IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 20, Issue 3 Ser. 3 (May. – June. 2025), PP 12-28
www.iosrjournals.org

"Drug Metabolism and Toxicology"

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Abstract:
Drug metabolism and toxicology are essential branches of pharmacological science that collectively contribute to the safe and effective use of medications. Drug metabolism involves the chemical alteration of pharmaceutical substances by the body, mainly in the liver, where lipophilic compounds are converted into more water-soluble forms to enable their elimination. This metabolic process occurs in two distinct phases: Phase I, which includes reactions like oxidation and hydrolysis often mediated by enzymes such as cytochrome P450, and Phase II, which involves conjugation with molecules like glucuronic acid to enhance solubility. Factors such as genetic variation, age, dietary habits, existing health conditions, and interactions with other drugs can significantly impact metabolic rates and the effectiveness of treatment. Simultaneously, drug toxicology focuses on identifying and understanding the negative effects of drugs on living systems. It explores the mechanisms by which drugs may cause harm, including organ-specific damage such as liver, kidney, or heart toxicity. The concept of the dose-response relationship guides toxicological analysis and includes both laboratory and clinical testing to determine safety thresholds, therapeutic indices, and potential lethal doses. Regulatory bodies, ethical standards, and monitoring systems play a crucial role in minimizing risks and ensuring safe drug use. Recognizing the relationship between metabolism and toxicity is vital in clinical practice, as it aids in optimizing dosage, preventing harmful interactions, and enabling personalized treatment through genetic screening. During the drug development process, these insights help detect potentially harmful metabolites early and guide the design of safer, more effective drugs. Therefore, a thorough understanding of both metabolism and toxicology is key to improving therapeutic outcomes and ensuring patient safety.

Key Words: *Hepatotoxicity, Nephrotoxicity, Neurotoxicity, Cardiotoxicity, Reproductive, Toxicology, Pharmaceuticals, Regulation, Terminology, Biomarkers, Screening, Mechanisms, Validation, Standards, ETC.*

Date of Submission: 09-06-2025 Date of Acceptance: 20-06-2025

LITERATURE OF PAPER:

- **BERT N. LA DU & H. GEORGE MANDEL (2025):** Fundamentals of Drug Metabolism and Drug Disposition.

- **FRANZ J. HOCK & MICHAEL K. PUGSLEY (SPRINGER NATURE, 2025**): Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays Approx. 2550 pages, ISBN 3031355296 / 978-3031355295

I. INTRODUCTION:

Drug metabolism is the biochemical process by which the body modifies pharmaceutical substances. It plays a critical role in determining the effectiveness, duration, and potential toxicity of drugs. Primarily occurring in the liver, drug metabolism transforms lipophilic (fat-soluble) compounds into more hydrophilic (water-soluble) forms that can be easily excreted from the body through urine or bile.

5.PHASES OF DRUG METABOLISM:

Drug metabolism generally occurs in two main phases:

5.1.PHASE I (FUNCTIONALIZATION REACTIONS):

In Phase I, enzymes introduce or expose functional groups on the drug molecule. This is often accomplished through oxidation, reduction, or hydrolysis reactions. The most important enzymes involved are the cytochrome P450 (CYP450) family. These enzymes oxidize drugs, making them slightly more water-soluble and often biologically inactive, although in some cases, they convert drugs into more active or toxic forms (e.g., prodrugs into their active metabolites).[1] Phase I metabolism, often referred to as the functionalization phase, marks the first crucial step in the biotransformation of pharmaceutical agents and various foreign substances (xenobiotics) within the human body. This metabolic stage is essential for converting lipophilic (fat-soluble) drug

molecules into more hydrophilic (water-soluble) forms, thereby promoting their elimination through bodily fluids such as urine or bile. The liver serves as the principal site for these reactions, where a vast array of enzymes alter the chemical structure of drug molecules to facilitate their further metabolism or excretion.[3] Primarily, Phase I reactions involve oxidation, but reduction and hydrolysis also contribute significantly. These processes work to introduce or reveal reactive functional groups, such as hydroxyl (-OH), amino (-NH_2), sulfhydryl (-SH), or carboxylic acid (-COOH), within the molecular structure of drugs. These functional groups make the drug more water-soluble and chemically suitable for subsequent Phase II conjugation or, in some cases, direct excretion.[6] The cytochrome P450 family of enzymes (commonly abbreviated as CYP450) plays a dominant role in catalyzing oxidative reactions during Phase I metabolism. These enzymes are predominantly found in the smooth endoplasmic reticulum of liver cells, although they are also present in other organs like the intestines, lungs, and kidneys. CYP450 enzymes utilize molecular oxygen to add an oxygen atom to drug substrates while reducing the second oxygen atom to water. This enzymatic activity can dramatically alter the drug's biological activity, potentially deactivating it, converting a prodrug into its therapeutic form, or sometimes forming harmful byproducts.[12] There are various types of oxidation reactions that CYP450 enzymes can catalyze, including hydroxylation of aliphatic or aromatic rings, N-oxidation, S-oxidation, and dealkylation reactions. In hydroxylation, a hydroxyl group is added to carbon chains or aromatic rings, increasing the compound's polarity. Dealkylation involves the removal of alkyl groups from atoms like nitrogen, oxygen, or sulfur. These changes not only improve solubility but also set the stage for Phase II metabolism. The specific outcomes of these reactions depend on both the chemical structure of the drug and the characteristics of the CYP450 isoenzymes involved. Variability among isoforms leads to differences in how individuals metabolize the same drug, often influenced by genetic polymorphisms.[4] Reduction reactions, although less frequent than oxidation, are still vital in certain physiological contexts, especially in areas of low oxygen supply such as the gastrointestinal tract. These reactions typically involve the enzymatic reduction of functional groups like nitro, azo, or carbonyl moieties within a drug molecule. The products are often pharmacologically inactive or less active, and like oxidation products, they are more easily eliminated from the body.[3] Hydrolysis is another important Phase I process, in which ester or amide bonds within drug molecules are cleaved by enzymes such as esterases and amidases. These reactions yield more polar compounds such as alcohols, carboxylic acids, and amines. Hydrolytic reactions are particularly important for the activation of prodrugs. For example, aspirin (acetylsalicylic acid) undergoes hydrolysis in the body to release salicylic acid, which is the active anti-inflammatory agent. Although the primary goal of Phase I metabolism is detoxification, the process can sometimes generate reactive intermediates with toxic potential. These metabolites may form covalent bonds with cellular macromolecules like proteins, nucleic acids, or membrane lipids, leading to cellular injury, oxidative stress, or even mutagenesis and cancer. This dual nature of metabolism underscores the importance of thoroughly studying Phase I reactions during drug development. Recognizing the potential for harmful metabolite formation allows pharmaceutical scientists to design safer drugs, modify molecular structures, or employ enzyme inhibitors to redirect metabolic pathways away from toxicity.[1] Several factors influence the efficiency and variability of Phase I metabolism. These include genetic differences, age, sex, liver health, environmental exposures, and the presence of other drugs that may either induce or inhibit metabolic enzymes. For instance, individuals with certain genetic polymorphisms in enzymes like CYP2D6 may metabolize drugs more slowly or rapidly, affecting therapeutic outcomes. Poor metabolizers may experience reduced efficacy due to limited conversion of prodrugs, while ultra-rapid metabolizers may encounter toxicity from the accumulation of active metabolites. Moreover, drug interactions frequently arise due to the modulation of CYP450 activity. Some drugs act as inducers, speeding up metabolism and reducing drug concentrations in the bloodstream, while others act as inhibitors, slowing metabolism and raising the risk of drug accumulation and toxicity. For example, rifampicin, a potent inducer of CYP3A4, can decrease the effectiveness of drugs metabolized by the same enzyme. Conversely, antifungal drugs like ketoconazole inhibit CYP3A4, potentially leading to toxic accumulation of co-administered medications.[12] Phase I metabolism is fundamental to the body's ability to manage and eliminate drugs. By introducing functional groups and increasing drug solubility, these reactions prepare compounds for further modification or excretion. However, the potential for generating reactive or harmful metabolites necessitates careful consideration in pharmacology and drug design. Understanding the complexities of Phase I functionalization reactions, the enzymes involved, and the individual variations in metabolic capacity is critical for ensuring both drug efficacy and safety in clinical practice.

5.1.1.Example: The conversion of codeine into morphine by CYP2D6 enzyme is a classic Phase I metabolic reaction:

Codeine is metabolized in the body through two main biochemical pathways. In the majority of individuals, approximately 80% of the administered dose is converted into codeine-6-glucuronide via a conjugation reaction, a metabolite that exhibits minimal analgesic properties. A much smaller fraction—generally less than 10%—is transformed into morphine through O-demethylation, a process primarily catalyzed by the enzyme CYP2D6. This morphine metabolite is largely responsible for codeine's therapeutic pain-relieving effect.[17] CYP2D6 is a member of the cytochrome P450 enzyme superfamily, which plays a crucial role in

metabolizing a wide range of pharmaceutical drugs and naturally occurring substances in the body. Although these enzymes are distributed throughout various tissues, they are predominantly found in the liver. Their activity and expression can be significantly influenced by genetic differences, which vary widely among different ethnic groups. For instance, alterations in CYP2D6 function are observed more frequently in individuals of Caucasian (5–10%) and African descent (up to 34%) compared to Asian populations, where such changes are rare (less than 1%).[1] These genetic differences can greatly impact how an individual metabolizes certain medications, including codeine. Depending on the efficiency of CYP2D6-mediated metabolism, codeine can be converted to morphine in varying amounts. In some individuals, this conversion may be insufficient, rendering the drug ineffective, while in others, excessive conversion can lead to toxicity. As a result, codeine may not be a suitable analgesic for everyone, and individual metabolic capacity should be considered when prescribing it.[23]

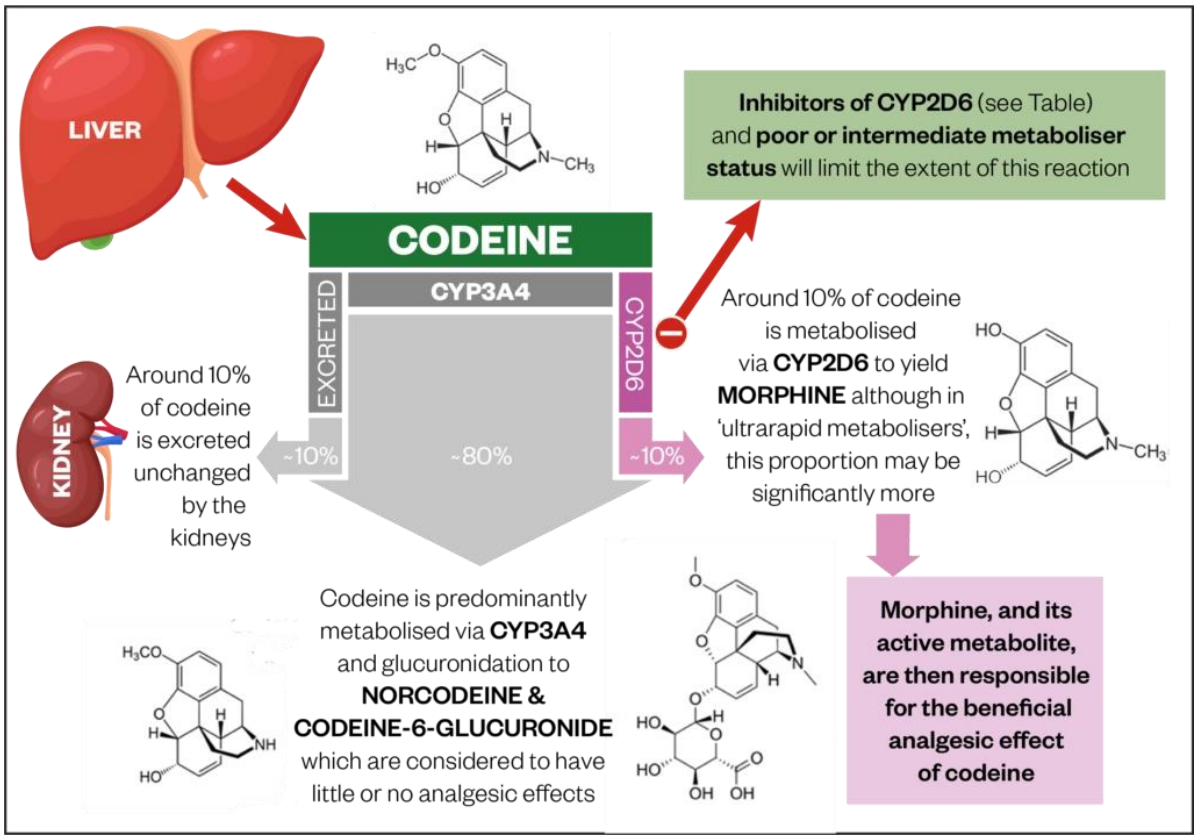

FIGURE: 1: THE HEPATIC METABOLISM OF CODEINE

5.2.PHASE II (CONJUGATION REACTIONS):

Phase II involves the conjugation of the drug or its Phase I metabolites with endogenous molecules such as glucuronic acid, sulfuric acid, or amino acids. This process increases the compound's water solubility significantly, facilitating its elimination.[24] Phase II metabolism, often referred to as conjugation reactions, represents a crucial step in the biotransformation of drugs and xenobiotics in the body. This phase typically follows Phase I reactions, which introduce or expose reactive functional groups on the drug molecule. While Phase I may involve oxidation, reduction, or hydrolysis to make the compound somewhat more polar, Phase II reactions further enhance the water solubility of the drug by covalently attaching endogenous, hydrophilic molecules. This increased hydrophilicity facilitates the efficient excretion of the drug or its metabolites through the kidneys or bile, thereby aiding in detoxification and clearance.[26] The primary purpose of Phase II metabolism is to detoxify potentially harmful intermediates produced during Phase I or to directly modify parent compounds that already contain functional groups amenable to conjugation. Unlike Phase I reactions, which often activate or deactivate drug molecules, Phase II conjugations generally result in pharmacologically inactive metabolites. These conjugated products are usually larger, more polar, and less likely to cross cellular membranes, minimizing their potential toxicity and facilitating elimination from the body.[29] Several types of conjugation reactions occur in Phase II metabolism, each involving different endogenous substrates. Common conjugates include glucuronic acid, sulfate, glutathione, amino acids, and acetate, among others. Each conjugation type is catalyzed by specific enzymes that recognize the functional groups on the drug or metabolite, ensuring efficient and selective modification.[3] One of the most prevalent and well-studied Phase II reactions is glucuronidation. This process involves the attachment of glucuronic acid, derived from uridine diphosphate glucuronic acid (UDPGA), to drugs

or metabolites containing hydroxyl, carboxyl, amino, or sulfhydryl groups. The enzyme family responsible for this reaction is the UDP-glucuronosyltransferases (UGTs), which are predominantly expressed in the liver but also found in other tissues such as the intestine and kidney. Glucuronides formed are highly water-soluble and generally inactive, making them ideal for renal or biliary excretion. Many commonly used drugs, including morphine, bilirubin, and acetaminophen, undergo glucuronidation as a major metabolic pathway.[17] Sulfation is another important conjugation reaction in Phase II metabolism. This process transfers a sulfonate group from the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to hydroxyl or amine groups on the substrate. Sulfotransferase enzymes catalyze this reaction, and sulfation is especially significant for the metabolism of endogenous compounds such as hormones and neurotransmitters, as well as certain drugs and environmental chemicals. Sulfate conjugates are highly polar and readily excreted in urine. Although sulfation is often a minor pathway compared to glucuronidation, it plays a crucial role in metabolizing compounds that are poor substrates for UGT enzymes.[21] Glutathione conjugation represents a unique Phase II pathway primarily involved in detoxifying reactive electrophilic compounds that may arise from Phase I metabolism or environmental exposure. Glutathione (GSH), a tripeptide composed of glutamate, cysteine, and glycine, acts as a nucleophile, binding to electrophilic centers on toxic metabolites. This reaction is catalyzed by glutathione S-transferases (GSTs), which help prevent the binding of these reactive species to cellular macromolecules such as DNA or proteins, thereby protecting cells from oxidative stress and damage. The glutathione conjugates formed are subsequently processed into mercapturic acids, which are excreted in urine. Glutathione conjugation is particularly important in the metabolism of drugs like acetaminophen, where toxic intermediates can cause severe liver damage if not neutralized.[17] Amino acid conjugation involves the covalent attachment of amino acids such as glycine, glutamine, or taurine to carboxylic acid groups on drugs or metabolites. This pathway is catalyzed by enzymes such as acyl-CoA synthetases and N-acyltransferases. Amino acid conjugation increases water solubility and usually results in pharmacologically inactive products. It is significant for the metabolism of aromatic acids and other xenobiotics, facilitating their excretion.[33] Acetylation and methylation are also considered Phase II conjugation reactions, although they differ slightly as they involve the addition of small alkyl groups rather than large polar conjugates. Acetylation, catalyzed by N-acetyltransferases (NATs), attaches an acetyl group to amines or hydrazine's on drug molecules, often decreasing their polarity and biological activity. Methylation, performed by methyltransferases, transfers a methyl group from S-adenosylmethionine (SAM) to hydroxyl, amine, or thiol groups. These reactions can influence drug activity and solubility, but are less commonly involved in drug clearance compared to glucuronidation or sulfation.[31] The efficiency and capacity of Phase II metabolism can vary widely between individuals due to genetic polymorphisms, age, diet, disease states, and environmental exposures. For example, genetic variations in UGT enzymes may lead to altered glucuronidation activity, impacting drug clearance and response. In neonates and elderly patients, Phase II metabolic pathways are often underdeveloped or impaired, which can result in slower elimination of drugs and increased risk of toxicity. Certain diseases, particularly liver diseases such as cirrhosis, can also compromise conjugation reactions. Additionally, dietary components, herbal supplements, and concurrent medications can induce or inhibit Phase II enzymes, leading to drug interactions.[12] Clinically, understanding Phase II metabolism is critical for predicting drug clearance, potential drug interactions, and adverse effects. Some drugs are designed as prodrugs that require Phase II conjugation for activation or deactivation. In other cases, toxic metabolites generated during Phase I are rapidly detoxified through Phase II conjugation, preventing harmful accumulation. For example, the analgesic acetaminophen undergoes Phase II glucuronidation and sulfation to form safe metabolites; however, in overdose situations, these pathways become saturated, leading to the formation of toxic intermediates that cause liver injury.[11] In drug development, Phase II metabolism is a key consideration when evaluating the safety and pharmacokinetics of new compounds. Identifying the conjugation pathways helps in anticipating potential metabolites, optimizing chemical structures for better metabolism, and minimizing toxicity. Furthermore, measuring Phase II metabolites in biological samples is valuable in therapeutic drug monitoring and toxicological assessments.[9] Phase II conjugation reactions are essential metabolic processes that transform drugs and their Phase I metabolites into more hydrophilic, less toxic, and excretable forms. This phase involves a variety of enzymatic pathways, each utilizing endogenous substrates to increase drug polarity and facilitate elimination. Variability in Phase II metabolism can significantly influence drug response and toxicity, highlighting the importance of this phase in clinical pharmacology and drug development.[4]

5.2.1.Example: Paracetamol (acetaminophen) undergoes glucuronidation and sulfation in Phase II metabolism for excretion:

At present, the detailed molecular mechanisms responsible for acetaminophen (APAP)-induced acute liver injury remain incompletely understood. Nonetheless, it is generally accepted that under therapeutic conditions, around 85% of APAP is metabolized through sulfation and glucuronidation pathways, resulting in water-soluble conjugates that are eliminated via urine or bile. The remaining 15% is processed by cytochrome P450 (CYP450) enzymes, predominantly by the isoenzyme CYP2E1, into a reactive and toxic intermediate known as N-acetyl-p-benzoquinone imine (NAPQI). Under normal physiological conditions, NAPQI is promptly neutralized by conjugation with glutathione (GSH), forming non-toxic sulfate and cysteine derivatives, which are

then excreted by the kidneys. Additionally, the CYP2A6 enzyme contributes to APAP metabolism by converting it into a non-toxic catechol derivative, 3-hydroxy-APAP (3-OH-APAP). However, during an overdose, the capacity of conjugation pathways becomes overwhelmed, leading to increased NAPQI formation. This results in liver damage through multiple processes, including oxidative stress, covalent binding to cellular macromolecules, disruption of mitochondrial function, and endoplasmic reticulum (ER) stress.[36]

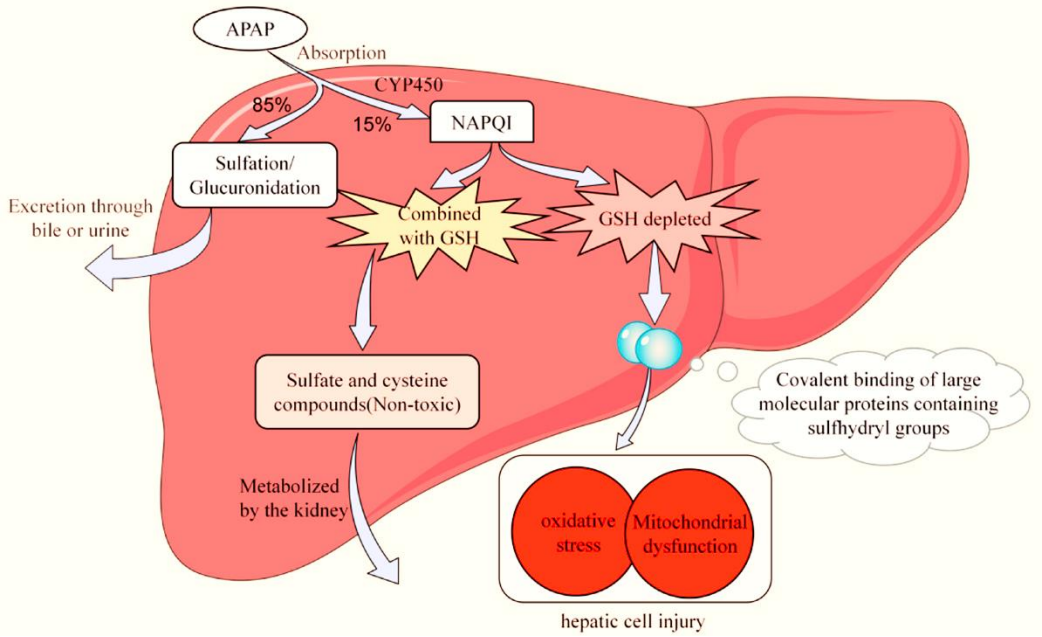

FIGURE: 2: ACETAMINOPHEN (APAP) IS PRIMARILY METABOLIZED IN THE LIVER BY CONJUGATION WITH SULFATE AND GLUCURONIDE, WHICH ARE EXCRETED THROUGH BILE OR URINE. A SMALLER PORTION IS PROCESSED BY CYTOCHROME P450 ENZYMES INTO A TOXIC METABOLITE CALLED NAPQI. NORMALLY, NAPQI BINDS TO GLUTATHIONE (GSH) AND IS SAFELY ELIMINATED BY THE KIDNEYS. HOWEVER, IF GSH IS DEPLETED EXCESSIVELY, NAPQI ACCUMULATES IN THE LIVER, CAUSING LIVER DAMAGE.

5.3.FACTORS AFFECTING DRUG METABOLISM:

Drug metabolism varies greatly among individuals due to several factors:

- **Genetic polymorphisms:** Genetic differences in CYP450 enzymes can lead to rapid or slow metabolism.
- **Age and sex:** Neonates and elderly patients may metabolize drugs more slowly.
- **Diet and environment:** Certain foods and chemicals (like grapefruit juice or cigarette smoke) can inhibit or induce metabolic enzymes.
- **Disease states:** Liver diseases such as cirrhosis can impair drug metabolism.
- **Drug interactions:** Some drugs can inhibit or induce enzymes that metabolize other drugs, leading to altered therapeutic effects or toxicity.

6.TOXICOLOGY:

Drug toxicology is the scientific study of the adverse effects, harmful properties, and toxic potential of drugs on biological systems. It is a crucial field in pharmacology and medicine, focusing on understanding how drugs can cause damage, determining their safety margins, and guiding appropriate therapeutic usage. The ultimate goal of drug toxicology is to ensure patient safety by identifying and minimizing the risks associated with drug use.[40]

6.1.DRUG TOXICOLOGY:

6.1.1.MECHANISMS OF TOXICITY:

Drugs can exert toxic effects by interacting with cellular proteins, DNA, or organ systems in undesirable ways. These effects may be dose-dependent (predictable and related to the drug's pharmacological action) or idiosyncratic (unpredictable and not related to dose or known mechanisms). Some drugs form reactive metabolites that bind to cellular components, causing cellular stress, apoptosis, or necrosis.[11]

6.1.2.TYPES OF TOXICITY:
Drug toxicity can affect various organs:

- **Hepatotoxicity** (liver damage) – e.g., acetaminophen overdose
- **Nephrotoxicity** (kidney damage) – e.g., aminoglycoside antibiotics
- **Neurotoxicity** (brain/nervous system) – e.g., chemotherapy drugs
- **Cardiotoxicity** (heart toxicity) – e.g., doxorubicin
- **Reproductive toxicity** – causing infertility or birth defects

6.1.3.DOSE-RESPONSE RELATIONSHIP:
Toxicology follows the principle that "the dose makes the poison." Even water can be toxic at high doses, while toxic drugs can be safe at therapeutic levels. The dose-response curve helps determine the drug's **therapeutic index (TI)** – the ratio between the toxic dose and the effective dose. A high TI indicates a safer drug.[4]

6.1.4.PRECLINICAL AND CLINICAL EVALUATION:
Before a drug reaches the market, it undergoes extensive toxicological testing:

- **In vitro studies** (cell-based assays)
- **In vivo studies** (animal models)
- **Phase I–IV clinical trials** in humans
  These studies help identify the no-observed-adverse-effect-level (NOAEL), lowest-observed-adverse-effect-level (LOAEL), and lethal dose (LD_{50}).

6.1.5.REGULATORY AND ETHICAL CONSIDERATIONS:
Drug toxicology is regulated by agencies such as the FDA (U.S.), EMA (Europe), and CDSCO (India). Toxicity data is essential for drug approval and labeling. Ethical standards ensure minimal harm to animal and human subjects during testing.[6]

6.1.6.THERAPEUTIC DRUG MONITORING (TDM) AND **POISON CONTROL:**
TDM helps in maintaining drugs within therapeutic levels and avoiding toxicity. Poison control centers and toxicologists play a crucial role in managing drug overdoses and accidental poisoning through antidotes and supportive care.[9]

7.TOXICOLOGY REDUCTION BY THE ANTIMETABOLITE METHOTREXATE:

Methotrexate is a well-established antimetabolite and antifolate drug widely used in the treatment of various cancers, autoimmune disorders, and inflammatory conditions. Despite its effectiveness, methotrexate is associated with potential toxic effects, particularly in high doses. However, several strategies have been developed to reduce its toxicological impact while maintaining therapeutic efficacy.[1][2] Methotrexate works by inhibiting the enzyme dihydrofolate reductase (DHFR), which is essential for DNA synthesis and cell replication. This action disrupts the formation of tetrahydrofolate, thereby blocking the production of purines and thymidylate, which are necessary for nucleic acid synthesis. This mechanism is especially effective against rapidly dividing cancer cells and overactive immune cells.[9] To minimize methotrexate-related toxicity, a well-known strategy involves the use of leucovorin rescue therapy. Leucovorin (folinic acid) is a reduced form of folic acid that bypasses the blocked DHFR pathway, replenishing folate stores in normal, healthy cells without interfering with methotrexate's action on malignant or overactive cells. Administering leucovorin shortly after high-dose methotrexate helps protect non-target cells from folate depletion and subsequent damage, significantly lowering the risk of severe adverse effects such as bone marrow suppression, gastrointestinal toxicity, and mucositis.[10] Additionally, therapeutic drug monitoring (TDM) is employed during methotrexate therapy, especially in oncology. Regular measurement of serum methotrexate levels helps guide dosing and the timing of leucovorin administration, ensuring the drug remains within a safe and effective range. This individualized approach reduces the risk of cumulative toxicity, particularly in patients with renal impairment, where methotrexate clearance is slower and toxicity risk is higher.[16] Another key method to reduce methotrexate toxicity is adequate hydration and urinary alkalinization, especially during high-dose regimens. Methotrexate and its metabolites can precipitate in acidic urine, causing nephrotoxicity. Alkalinizing the urine with sodium bicarbonate and maintaining good hydration helps enhance methotrexate solubility and promotes efficient renal excretion, thereby reducing renal damage.[3] Furthermore, dose adjustments based on patient-specific factors—such as age, liver and kidney function, and concurrent medications—play a vital role in minimizing toxic outcomes. Certain drugs like NSAIDs, penicillin's, and proton pump inhibitors can interfere with methotrexate excretion and increase its toxicity. Therefore, careful selection and timing of co-administered drugs are essential.[31] Although methotrexate carries toxic potential, especially at high doses, its safety profile can be significantly improved through clinical strategies such as leucovorin rescue, therapeutic monitoring, hydration with urinary alkalinization, and individualized dosing. These interventions

collectively help reduce methotrexate-induced toxicity, allowing it to be used safely and effectively in clinical settings.

7.1.METHOTREXATE SIDE EFFECTS:

7.1.1.RENAL TOXICITY ASSOCIATED WITH METHOTREXATE:

High-dose methotrexate (HD-MTX) treatment, while effective, carries a risk of acute renal failure, primarily due to acute tubular necrosis. Though this condition is relatively rare—occurring in about 2–4% of patients—it is a serious and potentially life-threatening complication. The primary mechanism involves the precipitation of methotrexate or its metabolites within the renal tubules. This crystallization leads to tubular obstruction, impaired renal clearance, and prolonged elevation of methotrexate levels in the bloodstream. As a result, the effectiveness of leucovorin (folinic acid) rescue therapy may diminish, and the risk of systemic toxicity from methotrexate increases significantly.[13] In addition to causing mechanical blockage, methotrexate can exert a direct toxic effect on renal tubular epithelial cells. It has also been shown to cause vasoconstriction of the afferent arterioles, further compromising renal perfusion and function. Importantly, methotrexate and its metabolites are not readily soluble in acidic urine. Raising the urinary pH improves solubility and reduces the likelihood of crystal formation in the renal tubules.[1] To prevent renal toxicity, it is essential to monitor renal function closely before, during, and after the administration of methotrexate. Standard preventive measures include vigorous intravenous hydration and alkalinization of the urine—both of which are initiated before and maintained throughout methotrexate therapy. These practices help facilitate renal clearance and minimize the accumulation of toxic metabolites.[3] Clinically, acute kidney injury resulting from methotrexate is often asymptomatic and typically presents as a non-oliguric form of renal failure. In most cases, kidney function recovers within two to three weeks. However, some patients may exhibit early warning signs such as nausea, vomiting, or diarrhoea, which precede the onset of renal dysfunction.[12] Managing methotrexate-induced nephrotoxicity relies heavily on timely urine alkalinization and the use of leucovorin rescue. In more severe cases where methotrexate levels remain elevated, renal replacement therapies like peritoneal dialysis, haemodialysis, or hemofiltration may be employed. While these techniques can help remove methotrexate from circulation, they are invasive and may lead to a rebound increase in methotrexate plasma concentration post-dialysis.[39] Additional pharmacological interventions have been explored. Thymidine, a naturally occurring nucleoside, offers protective effects by bypassing the metabolic blockade induced by methotrexate. Unlike folates, it enters cells through a separate mechanism and directly contributes to DNA synthesis by forming thymidine monophosphate. Another promising strategy involves the use of carboxypeptidase G2 (CPDG2), an enzyme that breaks down methotrexate into an inactive metabolite called DAMPA (4-amino-4-deoxy-N10-methylpteroic acid). This enzymatic conversion provides an alternative pathway for methotrexate detoxification and clearance.

7.1.2.NEUROTOXICITY ASSOCIATED WITH METHOTREXATE (MTX):

Methotrexate has been linked to a spectrum of neurotoxic effects, which can manifest in acute, subacute, or chronic forms. These adverse neurological outcomes are most commonly observed following intrathecal or high-dose intravenous administration of the drug. Although the precise mechanisms behind MTX-induced neurotoxicity are not yet fully established, several biological pathways have been proposed.[33] One hypothesis suggests that MTX disrupts transmethylation processes essential for synthesizing critical cellular components like proteins, lipids, and myelin. This interference can impair central nervous system (CNS) function. MTX has also been shown to reduce methionine and S-adenosylmethionine concentrations in the cerebrospinal fluid (CSF), while simultaneously elevating levels of homocysteine and S-adenosylhomocysteine. Increased homocysteine, in particular, is believed to contribute to vascular dysfunction within the brain, potentially exacerbating neurotoxic outcomes.[27] Among the most frequently reported acute neurologic complications is leukoencephalopathy. This condition can range from asymptomatic findings detectable only through magnetic resonance imaging (MRI) to more pronounced clinical presentations, including confusion, agitation, sleep disturbances, seizures, and even coma. Additional symptoms such as headache, nausea, vomiting, and aseptic meningitis can also occur shortly after intrathecal methotrexate administration. These signs are usually transient, emerging within 12 to 72 hours of treatment, and tend to resolve once the drug is withdrawn.[22] Subacute neurotoxic effects generally appear a few weeks after initiating methotrexate therapy. They may include paraplegia, cerebellar ataxia, and seizure activity. Although less common than acute toxicity, these manifestations can significantly impact patient quality of life and require prompt clinical attention.[37] Chronic methotrexate-induced neurotoxicity typically develops several months or even years after treatment. This form is often irreversible and more likely in patients who have also received cranial radiation as part of their therapeutic regimen. The most severe chronic complication is necrotic leukoencephalopathy, a progressive and debilitating condition characterized by worsening cognitive impairment, persistent seizures, gait abnormalities, muscle stiffness, and potentially coma.[38] Treatment options for methotrexate neurotoxicity include supportive interventions such as the administration of leucovorin (folinic acid), which helps to mitigate the drug's adverse effects on folate metabolism. Aminophylline has also been employed in some cases, particularly in the management of acute symptoms. Methotrexate's neurotoxicity spans

a broad clinical spectrum and can affect patients at various stages of treatment. Early recognition of neurological signs, careful monitoring, and appropriate intervention are essential in minimizing long-term damage and improving patient outcomes.

8.HAZARD IDENTIFICATION AND RISK ASSESSMENT:

8.1.TARGET ORGAN TOXICITY AND EXPERIMENTAL MODELS:

A significant portion—around 70%—of toxic effects relevant to humans can be identified using experimental animal models. However, the accuracy and clinical relevance of these findings are largely dependent on the specific organ targeted by the drug. Among adverse drug reactions (ADRs) that pose the greatest risk to humans, the liver, heart, and central nervous system are the most frequently affected.[22] Current methodologies for identifying dose-related liver toxicity, particularly hepatocellular damage, are well-established in both cell-based in vitro systems and animal models. Despite these capabilities, drug-induced liver injury (DILI) remains complex and multifactorial. In addition, notable differences in how species respond to drugs emphasize the need for more predictive, human-relevant models—especially those that can account for patient-specific immune responses or genetic susceptibilities.[23] In the context of cardiovascular safety, the combined application of in vitro assays and animal studies is a standard practice. For instance, interactions with cardiac ion channels can be reliably assessed through techniques like patch-clamp studies. However, more intricate processes—such as how a drug affects heart rate, cardiac output, and blood pressure—typically require whole-animal models to fully understand potential downstream effects, including organ damage.[12] Neurological adverse drug reactions (ADRs) are even more difficult to predict early in development. Many of these effects are only identified during human clinical trials because they often involve rare molecular targets or emerge only after long-term use—conditions that are challenging to replicate in cell-based assays.[19] As for other toxic effects, such as blood disorders or the potential for cancer development, there are currently few reliable in vitro models available. This is largely due to the complexity and variability of these disease processes. Nevertheless, research is rapidly advancing in the field of organ-specific and multi-organ technologies. Emerging systems, such as organotypic cultures and micro-physiological systems (MPS), hold significant promise. These three-dimensional (3D) models aim to replicate the structure and function of human tissues more accurately and may greatly improve the ability to detect drug-induced toxicities during the preclinical safety assessment phase.

8.2.DISEASE MODELS:

Disease models are essential tools for mimicking organ-level functions and replicating the characteristic features of human pathologies. These models are developed using both cell or tissue-based systems and traditional or genetically modified animal models. The presence of disease within a biological system can significantly influence how a substance behaves, particularly in terms of toxicity. Therefore, understanding disease-specific responses is critical when evaluating the safety and efficacy of new drug candidates in their intended patient populations.[1] Many animal models designed to simulate human diseases have shown limited effectiveness in predicting clinical outcomes. This shortcoming often contributes to the gap between preclinical success and failure in later-stage human clinical trials. The lack of accurate disease representation can lead to misleading results, reducing the reliability of both efficacy and safety predictions. To address these limitations, future toxicity assessments are expected to increasingly integrate humanized in vitro disease models. These advanced systems can bridge the gap between laboratory studies and real-world clinical responses. By closely replicating human disease phenotypes, such models can enhance both drug discovery and safety evaluation processes. Moreover, they allow for better prediction of how various patient subpopulations may respond to a treatment, thus supporting the development of personalized and mechanism-based therapeutic strategies.[22] Incorporating these models into the early stages of drug testing can improve the accuracy of safety margin determinations and potentially reduce late-stage clinical trial failures, ultimately streamlining the drug development pipeline.

TABLE 1: CATEGORIES OF SAFETY ATTRITION CHALLENGES:

ATTRITION ORGAN/SYSTEM	DISCOVERY (CELL & TISSUE-BASED MODELS)	NON-CLINICAL (TOX SPECIES)	TRANSLATIONAL VALUE & GAPS	HAZARD IDENTIFICATION	RISK ASSESSMENT	PREDICTIVE NOTES
Heart	•	•	Functional changes detectable; limited for structural alterations	•	•	(1)
Liver (Hepatocellular)	•	•	Good in vitro-in vivo correlation for hepatocellular injury	•	•	(2)
Liver (Cholestasis)	•	x	Animal models poorly predictive of cholestasis	•	x	

CNS (Neurological)	o	x	Models exist for behaviour, seizures, abuse; lacking for cognition/suicidal ideation	x	•	(1,2)
Gastrointestinal	o	x	Limited and poorly predictive models	x		
Kidney	o	x	Translational biomarkers useful for detecting injury	x	•	(1,2)
Immune System	x	x	Poor predictivity for hypersensitivity; no early testing models available	x	x	
Lung	•	•	Relatively predictive models	•	•	(1,2)
Haematology	x	x	No in vitro model; good animal-human correlation	x	•	(1,2)
Haemopoiesis	x	x	No in vitro model; animal models correlate well with human data	x	•	(1,2)
Skin (Irritation/Sensitization)	•	•	In vitro models available; confirmed by in vivo where needed	o	•	(2,3)
Reproductive Organs & Embryo-Foetal Development	x	x	Effective for embryo-foetal toxicity; limited for reproductive organ effects	x	•	(2)
Genetic Toxicity & Carcinogenicity	• / o	x	Good for genotoxicity; limited carcinogenicity prediction	• / o	•	(2)

Legend:

- • = Models routinely available/in use
- x = No model available
- o = Models under evaluation for R&D applicability
- **Predictive Reference Notes**:
  - (1) = Large species (e.g., dog or monkey)
  - (2) = Rodent models
  - (3) = Lagomorphs

8.3.SAFETY MARGINS:

Safety margins play a critical role in evaluating the risk–benefit profile of a drug candidate. These margins are often defined by the dose-limiting toxicity and the No Observed Adverse Effect Level (NOAEL). The NOAEL is used to determine the highest dose at which no harmful effects are observed, thereby helping to estimate the therapeutic window of a drug. However, establishing the NOAEL can sometimes be subjective, as it is typically based on biochemical or histological changes that may not be consistently interpreted across studies.[33] One of the major challenges in accurately assessing safety margins is the current lack of highly specific and validated safety biomarkers that are both mechanistically informative and reliably translatable to humans. Without such biomarkers, findings from preclinical animal models offer only approximate estimations of human safety margins. Therefore, it becomes essential to prioritize drug candidates with broad safety margins during early development stages to enhance their chances of success in clinical trials.[32] Despite these efforts, some adverse toxicological findings can still emerge during the clinical phase, revealing narrower safety margins than anticipated. These discrepancies highlight the need for more predictive tools in early-stage testing. Recent progress in identifying novel biomarkers with improved sensitivity and mechanistic relevance offers promising solutions. Such biomarkers could enhance real-time monitoring of drug-induced toxicities and support better decision-making in both preclinical and clinical environments.[39] Incorporating mechanistic insights from toxic kinetics and toxic dynamics (TKTD), along with pharmacokinetic and pharmacodynamics (PKPD) modelling, can strengthen the quantitative evaluation of safety margins. This integrated approach has the potential to refine the risk assessment process by enabling more accurate predictions of how drugs will behave in human systems.

9.PREDICTING HUMAN SAFETY WITH MECHANISTIC INSIGHT:

9.1.ADVERSE OUTCOME PATHWAYS AND MECHANISMS OF TOXICITY:

Mechanistic insights are often organized into structured sequences of biological events, such as Adverse Outcome Pathways (AOPs), Modes of Action (MoA), and Pathways of Toxicity (PoT). AOPs provide a logical framework outlining the progression from a molecular initiating event to an adverse health outcome across multiple levels of biological complexity. While AOPs primarily organize qualitative information, PoTs focus more on the dynamic and molecular-level interactions, providing a quantitative perspective. Integrating AOPs with mechanistic understanding enhances the potential for adopting novel biomarkers to identify and monitor early toxicity signals. However, their practical utility depends on linking in vitro or animal model signals to actual adverse drug reactions (ADRs) observed in humans. This connection, supported by target- or phenotype-based assessments, strengthens the weight-of-evidence (WoE) approach used in human risk assessment for organ-specific toxicity.

9.2.HYPERSENSITIVITY AND IDIOSYNCRATIC REACTIONS:

Hypersensitivity and idiosyncratic reactions are particularly challenging to detect due to their occurrence at standard therapeutic doses and in only a small subset of patients during late-stage trials or post-marketing. These reactions lack clear dose dependency and often involve complex immune mechanisms that are not well represented in current preclinical models.

There remains a critical need for innovative diagnostic tools and a deeper understanding of these mechanisms to improve the safety of drug development. A promising advancement is the case of skin sensitization, where applying the AOP framework has led to the development of validated in vitro assays that now replace traditional animal testing, as recognized by international regulatory agencies (e.g., OECD).

9.3.BRIDGING THE TRANSLATIONAL GAP:

Despite advances in model development, fully validated human-specific in vitro models for organ toxicity testing are still lacking. The successful implementation of such models depends on a robust set of mechanistically anchored biomarkers that can bridge preclinical and clinical safety assessments. Currently, toxicity predictions often rely on observational data and WoE rather than mechanistic insights.[28] Recent developments in gene-editing technologies like CRISPR/Cas9 have enabled the creation of more accurate animal disease models. For instance, pigs genetically engineered to model cystic fibrosis display clinical features more closely resembling human pathology than traditional rodent models. These innovations can significantly improve the translatability of preclinical findings to human applications.

9.4.TRACKING SAFETY SIGNALS:

Safety biomarkers, which span molecular and imaging-based technologies, are vital for identifying and monitoring toxicological signals. While the use of 'omics platforms (genomics, proteomics, transcriptomics, etc.) has surged, their routine application remains limited by challenges such as low predictive value and invasive sampling methods.

9.5.PROMOTING DATA TRANSPARENCY:

Concerns persist regarding the reproducibility and completeness of published experimental data. Inconsistent data standards, lack of clear definitions, and poor use of ontologies hinder data integration and computational modelling efforts.[40]Efforts to address these challenges have led to the establishment of data governance frameworks aimed at harmonizing study design, data curation, and control mechanisms. Increased emphasis on data sharing—both publicly and through public-private partnerships—is transforming how pharmaceutical companies leverage existing datasets. Initiatives like those led by EFPIA promote collaborative data sharing, encouraging the standardization of protocols and enhancing the overall quality and impact of preclinical and clinical research in drug development.[42]

10.SILICO TOOLS AND MODELING:

10.1.THE ROLE OF AOPS IN NETWORK-BASED MODELLING:

Although Adverse Outcome Pathways (AOPs) have traditionally found more extensive application in evaluating the safety of chemicals, their incorporation into drug discovery pipelines remains limited. AOPs are typically structured as linear progressions of biological events, emphasizing the identification of measurable key events (KEs) that are critical in determining toxicological outcomes.[25] However, with the advancement of quantitative AOPs (qAOPs), there is growing potential to use them as frameworks for developing in silico prediction tools and in vitro test batteries in pharmaceutical research (Hartung, 2017b). Emerging AOP networks are being constructed by mapping overlapping KEs across different pathways, forming interconnected models that can better represent complex biological responses (Knapen et al., 2018). [21] Systems biology approaches—such as neural networks—have gained prominence in drug development for modelling these complex relationships. However, their implementation demands robust computational tools and detailed datasets for accurate quantification (Hartung et

al., 2012; 2017). Toxicokinetic-Toxicodynamic (TKTD) modelling—though not yet widely adopted in this context—offers significant promise. These models simulate dynamic biological processes by:

- Estimating the uptake and elimination rates of new chemical entities (NCEs) or biologics (NBEs) to understand how a compound reaches and affects target sites (e.g., Molecular Initiating Events or MIEs).
- Quantifying the rates of damage accumulation and recovery at various biological levels to simulate time-dependent toxic effects (Tsaioun et al., 2016; Kretschmann et al., 2012).

10.2.QIVIVE AND PHYSIOLOGICALLY BASED PHARMACOKINETIC/PHARMACODYNAMICS MODELLING (PBPK/PD):

To enhance risk assessment, it is essential to quantitatively understand how biological events progress from molecular initiation to adverse outcomes. Organ-specific in vitro assays allow for the determination of Points of Departure (PoDs)—biological thresholds that signify the onset of perturbation in toxicity pathways.[33] PoDs are foundational to Quantitative In Vitro to In Vivo Extrapolation (QIVIVE) strategies, helping define safe exposure limits by correlating in vitro findings with in vivo outcomes (Hartung, 2017a). This process requires an understanding of:

- The threshold levels of molecular activity that push a biological pathway forward, and
- The internal concentration of the compound that influences the probability and severity of adverse effects.

Physiologically Based Pharmacokinetic (PBPK) models are critical enablers of QIVIVE. Particularly, reverse dosimetry PBPK models estimate human exposure scenarios that result in concentration-time profiles comparable to active in vitro concentrations (Basketter et al., 2012; Leist et al., 2014).[37] For example, the US EPA's Toxicant program has demonstrated the use of PBPK modelling for integrating in vitro bioactivity data. In this approach, experimentally derived values such as hepatic clearance and plasma protein binding inform toxicokinetic models to predict steady-state plasma concentrations (Css) resulting from repeated exposure. Reverse PBPK modelling then estimates the corresponding human equivalent doses (in mg/kg/day) required to reach those Css levels—bridging laboratory assays and real-world human exposure predictions (Louisse et al., 2017).

11.SAFETY BIOMARKERS

11.1.CARDIOVASCULAR TOXICITY (HEART):

Cardiovascular toxicities contribute significantly to adverse drug reactions (ADRs), drug development failures, and post-market withdrawals, affecting various components of the cardiovascular system (Laverty et al., 2011; Valentin et al., 2010). *These toxicities can be broadly classified into three categories:*

1. Structural Damage: Physical injury or degeneration of cardiac tissues.
2. Functional Impairment: Altered heart function which may or may not be accompanied by histopathological changes.
3. Disruption of Cellular or Tissue Homeostasis: Changes in cellular or tissue equilibrium without obvious structural or functional abnormalities (Wallace et al., 2004).

Given the wide spectrum of cardiovascular ADRs, a diverse set of biomarkers is required to effectively detect, predict, and monitor these effects during preclinical and clinical testing phases.[40] Hemodynamic biomarkers include measurements such as blood pressure, heart rate, and cardiac ejection fraction, often assessed using semi-invasive techniques.[22] Electrophysiological changes—such as prolongation or shortening of the QTc interval, widening of the QRS complex, PR interval prolongation, and arrhythmias including Torsades de Pointes and ventricular fibrillation—can be identified through electrocardiogram (ECG) monitoring. For many of these electrophysiological endpoints, robust in vitro screening methods exist. Notably, there is a strong correlation between the free plasma concentration that induces significant QT prolongation or Torsades de Pointes in clinical settings and the in vitro half-maximal inhibitory concentration (IC50) for the IKr channel blockade (Webster et al., 2002).[46]

11.2.LIVER (HEPATOTOXICITY):

Drug-induced liver injury (DILI) represents one of the most critical adverse drug reactions encountered during drug development, often leading to the discontinuation of promising drug candidates in both preclinical and clinical phases (Clarke et al., 2016; Pognan, 2018). DILI is categorized into two primary types: intrinsic DILI, which exhibits a clear dose-dependent pattern of hepatocellular damage (Corsini et al., 2012), and idiosyncratic DILI, which is less common, unpredictable, and challenging to detect using current in vitro or animal models.[1] In clinical settings, the phenotypic diagnosis of DILI largely depends on measuring serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin (BIL). Although these markers are widely used, ALT, AST, and ALP lack specificity for liver injury, while elevated bilirubin levels typically indicate significant, often advanced, hepatic damage (Church et al., 2018). In preclinical

studies, histopathological examination remains a cornerstone for detecting DILI (Weaver et al., 2017).[39] Because traditional biomarkers like ALT, ALP, and bilirubin have limitations in sensitivity and prognostic value, research has focused on identifying novel biomarkers that provide mechanistic insights. For example, High Mobility Group Box 1 protein (HMGB1) is recognized as a marker for necrosis (Scaffidi et al., 2002), and its acetylated form is linked to immune-mediated DILI (Lu et al., 2012). Other emerging biomarkers such as Keratin-18 and microRNA-122 (miR-122) show promise in enhancing the detection and understanding of liver injury (Antoine et al., 2013; Clarke et al., 2016; Ward et al., 2014). These biomarkers are considered 'response biomarkers' and further validation is required to establish their translational and predictive value in clinical practice (Matheis et al., 2011). The development of translationally relevant biomarkers holds considerable promise for improving prognosis and management of DILI in patients (Ozer et al., 2008).

11.3.CENTRAL NERVOUS SYSTEM (NEUROTOXICITY):

There is an ongoing need to develop biomarkers that are both sensitive and specific for diagnosing and predicting neurotoxicity, with relevance that spans animal models and clinical translation (Schmidt et al., 2017). Traditional functional biomarkers such as electroencephalogram (EEG), electroretinogram (ERG), and brainstem auditory evoked potential (BAEP) have demonstrated utility in bridging preclinical findings to clinical outcomes.[7] Fluid-based biomarkers are particularly attractive due to ease of collection and include candidates such as microRNAs (miRNAs), F2-isoprostanes, translocator protein, glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase L1 (UCH-L1), myelin basic protein, microtubule-associated protein-2 (MAP-2), and total tau protein. However, some biomarkers, especially those requiring cerebrospinal fluid (CSF) samples, involve invasive procedures, and others may be disease-specific—such as those linked exclusively to Alzheimer's or Parkinson's disease—and need further validation for broader neurotoxicity applications.[23] Neuroimaging techniques also offer potential biomarker roles. When combined with functional assessments and molecular biomarkers—spanning genetics and proteomics—these tools provide a promising integrated approach to improve the detection, monitoring, and prediction of drug-induced neurotoxicity.[47]

12.NOVEL CELL MODELS:

Developing physiologically relevant cell models offers significant potential to enhance the detection and prediction of drug-induced toxicity, as well as to deepen our understanding of the underlying mechanisms. Consequently, there is a growing interest in shifting away from traditional immortalized cell lines, which have been commonly used in screening cascades during drug discovery, towards primary cells, despite their well-known challenges such as limited availability and batch-to-batch variability (Eskes et al., 2017; Pamies et al., 2017, 2018; Coecke et al., 2007).[24] An ideal solution for toxicity testing would involve a reliable, reproducible, and virtually inexhaustible source of cells with well-defined phenotypic and genotypic characteristics. The differentiation of various human stem cells into desired somatic cell types could fulfill this need (Pamies and Hartung, 2017). Adding further biological complexity by culturing cells in three-dimensional (3D) structures, micro physiological systems, or organoids is gaining traction in the field of investigative toxicology (Alépée et al., 2014). These 3D and organoid models better mimic physiological conditions, including proper cell polarization and intricate cell–cell or cell–microenvironment interactions, which are critical for tissue differentiation and function (Anton et al., 2015; Duval et al., 2017; Retting et al., 2018). Techniques such as microfluidics and bioprinting have been employed to further enhance these tissue models by enabling co-culture of multiple cell types, compartmentalization, formation of gradients, fluid flow, and mechanical stress. This increased complexity generally improves tissue functionality and has been demonstrated in various hepatocyte models, including 3D spheroids (Messner et al., 2013; Bell et al., 2016; Proctor et al., 2017), bio printed systems (Retting et al., 2018), organoids (Huch et al., 2015), and micro physiological systems (Huh et al., 2010; Vernetti et al., 2016). While these approaches show considerable promise in terms of biological relevance, their routine adoption in toxicology laboratories is still emerging.

12.1.STEM CELL MODELS:

Traditionally, cell-based experiments have relied on immortalized cell lines and primary cells. While cell lines offer a consistent and renewable source of biological material, they often lose the characteristics of their tissue of origin over time. In contrast, primary cells freshly isolated from tissues are considered the gold standard for physiological relevance but have a limited lifespan and their properties may degrade rapidly depending on storage and handling. Additionally, the isolation process can be complex, and variability in cell quality is common.[31] Induced pluripotent stem cells (iPSCs) offer a promising alternative, providing a renewable source of cells that can be generated in large quantities with well-defined physiological and genetic profiles, potentially reflecting specific individuals. This makes iPSCs highly valuable as toxicity models, offering a nearly unlimited supply of human cells with controlled phenotypes.[12] iPSCs can be generated from various starting cell types, including germ cells, hepatocytes, skin cells, and lymphocytes (Takahashi and Yamanaka, 2006; Yu et al., 2007; Gadue and Cotsarelis, 2008; Okita et al., 2007; Loh et al., 2009; Aoi et al., 2008). Numerous protocols have been

developed to guide their differentiation into specific lineages, such as endothelial cells, smooth muscle cells, neurons, cardiomyocytes, and hepatocyte-like cells, often using tailored growth factors and supplements (Patsch et al., 2015; Hu et al., 2011; Mauritz et al., 2008). Furthermore, iPSC-derived hepatic cells have been used to model diseases like familial hypercholesterolemia, Wilson's disease, and alpha-1 antitrypsin deficiency, supporting the study of disease mechanisms and drug development (Cayo et al., 2012).[8] Among toxicity testing applications, iPSC-derived cardiomyocytes are the most established (Millard et al., 2018), followed closely by neurons (Wevers et al., 2016). Hepatocyte differentiation protocols are improving, though iPSC-derived cells still generally exhibit lower expression of xenobiotic metabolism enzymes compared to primary liver cells. Additional challenges include variability between iPSC lines, incomplete programming of some cell populations, and atypical responses to toxicants.[1] Despite these limitations, iPSC-derived cells are increasingly recommended as an alternative human cell source for toxicology screening and may provide insights into individual susceptibility to adverse drug reactions (van Hasselt and Iyengar, 2017). A notable example is the FDA's Comprehensive In Vitro Pro-arrhythmic Assay (CiPA) initiative, which uses iPSC-derived cardiomyocytes to assess pro-arrhythmic risk (Wallis et al., 2018).[30] Fully functional organ-specific cells derived from iPSCs hold great potential to advance drug development and to investigate how genetic variability influences drug response. This technology also offers opportunities to explore differences related to sex, ethnicity, and disease background. While the field is progressing rapidly, standardization, validation, and regulatory frameworks are still needed to fully integrate these models into routine toxicological and pharmacological research.

12.3.FUTURE PERSPECTIVES ON IN VITRO MODELS:

Recent advancements in generating human induced pluripotent stem cells (iPSCs) at large scale, their differentiation into diverse immature organ-specific somatic cells forming artificial organoids, and the increasing integration of multiple organ models on micro physiological systems (MPS) platforms have together opened a groundbreaking opportunity for humanized safety assessment models (Miller and Shuler, 2016; Xiao et al., 2017; Edington et al., 2018).

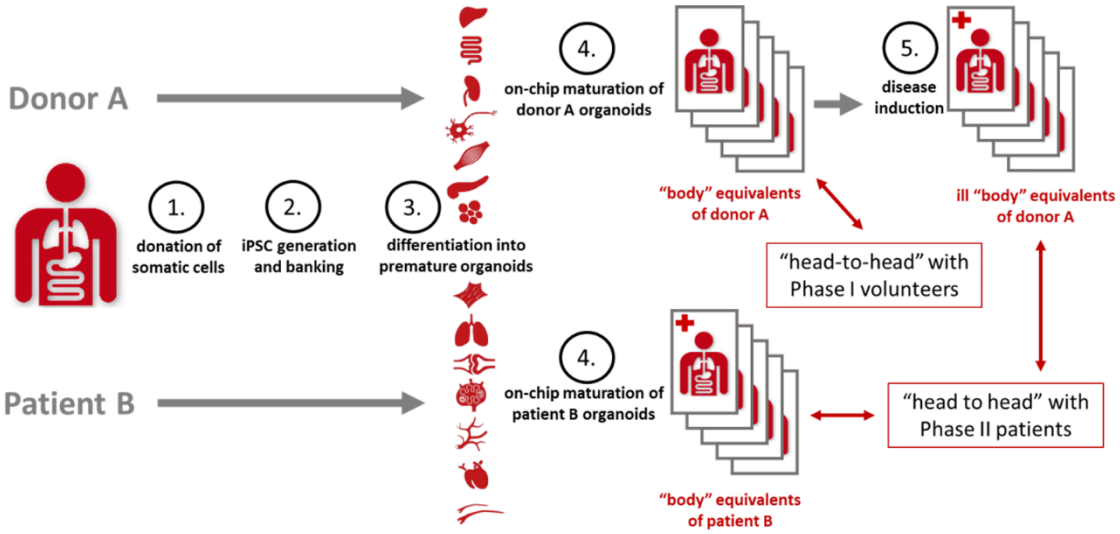

FIGURE: 3: SKETCHING A ROADMAP TOWARDS "CLINICAL TRIALS" ON A CHIP

The integration of these three technologies holds the promise of creating personalized, miniature versions of healthy donors or patient "bodies-on-a-chip." Figure 7 illustrates the long-term vision of utilizing such MPS-based personalized models to simulate Phase 1 and Phase 2 clinical trials. Success in this endeavor could allow for individualized experimental studies that replicate clinical trials by using statistically significant numbers of near-identical miniaturized donor or patient models on chips.[26] This concept parallels the current use of genetically identical inbred laboratory animals in preclinical research, with the key difference being that these miniaturized "bodies-on-a-chip" originate from personalized human cells. Additionally, this approach may enable direct comparison of outcomes between real patients and their corresponding body-on-a-chip models.[30] Employing "body" equivalents derived from donors and patients of varying genders, ethnicities, and genetic backgrounds could allow precise evaluation of how these factors influence the safety and efficacy of new chemical entities (NCEs) or new biological entities (NBEs) during preclinical development. This highlights the tremendous potential of such advanced in vitro platforms to transform and optimize the drug development process (Marx et al., 2016).

13.IMAGING TECHNOLOGIES:

Over recent decades, investigative toxicology has greatly benefited from significant advances and widespread adoption of high-content imaging techniques (van Vliet et al., 2014; Uteng et al., 2014). The use of various small molecule fluorescent probes enables the detection of numerous biochemical disturbances and assessments of cell viability. For instance, fluorescent probes have been utilized to monitor intracellular fatty acid accumulation, a hallmark of steatosis and a key endpoint in drug-induced liver injury (DILI) (Germano et al., 2015). Similarly, fluorescent bile acid analogues help track bile acid buildup caused by impaired bile acid transport, aiding the identification of compounds that may induce cholestasis (Germano et al., 2015). Other fluorescent markers are employed to evaluate phospholipids (Morelli et al., 2006), oxidative stress, and changes in mitochondrial membrane potential (Billis et al., 2014).[2] Pharmaceutical research, high-content imaging has become indispensable in predictive toxicology, supporting the design and prioritization of drug candidates with improved safety profiles (Persson and Hornberg, 2016). Traditionally, these methods have been applied mainly to two-dimensional (2D) cell cultures—either established cell lines or primary cells (Pampaloni et al., 2007). A key challenge moving forward is adapting these imaging approaches to more complex three-dimensional (3D) cell models, enabling high-resolution, single-cell analysis of probe activities within multicellular environments.[38] Current high-content imaging platforms face difficulties in capturing fluorescence signals from cells located in the core of 3D spheroids. Future progress is anticipated through the integration of advanced microscopy techniques such as light-sheet microscopy into high-content screening workflows, which would facilitate detailed biochemical analyses in complex micro physiological systems (MPS) (Joshi and Lee, 2015). Additionally, phenotypic screening that quantifies numerous cellular morphology parameters in parallel is an emerging approach, enhancing the understanding of compound-induced changes (Joshi and Lee, 2015; Leary et al., 2018).[39] Further development aims to incorporate mechanistic biomarkers linked to adverse outcome pathways (AOPs) into high-content imaging assays, thereby enriching their predictive power. Beyond fluorescence-based methods, label-free molecular imaging techniques such as mass spectrometry (MS)-based bioimaging are evolving rapidly. These approaches allow visualization of tissues, cells, and even subcellular structures without the need for external labels, offering promising applications for toxicity assessment and mechanistic studies (Passarelli and Ewing, 2013).

14.CHALLENGES IN INVESTIGATIVE TOXICOLOGY:

14.1.TERMINOLOGY, AND ONTOLOGIES:

Toxicology spans multiple industries—including pharmaceuticals, chemicals, cosmetics, consumer products, food, and environmental health—leading to varied terminology and inconsistent use of key terms like *Investigative Toxicology*. This lack of harmonization complicates communication and regulatory integration. A shared, precise vocabulary is essential, especially to align prospective (early screening) and retrospective (mechanistic elucidation) approaches in drug development. Harmonizing definitions of critical terms (e.g., biomarkers, safety assessment, mode of action) is vital to integrate emerging technologies effectively. Ontologies such as the Histopathology Ontology (IMI eTOX Project) have helped standardize data, enabling better data mining and in silico/in vitro model development.[14] Clear performance standards and proof-of-concept validation studies are needed to demonstrate the reliability and relevance of new models, aiding regulatory acceptance. Despite widespread use of concepts like translational biomarkers and adverse outcome pathways (AOPs), their definitions and quantitative thresholds remain inconsistent, limiting practical application in risk assessment.

14.2.STUDY DESIGN:

Investigative studies must be clearly planned with defined expectations and scientifically justified protocols. While full regulatory validation may not always be necessary, robustness—proven through multi-center pre-validation—is crucial for advanced models. Iterative investigation cycles often generate hypotheses and mechanistic insights into toxicity. The choice to incorporate disease models is complex but potentially valuable for identifying key adverse events and dose thresholds. Advances in human and gene-edited animal disease models offer promising translational tools.[37] Patient-derived cell models may become integral to clinical trials, although the broader applicability of AOPs across organs and systems challenges organ-specific toxicity paradigms. A critical gap remains in defining quantitative cutoffs from AOPs to determine safe human dosing.

15.CONCLUSION:

Drug metabolism is a complex but vital process that ensures drugs are effectively processed and eliminated from the body. It determines the pharmacokinetic behaviour of medications and influences both therapeutic outcomes and the potential for adverse drug reactions. Knowledge of drug metabolism is fundamental in pharmacology, toxicology, and clinical medicine for safe and effective drug use. Drug toxicology is a vital discipline that bridges medicine, chemistry, and biology. It ensures that the drugs we use are not only effective but also safe when administered properly. As pharmaceutical research advances, toxicological studies continue to play

a foundational role in drug development, risk assessment, and patient care. while high-dose methotrexate therapy is effective in many clinical scenarios, it must be carefully managed to prevent renal toxicity. Early identification of at-risk patients, aggressive hydration, urine alkalinization, timely leucovorin administration, and alternative detoxification methods such as CPDG2 or thymidine use are critical tools in reducing the risk and severity of methotrexate-induced renal complications. The development of "bodies-on-a-chip" through the integration of microphysiological systems holds transformative potential for personalized clinical trials, enabling replication of Phase 1 and 2 trials on miniaturized human models derived from diverse donor cells. This innovative approach promises more precise, individualized drug safety and efficacy assessments while reflecting genetic and demographic variability. Advances in high-content imaging and label-free molecular techniques further enhance the predictive power and physiological relevance of in vitro toxicology models. However, challenges remain in harmonizing terminology, validating new models, and integrating disruptive technologies such as big data and in silico modeling. Overcoming these hurdles is essential to fully realize the promise of investigative toxicology in optimizing drug development and reducing reliance on animal testing. Advances in in silico modeling and network-based approaches, such as quantitative Adverse Outcome Pathways (qAOPs), Toxicodynamic models, and physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) modeling, are progressively enhancing the predictive power of drug safety assessments. These computational tools allow for more accurate simulations of biological processes and help bridge in vitro data with in vivo human outcomes through Quantitative In Vitro to In Vivo Extrapolation (QIVIVE). Meanwhile, safety biomarkers across critical organ systems—particularly cardiovascular and hepatic—remain essential for early detection and monitoring of drug-induced toxicities. The integration of computational modeling with robust biomarker strategies promises to improve risk assessment, optimize drug development pipelines, and reduce attrition rates by identifying adverse effects more efficiently and reliably. Continued refinement and validation of these tools are vital to fully realize their potential in transforming preclinical toxicology and enhancing patient safety.

16.ABBREVIATIONS TABLE:

S.	ABBREVIATION	FULL FORM / MEANING	S.	ABBREVIATION	FULL FORM
1	**CYP450**	Cytochrome P450 enzyme family	**27**	ADRs	Adverse Drug Reactions
2	**CYP2D6**	Cytochrome P450 2D6 isoenzyme	**28**	QTc	Corrected QT Interval
3	**DILI**	Drug-Induced Liver Injury	**29**	ECG	Electrocardiogram
4	**IC_{50}**	Half-maximal Inhibitory Concentration	**30**	IC50	Half-Maximal Inhibitory Concentration
5	**OH**	Hydroxyl Group	**31**	DILI	Drug-Induced Liver Injury
6	**NH_2**	Amino Group	**32**	ALT	Alanine Aminotransferase
7	**SH**	Sulfhydryl Group	**33**	AST	Aspartate Aminotransferase
8	**COOH**	Carboxylic Acid Group	**34**	ALP	Alkaline Phosphatase
9	**MIE**	Molecular Initiating Event	**35**	BIL	Bilirubin
10	**NCE**	New Chemical Entity	**36**	HMGB1	High Mobility Group Box 1
11	**NBE**	New Biological Entity	**37**	miR-122	MicroRNA-122
12	**PBPK**	Physiologically Based Pharmacokinetics	**38**	GFAP	Glial Fibrillary Acidic Protein
13	**PD**	Pharmacodynamics	**39**	UCH-L1	Ubiquitin C-terminal Hydrolase L1
14	**QIVIVE**	Quantitative In Vitro to In Vivo Extrapolation	**40**	MAP-2	Microtubule-Associated Protein 2
15	**qAOP**	Quantitative Adverse Outcome Pathway	**41**	EEG	Electroencephalogram
16	**TKTD**	Toxicokinetic-Toxicodynamic	**42**	ERG	Electroretinogram
17	**Css**	Steady-State Plasma Concentration	**43**	BAEP	Brainstem Auditory Evoked Potential
18	**ECG**	Electrocardiogram	**44**	CSF	Cerebrospinal Fluid

19	**QTc**	Corrected QT Interval	**45**	iPSC	Induced Pluripotent Stem Cell
20	**QRS**	QRS Complex (ventricular depolarization on ECG)	**46**	3D	Three-Dimensional
21	**PR**	PR Interval (atrial to ventricular conduction time)	**47**	MPS	Microphysiological Systems
22	**ADR**	Adverse Drug Reaction	**48**	CiPA	Comprehensive In Vitro Pro-arrhythmic Assay
23	**DNA**	Deoxyribonucleic Acid	**49**	NCEs	New Chemical Entities
24	**RNA**	Ribonucleic Acid	**50**	NBEs	New Biological Entities
25	**FDA**	Food and Drug Administration (U.S.)	**51**	MS	Mass Spectrometry
26	**GI Tract**	Gastrointestinal Tract	**52**	AOPs	Adverse Outcome Pathways

16.ACKNOWLEDGMENT:
The author sincerely acknowledges the faculty and staff of **Ravishankar College of Pharmacy, Bhopal (462037)** for their valuable support and encouragement throughout the course of this research. Special thanks to the Department of Pharmacology for providing the necessary resources and guidance. Gratitude is also extended to peers and mentors whose constructive feedback contributed to the improvement of this work.

17.AUTHOR CONTRIBUTIONS:
Manish Kumar Vishwakarma independently conceptualized the study, conducted the literature review, and drafted the initial manuscript. He also carried out the data analysis and interpretation of findings, along with critical revisions of the manuscript.

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