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Chapter 9: Preclinical Evaluation of Nanocarriers: *In Vitro, Ex vivo,* and *In vivo* Models

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Abstract

The preclinical assessment of nanocarriers represents a vital stage in the creation of innovative drug delivery systems, ensuring their safety, effectiveness, and potential for translation into human clinical trials. This chapter explores the three main preclinical testing methods: *in vitro, ex vivo,* and *in vivo*, each providing unique benefits for evaluating the pharmacokinetic and pharmacodynamic properties of nanocarrier formulations. *In vitro* models, which include 2D cell cultures, co-culture systems, and sophisticated 3D spheroids or organoids, offer controlled settings for examining cytotoxicity, cellular uptake, and drug release kinetics. *Ex vivo* models, such as isolated perfused organs, excised tissues, and organ-on-chip platforms, serve as a link between cell culture and whole-animal studies by maintaining physiological structure and function, facilitating localized toxicity and penetration assessments. *In vivo* models, which involve both rodent and non-rodent species, allow for a thorough evaluation of biodistribution, metabolism, immunogenicity, and therapeutic effectiveness within a systemic framework. Particular attention is given to the selection of animal models based on the type of disease and the characteristics of the nanocarrier. The chapter also emphasizes the importance of imaging technologies (fluorescence, PET, MRI) and biomarkers for real-time monitoring and the development of predictive modeling systems. Furthermore, it addresses regulatory considerations and ethical issues, along with emerging trends in preclinical personalized models, such as patient-derived xenografts and humanized animals. These preclinical strategies provide the basis for the optimization of nanocarrier design and translation in clinical applications.

Keywords: *Nanocarriers, Preclinical Evaluation, Biodistribution, Toxicity, Organoids, Animal Models, Imaging Techniques, Translational Research.*

1. Introduction

Over the past few years, nanocarrier systems have become a ground-breaking approach in the field of pharmaceutics, providing novel solutions to long-standing drug delivery problems. Nanoscale delivery systems provide a large advantage over traditional methods of drug delivery by enhancing the therapeutic effects and safety of drugs via improved solubility, stability, and bioavailability(1). Along with establishing bioavailability, nanocarriers can also be designed to enable controlled and/or targeted release, limiting potential side effects throughout the body where the drug may be inactive, while allowing for an increased concentration of the drug at the site of action. They also allow for combinations of high versatility in payloads, can encapsulate any therapeutic agent, poorly water-soluble drugs, peptides, nucleic acids, etc(2). Because of this, nanocarrier systems have become integral to modern health care in administering advanced drug formulations and personalized medicine. In the development of any new therapeutic modalities, of course, the pre-clinical phase can be considered the first phase where the safety, efficacy, pharmacokinetics and toxicity profiles are established before clinical evaluation in humans. An effective preclinical strategy is imperative to lessen the chance of substantial late-stage failure, which can be costly financially and ethically(3). The process of drug design is becoming more intricate, and the expectations from regulatory bodies are rising as well; as a result, traditional models frequently fall short in accurately forecasting clinical success. This highlights the importance of incorporating next-generation *in vitro* systems, in silico modeling, and relevant *in vivo* techniques. Merging these technologies into a more predictive and all-encompassing preclinical framework will yield higher quality preclinical data and enhance decision-making during the initial stages of drug development (4).

The progression of pharmaceutical and biomedical research relies to a large extent on the ability to leverage experimental models that reproduce certain biological systems of interest. In this category, in vitro, ex vivo and in vivo models represent important tools in the preclinical evaluation of drugs and therapeutic interventions. In vitro models utilize isolated cells or tissues maintained outside of their respective biological context to provide a controlled environment to assess cellular responses, toxicity, mechanisms of drug action, etc(5). The use of in vitro models is effective, cost-efficient, reproduceable, and allows for high-throughput screening, however, they subjectively lack the whole organism complexity. Ex vivo models utilize organs or tissues isolated from an organism but allow researchers to conserve the original physiological architecture and function of the tissue or organ. This type of model allows researchers to study the organ-specific response in ex vivo experiments at near-physiological conditions. Nonetheless, they are only good until the viability is reached. *In vivo* models, which is experimentation in a living organism provide the fullest understanding of ADMET (absorption, distribution, metabolism, excretion, toxicity)(6). While *vivo*

models are required for transferring lab-based findings into clinical practice, they tend to be costly, ethically challenging, and slow. Throughout the development process, each model system has a unique and complementary role from basic science to safety and efficacy. The combination of model systems provides more insight into disease processes and potential therapies further bridging experimental data to clinical outcomes. Therefore, it is essential to choose the right model to produce a significant translatable outcome in drug discovery and biomedical innovation (6).

2. *In Vitro* Evaluation Models

In Vitro Evaluation Models are essential instruments in pharmaceutical and biomedical research. They enable researchers to comprehend cell-based responses (whether intrinsic or extrinsic to the specific treatment), the actions of drugs, and the physical/biological consequences of drug toxicity, all while avoiding the ethical dilemmas or restrictions linked to *in vivo* evaluations. These evaluation model systems utilize isolated cells, tissues or organoids that have been cultured outside of their biological milieu (typically), to mimic existing physiological/pathological conditions of interest for the specific evaluative need(7). The use of in vitro model systems allows for direct and detailed observation of biochemical, cellular and microenvironmental mechanisms responsible for disease progression or new drugs' therapeutic effects in vitro, along with high-throughput drug screening. With continual evolutions of cell culture techniques, creating in vitro models is becoming more accomplished through co-culture systems, 2D in vitro models, 3D in vitro models and organ-on-chip technologies. There is a distinct academic strength for each type of in vitro model 2D in vitro models are simple and inexpensive, while 3D in vitro and microfluidic models are more physiologically accurate than traditional 2D in vitro models with regards to the intrinsic architecture and function of native tissues(8). Because they are derived from human tissues, human-derived cells used in in vitro systems may lessen the need for animal-based models and increase the translational relevance of experimental results. In the development of drugs or other chemical agents, in vitro assays are useful for early-stage screening, cytotoxicity testing, and ADME (absorption, distribution, metabolism, and excretion) profiling. Regulatory agencies frequently require *in vitro* data as part of any preclinical *in vivo* evaluation aimed at evaluating safety margins in a human population prior to moving into clinical testing(9). Although valuable contributions to the scientific process, *in vitro* models cannot yet fully replicate any living organism's complexity. Therefore, while *in vitro* approaches cannot entirely eliminate the use of *in vivo* testing, they should be viewed as important tools for hypothesis development and testing, mechanistic insights, and preliminary assessment of safety and efficacy in contemporary biomedical research(10).

2.1. Cell Culture Models

Cell culture models are valuable tools in biomedical research, where researchers can control many environmental conditions to study the physiology, behavior, and biochemistry of cells outside of their natural context. Cell culture models involve the growth of cells in a controlled way in the lab, directly outside of the human body, growing them in nutrient-rich media in petri dishes or flasks. Cell culture systems allow the investigator to precisely and reproducibly investigate how cells respond to drugs, toxins, pathogens, and genetic changes(11). While cell culture models vary based on the environmental composition, they can mostly be divided into two-dimensional (2D) monolayer and three-dimensional (3D) cultures. 2D monolayer cultures are simpler and more widely adopted because of the ease of handling and associated lower costs, while the 3D cultures are more physiological and ideally suited to maintain the in vivo environment to support cell–cell and cell–matrix interactions. Technological advancements have led to organoids, spheroids, and lab-on-a-chip/systems that help improve the physiological relevancy of in vitro studies. These values models have value particularly for use in cancer research, drug screening, toxicology, and regenerative medicine(12). The even better highlight of using human-derived cells is that it helps reduce the need to utilize animal models and enhances the ability to more specifically predict the human response from said studies. Culture models of any kind are associated with inherent limitations such as loss of tissue architecture and function over time, contamination, and variability between cell lines. Nonetheless, new developments in culture techniques and biomaterials allows for increased complexity and provide additional relevance of the models used in biomedical research. In summary, cell culture continues to contribute significantly to modern experimental biology and translational medicine(13).

2.1.1 D monolayer cultures

Two-dimensional (2D) monolayer cell cultures are one of the most widely used in vitro models for biological research and pharmaceutical testing. In this methodology, cells are grown on flat, rigid surfaces (plastic or glass), where they can attach and flatten into a monolayer. 2D monolayer cultures are easy to use, economically viable, and reproducible; therefore, they are favorable for high-throughput applications for screening and drug cytotoxicity testing, and fundamental studies in cell biology(14). 2D experimental cell cultures facilitate the uniform exposure of cells to nutrients and drugs, easily observed under a microscope, and there is clear and easy data analysis of cellular responses to drugs and environmental conditions. However, 2D monolayer cultures, despite widespread use, can never provide true complexity or 3D architecture and microenvironment features of tissues found in vivo. 2D cultures offer little or no spatial cell−cell or cell−matrix interactions, which can affect specific cellular behaviors, which

can result in different patterns of gene expression, proliferation, and drug response from in vivo systems(15). This drawback has spurred the emergence of more advanced models, such as 3D cultures and organoids, that more closely simulate physiological environments. However, 2D cultures should not be completely disregarded as a significant force in early-stage research and are commonly paired with complex systems for validation purposes. The speed and convenience, as well as compatibility with established laboratory methods, will ensure that 2D monolayer cultures will be a mechanism to support biomedical research for the foreseeable future, just for mechanistic studies, gene editing studies, and early pharmacological screening(16).

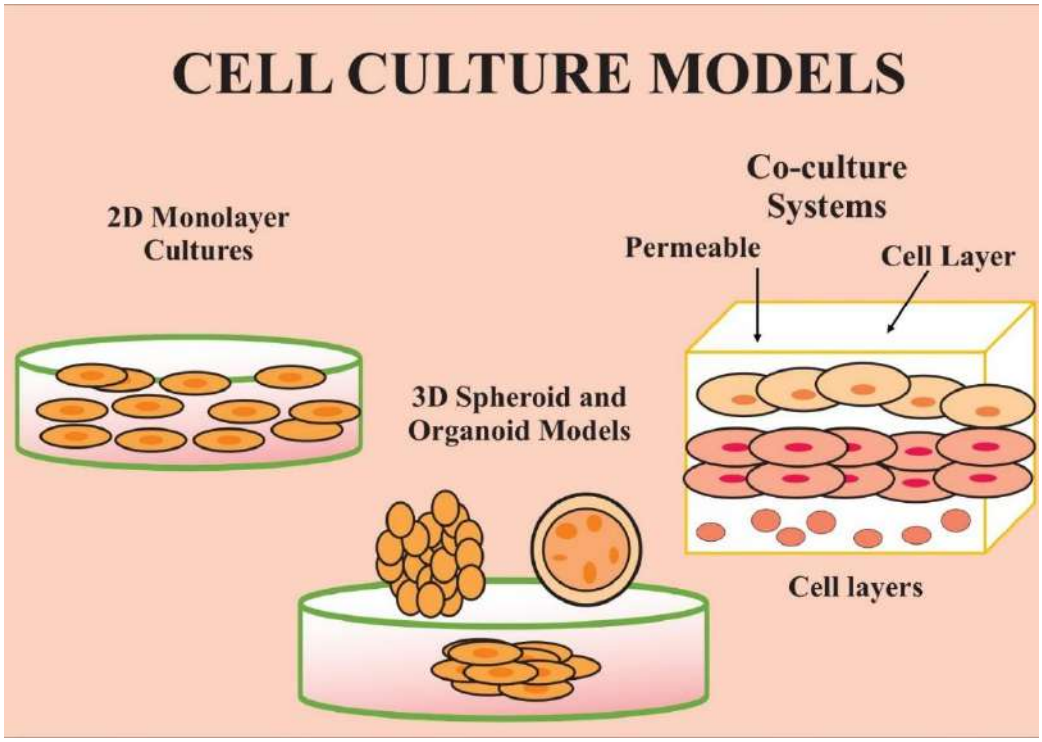

Fig.9.1: Overview of different cell culture models, including 2D monolayer cultures, 3D spheroid/organoid models, and co-culture systems with multiple cell layers.

2.1.2D spheroid and organoid models

Three-dimensional (3D) spheroid and organoid models have become innovative tools in the field of cell culture, encompassing a much more physiologically relevant systems compared to classic two-dimensional (2D) cultures. Spheroids can be defined as spherical clusters of cells which self-assemble, with cell-cell and cell-matrix interactions occurring much like they do in vivo. Spheroids are used extensively in cancer research, drug screening, and toxicity studies, as they can represent nutrient gradience, hypoxia,

and different proliferation zones, which are important features to study(17). Organoids are derived from stem cells and culture systems to create miniaturized versions of organs, with similar structural and functional aspects. Organoids can be developed from pluripotent or adult stem cells, and may contain different cell types, that build an organ-like architecture. As they can mimic organ structure, they are also useful for the study of organ development, disease modeling, and precision or personalized medicine. Organoids offer advancement research into tissue-specific responses or genetic variability(18). Overall, spheroids and organoids facilitate the examination of intricate cellular behaviors, including differentiation, migration, and signaling, in a more regulated way compared to traditional models. By closely replicating the *in vivo* microenvironment, they enhance the predictive power of experimental results. Furthermore, innovations in bioengineering and scaffold designs yield more consistent and scalable models that serve as valuable alternatives to animal models. These developments will enable spheroid and organoid models to engage future research efforts as precision medicine and regenerative therapeutics gain prominence. Although they will not entirely bridge the gap between *in vitro* studies and clinical applications, they are well-positioned to play a crucial role in pre-clinical and translational research. They not only revolutionize experimental biology but also re-evaluate all past and future biomedical studies (19).

2.1.3 Co-culture systems

Co-culture systems involve the use of distinct cell types clustered or cultured together and are considered sophisticated in vitro models of biologic interest and functionality. They allow for the exploration of cellular interactions in terms of paracrine cell signaling, cell-cell contact, and matrices. Co-cultures are useful for studying physiologic and pathologic processes such as tumor development and immune-mediated tissue damage, regenerative medicine, metabolism of drugs, etc. Dependent on the focus of study, co-culture systems can be direct (cells in contact with one another) or indirect (by a permeable membrane). Due to the above-mentioned attributes, co-culture systems are incredibly useful in developing an improved understanding of cellular behavior earlier observed by their application in stark and potentially less physiologic conditions of cell culture or Dutch practices of monoculture(20).

3. Evaluation Parameters

Table 9.1: *In Vitro* Evaluation Parameters for Nanocarrier-Based Drug Delivery Systems(21)

Parameter	Purpose	Common Methods/Techniques	Remarks
Cytotoxicity	To assess the toxic effects of nanocarriers on cell viability	MTT, XTT, LDH, Alamar Blue, Trypan Blue exclusion	Essential for preliminary safety profiling
Cellular Uptake	To determine the internalization of nanocarriers by cells	Fluorescence microscopy, Flow cytometry, Confocal microscopy	Indicates efficiency of nanocarrier delivery to target cells
Endocytosis Pathways	To identify the specific internalization mechanisms	Inhibitor-based studies, siRNA knockdown, Co-localization	Helps in understanding cellular entry routes (clathrin, caveolae, etc.)
Intracellular Trafficking	To trace nanocarrier movement and final destination within the cell	Fluorescent labeling, Live-cell imaging, Co-localization studies	Assists in understanding endosomal escape or lysosomal degradation
Drug Release Kinetics	To analyze the release profile of the loaded drug inside or outside cells	Dialysis method, HPLC, UV-Vis spectroscopy	Determines the controlled and sustained release potential
Hemocompatibility	To evaluate interaction with blood components (e.g., RBCs, platelets)	Hemolysis assay, Coagulation assays (PT, aPTT), Aggregation tests	Critical for IV administration and systemic circulation compatibility
Oxidative Stress Assays	To measure ROS generation or oxidative damage caused by nanocarriers	DCFH-DA assay, GSH/GSSG ratio, Lipid peroxidation (MDA assay)	Indicates potential pro-inflammatory or cytotoxic effects

3.1 Cytotoxicity (MTT, XTT, LDH assays)

Assessment of cytotoxicity is an essential component of evaluating the safety and biological responses of drug candidates, biomaterials, and environmental chemicals as determined using cell culture models. The MTT, XTT, and LDH cytotoxicity assays are the most common assays utilized for determining cytotoxicity, with each offering advantages based primarily on its sensitivity, reliability, and ease of use. The MTT assay measures cellular viability as determined by the conversion of yellow tetrazolium salt (MTT) to purple formazan crystals by mitochondrial enzymes in only metabolically active cells, where bioluminescence is generated. The XTT is a similar tetrazolium-based method that builds on some of the challenges of utilizing MTT by producing a soluble formazan product, thus eliminating a solubilization step, making it easier to work with for more efficient and high-throughput analysis(22).

The LDH (lactate dehydrogenase) assay, on the other hand, assesses membrane integrity by measuring cytoxic release of the cytosolic enzyme LDH into the culture medium. Release of LDH indicates damage or lysis of the cytosolic membrane. A LDH assay is also valuable for observing cytotoxicity for early-stage effects and is often used in combination with metabolic activity-based assays during evaluation. Both assays allow for analysis of dose-response and time-response of a test substance with cultured cells for either pre-clinical screening or toxicity profiling(23). By utilizing vitro testing methods, the use of animal models and associated ethical concerns can be reduced, and controlled conditions can be created to better assess cellular responses. LDH assays and similar, metabolic activity-based assays are still an approach utilized routinely in pharmacology, toxicology, and biomedical research. These assays provide uncomplicated, precise, and reproducible results and are a routine portion of information submitted to support cell health and viability(24).

3.2 Cellular uptake (fluorescence microscopy, flow cytometry)

Cellular uptake is a critical measurement parameter in cell culture models, especially for evaluating the intracellular delivery and biodistribution of nanocarrier systems. The cellular uptake parameter gives a measure of how much of the drug or nanoparticle is internalized by the cell, as delivery and biodistribution, will help dictate whether the therapeutic outcome is achieved. Fluorescent microscopy and flow cytometry are the two most common methodologies employed to study cellular uptake. Fluorescent microscopy provides qualitative data as it gives a visual representation of where ideally the fluorescently labelled carriers are located within the cellular compartments(25). Fluorescence microscopy enables the localisation of the cellular deposits of the drugs or nanoparticles to determine whether they are concentrated in the cytoplasm, nucleus or endosomes as well as to observe kinetic events as a function of time. Conversely, flow

cytometry provides a quantitative measurement, flow gives a measure of cellular internalization based on the fluorescent signal from a single cell amongst a large population of cells. Flow cytometry allows assessment of many cells in a short duration and analytical power to observe minor differences in uptake efficiencies between different formulations, concentrations, or incubation periods. To perform these analyses, nanocarriers are generally labeled with fluorescent dyes such as rhodamine, FITC, or coumarin-6(26). Subsequently, the cells are incubated with the nanocarriers and, after an adequate time for incubation, inoculated cells are washed to remove any contaminants (nanocarriers) which were not internalized. Typically, the experimental procedures for fluorescence microscopy (FM) and flow cytometry (FCM) are performed in a reproducible manner to ensure that all environmental variables remain fixed. Imaging techniques such as FM and FCM provide significant complementary data; FM generating images that allow high-resolution cellular and sub-cellular localization of the nanocarriers, while FCM generates utilization and high resolution and statistics at the population level. Both FM and FCM are important in the preclinical assessment of drug delivery from nanocarrier systems. They help determine whether the nanocarrier constructs designed achieve endocytosis, internalization to the desired intracellular location, and the wanted interaction with cellular components (molecular or sub-cellular) in the target cells for the therapeutic design(27).

3.3 Endocytosis pathways

Investigating endocytosis pathways in cell culture models is essential to understand the intracellular delivery and fate of nanocarrier systems. Endocytosis is the term for the range of mechanisms that cells use to internalize material; clathrin-mediated endocytosis, caveolae-based uptake, macropinocytosis, and phagocytosis all fall under this classification of biological processes. These pathways allow cells to internalize nanocarrier systems with variable efficiency and specificity that ultimately impact therapeutic delivery(28). Endocytosis is studied in vitro using a variety of models—examples include epithelial, endothelial, or cancer cell lines. It is common practice to fluorescently label nanocarriers and pair them with confocal microscopy, flow cytometry and pathway specific inhibitors to identify and quantify predominating mechanisms of non-carrier internalization. In this context, experimental design is a critical step-in understanding and monitoring endocytosis and therefore, pathways which allow nanoparticles to be design and optimized for mechanism selectivity and off-target delivery potential control would benefit from these evaluations(29).

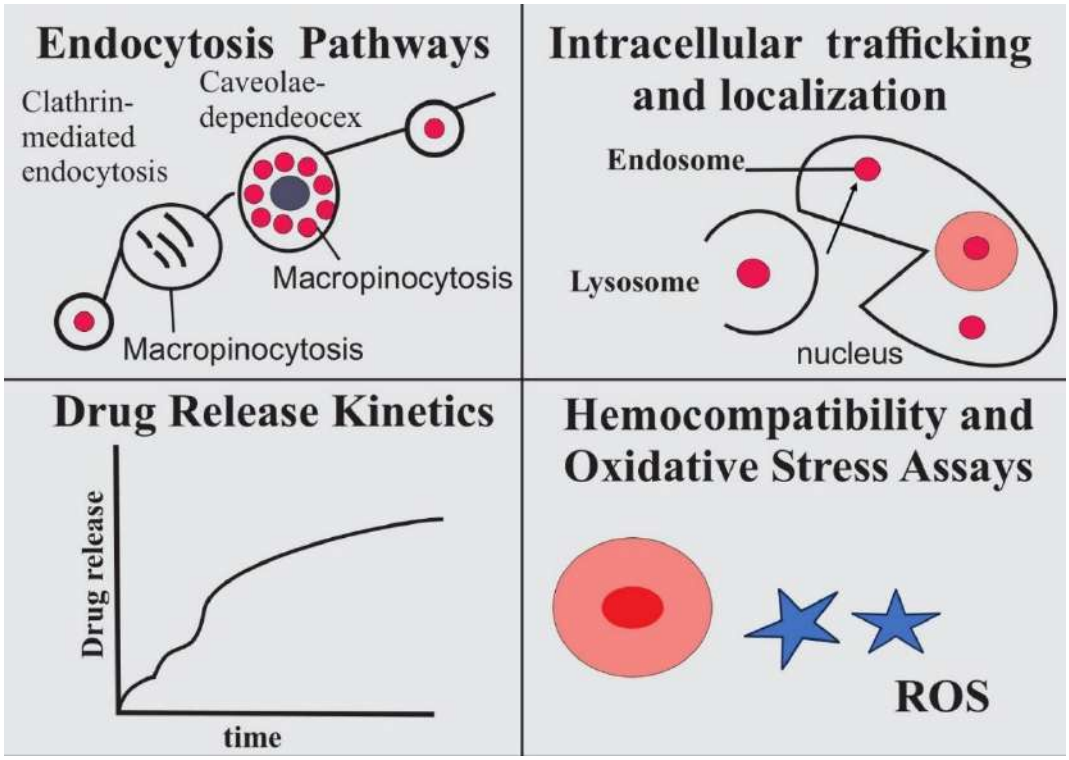

Fig.9.2: Illustration of cellular uptake mechanisms, intracellular trafficking, drug release kinetics, and biocompatibility assays for nanocarrier evaluation.

3.4 Intracellular trafficking and localization

Therapeutically and concerning safety, the intracellular destiny of the nanocarriers must be transparent. Cell culture systems constitute a controlled system to examine the internalization, trafficking, and regulation of nanocarriers within cells of a specialized nature. Among common methods used, there is confocal laser scanning microscopy, transmission electron microscopy (TEM), or fluorescence tracking, through which it is possible to examine the transport of nanocarriers from the plasma membrane to organelles like endosomes, lysosomes, or even the nucleus(30). These investigations will clarify if the process involves endocytosis, endosomal escape, and accumulation at action sites, all of which are crucial steps in optimizing delivery systems. Moreover, co-staining with organelle-specific markers enables the identification of their locations, offering insights into nanocarrier interactions with intracellular pathways. This research contributes to laying the groundwork for the synthesis of nanocarriers that possess improved target affinity and reduced off-target effects (31).

3.5 *In-vitro* drug release studies

The kinetics of drug release is a crucial factor assessed through cell culture models to investigate the effects of nanocarrier systems *in vivo*. These models mimic the biological environment and help in establishing the time-dependent release profile of a drug from its carrier. *In vitro* release studies are typically conducted using methods such as dialysis membranes, cell monolayers, or Franz diffusion cells, which act as tissue barriers. The release profile provides essential insights into the drug release rate, the underlying mechanism, and the degree of drug diffusion, all of which are critical for predicting *in vivo* results (32). The mechanism of release is defined as being either diffusion-controlled, erosion-based, or a hybrid of both, utilizing specific mathematical models such as zero-order, first-order, Higuchi, and Korsmeyer-Peppas. Evaluating drug release kinetics in cell culture environments is essential for optimizing formulation parameters and ensuring the effective controlled and sustained release of the drug for clinical applications (33).

3.6 Studies for oxidative stress and hemocompatibility

Cell culture models play a crucial role in the preclinical evaluation of nanocarrier systems, especially in assessing hemocompatibility and oxidative stress. Hemocompatibility focuses on the interaction between nanocarriers and blood components, ensuring they do not induce harmful effects like hemolysis, platelet aggregation, or coagulation. *In vitro* hemolysis assays using red blood cells determine the safety of nanocarrier formulations for intravenous use. Additionally, tests for complement activation and coagulation times (PT, aPTT) provide further validation of their compatibility with the bloodstream (34). Concomitantly, oxidative stress assays examine both the generation of ROS and their impact on the cellular redox state. These assays typically measure oxidative stress markers such as glutathione (GSH), malondialdehyde (MDA) or employ fluorescent probes like DCFH-DA to detect ROS. We must monitor oxidative stress because if ROS levels are too high they can induce cytotoxicity, inflammatory responses, or apoptosis. Together, these measures provide key information on the safety and biocompatibility of nanocarrier systems in vitro before moving to in vivo models(35).

4. Ex Vivo Evaluation Models

4.1. Tissue-Based Assays

Tissue-based ex vivo assays provide an important middle ground between in vitro cell culture and in vivo animal models. These assays often utilize freshly isolated tissue slices or organ slices, preserving the native tissue architecture and cellular interactions.

Standard tissues are skin, intestinal mucosa, liver slice, and ocular tissues, depending upon the route of administration or target organ. Such models allow evaluation of parameters including drug permeability, retention, metabolism, and local toxicity. For example, excised skin in Franz diffusion cells is widely used to investigate transdermal drug delivery. The preservation of cell–cell and cell–matrix interactions within the tissue enhances the predictive capacity of these assays, making them invaluable tools for evaluating the performance and safety of nanocarrier systems before progressing to animal studies(36).

Table 9.2*: Ex Vivo* Evaluation Models for Nanocarrier Systems(37)

Model Type	Examples	Applications	Advantages	Limitations
Isolated Perfused Organs	Liver, Lungs	- Study of organ-specific distribution - Metabolism	- Maintains organ architecture and microcirculation - Real-time assessment	- Technically complex - Short viability period
Mucosal Tissue Models	Intestinal, Buccal, Nasal mucosa	- Absorption studies - Drug permeability	- Mimics physiological barrier - High relevance to mucosal delivery	- Limited tissue availability - Inter-donor variability
Skin Penetration Models	Human/animal skin using Franz diffusion cells	- Transdermal delivery - Skin retention and permeation	- Preserves skin barrier function - Standardized protocol available	- Restricted to passive diffusion studies - Short-term evaluations only

4.2 Isolated perfused organs (e.g., liver, lungs)

Ex vivo models using isolated perfused organs provide a controlled environment to assess the pharmacokinetics, metabolism, and toxicity of nanocarrier systems without the complexity of whole-animal interactions. In this method, organs like the liver or lungs are surgically extracted from the animal and preserved in a perfusion system that simulates physiological conditions. The organ stays functional for several hours, enabling real-time examination of drug distribution, absorption, and metabolic changes (38). While the isolated perfused lung paradigm makes it easier to assess pulmonary targeting and retention, the isolated perfused liver (IPL) model is especially useful for researching hepatic metabolism and biliary excretion of nanoparticles. By lowering the

number of animals needed, these models provide great repeatability and ethical benefits, bridging the gap between *in vitro* and *in vivo* research (39).

4.3 Models of mucosal tissue (oral, buccal, and intestinal)

When examining medication absorption, permeability, and retention across biological membranes, *ex vivo* mucosal tissue models are essential resources. In order to more accurately resemble the physiological environment than in vitro systems, these models incorporate recently removed animal or human tissues, usually from the colon, buccal cavity, or nasal tube. Intestinal mucosal tissues are often utilized to evaluate oral drug delivery and forecast patterns of absorption throughout the gastrointestinal tract. Buccal mucosa, which is provided with a non-keratinized epithelial lining and adequate vascularity, provides an excellent interface for the evaluation of transmucosal drug delivery free from hepatic first-pass effects(40). At the same time, nasal mucosal tissues are utilized to investigate intranasal formulations because they have the potential for rapid systemic absorption and nose-to-brain delivery routes. Such models enable studying drug transport, integrity of tissues, enzymatic activity, and toxicity under near-physiological conditions, which renders them extremely useful for preclinical evaluation of nanocarrier-based delivery systems(41).

4.4 Skin penetration studies (Franz diffusion cells)

Ex vivo skin penetration experiments are a key measure in assessing the transdermal delivery ability of nanocarrier systems. Franz diffusion cells are most commonly used for this application, providing an excellent source of a valid and reproducible means of determining the permeation profile of active pharmaceutical ingredients across excised animal or human skin. The nanocarrier formulation is typically placed on the donor side of a patch of skin that is positioned between the donor and receptor chambers, while the bottom chamber contains a receptor medium that simulates physiological conditions. (42). To determine the rate and degree of drug penetration, samples from the receptor compartment are periodically collected and examined. This model is a crucial stage in the preclinical assessment of transdermal drug administration because it offers valuable information about the diffusion kinetics, reservoir phenomena inside the layers of skin, and formulation effectiveness (43).

4.5 Advantages and Limitations

Ex vivo models, which offer an environment that is calibrated to a biological system but less complex than a full organism, bridge the gap between *in vitro* experiments and *in vivo* research. One of their advantages is being able to assess drug responses, toxicity, or the behavior of nanocarriers in a more precise environment because of the preservation

of the native tissue architecture and functional integrity. With ex vivo models, it is possible to investigate cell–cell and cell–matrix interactions, which are neglected in monolayer cultures(44). However, ex vivo systems are generally constrained to short-term evaluations because of the gradual loss of tissue viability and function. Their short lifespan, absence of systemic immune responses, and metabolism interactions limit their ability to predict chronic effects and pharmacokinetics. Thus, although the insights obtained and the reduction in animal testing reliance is significant, ex vivo models have to be supplemented with in vivo studies to achieve thorough preclinical evaluations(45).

5. *In Vivo* Evaluation Models

Although *in vivo* models remain the gold standard for assessing the therapeutic efficacy, pharmacokinetics, and toxicity of novel drug delivery systems, including nanocarriers, cell culture models offer valuable preliminary data that can support and refine *in vivo* experimentation. These *in vitro* platforms are designed to mimic specific physiological and pathological conditions, enabling early prediction of *in vivo* responses (46). For instance, 3D spheroid cultures, organ-on-chip systems, and co-culture models replicate aspects of the tissue microenvironment, allowing researchers to evaluate cellular uptake, metabolism, and potential cytotoxicity prior to animal studies. By closely simulating the complexities of *in vivo* conditions, these cell culture models help reduce the number of animals required for testing, refine the experimental design, and enhance the translational relevance of preclinical data. Thus, they serve as an essential bridge between *in vitro* assays and full-scale *in vivo* evaluations (47).

Table 9.3*: In Vivo* Evaluation Models and Parameters for Nanocarrier Systems(48)

Category	Subcategory	Description / Application
Animal Models Used	Rodents (mice, rats)	Commonly used due to low cost, ease of handling, and well-established disease models.
	Zebrafish	Transparent body allows real-time imaging; suitable for rapid toxicity and biodistribution studies.
	Rabbits, dogs, non-human primates	Employed in advanced stages for closer physiological relevance to humans.
Pharmacokinetics and Biodistribution	ADME Profiling	Assesses Absorption, Distribution, Metabolism, and Excretion of nanocarriers.
	Imaging Techniques	Includes MRI, PET, and fluorescence imaging to track nanocarrier distribution in vivo.
	Tissue Distribution and Accumulation	Measures localization and retention in organs or target tissues using imaging or dissection.

Therapeutic Efficacy Evaluation	Tumor Regression	Evaluated in oncology models to assess reduction in tumor size post-treatment.
	Disease-specific Models	Includes models for neurological, cardiovascular, and infectious diseases to evaluate efficacy.
Toxicity Assessment	Acute and Chronic Toxicity	Determines immediate and long-term adverse effects of nanocarrier systems.
	Histopathological Analysis	Microscopic examination of tissues to detect cellular and structural toxicity.
	Hematological and Biochemical Profiling	Blood and serum analysis for organ function and systemic toxicity markers.

5.1. Animal Models Used

5.1.1 Rodents (mice, rats)

Mice and rats serve as popular models in biomedical research because of their similarities to humans, shorter generations, and convenience to work with. Preclinical testing of nanocarrier systems is aided by their genetically modified strains and well-characterized physiology. In cell culture model validation, primary and established cell lines derived from rodents are commonly used to investigate toxicity and therapeutic responsiveness. Transgenic and knockout mice provide in vivo models for the study of various biological processes and diseases, while rats are preferred for pharmacokinetic and pharmacodynamic studies because of their size and the sampling routes. These rodent models are crucial in translating in vitro research to the clinic(49).

5.1.2 Zebrafish

Zebrafish (*Danio rerio*) have emerged as a powerful vertebrate model for preclinical evaluation of drug delivery systems, including nanocarriers. Their small size, transparent embryos, rapid development, and high genetic similarity to humans make them an attractive alternative to traditional mammalian models. In nanomedicine research, zebrafish offer unique advantages for real-time imaging of biodistribution, toxicity, and therapeutic effects due to their optical transparency during early developmental stages. Additionally, their amenability to high-throughput screening allows researchers to assess multiple formulations efficiently(50). Zebrafish models are particularly valuable for studying vascular targeting, organ-specific accumulation, and developmental toxicity of nanosystems. The ability to genetically modify zebrafish further enhances their utility in mechanistic studies and disease modeling. Thus, the zebrafish model serves as a versatile and cost-effective platform bridging the gap between in vitro cell-based assays and more complex mammalian models in the evaluation of nanocarrier-based therapeutics(51).

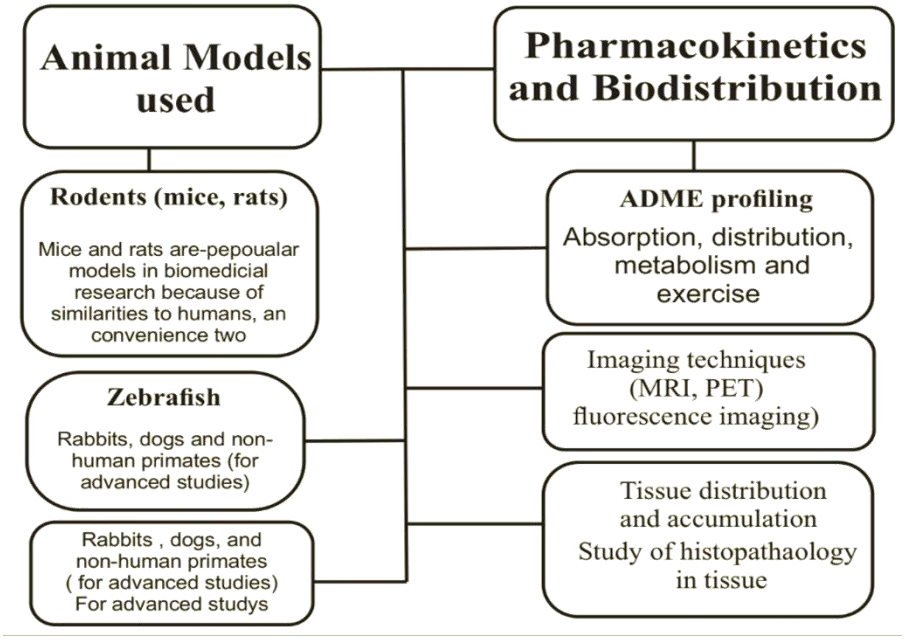

Fig.9.3: Overview of animal models used and key pharmacokinetic and biodistribution assessments in preclinical drug evaluation.

5.1.3 Rabbits, dogs, and non-human primates (for advanced studies)

In advanced stages of preclinical evaluation, animal models such as rabbits, dogs, and non-human primates play a pivotal role in bridging the gap between in vitro findings and human clinical trials. These species are selectively employed due to their physiological and anatomical similarities to humans, which enable a more accurate prediction of pharmacokinetic, pharmacodynamic, and toxicological outcomes. Rabbits are commonly used in ocular, dermal, and pyrogen testing, offering ease of handling and well-established baseline data(52). Dogs, particularly beagles, are favored in cardiovascular, gastrointestinal, and metabolic studies due to their size and consistent response to pharmacological agents. Non-human primates, although used sparingly and under strict ethical regulations, provide invaluable insights in immunological, neurological, and complex systemic studies because of their close genetic and physiological resemblance to humans. The use of these animal models is essential in validating safety, efficacy, and dosing strategies before proceeding to human trials(53).

6. Pharmacokinetics and Biodistribution

6.1 ADME profiling

Estimating the therapeutic potential and safety profiles of nanocarrier systems requires an understanding of their pharmacokinetic mechanisms and biodistribution. Detailed information about how these systems interact with one another and how they are absorbed in biological systems can be obtained by profiling ADME, or absorption, distribution, metabolism, and excretion. Distribution studies show the location of the carrier in tissues and organs, including potential off-target accumulation, while assessment of absorption assesses the nanocarrier and its payload, indicating how far and how quickly they reach circulation levels (54). The evaluations of metabolism offer insights into the enzymatic mechanisms involved in the bioconversion of the nanomaterial or its associated cargo. This information is crucial for understanding both efficacy and toxicity. Additionally, excretion studies yield data on the biokinetic parameters of the systems concerning the clearance from the body, typically through renal or hepatobiliary pathways. All these parameters are vital for optimizing the formulation and dosage, and they play a significant role in the regulatory approval processes (55).

6.2 Imaging techniques (MRI, PET, fluorescence imaging)

The development of non-invasive imaging modalities like Ultrasound, MRI, PET, and Fluorescence imaging has proven fruitful for the pharmacokinetics and biodistribution studies of nanocarrier-enabled drug delivery systems. MRI permits the visualization of nanocarrier-associated contrast agents in different organs and provides high spatial resolution and soft-tissue contrast for real time imaging. PET is famous for its high sensitivity and offers quantitative measurements of radio-labeled formulations(56). Moreover, PET provides information concerning the absorption and distribution as well as clearance of the drug over time. Fluorescence imaging has the advantage of detecting fluorescently labeled particles in small animal models. Despite restriction on penetration depth, this imaging technique still provides high sensitivity and specificity for visualization. Collectively, the above imaging techniques enhance overall understanding of the in vivo behavior of nanocarriers which in turn helps optimize the formulation design, targeting, safety features and efficiency(57).

6.3 Tissue distribution and accumulation

Understanding tissue distribution and accumulation is important for determining the in vivo behavior of nanocarrier-administered drug delivery systems. After administration of the nanocarrier, these systems will take time to circulate within the blood stream and

could interact with various physiological barriers, leading to tissue distribution within the organs and tissues in the body. The amount and type of tissue distribution will be impacted by characteristics of the nanocarrier itself such as the size, surface charge, shape, and surface modification characteristics of the system(58). Tissues such as liver, spleen, and lungs are highly vascularized and have extensive mononuclear phagocyte system (MPS) activity, and thus would be associated with a greater accumulation of nanoparticles. While extended retention and accumulation in non-target tissues has significance for potential toxicity, accumulation in target tissues can represent the desired effects of enhanced therapeutic efficacy. It is therefore imperative that in the preclinical development of nanocarrier systems that the biodistribution is assessed with radiolabel imaging, quantitative determination of accumulation levels in tissues, and through histopathological assessments(59).

7. Therapeutic Efficacy Evaluation

7.1 Tumor regression (oncology models)

In cancer models of therapeutic efficacy, the extent of tumor regression, or reduction in tumor size or mass after treatment, is considered a direct metric of the anticancer effects produced by a nanocarrier drug formulation. As a primary measure of efficacy, tumor regression is often assessed during preclinical studies via an increase in anti-cancerous pharmacist company product by utilizing orthotopic, genetically modified, and xenografted murine tumor models. The investigators used calipers or measures of tumor volume at regular intervals, and in most instances used measure of imaging techniques (e.g., MRI, PET, or bioluminescence) or histopathology(60). Researchers used key metrics, including percentage tumor growth inhibition (TGI), tumor doubling time, and complete and/or partial response rates to quantitate efficacy. These studies assist in quantifying pharmacological activity, optimal dosing schedule to assess bioavailability, and measurements of nanocarrier changes that will influence drug delivery of the regime. In conclusion, during tumor regression studies that assess the translational GLP relevance of nanomedicine in oncology, where a demonstrable therapeutic benefit is observed in vivo models, the studies allow researchers to demonstrate a clear advantage, prodrug activity demonstrated by anticancerous agent given with a nanocarrier(61).

7.2 Disease-specific models (neurological, cardiovascular, infectious)

Therapeutic efficacy assessment is important in preclinical studies assessing the use of nanocarrier systems for targeted drug delivery. Disease-related models offer a powerful means to assess the ability of formulations to duplicate the pathophysiology exhibited by human disorders. For example, neurological models related to Alzheimer's and Parkinson's disease, and models mimicking seizures allow for assessment of the ability

of nanocarriers to cross the blood-brain barrier and to deliver drugs to desired regions of the brain(62). Cardiovascular models of disease related to myocardial infarction, atherosclerosis or hypertension, can be examined for therapeutic outcomes including plaque regression, cardiac output or vascular remodeling following treatment. Infectious disease models related to bacterial sepsis, or a viral infection, or a parasitic disease to assess the ability of drug loaded nanocarriers to reduce microbial load, change immune response, and increase overall survival. Collectively, all models provide evidence of pharmacodynamic response, effectiveness of drug delivery targeting, and overall, the desired therapeutic benefit to aid in the advancement of interventions using nanomedicines(63).

7.3. Toxicity Assessment

A thorough assessment of toxicity is an important part of the preclinical evaluation of nanocarrier systems, as it evaluates these systems for safety and tolerability prior to potential translation into clinical studies. Acute toxicity studies aim to identify the negative effects of a single or short-term dosage of a nanocarrier system. Generally, acute toxicity studies examine the effects of being exposed to a substance/make observation in a short period of time (typically, the maximum in 24-72 hours post-exposure). Acute toxicity studies examine many factors (mortality, effects on characteristics such as activity levels, behaviour, or physical condition (such as changes in breathing rates or discoloration of paws), potential effects and damages on organ/tissue structures and functions after an exposure period, or toxicological evaluations)(64). Chronic toxicity studies evaluate potential cumulative or delayed toxic effects of the nanocarrier system on organ systems and associated physiological functions after being exposed to doses over long periods of time (often from weeks to months). Histopathological assessments (to evaluate tissue structure) are a component of toxicity assessments by performing a histopathological evaluation of excised tissues from organs exploring potential damages. Organs that are often examined for histopathology include the liver, kidney, heart, and spleen. Hematological/tissue/biochemical profiling can provide meaningful insights of potential systemic toxicity, from observed toxicities/tissues including blood parameters and blood and tissues signals of (for example) red and/or white blood cell counts, hemoglobin levels, liver enzymes levels (AST, ALT), kidney indications (creatinine levels, urea levels), blood/lipid profiles, etc. Collectively, these components contribute to an overall understanding of the potential safety of the nanocarrier system and guide dosage selection for the next steps(65).

8. Translational Relevance and Regulatory Perspective

Toxicity evaluation is an important part of the preclinical exploration of nanocarrier-based drug delivery systems to assess the safety profile of a formulation. Acute toxicity refers to the toxic effects that occur in a short duration (usually 24 - 72-hour period) after a single or multiple exposure to a testing substance. The impact of acute toxicity evaluation is typically what is put forward in ordering an estimate for lethal dose (LD_{50}), and indications of the organ that could be affected. While it may differ somewhat amongst organisations, there are standard protocols, e.g. OECD, that ensure the animal model employed serves to assess evidence of toxicity, such as behaviour, changes in weight, mortality, necropsy and histopathology examinations. Chronic toxicity involves longer studies (i.e., for weeks/months). The principal focus of chronic toxicity is long-term toxic effects such as carcinogenicity, irreversible organ dysfunction, or developmental/reproductive toxicity (or other long-term effects). Within the chronic assessments will also case the none-observed-adverse-effect level (NOAEL), which is an important endpoint relevant for risk and dosage assessments in humans. The observations from acute and chronic toxicity assessments together provide a snapshot of the potential safety and tolerability issues along the way as we assess the utility of the nanocarrier systems for clinical application(66).

Assessing nanomedicines presents distinct challenges; they differ from conventional pharmaceuticals in both their physicochemical properties and the evaluation methods required. The U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have recognized that evaluating nanoscale formulations necessitates a specialized regulatory approach to guarantee safety and effectiveness. The FDA has proposed an evaluation strategy based on a case-by-case approach, with a focus on full characterization (size, shape, surface charge, composition) of the nanoparticles, along with in vitro and in vivo toxicity data. The FDA's guidance suggests reconsidering various aspects of the device or product as early as possible with regulators before commercialization to address early-stage concerns(67). The EMA similarly outlined some specific issues related to medicinal products utilizing nanotechnology, such as biodistribution, immunogenicity, and long-term toxicity. The EMA encourages a total risk-based approach; this should include standard toxicity testing, as well as additional studies targeting the potential hazards unique to nanoparticles. Together, the FDA and EMA efforts are designed to create a framework to promote the considered development of novel and innovative nanomedicines for the future of health care without compromising safety(68).

Toxicity evaluation is an important part of preclinical testing for the safety of new drug products (including nanocarrier systems). Standardization and Good Laboratory Practices (GLP) are crucial to achieve consistent, reproducible, and reliable studies. GLP is part of a systematic quality management controls and technical standards that encourage the integrity and validity of nonclinical laboratory studies. GLP principles are

used to determine how the study is to be planned, performed, monitored, recorded, reported, and archived so that the data can be traced, thus, compliant with current government regulations or industry standards(69). Standardization emphasizes the uniformity of acceptable validated procedures, reference materials, and assay controls to reduce laboratory-to laboratory variability and increase comparability of results. Combined, GLP and standardization help to create acceptable scientific confidence in toxicity data to gain subsequent market approval, and when candidate formulations have met a predetermined safety threshold, the advancement and ultimate success through clinical trials(70).

9. Challenges and Future Perspectives

Despite the considerable advancements made in nanocarrier-based drug delivery systems, some challenges remain focused on preclinical evaluation. One main obstacle is the limited predictability of the current *in vitro, ex vivo,* and *in vivo* models that are often used to evaluate nanocarriers. The reason for this is that the models used do not adequately simulate the complexity of human systems which will lead to inconsistent outcomes of preclinical investigations and subsequent clinical efficacy or safety in patients. Investigations are currently underway to find solutions to this problem, potentially involving microfluidic (organ-on-a-chip) systems. These dynamic systems facilitate a more human-relevant physiological environment (71). In addition, the ethical considerations of working with animals have encouraged researchers to develop and adopt the 3Rs (Replace, Reduce, Refine) approach when evaluating clinical efficacy in spite of maxing the use of animals and improve their welfare during the development of alternative testing strategies. Looking forward, there is a demonstrated need for integrating a very complex, multi-model approach using integrated data across multiple platforms from studies in traditional computational, *in vitro, ex vivo,* and *in vivo* - to increase the predictability of translational outcomes while simultaneously speeding up the safe and effective development of nanocarrier systems for clinical application(72).

Conclusion

Preclinical assessment is a necessary step to demonstrate safety, efficacy, and translation of any nanocarrier product to the clinic. A systematic approach, with well-defined stages, commencing with assessment of cytotoxicity and cellular uptake *in vitro*, followed by *ex vivo* tissue studies, and into *in vivo* investigations, provides a useful outline for assessing these systems. Each of the models used serves a unique purpose, including, for example, the *in vitro* systems to screen metabolic impact quickly and mechanistically; *ex vivo* models to retain the tissue architecture and focus on cells while bridging the *in vitro to in vivo; and in vivo* to procure data on pharmacokinetics, biodistribution, immunogenicity, and efficacy. Utilizing a comprehensive approach across multiple preclinical models will expand the predictive capabilities of preclinical results, while ideally limiting the reliance on animals models through forward planning and adherence to the 3Rs (Replacement, Reduction, Refinement). With the field evolving through the introduction of advanced models such as organ-on-chip platforms and computational modeling systems, it is presumed that preclinical development will further underscore compliance and efficiency, while being more ethical regarding the use of animals and with more alignment to not only regulatory expectations, but to scientific expectations. Collectively, this combination of models should provide required evidence of translational capacity for in clinic/nanocarrier delivery therapeutics in early drug development in a timely manner.

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