in vivo experimentation was mostly used to screen at an early point in the drug development phase before target-directed methods became the standard. Many essential drugs (e.g., thiazide diuretics, benzodiazepines, cyclosporin) were discovered on the basis of their in vivo effects. In vitro assays provide valuable data related to pharmacological mechanisms of action, which is helpful in decision-making during the process of drug development.

However, the relevance to human toxicity and risk assessment is limited without corelating in vitro toxicodynamic results with the in vivo toxicokinetics findings, as in vivo systems accurately mimic a live biological system (Sewell et al. 2017). In vitro studies can anticipate organ and organ system interactions with drugs, as well as drug-drug interactions inside a system, and give a quantitative data of ADME in animal and human models (Singhvi and Singh 2011; Pellegatti 2012).

In vitro studies are unable to accurately mimic the system's micro- and macroenvironment; therefore, they are unable to translate in vivo at the preclinical stage in the case of metabolic malignancies (Amoedo et al. 2017). Anticancer activity of benzimidazole derivatives, amidino-substituted benzimidazole and benzimidazo[1,2-a]quinoline, has shown 2D cell cultures were comparable to 3D cell cultures, but significant discrepancies revealed false-positive findings that ultimately require in vivo profiling for validation (Brajsa et al. 2015). In vivo research is essential to assess various parameters such as safety, dosage schedule, bioequivalence, effects of the drug, side effects, and drug interactions to develop a safe and

effective drug (Pellegatti 2012). In vivo findings are multifactorial, combining the effects of permeability, distribution, metabolism, and excretion to produce a valuable data related to pharmacokinetic parameters and toxicological endpoints. Although in vitro assays are useful to determine various parameters, animal studies are essential to analyze the therapeutic effect and potential toxicities associated with the drugs (Sewell et al. 2017). There are a wide variety of animals used in preclinical in vivo studies. Rodents are commonly used in animal testing, particularly mice and rats. Since they are low cost and only need a little quantity of test chemical, they are the first animal species utilized to assess drug exposure. Laboratory rats and mice provide ideal animal models for drug development because the anatomy and physiology of rats and mice are more similar to humans. Similarly, rats, mice, and humans each contain about 30,000 genes, with 95% of them being shared by all three species (Waterston et al. 2002; Bryda 2013). In vivo rat investigations can highlight ADME issues with a novel chemical series, for example, whether there is a low absorption level or high level of clearance, resulting in unacceptable pharmacokinetics (PK).

2.2 *Challenges to Design In Vivo Studies for Drug Discovery and Development*

Drugs, chemical drugs, or biologics such as antibodies, vaccines, and peptides can be administered into the body through different routes of administration such as mouth (gastrointestinal lining), upper respiratory airway (pulmonary epithelium), and intravenous (vascular endothelium). Intravenous route is particularly used for tumor vasculature and blood-brain barrier targeting. Biological barriers typically occur during the delivery of lead drugs to target areas, and these barriers have a considerable impact on drug bioavailability and potential therapeutic action. To reach the blood compartment, the lead molecule(s) must pass through epithelia of the lung or gastrointestinal (GI) tract, tumoral vascular endothelial lining, or the blood-brain barrier (BBB) (Sjogren et al. 2014). The pharmacokinetic parameters are influenced by the in vivo effect of the drug and the biological barriers related to drug delivery.

2.3 *Limitations of In Vivo Studies*

In vivo studies provide many detailed information in the drug development process, but there are few limitations that warrant attention. About 75% of drugs flop in phase II or phase III human clinical trials owing to lack of efficacy or safety data (Van Norman 2019). Dependence on non-human animal models in preclinical investigations remains a major factor in this failure. It is difficult to anticipate the efficacy and safety of a drug in small animals like mice because of fundamental biological

differences (Pound and Ritskes-Hoitinga 2018; Weaver and Valentin 2019; Khalil et al. 2020; Ferreira et al. 2020). The shape, size, and regenerating capability of tissues and organs, along with physiological variations in immunology, metabolism, and drug transportation, all affect drug development in humans and small animals (Pound and Ritskes-Hoitinga 2018; Weaver and Valentin 2019; Khalil et al. 2020). Large animal models, for instance, dogs, pigs, and non-human primates, are more identical to human anatomy and physiology and therefore can ameliorate the predictive worth of in vivo models (Tsang et al. 2016; Ziegler et al. 2016; Khalil et al. 2020). Nevertheless, large animal models increase cost, time, and more ethical considerations significantly. Additionally, there is a remarkable difference between humans and animals at molecular, genetic, cellular, anatomical, and physiological levels (Pound and Ritskes-Hoitinga 2018; Weaver and Valentin 2019; Khalil et al. 2020). Therefore, there is requirement of biological models based on human tissue for better representation of human biology (Khalil et al. 2020; Pound 2020). Novel in vitro and in vivo preclinical models that mimic human tissues are needed to address this constraint.

3 Preclinical Animal Models Used for ADME Parameter Optimization in Drug Discovery and Development

Animal models are required for bridging the preclinical-clinical research gap. Preclinical research in fit-for-purpose animal models may improve success rates of drugs during clinical development (Pound and Ritskes-Hoitinga 2020). In vivo experiments can be designed to determine efficacy in a specific biological model based on early findings from in vitro and ex vivo research, in addition to information regarding therapeutic target, clinical symptoms, and pharmacokinetic profile of the drug candidate. Furthermore, the scientifically relevant in vivo studies will be selected on a case-by-case manner. There are three types of disease models to choose from: physiological, pharmacological, and genetically engineered animal models (genetic) (Andrade et al. 2016). All of these models are intended to develop abnormalities that are comparable to those seen in the disease under investigation. Furthermore, depending on the duration of the disease, the in vivo models may be classified as acute or chronic (Andrade et al. 2016).

When evaluating the efficacy of a new chemical entity in preclinical in vivo studies, it is also crucial to establish the therapeutic target/protein. In the case of *Mus musculus* and *Rattus norvegicus*, proof-of-principle assays (proof-of-concept testing) are usually done, and if no association with the target is detected, the animal studies will not give significant results. Using several animal species during the drug development process is one of the primary reasons of failure because of the variances between species and the complications in translating the findings to humans (Oreff et al. 2021). Indeed, the pathophysiology of a variety of diseases varies significantly between species (Mestas and Hughes 2004; Wang and Urban 2004). Furthermore,

the ADME profile in animals and humans is frequently different, which might cause variations in the duration of the test substance action, influencing both pharmacology and toxicology and ultimately leading to ambiguous findings (Martignoni et al. 2006).

3.1 The Role of Animal Models

Animal models are frequently used in drug absorption, metabolism, distribution, and excretion investigations, and the scientist's ability to increase and improve human and animal well-being is wholly dependent on breakthroughs in research employing animal models for evaluating pharmacological properties in vivo (Landskroner et al. 2011). Although animal models can provide helpful information regarding a substance's nonclinical efficacy, they are not able to replicate all of the indications and symptoms of human disease pathology, and ultimate efficacy confirmation can only be validated when phase II clinical trials are completed. Before a medicine is investigated in humans, it must first be tested in an animal model to record toxicity, side effect, and drug interactions, among other pharmacokinetic parameters. Furthermore, animal studies are required to assist development in the early stages, especially when deciding whether to precede with the human research studies (go/no-go choice).

Human disease-based animal models are only considered significant if these models aid in the improvement of intervention and therapy techniques by recapitulating disease pathophysiology. A model must be able to precisely represent the morphological and biochemical components of the pathophysiology and also able to imitate the typical physiology and anatomy of human organs as well as tissues of interest.

Scientists use models to create an artificial condition in a lab animal that mimics the etiology of human disease. There are several animal models, both vertebrates and invertebrates that may be used to study disease pathogenesis that affects both humans and animals. Invertebrate animal models including zebrafish, *Caenorhabditis elegans*, and *Drosophila melanogaster* have been widely used in neuroscience, genetics, and metabolic and cancer research. A variety of vertebrate animal including mice, rats, guinea pigs, rabbits, cats, dogs, and primates are essential for translational research in biomedical sciences (Harman et al. 2020).

3.2 Choosing an Animal Model

Animal models are of three main types: homologous, isomorphic, or predictive, which is largely determined by the study objective (Davidson et al. 1987; Brake et al. 2017). An appropriate model for preclinical in vivo studies would be the disease-based animal model that shares the same pathophysiology as humans and

recapitulates the disease phenotype and responds to human treatments in a way that is analogous to humans. In every way, human physiology, pathology, and treatment are replicated in homologous animal models (Davidson et al. 1987; Brake et al. 2017). Isomorphic models have identical symptoms to humans, although they are not represented by the same events (Davidson et al. 1987; Brake et al. 2017). Predictive animal models are not similar to human disorders; yet they do allow for some comparisons or predictions of human disease, treatment, and treatment effect (Davidson et al. 1987; Brake et al. 2017). Mechanisms of actions, pharmacokinetics, and pharmacodynamics along with biomarkers and safety and toxicity of prospective therapies must also be determined using animal models that correctly mimic disease pathophysiology (Franklin et al. 2022). In therapeutic studies, such animal models might potentially help in assessing human dose (Sim and Kauser 2015). Availability, cost-effectiveness, ethical concern, ease of handling, housing requirements, and disease vulnerability are all factors to consider while choosing an animal model (Brake et al. 2017).

Various animal models including invertebrate and vertebrate are being used in preclinical drug testing. In pharmacological research involving neurological, genetic, and developmental problems, invertebrate animal models are frequently employed (Wilson-Sanders 2011). The zebrafish is one such creature that is frequently utilized (Zon and Peterson 2005; Takaki et al. 2018). This model is particularly useful when researchers are looking for a disease model that is both embryologically and genetically docile (Lieschke and Currie 2007). Traditionally, mice, rats, guinea pigs, rabbits, dogs, baboons, cows, and macaques have been used as vertebrate models. In translational research, these models may be the most useful (Hickman et al. 2017).

When selecting an animal model for preclinical studies, general principles such as a large number of results and a related life cycle must be considered. If a large number of findings are required, an invertebrate would be an excellent candidate; nevertheless, relevancy of life cycle and ability of the biological sample must be considered. A pair of zebrafish may produce a large number of embryos every week, leading to the generation of huge results and findings at low cost (Kari et al. 2007). Although the zebrafish is a more popular animal model, genetically engineered mice, rats, dogs, and non-human primates are commonly used animal models in drug testing (Hickman et al. 2017; Khan et al. 2018; Sobczuk et al. 2020). Furthermore, biochemical and physiological resemblance between animal models and humans, as well as the underlying mechanism of drug ADME in animals, should be considered while choosing the suitable animals (Tang and Prueksaritanont 2010). There are several instances of well-established animal models that have been utilized to study particular diseases (Khan et al. 2018). In addition, when compared to human physiological and biochemical parameters, including blood pH, blood volume, organ blood flow, tissue distribution, localization of metabolizing enzymes, and drug transporters, are used for selecting an appropriate animal model (Tang and Prueksaritanont 2010).

4 Human Disease Models for the Preclinical In Vivo Studies

Human disease animal models are extremely useful for the advancement of innovative and effective diagnostic and therapeutic approaches. These models are the valuable tool to understand the disease pathology and also helpful for assessing the safety of novel drugs. Animal models have provided new insights and an extensive knowledge of the onset and diagnosis of human disease. They have been used to evaluate new chemical entity and other biologics, like vaccines, hormones, and antibodies (Gong et al. 2020).

Methodological advancements in recombinant DNA technology now allow for precise manipulation of laboratory animals, such as the introduction or the deletion of a gene. These advances have resulted in the production of transgenic and knockout (KO) mice, which are useful tools for understanding the molecular basis of various human diseases and also for the development of novel medication and therapeutic procedure for the treatment of diseases. Current trends in animal models indicate the inclusion of advance technologies like genetic manipulations and stem cell technology, which may be even more potent in the development of successful drugs, vaccines, and medical devices (Gong et al. 2020; Gong et al. 2021). The mostly used models are listed below.

4.1 Mouse Model

The drug discovery and development process has been transformed due to advancement in genetic engineering. As a result, genetic engineered mouse models have emerged as precious tools for modeling of human disease and drug development. Transgenic mouse models with knock-in and knockout technologies have proven effective in basic and applied research to find answers to fundamental questions (Table 1). Furthermore, more advanced mouse models are essential for cutting-edge research.

LDLR$^{2/2}$ Mouse Model

The LDLR$^{2/2}$ mice have been used to study familial hypocholesterolemia (low-density lipoprotein (LDL) receptor). The plasma lipoprotein profile of these mice is comparable to that of humans because of the mutation in LDLR. On a typical chow diet, the genetic abnormality causes a delay in the disposal of very low-density lipoprotein (VLDL) and LDL from the plasma, resulting in an elevated plasma level of cholesterol (Bentzon and Falk 2010). A high-cholesterol and high-fat diet worsens lesions associated with atherosclerosis and hypercholesterolemia in LDLR$^{2/2}$ mouse model (Knowles and Maeda 2000).

Table 1 Disease-based mouse models for preclinical in vivo studies (modified from Khan et al. 2018)

Diseases	Mouse models	References
Atherogenesis	ApoE$^{2/2}$ mice	Plump et al. (1992)
Hypercholesterolemia	Calcium chloride–induced abdominal aortic aneurysm (AAA)	Freestone et al. (1997)
Aneurysm	Spontaneous mutant mouse strain	Brophy et al. (1998)
Hyperlipidemia and atherosclerosis	Mutant E3L, ApoE mice	Leppanen et al. (1998)
Colon cancer	Human colorectal cancer (CRC) cell lines in mice	Rashidi and Gamagami (2000)
Familial hypocholesterolemia	LDLR$^{2/2}$ mice	Jawien et al. (2004)
Diabetic cardiomyopathy and atherosclerosis	LDLR$^{2/2}$ mice and ApoE$^{2/2}$ mice	Hayek et al. (2005)
Colon cancer	C57BL/6 mice applying murine colon adenocarcinoma (MCA) cells	de Jong and Aarts (2009)
Liver diseases	Fatty liver disease-associated mouse model	Chung et al. (2010)

On a normal chow diet, LDLR$^{2/2}$ and ApoE double-deficient mice (LDLR$^{2/2}$ApoE$^{2/2}$) could indeed develop severe atherosclerosis and hyperlipidemia. As a result, these models make it easier to study diseases without having to worry about feeding atherogenic diets to the mice (Jawien et al. 2004).

ApoE$^{2/2}$ Mouse Model

In 1992, two different embryonic stem cell research groups employed the homogeneous recombination technique to produce ApoE mice (Zhang et al. 1992; Plump et al. 1992; Zhang et al. 1992). A homogeneous loss in the ApoE gene causes plasma levels of VLDL and LDL to rise, resulting in the inability of the LDL receptor and associated proteins to function. It was the first mouse model to display a wide range of atherogenesis lesions, making it the first mouse model to resemble human-like lesions (Plump et al. 1992).

Transgenic Mouse Model

The use of transgenic mice in the research of hyperlipidemia and atherosclerosis is common, and the mutant ApoE3 Leiden (E3L) and ApoE (Arg 112-Cys-142) are often utilized transgenic mice in such studies. These transgenic mice have a lipoprotein profile that is analogous to the profile of people having dysbetalipoproteinemia (Hofker et al. 1998). The E3L mice exhibit the features of human vasculopathy in mild, moderate, and severe atherosclerotic plaques (Leppanen et al. 1998).

Diabetes-Associated Atherosclerosis Model

One of the primary causes of cardiovascular disease is diabetes. The $LDLR^{2/2}$ and $ApoE^{2/2}$ mouse models are frequently used to examine diabetes-related cardiomyopathy and atherosclerosis. Injecting the models with viral injections or streptozotocin causes them to develop type 1 diabetes (Shen and Bornfeldt 2007). Streptozotocin injections cause calcification in the proximal aorta as well as atherosclerosis inside aortic sinus, abdominal aorta, and carotid artery in mice (Khan et al. 2018).

Abdominal Aortic Aneurysm (AAA) Calcium Chloride-Induced Model

This model was created initially in rabbits and subsequently in mice. Calcium chloride was injected intravenously into the region between the iliac bifurcation and the renal artery during the model's development. The aorta dilates significantly after 14 days, resulting in the formation of an aneurysm. Calcium chloride and thioglycolate can be used to augment the severity. The animals can also be fed a high-cholesterol diet to get similar outcomes (Freestone et al. 1997).

Spontaneous Mutant Mouse Model

In the X chromosome, a spontaneous mutation was done to create the blotchy mouse model, which results in an abnormal shift in the rate of intestinal copper absorption. This mutant model develops aneurysms in the thoracic aorta, aortic arch, and abdominal aorta because of inadequate cross-linkage within collagen and weaker elastin tissues. However, as mutation leads to several effects, besides aneurysm, it becomes difficult to interpret the results drawn from such models (Brophy et al. 1998).

Liver Metastasis Mouse Model

In roughly 50% to 60% of patients, liver metastasis develops in the colorectal area. Better treatment options are urgently needed to extend the life span of patients suffering from this condition. A system for animal trials on rodents was devised for this purpose (de Jong and Aarts 2009). Immunocompetent rodents were used in this study because they have an advantage in that their immune systems are similar to those of patients with colorectal cancer that develop metastases. Therefore, to induce liver metastases, first, the mice were examined for immunotherapy effects, and then the human colorectal cancer (CRC) cells were inoculated in five different locations of the animal, including the colonic wall, subcutaneous, intraportal, intrasplenic, and intrahepatic (Kobaek-Larsen and Thorup 2000). The advantage of employing this model is believed that they exhibit pathologic behavior that is quite comparable to human pathological behavior.

Colon Cancer Mouse Model

To establish a hepatic tumor model, scientists used orthotopic injection of tumors into the cecal walls. The intrasplenic or intrahepatic injection of tumor cells is similar to the hematogenous spread of tumor cells in the liver. Moreover, these models are useful to create macroscopic metastases about in all cells within the entire body of the animal. The C57BL/6 mouse model with MCA cells and Wistar, WAG/Rij, or BDIX rat models having N-methyl-N-nitrosoguanidine-induced adenocarcinoma cells, CC531, or DHDK12/TR colon cancerous cells are the most useful animal models of hepatic tumor (Burtin et al. 2020). Most of the desired qualities are covered by injecting heterotopic syngeneic tumor cells into immunocompetent animals (de Jong and Aarts 2009; Ben-david et al. 2019; Guerin et al. 2020).

Fatty Liver Disease Mouse Model

The complication of the metabolic syndrome is non-alcoholic steatohepatitis (Zivkovic et al. 2007). Choline- and methionine-deficient diet is provided to the non-alcoholic steatohepatitis mouse models. The particular diet causes an increase in liver triglycerides and total bilirubin levels in the blood, fibrosis, and hepatic steatosis. Ultimately, mice not only had dramatically reduced overall weight but also liver weight and total protein concentration. Non-alcoholic steatohepatitis has been developed in these mice without showing any other indications of metabolic syndrome (Chung et al. 2010).

Neurodegenerative Disease Mouse Model

Parkinson's Disease

In the study of neurodegenerative illnesses, mouse models have shown to be invaluable. They have been shown to be a good model organism for Parkinson's disease (PD). PD is a degenerative neurological condition characterized by a deficiency of dopaminergic neurons (DNs) within the substantia nigra as well as extensive buildup of the protein α-synuclein, which results in motor deficits and eventually cognitive dysfunction (Youssef et al. 2019; Shadrina and Slominsky 2021).

Alzheimer's Disease Model

A new transgenic mouse model, APPPS1, has been developed with strain C57 black 6/Jackson (C57BL/6 J) genetic background. The transgenic mouse model has been co-expressed with KM670/671NL-mutated amyloid precursor protein (APP) and

Table 2 Transgenic mouse models of neurodegenerative diseases (modified from Khan et al. 2018)

Disease	Name of the model	Target gene	References
Alzheimer's disease	APPPS1	Co-expression of KM670/671NL-mutated APP and L166P-mutated presenilin 1	Francis et al. (2009)
Parkinson's disease	KO mice	Overexpression of α-synuclein with mutations in familial A53T or A30P	Janus and Welzl (2010)

L166P-mutated presenilin 1 controlled by a neuron-specific Thy1 promoter element. The APPPS1 mouse models are suitable tools for Alzheimer's disease research due to the early development of amyloid plaques, known genetic background, and ease of breeding (Francis et al. 2009). In Table 2, transgenic mouse models of Alzheimer's and Parkinson's disease are summarized.

Heart Failure Models

The ligation of the left coronary artery is a way of producing myocardial injury in rats and mice that permanently occludes arteries. Partially obstructed arteries have been found in recent investigations to generate comparable effects (Michael et al. 1995). As this method has proven to be effective, cryoinjuries are currently being used to cause cardiac injury in rat and mouse models (Ryu et al. 2010).

4.2 Rat Models

Rat models have speeded preclinical in vivo cardiovascular disease research. To generate myocardial injury in the rat heart, three methods are typically used: surgical, electrical, and pharmacological. Myocardial damage is caused in the rat by ligating the left coronary artery (Pfeffer et al. 1979). Isoproterenol, an agonist of the β-1 adrenergic receptor, was first used to inflict pharmacological damage in the heart tissue in 1963. Isoproterenol has a cardioprotective effect when given before ischemia, but when given at a proper dose, it produces myocyte necrosis, severe hypertrophy, and left ventricular dilatation. This method has been used to investigate the fundamental mechanisms of heart attacks (Zbinden and Bagdon 1963) as well as to better understand the role of potential heart attack prevention drugs.

In order to cause electrically induced myocardial damage in rats, an electric shock is delivered to the left ventricle of the heart. Though this is a highly validated method for causing cardiac injury, its results are not shown to be consistently reproducible (Adler et al. 1976).

Celiac Disease Rat Model

Celiac disease is classified as "immune-mediated small intestinal enteropathy." It is caused by the intake of gluten in the diet. Gluten causes this reaction exclusively in people who are genetically prone to the condition. The condition is diagnosed by looking for serum antibodies produced by the body's reaction to the enzyme tissue transglutaminase 2. Gluten-dependent enteropathies are studied in vivo using gluten-sensitized rat models. There are two types of rat models: HLA independent and HLA dependent. An HLA-independent model was developed based on the T-cell transfer colitis model that was used to investigate chronic inflammatory bowel disease of the colon in a rat (Freitag and Rietdijk 2009). In RAG1 mice, expansion of crypt hyperplasia and villous atrophy was induced by giving gluten orally and transferring in vitro gliadin primer. When Wistar rats were given gluten orally plus INF-γ intraperitoneal injection, they exhibited lower villus height, higher TNF levels, and cellular infiltrates within the small intestinal lamina propria (Laparra and Olivares 2012). As a result, the progression of disease-based animal models provided us a plethora of novel therapeutic targets and numerous pathways for testing that could ultimately lead to prevention of celiac disease and support to the discovery leading to the chain of events accountable for the disease (Laparra and Olivares 2012; Costes and Meresse 2015).

Nile Rat

Nile rat (*Arvicanthis niloticus*), also branded as African grass rat, has been used as an animal model for obesity and diabetes studies. Metabolic disease develops in these rats when a high-fat diet is given to them, but wild type rats do not develop diabetes (Noda et al. 2010). These rats show signs of dyslipidemia and hyperglycemia at the age of 1 year. Other symptoms, including abdominal fat deposition, hypertension, hyperinsulinemia, and liver steatosis, have also been shown in these animal models. They provide significant results in case of metabolic diseases when a regular diet is given to them, in contrast to people who are fed a high-fat, high-carbohydrate diet (Noda et al. 2010; Chaabo et al. 2010).

4.3 Porcine Model

Pigs are particularly valuable model organisms in the preclinical stage of drug development, especially for research on neurobiology, as anatomical and physiological properties of pigs are similar to humans. Different technologies have been utilized to generate genetically modified pigs, including DNA microinjection into pronuclei for zygote collected from super-ovulated women, lentivirus and retrovirus gene translation into swine oocytes, sperm-mediated gene transfer, and nuclear

Table 3 Transgenic pig models for Alzheimer's disease, Huntington's disease, Parkinson's disease, and amyotrophic lateral sclerosis (modified from Khan et al. 2018)

Disease	Name of the model	Mutation in genes	References
Alzheimer's disease	Gottingen minipig model for AD	Mutation in amyloid precursor protein (APP) gene	Swindle et al. (2012)
Parkinson's disease	Minipig models for PD	Homolog of FBXO7 gene	Swindle et al. (2012)
Huntington's disease	Transgenic HD (TgHD) minipig model for HD	Mutation in N-terminal HTT (huntingtin) fragment (208 amino acids and 105 Q)	Bassols et al. (2014)
Amyotrophic lateral sclerosis (ALS)	Transgenic pig model for ALS	Mutant G93A hSOD1 gene expression	Yang et al. (2014)

Table 4 Zebrafish models used in neurodegenerative diseases (modified from Khan et al. 2018)

Disease	Name of the model	Genes involved	References
Alzheimer's disease	Zebrafish model for AD	Two homologs of APP	Newman et al. (2007)
Parkinson's disease	Zebrafish model for PD	*DJ-1* gene expression (DJ-1 knockdown zebrafish)	Best and Alderton (2008)
Epilepsy	Zebra fish models for epilepsy	Expression of early proto-oncogenes, e.g., *c-fos* *Mind-bomb* mutant zebrafish, zebrafish Nav1.1 mutants	Hortopan and Baraban (2011), Kalueff et al. (2013)

transfer and cloning (Dolezalova et al. 2014). Pigs are also a perfect model for research on accelerated atherosclerosis in the presence of diabetes and hypercholesterolemia because they resemble the instability of human plaques (Gerrity et al. 2001). The porcine models for coronary atherosclerosis make it easier to study vascular remodeling, adventitial neovascularization, and the makeup of atherosclerotic plaque (Alviar et al. 2010). Table 3 summarizes transgenic pig models utilized in in vivo research.

4.4 Zebrafish Model

Zebrafish has become quite prominent for neurological research. Brain cell processes in both normal and diseased conditions have been studied using adult and larval zebrafish as models. The commonly used zebrafish models for preclinical in vivo research are tabulated in Table 4.

4.5 Rabbit Models

Rabbit models have largely been utilized in cardiovascular disease research to see how statins or diet affects cholesterol levels and plaque formation. These findings increased our understanding of pathways involved in atheroma inflammatory processes including accumulation of macrophage and lipid reduction, as mentioned further below (Khan et al. 2018).

Inflammation-Associated Atherosclerosis Rabbit Models

In magnetic resonance imaging (MRI) quantification studies, rabbits are employed as animal models to determine and image the atherosclerotic aortic component (Helft et al. 2001). Although aortic arteries of rabbits are lesser in diameter than human carotid arteries, they are extensively used in developing endovascular therapies. Furthermore, rabbits have numerous benefits as models for cardiovascular disease research, the most notable of which is the high degree of resemblance between the appearance of aneurysm in rabbits and the incidence of aneurysm in humans. Because they can be readily checked in the femoral artery, rabbit aneurysms are useful models for researching endovascular treatments (Dai et al. 2008).

Myocardial Damage Rabbit Model

Rabbits are useful models for studying myocardial damage because their sarcomere protein composition is comparable to that of humans. The rabbit strain WHHLMI serves as a non-surgical model of spontaneous myocardial infarction. The strain was created via selective breeding of WHHL rabbits with coronary atherosclerosis. A fundamental flaw in this model is the deficiency in plaque formation, conflicting with true myocardial infarction and related with coronary plaque rupture and intravascular thrombosis (Kuge et al. 2010; Shin et al. 2021).

5 In Vivo Research Techniques

As mentioned earlier, before a drug can be approved, its metabolism and drug interactions must be properly studied. For analyzing specific in vivo properties of a drug, a variety of methodologies and sampling protocols are available. Many approaches including equilibrium dialysis, microdialysis, isolated lung perfusion, and imaging techniques are widely employed for determining the distribution of a drug of interest. Advanced techniques, for example, microdialysis, positron emission

tomography (PET), and magnetic resonance spectroscopy (MRS), provide a number of advantages over traditional approaches such as saliva sampling, tissue biopsy, and skin blister fluid sampling, to name a few. These methods have a number of advantages, including a semi-invasive method, direct concentration measurement, multiple location measurement, continuous monitoring, low technological complexity, and low cost (Brunner and Langer 2006). These techniques are briefly exemplified for their functional roles.

5.1 Equilibrium Dialysis

Equilibrium dialysis is utilized for determining how much ligand is bound to a macromolecule (Lanao and Fraile 2005). Despite the fact that there is no standard for measuring in vitro protein binding, equilibrium dialysis remains routinely employed to determine therapeutic protein binding characteristics (Zeitlinger et al. 2011).

5.2 Isolated Organ Perfusion

By using a single pass or recirculation with the medium, the isolated organ perfusion technique can keep an organ alive. Distribution studies use a single pass, whereas metabolism and excretion investigations benefit from recirculation. This technique is often employed in distribution investigations involving various organs, including kidney, lung, and brain (Lanao and Fraile 2005). Chemotherapy is given to the target organ without disrupting the functionality of other organs using these isolated organ perfusion techniques.

5.3 Microdialysis

Microdialysis remains a preferable technique for assessing the pharmacokinetics of a drug as it is extremely valuable to determine in vivo protein bonding (Zeitlinger et al. 2011). It is a powerful semi-invasive sampling technique especially effective for explaining drug distribution and receptor phase pharmacokinetics (Brunner and Langer 2006). Microdialysis enables simultaneous monitoring of a number of physiological parameters including locomotor activity, convulsive activity, and blood pressure, making it an appropriate tool for drug pharmacokinetic-pharmacodynamic studies. The reverse microdialysis approach (Hocht et al. 2004; Rudin and Weissleder 2003) is a strong and effective tool for studying local drug effects in diverse tissues, particularly liver, brain nuclei, and skeletal muscle.

5.4 *Imaging Techniques*

Non-invasive imaging techniques such as autoradiography, magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), and positron emission tomography (PET) are routinely utilized in in vivo drug distribution research (Brunner and Langer 2006). Because imaging technologies are non-invasive, they may be used to conduct longitudinal investigations on a single animal, and that statistically enhances the significance of a study (Rudin and Weissleder 2003).

Neuroimaging techniques give precise anatomical, functional, and metabolic details of the human or animal brain in real time, which helps researchers better understand drug impacts on brain systems. MRI and PET can be used to explore disease pathogenesis in vivo, diagnose patients, and offer quantitative markers for disease status assessment (Wise and Tracey 2006; Gustafsson et al. 2017). Early biomarkers associated with neurological diseases, for example, epilepsy, brain tumors, PD, schizophrenia and Alzheimer's disease (AD), are commonly identified using these techniques (Wise and Tracey 2006; McGuire et al. 2008; Bertoglio et al. 2017; Zhao et al. 2017).

6 Conclusion

Preclinical in vivo studies are essential for assessing the pharmacokinetic and pharmacodynamic properties of drugs during development. These studies are necessary since in vitro research cannot provide quantitative data on absorption, distribution, metabolism, and excretion in animal and human models. The animal models are crucial in the process of drug discovery and development, and they have played a critical role in elucidating the critical processes behind many deadly human diseases. Animal models that are more similar to the human genome have shown to be very useful in drug development and discovery. These animals were chosen for their physiological and biochemical parallels to humans, as well as their underlying drug absorption, distribution, metabolism, and excretion systems. Transgenic models can modify the genetic composition of animal models, which is beneficial to examine the molecular mechanisms of human genome-related activities and develop new medications and testing procedures. Many modern techniques, such as MRI and microdialysis, have gradually superseded traditional approaches, such as skin blistering, in in vivo investigations. Undeniably, in vivo studies are the required stage in the drug discovery and development process; however, considerable effort remains to be done in order to make animal study results more comparable to human clinical trials.

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