Chapter 2

2. LITERATURE REVIEW

2.1 Drug-Induced Liver Injury (DILI)

Drug-induced liver injury (DILI) represents a significant clinical and regulatory challenge worldwide, accounting for a major proportion of acute liver failure cases and being a frequent cause of drug withdrawal or restricted use in the post-marketing phase. The liver, as the primary site of xenobiotic metabolism, is particularly susceptible to injury from pharmaceuticals, herbal supplements, and other exogenous chemicals. The incidence of DILI varies geographically, with population-based studies reporting rates between 10 and 15 cases per 100,000 individuals per year, though underreporting and diagnostic variability suggest the true incidence may be higher [27–29]. DILI is a leading cause of acute liver failure in several countries, surpassing viral hepatitis in certain Western nations [30].

From a clinical classification perspective, DILI is broadly categorized into **intrinsic** and **idiosyncratic** types. **Intrinsic DILI** is predictable, dose-dependent, and reproducible across individuals; acetaminophen overdose is the prototypical example, resulting in massive hepatocellular necrosis through oxidative stress and mitochondrial failure [31,32]. In contrast, **idiosyncratic DILI** is unpredictable, not clearly dose-dependent, and often influenced by genetic and environmental factors, immune responses, and metabolic idiosyncrasies [33]. Idiosyncratic DILI accounts for the majority of clinical cases and presents a significant challenge in drug safety evaluation, as it is rarely detected in preclinical testing and often occurs weeks to months after drug exposure [34].

Histologically, DILI can present as **hepatocellular**, **cholestatic**, or **mixed** injury patterns, determined by the R-value (ratio of alanine aminotransferase [ALT] to alkaline phosphatase [ALP] elevation). Hepatocellular injury is more likely to progress to acute liver failure, while cholestatic patterns are often associated with prolonged recovery times [35]. Laboratory markers such as ALT, aspartate aminotransferase (AST), ALP, and total bilirubin remain the primary diagnostic indicators, with the **Hy's Law** criteria serving as a regulatory benchmark for identifying drugs with high fatality risk when ALT/AST elevations are accompanied by hyperbilirubinemia [36].

Pathophysiologically, DILI involves a complex interplay between drug metabolism, reactive metabolite formation, mitochondrial injury, oxidative/nitrosative stress, and immune-mediated mechanisms. Mitochondrial dysfunction has emerged as a central feature in both intrinsic and idiosyncratic hepatotoxicity, contributing to impaired ATP generation, increased reactive oxygen species (ROS) production, and activation of cell death pathways [37,38]. The high metabolic activity of hepatocytes, coupled with the liver's extensive mitochondrial population, makes it particularly vulnerable to drugs that impair oxidative phosphorylation or mitochondrial DNA integrity [39].

Epidemiological analyses reveal that **antibiotics, antiepileptics, and non-steroidal anti-inflammatory drugs (NSAIDs)** are among the most common therapeutic classes implicated in DILI. For example, amoxicillin–clavulanate is the leading cause of idiosyncratic DILI in Western countries, often presenting with cholestatic or mixed injury patterns [2]. Importantly, mitochondrial liabilities have been identified as key factors in the post-marketing withdrawal of several drugs, highlighting the need for mitochondrial toxicity assessment during drug development [40].

From a public health perspective, the burden of DILI extends beyond clinical morbidity and mortality to substantial healthcare costs, loss of therapeutic options, and diminished patient trust in pharmacotherapy. Regulatory agencies, including the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), have implemented stringent safety monitoring programs, such as the FDA Adverse Event Reporting System (FAERS) and EMA's EudraVigilance, to improve early detection of hepatotoxic signals [41,42]. Despite these efforts, the unpredictable nature of idiosyncratic DILI underscores the importance of mechanistic research, biomarker development, and the exploration of protective strategies such as antioxidant therapies.

Mechanisms of Drug-Induced Liver Injury (DILI)

The pathogenesis of drug-induced liver injury (DILI) is complex and multifactorial, involving the interplay of metabolic activation, oxidative stress, mitochondrial dysfunction, immune-mediated responses, and genetic susceptibility. While different drugs may trigger DILI through distinct pathways, a unifying concept is that hepatotoxicity arises when the balance between cellular defense mechanisms and injury-inducing processes is disrupted [43].

Mechanism of Drug Entry into Cells and Mitochondria

Plasma Protein Binding

Drugs in systemic circulation often bind to plasma proteins like albumin. Only unbound drugs can cross membranes, influencing toxicity and mitochondrial uptake. Highly protein-bound drugs may exhibit altered pharmacokinetics and toxicity when displaced by competing compounds. Moreover, shifts in protein binding dynamics modulate the fraction of unbound drug available for cellular and mitochondrial uptake, thereby influencing both pharmacodynamic and toxicological outcomes [44-47].

Passive Diffusion and Physicochemical Properties

Lipophilic drugs can diffuse passively across cell membranes. This includes many antibiotics, antidepressants, and NSAIDs. Lipophilic cations accumulate in mitochondria due to their negative membrane potential [48].

Transporter-Mediated Entry

Solute carrier (SLC) and ATP-binding cassette (ABC) transporters are pivotal in regulating drug uptake and efflux. For instance, SLC transporters such as organic anion transporting polypeptides (OATPs) and organic cation transporters (OCTs) facilitate the entry of drugs into hepatocytes and renal cells. Conversely, ABC transporters like P-glycoprotein actively expel drugs, thereby limiting their intracellular accumulation. Additionally, the presence of these transporters on mitochondrial membranes suggests they play a role in modulating the distribution of toxic compounds, which may contribute to tissue-specific drug toxicity [49-50]. (Figure2)

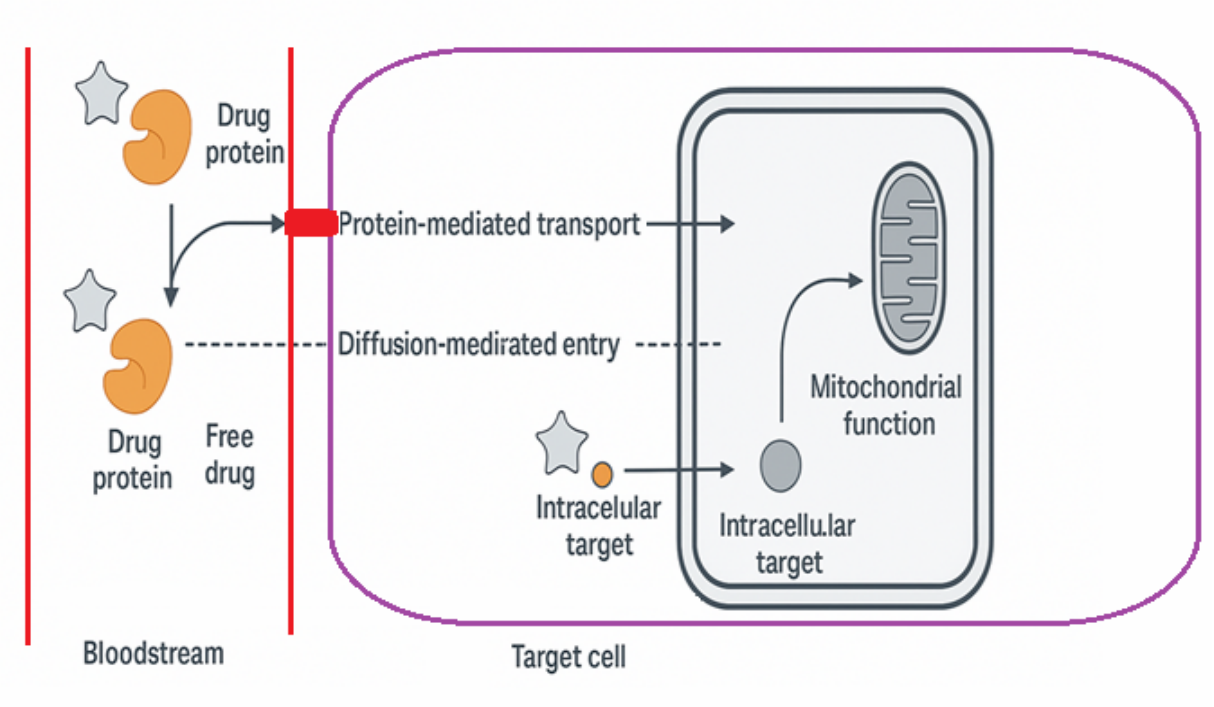

Figure 2: General mechanisms of drug delivery and their influence on mitochondrial function within target cells

2.1.1 Main Consequences of Hepatic Mitochondrial Dysfunction

Mitochondria are vital organelles responsible for ATP production via oxidative phosphorylation, regulation of metabolic pathways, and initiation of apoptotic signaling. In hepatocytes, mitochondrial dysfunction can lead to several detrimental outcomes:

- **Impaired Oxidative Phosphorylation**: Disruption of the electron transport chain hampers ATP synthesis, resulting in energy deficits that impair essential cellular functions.
- **Increased Reactive Oxygen Species (ROS) Production**: Dysfunctional mitochondria can leak electrons, leading to excessive ROS formation. Elevated ROS levels cause oxidative stress, damaging lipids, proteins, and DNA, and further compromising mitochondrial integrity.
- **Induction of Apoptosis and Necrosis**: Opening of the mitochondrial permeability transition pore (MPTP) can lead to the release of pro-apoptotic factors like cytochrome c, triggering apoptotic pathways. Severe mitochondrial damage may result in necrotic cell death due to the inability to maintain ionic homeostasis.

These events culminate in hepatocellular damage, manifesting as steatosis, inflammation, and fibrosis, which are hallmark features of drug-induced liver injury (DILI) [51-53]. (Figure 3)

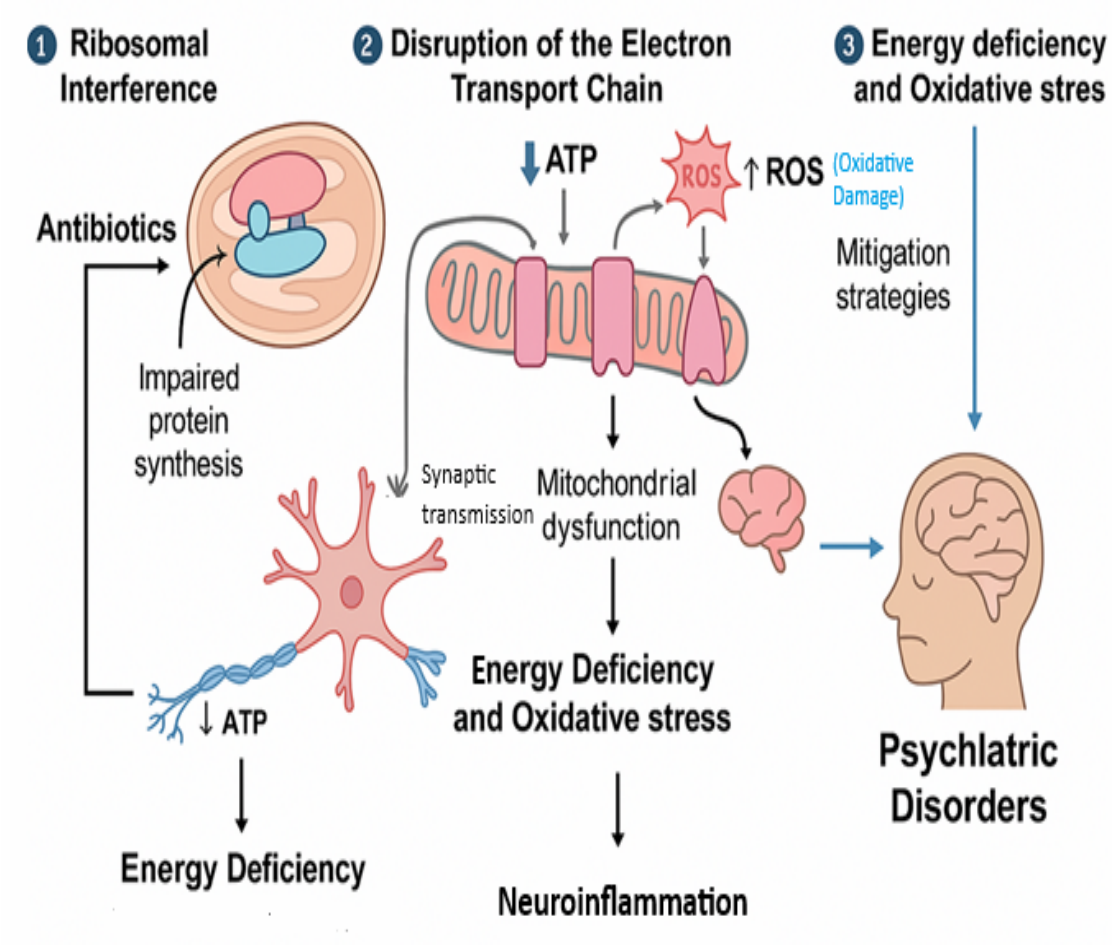

Figure 3: Mechanistic pathway by which certain antibiotics (e.g., tetracyclines, aminoglycosides) may contribute to mitochondrial dysfunction

2.1.2 Intrinsic vs. Idiosyncratic Mechanisms

Intrinsic DILI is typically dose-dependent, reproducible across individuals, and has a short latency period. It results from direct hepatotoxic effects of the parent drug or its reactive metabolites, as exemplified by acetaminophen overdose [54]. The reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) covalently binds to hepatic proteins, depletes glutathione (GSH), and induces mitochondrial oxidative stress, culminating in hepatocellular necrosis [55].

Idiosyncratic DILI (iDILI), in contrast, is unpredictable, occurs in a minority of exposed individuals, and often has a delayed onset. Proposed mechanisms for iDILI include individual differences in drug metabolism, mitochondrial susceptibility, immune recognition of drug-protein adducts (hapten hypothesis), and alterations in bile acid homeostasis [56]. Many iDILI cases have an immunoallergic component, with features such as fever, rash, eosinophilia, or autoantibody formation [57].

2.1.3 Mitochondrial Dysfunction in DILI

Mitochondria are central to hepatocyte energy metabolism and apoptotic signaling, making them a prime target in DILI. Drugs can impair mitochondrial function via:

- **Inhibition of the electron transport chain (ETC):** For example, chloramphenicol inhibits mitochondrial protein synthesis, impairing complexes I–IV and reducing ATP output [58].
- **Disruption of β-oxidation:** Valproic acid and tetracyclines impair fatty acid metabolism, leading to microvesicular steatosis [59].
- **Induction of mitochondrial permeability transition (mPT):** Acetaminophen-induced oxidative stress activates c-Jun N-terminal kinase (JNK), promoting mPT pore opening, membrane depolarization, and release of pro-apoptotic factors [60].
- **mtDNA depletion or damage:** Nucleoside reverse transcriptase inhibitors (NRTIs) inhibit DNA polymerase γ, resulting in mtDNA loss and impaired OxPhos [61].
- These mitochondrial insults initiate a cascade of energy failure, ROS accumulation, and activation of cell death pathways, either apoptotic (caspase-dependent) or necrotic (unregulated cell lysis with inflammation) [62].

2.1.4 Oxidative Stress and Redox Imbalance

Oxidative stress plays a dual role in DILI: as both a direct cytotoxic factor and as a signaling mechanism for immune activation. Drug metabolism via cytochrome P450 enzymes, especially CYP2E1 and CYP3A4, generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) [63]. Excess ROS oxidizes lipids, proteins, and DNA, compromising membrane integrity and enzymatic function. Mitochondria are both a major source and target of ROS in hepatocytes, creating a vicious cycle of oxidative damage and functional impairment [64].

When ROS overwhelms antioxidant defenses such as GSH, superoxide dismutase (SOD), and catalase, redox homeostasis is lost, precipitating mitochondrial dysfunction and cell death [65]. Notably, antioxidants have shown protective effects in experimental DILI models by restoring GSH levels, scavenging free radicals, and stabilizing mitochondrial membranes [66].

2.1.5 Immune-Mediated Injury

Immune mechanisms contribute significantly to many iDILI cases. The hapten hypothesis posits that reactive drug metabolites covalently bind to cellular proteins, forming neoantigens that are recognized by the immune system [67]. This leads to activation of antigen-presenting cells, T lymphocyte recruitment, and cytokine-mediated hepatotoxicity. In some cases, innate immunity via pattern recognition receptors (e.g., TLR4) amplifies injury through inflammasome activation and secretion of pro-inflammatory cytokines like TNF-α and IL-1β [68].

2.1.6 Genetic and Epigenetic Susceptibility

Genetic polymorphisms in drug-metabolizing enzymes (e.g., NAT2, CYP2C9), transporters (e.g., BSEP), and HLA alleles influence susceptibility to DILI [69]. For example, HLA-B*57:01 is strongly associated with flucloxacillin-induced liver injury, while HLA-A*33:01 is linked to terbinafine hepatotoxicity [70]. Epigenetic modifications, including microRNA dysregulation, may also modulate hepatic response to drugs [71].

2.2 Role of Plasma Binding, Transporters, and Mitochondrial Cytochrome P450 Enzymes in Drug-Induced Mitochondrial Toxicity

Plasma protein binding is a key determinant of drug bioavailability, influencing the distribution of drugs to hepatic mitochondria. Drugs with high protein binding tend to have lower free fractions, resulting in reduced mitochondrial uptake, whereas those with lower binding are more readily available to interact with mitochondrial targets. Membrane transporters such as organic anion-transporting polypeptides (OATPs) and ATP-binding cassette (ABC) transporters further modulate intracellular concentrations of drugs and their metabolites, thereby impacting mitochondrial exposure and potential toxicity [72,73]. Additionally,

mitochondrial cytochrome P450 enzymes, exemplified by CYP2E1, are instrumental in the bioactivation of xenobiotics; they generate reactive metabolites that can bind to mitochondrial macromolecules, impair electron transport chain function, and induce oxidative stress. Understanding the interplay between plasma protein binding, transporter activity, and mitochondrial cytochrome P450-mediated bioactivation is essential for elucidating the mechanisms underlying certain forms of drug-induced liver injury. [72-74].

2.3 Evaluating Mitotoxicity as Either a Single or Multi-Mechanistic Insult in the Context of Hepatotoxicity

Mitotoxicity may act via single or multiple mechanisms, often interlinked with broader hepatotoxic pathways. For instance, some drugs may cause mitochondrial damage through a single mechanism, such as inhibition of the electron transport chain, while others may exert multi-mechanistic insults involving oxidative stress. [75]

2.4 Mitochondrial Dysfunction in Hepatotoxicity

Mitochondrial dysfunction has emerged as a central mechanism in both intrinsic and idiosyncratic drug-induced liver injury (DILI). As the primary site of oxidative phosphorylation (OxPhos) and ATP production, mitochondria are critical for hepatocyte survival, metabolic homeostasis, and detoxification processes. Disruption of mitochondrial function not only compromises hepatocellular energy balance but also precipitates oxidative stress, bioenergetic failure, and activation of programmed or unregulated cell death pathways [76].

2.4.1 Structural and Functional Features of Hepatic Mitochondria

Hepatocytes are among the most mitochondria-rich cells in the human body, with mitochondria comprising approximately 18% of cell volume [77]. These organelles play pivotal roles in β-oxidation of fatty acids, the tricarboxylic acid (TCA) cycle, amino acid metabolism, urea cycle function, and steroid biosynthesis [78]. The electron transport chain (ETC), located in the inner mitochondrial membrane, drives ATP production through sequential redox reactions across complexes I–IV, coupled to proton pumping and ATP synthesis via complex V (ATP synthase)

[79]. Mitochondrial DNA (mtDNA), which encodes 13 essential ETC proteins, is particularly vulnerable to oxidative damage due to its proximity to ROS production sites and lack of protective histones [80].

2.4.2 Mechanisms of Mitochondrial Injury in DILI

1. Direct Inhibition of OxPhos

Several drugs directly impair ETC function, leading to reduced ATP synthesis and enhanced ROS generation. Chloramphenicol, for instance, binds to the mitochondrial 55S ribosome, inhibiting translation of mtDNA-encoded ETC subunits such as cytochrome *c* oxidase I, thereby reducing complex IV activity [80]. This inhibition limits electron transfer, causing upstream accumulation of reducing equivalents and increasing ROS leakage from complexes I and III [81].

2. Induction of Mitochondrial Permeability Transition (mPT)

The mPT pore, a multiprotein complex spanning the inner and outer mitochondrial membranes, opens in response to calcium overload, oxidative stress, or toxic metabolites [60]. mPT pore opening leads to loss of mitochondrial membrane potential ($\Delta\Psi m$), matrix swelling, outer membrane rupture, and release of pro-apoptotic factors such as cytochrome *c* and apoptosis-inducing factor (AIF) [83]. Drugs such as acetaminophen, amiodarone, and valproic acid have been shown to induce mPT, amplifying hepatocellular injury [84].

3. mtDNA Damage and Depletion

Nucleoside reverse transcriptase inhibitors (NRTIs), such as zidovudine and didanosine, inhibit DNA polymerase γ, leading to mtDNA depletion, impaired ETC function, and lactic acidosis [87]. Additionally, oxidative damage to mtDNA can result from excessive ROS generation during drug metabolism, as seen with diclofenac and isoniazid [88].

i. Oxidative Stress as a Downstream Effect

A common downstream consequence of mitochondrial dysfunction is excessive ROS production, particularly superoxide anions from complexes I and III. Superoxide is rapidly converted to hydrogen peroxide by mitochondrial superoxide dismutase (SOD2), but when antioxidant defenses are overwhelmed, hydrogen peroxide can form hydroxyl radicals via the

Fenton reaction [89]. These radicals cause lipid peroxidation, protein oxidation, and mtDNA strand breaks, further impairing mitochondrial function [90]. In hepatocytes, oxidative stress also activates redox-sensitive kinases such as JNK, which can translocate to mitochondria and exacerbate dysfunction [91].

ii. Mitochondrial Dynamics and Quality Control in DILI

Mitochondria are dynamic organelles that constantly undergo fusion and fission to maintain functional integrity. Disruption of this balance contributes to DILI pathogenesis [92]. Excessive mitochondrial fission, mediated by dynamin-related protein 1 (Drp1), has been observed in hepatocytes exposed to drugs like acetaminophen and chloramphenicol, leading to mitochondrial fragmentation and loss of function [93]. Conversely, impaired mitochondrial fusion, regulated by mitofusins (Mfn1, Mfn2) and optic atrophy protein 1 (OPA1), hinders the exchange of mitochondrial contents and repair of damaged components [72]. Mitophagy, the selective autophagic removal of damaged mitochondria, is another key protective mechanism that can be overwhelmed in severe DILI [94].

iii. Clinical Implications of Mitochondrial Dysfunction in DILI

Mitochondrial injury is a hallmark of both intrinsic and idiosyncratic DILI, but its clinical manifestations vary. Acute mitochondrial failure often presents as severe hepatocellular necrosis and can lead to acute liver failure, while chronic mitochondrial impairment may result in progressive steatohepatitis, fibrosis, or cirrhosis [96]. Biomarkers such as serum glutamate dehydrogenase (GLDH), mitochondrial DNA fragments, and circulating microRNAs (e.g., miR-122) are under investigation for early detection of mitochondrial DILI [97].

2.5 Regulatory Perspective on Mitochondrial DILI

The regulatory landscape surrounding drug-induced liver injury (DILI), especially when driven by mitochondrial dysfunction, has evolved significantly over the past two decades. Recognition of the severe, sometimes fatal, consequences of mitochondrial hepatotoxicity has led to the withdrawal of multiple drugs from the market, the implementation of stricter preclinical safety testing, and the addition of black box warnings to product labeling [98].

2.5.1 FDA Black Box Warnings and Drug Withdrawals

The U.S. Food and Drug Administration (FDA) mandates **black box warnings** for drugs associated with serious or life-threatening risks, including hepatotoxicity. Mitochondrial toxicity–linked hepatic injury has been the basis for both post-marketing drug withdrawals and boxed warnings in multiple therapeutic classes [99].

- **Troglitazone** – Approved in 1997 for type 2 diabetes, withdrawn in 2000 after reports of acute liver failure. Mechanism: oxidative stress and mitochondrial injury from quinone metabolites [100].
- **Trovafloxacin** – A fluoroquinolone antibiotic withdrawn from general use in 2000 after >100 reported cases of acute hepatic necrosis, likely involving mitochondrial dysfunction [101].
- **Ximelagatran** – An oral thrombin inhibitor withdrawn in 2006 due to hepatotoxicity with elevated liver enzymes and suspected mitochondrial injury [80].
- **Valproic Acid** – Retains market approval but carries a black box warning for hepatotoxicity, particularly in pediatric patients, due to inhibition of mitochondrial β-oxidation [103].
- **Fialuridine (FIAU)** – An investigational antiviral terminated in phase II trials after causing fatal lactic acidosis and liver failure via mtDNA depletion [104].

These cases underscore the fact that mitochondrial liabilities can evade detection during preclinical development and manifest only after widespread clinical use.

Drug Name	Indication	Regulatory Action	Liver Toxicity Mechanism
Chloramphenicol	Bacterial Infections	Restricted Use	Mitochondrial protein synthesis inhibition
Diclofenac	Pain/Inflammation	Warning/Restricted Use (2006)	Immune-mediated hepatotoxicity
Felbamate	Epilepsy	Black Box Warning (1994)	Idiosyncratic hepatotoxicity
Isoniazid	Tuberculosis	Black Box Warning (2004)	Hepatocellular injury (CYP2E1 activation)
Ketoconazole	Fungal Infections	Black Box Warning (2013)	Dose-dependent hepatotoxicity
Nefazodone	Depression	Black Box Warning	Hepatocellular necrosis

		(2001)	
Pemoline	ADHD	Withdrawn (2005)	Acute liver failure
Troglitazone	Type 2 Diabetes	Withdrawn (2000)	Idiosyncratic hepatocellular injury
Trovafloxacin	Bacterial Infections	Withdrawn (2000)	Mitochondrial toxicity
Ximelagatran	Anticoagulant	Withdrawn (2006)	Mitochondrial dysfunction

Table 1: Listing of drugs that received FDA black box warnings or were withdrawn from the market due to hepatotoxicity

2.5.2 Regulatory Guidelines for Mitochondrial Safety Assessment

Regulatory authorities now emphasize mitochondrial safety in preclinical development. The **ICH S7A/S7B** guidelines, while primarily focusing on safety pharmacology, encourage evaluation of organellar toxicity in relevant systems [105]. The FDA and EMA recommend inclusion of **mitochondrial function assays** early in development, particularly for compounds with high lipophilicity, extensive metabolism, or structural alerts for mitochondrial toxicity [106].

Suggested preclinical evaluations include:

- Assessment of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in hepatic cells.
- Galactose-adapted hepatocyte models to reveal hidden mitochondrial toxicants.
- Measurement of mitochondrial membrane potential (ΔΨm), ATP levels, and ROS generation.
- mtDNA copy number quantification and polymerase γ inhibition assays for nucleoside analogues.

2.6 Overview of Mitochondrial-Toxic Drugs

Numerous pharmaceutical agents across different therapeutic classes have been identified to impair mitochondrial function, contributing to hepatotoxicity and DILI. The mechanisms include inhibition of the electron transport chain (ETC), disruption of mitochondrial membrane potential (Δψm), oxidative phosphorylation uncoupling, or direct damage to mitochondrial DNA and ribosomes [108].

Several of these drugs have received black-box warnings, usage restrictions, or have been withdrawn from the market due to their hepatotoxic potential. Regulatory bodies like the FDA have emphasized the need for mitochondrial toxicity screening in preclinical drug development stages [107].

1. Acetaminophen (Paracetamol)

Acetaminophen is a widely used analgesic and antipyretic agent. While safe at therapeutic doses, overdose can lead to severe hepatotoxicity. The liver metabolizes acetaminophen primarily via conjugation pathways; however, a small fraction is oxidized by cytochrome P450 enzymes to form N-acetyl-p-benzoquinone imine (NAPQI), a reactive metabolite [109].

Under normal circumstances, NAPQI is detoxified by glutathione; however, in overdose situations, glutathione stores become depleted, allowing NAPQI to bind covalently to cellular proteins, particularly in mitochondria. This binding induces severe mitochondrial impairment, characterized by oxidative stress, disruption of the electron transport chain, and the formation of mitochondrial permeability transition pores. The resulting energy crisis and cell death cascade contribute significantly to liver necrosis. Additionally, this review discusses the therapeutic role of Nacetylcysteine (NAC) in replenishing glutathione levels and mitigating mitochondrial damage, and it highlights emerging strategies aimed at protecting or restoring mitochondrial integrity to improve clinical outcomes in APAP-induced hepatotoxicity [110-112].

2. Methotrexate

Methotrexate is an antimetabolite used in oncology and autoimmune diseases. Its hepatotoxic potential is well-documented, especially with long-term use. Methotrexate inhibits dihydrofolate reductase, affecting DNA synthesis and repair. Chronic administration can lead to hepatic fibrosis and, in some cases, cirrhosis. Risk factors include cumulative dose, alcohol consumption, obesity, diabetes, and pre-existing liver disease. Regular monitoring of liver function tests and, in some protocols, liver biopsies are recommended to detect early signs of hepatotoxicity. Folate supplementation may mitigate some hepatic side effects [112, 114].

3. Statins

Statins (e.g., Atorvastatin, Simvastatin) are lipid-lowering agents that inhibit HMG-CoA reductase. While generally safe, they have been associated with elevations in liver enzymes and, rarely, serious hepatotoxicity. The mechanism is not fully understood but may involve mitochondrial dysfunction and oxidative stress. Most cases are asymptomatic and reversible upon discontinuation. Routine monitoring of liver enzymes is advised, especially during the initial months of therapy. The benefits of statins in cardiovascular risk reduction often outweigh the risks of hepatotoxicity, but vigilance is necessary [115,116].

4. Diclofenac

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) used for pain and inflammation. It has been implicated in idiosyncratic hepatotoxic reactions, presenting as hepatocellular, cholestatic, or mixed liver injury. The proposed mechanisms include metabolic activation to reactive intermediates, mitochondrial dysfunction, and immune-mediated responses. Hepatotoxicity can occur within weeks of initiation and may be severe. Monitoring liver function tests during therapy is prudent, especially in patients with existing liver conditions [116,117].

5. Nitrofurantoin

Nitrofurantoin is an antibiotic commonly used for urinary tract infections. While effective, it has been associated with both acute and chronic liver injury. The hepatotoxicity is thought to be immune-mediated, with features resembling autoimmune hepatitis. Risk factors include prolonged use, especially in the elderly and those with renal impairment. Liver injury can manifest weeks to months after initiation and may be resolved upon discontinuation. Regular monitoring is recommended for long-term therapy [118,119].

6. Valproic acid

Is a widely prescribed antiepileptic drug that, because of its structure resembling a simple fatty acid, enters the liver's metabolic pathways like those used for fatty acid oxidation. In the liver, VPA is processed mainly through three pathways: glucuronidation (approximately 50%), mitochondrial β-oxidation (around 40%), and a minor pathway known as ω-oxidation (about 10%) [120]. The review stresses that the mitochondrial β-oxidation pathway is not only essential for energy production but also a critical route where VPA may interfere with normal lipid metabolism.

During β-oxidation, VPA competes with natural fatty acids for the enzymes and cofactors (notably carnitine and coenzyme A) needed for effective energy production. The metabolism of VPA can lead to the formation of toxic intermediates (such as the 4-ene metabolite) that further deplete cellular reserves of carnitine and CoA. This depletion disrupts normal mitochondrial fatty acid oxidation, resulting in reduced energy production and increased oxidative stress. Such mitochondrial dysfunction is a key contributor to the development of liver steatosis (fatty liver) and, in severe cases, acute liver toxicity. Moreover, individuals with underlying inborn errors of metabolismwhere mitochondrial FAO may already be compromised—can be even more susceptible to these toxic effects. Understanding these pathways opens potential therapeutic avenues. For instance, supplemental carnitine is sometimes considered to help restore mitochondrial function by replenishing depleted cofactor levels. Further research into genetic predispositions (inborn errors of metabolism) may also aid in identifying patients at higher risk for VPA-induced liver dysfunction [121-123]. (Figure 4)

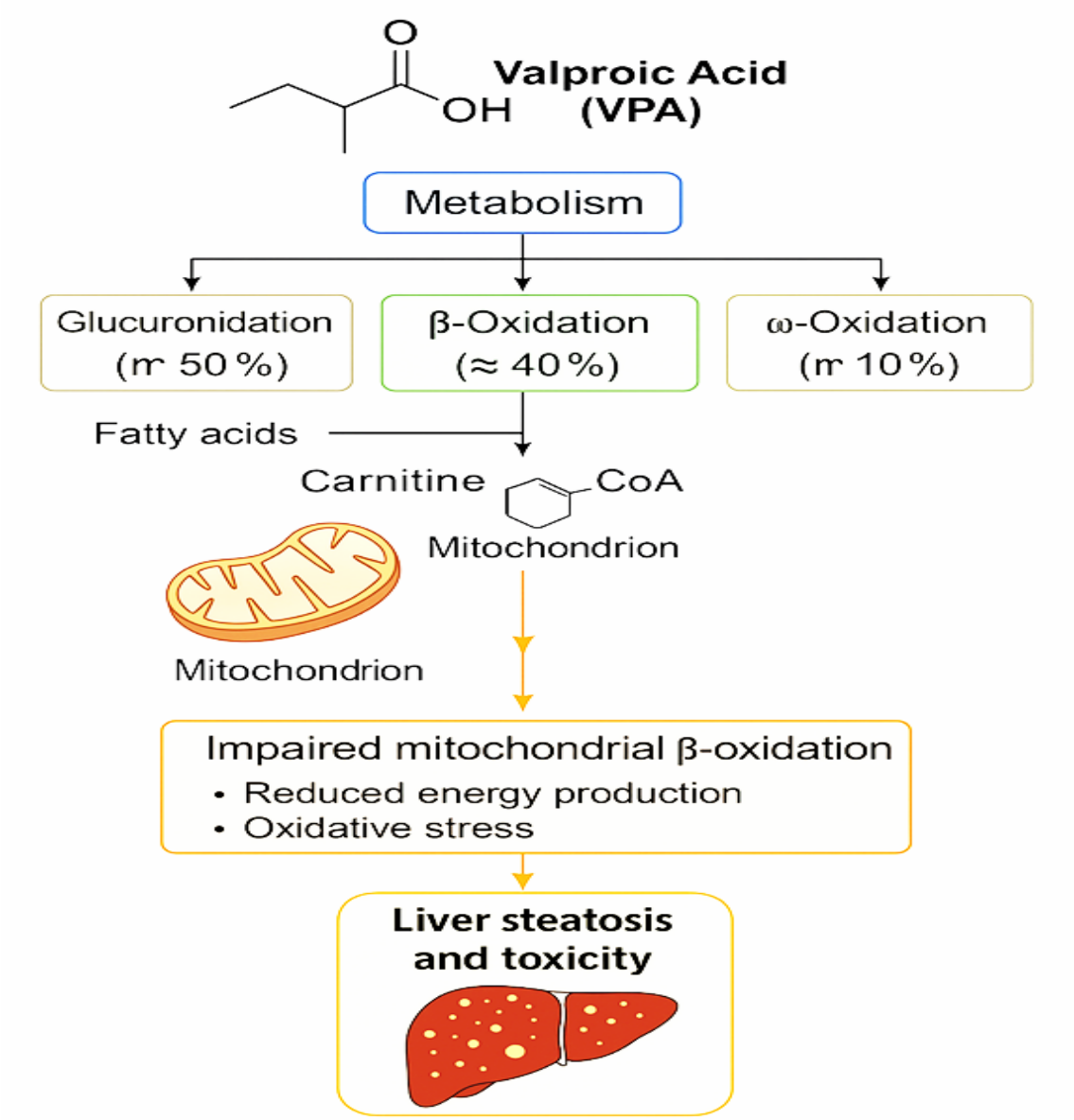

Figure 4: Metabolic pathways of Valproic Acid (VPA) and its role in liver toxicity

2.7 Post-Marketing Surveillance and Risk Management

For drugs with known or suspected mitochondrial hepatotoxicity, regulatory agencies mandate **Risk Evaluation and Mitigation Strategies (REMS)** and stringent post-marketing safety monitoring [124]. Pharmacovigilance systems such as the **FDA Adverse Event Reporting System (FAERS)** and **EudraVigilance** collect and analyze spontaneous adverse event reports to detect hepatic signals [125]. These systems have been instrumental in linking mitochondrial injury to hepatotoxicity for drugs like valproate and certain antibiotics.

2.7.1 Regulatory Gaps and Future Directions

While regulatory frameworks have improved, challenges remain in predicting idiosyncratic mitochondrial DILI before market entry. Current in vitro assays may fail to capture immune-mediated or metabolic idiosyncrasies [126]. There is growing advocacy for integrating **biomarkers**—such as serum glutamate dehydrogenase (GLDH), fibroblast growth factor 21 (FGF21), and circulating mtDNA fragments—into clinical trials to detect early mitochondrial stress [127]. Additionally, the use of patient-derived hepatocytes and induced pluripotent stem cell (iPSC) models may enhance preclinical predictivity [128].

From a regulatory science perspective, ensuring drug safety will increasingly rely on a combination of mechanistic in vitro assays, sensitive biomarkers, and active post-marketing surveillance. For drugs that remain clinically indispensable but carry mitochondrial liabilitysuch as chloramphenicolco-administration with protective agents (e.g., antioxidants) could offer a viable mitigation strategy, provided robust efficacy and safety data are presented to regulators [129].

2.8 Drugs Associated with Mitochondrial Hepatotoxicity

Numerous drugs from diverse therapeutic classes have been implicated in hepatotoxicity via mitochondrial dysfunction. This can occur through **direct inhibition of mitochondrial protein synthesis**, **impairment of electron transport chain (ETC) activity**, **disruption of β-oxidation**, **mtDNA damage**, or **induction of oxidative stress**. In many cases, these mitochondrial effects have prompted **market withdrawal** or **black box warnings** due to serious hepatic adverse events [130].

2.8.1 Antibiotics

Antibiotics are one of the most frequently implicated drug classes in DILI, with several members exhibiting mitochondrial toxicity.

- **Chloramphenicol:** Inhibits mitochondrial 55S ribosomes, reducing synthesis of ETC components and impairing OxPhos. Leads to ATP depletion, ROS overproduction, and hepatocyte injury [131]. Clinically associated with bone marrow suppression and hepatotoxicity, particularly at high doses or in prolonged therapy.

- **Tetracyclines:** Inhibit mitochondrial translation and β-oxidation, causing microvesicular steatosis [132].
- **Linezolid:** Causes mitochondrial protein synthesis inhibition, lactic acidosis, and hepatic injury [133].
- **Fluoroquinolones (e.g., trovafloxacin):** Induce mitochondrial membrane depolarization, impairing ATP synthesis and triggering apoptosis [134].

2.8.2 Antivirals

- **Fialuridine (FIAU):** Nucleoside analogue causing fatal mtDNA depletion and liver failure in clinical trials [134].
- **NRTIs (e.g., zidovudine, didanosine):** Inhibit DNA polymerase γ, leading to mtDNA loss and mitochondrial dysfunction [7].

2.8.3 Antiepileptics

- **Valproic Acid:** Inhibits CPT-I and fatty acid β-oxidation, resulting in microvesicular steatosis and hepatotoxicity [136].
- **Carbamazepine, Phenytoin:** Linked to oxidative mitochondrial injury and immune-mediated hepatotoxicity [137].

2.8.4 NSAIDs

- **Diclofenac:** Causes mitochondrial permeability transition and oxidative stress in hepatocytes [138].
- **Nimesulide:** Linked to mitochondrial dysfunction and cholestatic hepatitis [139]

2.8.5 Anticancer Agents

- **Doxorubicin:** Induces ROS generation and mitochondrial DNA damage [140].
- **Tyrosine Kinase Inhibitors (e.g., sunitinib):** Inhibit ETC complexes and cause mitochondrial swelling [141].

2.8.6 Relevance to Current Study

Among these drugs, chloramphenicol holds a unique position. It remains clinically indispensable in certain settings (e.g., typhoid fever, meningitis, rickettsial infections in resource-limited regions) but has a well-characterized mechanism of mitochondrial toxicity [131]. Its predictable effects on mitochondrial protein synthesis make it an ideal model drug for investigating antioxidant mitigation strategies in both *in vitro* and *in vivo* systems.

Drug/Class	**Primary Mitochondrial Target**	**Hepatic Outcome**	**Regulatory Status**
Chloramphenicol	Inhibits mitochondrial 55S ribosome → ↓ ETC protein synthesis	Hepatocellular injury, oxidative stress	Restricted use, no FDA black box warning
Tetracyclines	Inhibit mitochondrial translation, β-oxidation	Microvesicular steatosis	Minocycline-associated warnings
Linezolid	Inhibits mitochondrial protein synthesis	Lactic acidosis, hepatotoxicity	Boxed warning for myelosuppression
Trovafloxacin	Mitochondrial membrane depolarization	Acute hepatic necrosis	Withdrawn from market
Fialuridine (FIAU)	Inhibits DNA polymerase γ → mtDNA depletion	Fatal liver failure	Development halted
Zidovudine, Didanosine	DNA polymerase γ inhibition	Hepatic steatosis, lactic acidosis	Boxed warning
Valproic Acid	CPT-I inhibition → impaired β-oxidation	Steatosis, hepatic failure	Boxed warning
Diclofenac	Induces mPT, oxidative stress	Hepatocellular/cholestatic injury	Warning for hepatotoxicity
Doxorubicin	ROS generation, mtDNA damage	Hepatotoxicity with high-dose regimens	Active, boxed warnings for cardiac toxicity

Table 2: Examples of Drugs Associated with Mitochondrial Dysfunction-Mediated Hepatotoxicity

2.9 Chloramphenicol and Mitochondrial Dysfunction

Chloramphenicol is a broad-spectrum antibiotic originally derived from *Streptomyces venezuelae*, later produced synthetically, and historically regarded as one of the most effective agents for treating severe bacterial infections, including typhoid fever, meningitis, and rickettsial diseases [142]. Despite its clinical utility, particularly in resource-limited regions, chloramphenicol's safety profile has been marred by severe adverse effects, most notably bone marrow suppression and hepatotoxicity, the latter largely attributable to mitochondrial dysfunction [143].

2.9.1 Mechanism of Antibacterial Action vs. Mitochondrial Toxicity

The primary antibacterial mechanism of chloramphenicol involves binding to the 50S subunit of bacterial ribosomes, thereby inhibiting peptidyl transferase activity and halting protein synthesis [144].

However, because mitochondria are evolutionarily derived from α-proteobacteria, their ribosomes (55S, composed of a 39S large subunit and a 28S small subunit) share structural similarities with bacterial ribosomes. As a result, chloramphenicol can bind to the mitochondrial 39S subunit, inhibiting mitochondrial protein synthesis [145].

This off-target binding suppresses translation of the 13 essential polypeptides encoded by mitochondrial DNA (mtDNA), which form critical components of the electron transport chain (ETC) complexes I, III, IV, and V [146]. The downstream effects include:

- Impaired oxidative phosphorylation (OxPhos) and ATP depletion.
- Accumulation of NADH and increased ROS leakage from ETC complexes I and III.
- Activation of mitochondrial permeability transition (mPT) and release of apoptogenic factors.

These mitochondrial effects are amplified under metabolic conditions where cells rely heavily on oxidative phosphorylation, such as galactose-based culture media in HepG2 cells, making such systems particularly sensitive for in vitro detection of chloramphenicol-induced mitotoxicity [147].

2.9.2 Evidence from In Vitro Studies

Galactose-adapted HepG2 cells, which cannot rely on glycolysis for ATP generation, exhibit pronounced sensitivity to chloramphenicol exposure [148]. Key observations include:

- ATP depletion within 48–72 hours of exposure at micromolar to millimolar concentrations.
- ROS overproduction detected via fluorescent probes and luminescent assays.
- Altered expression of mitochondrial biogenesis regulators, such as *NRF1*, *TFAM*, and *SOD2*.

These findings reinforce the hypothesis that mitochondrial ribosomal inhibition is a primary driver of hepatocellular toxicity.

2.9.3 Clinical Manifestations

In humans, chloramphenicol-associated hepatotoxicity is relatively rare but potentially severe. Clinical cases report:

- Hepatocellular or mixed-pattern enzyme elevations.
- Acute hepatitis-like presentations in some patients.
- Risk factors including prolonged high-dose therapy, pre-existing liver disease, and concurrent mitochondrial toxicants [151].

While bone marrow toxicity remains the most recognized adverse effect, mitochondrial hepatotoxicity warrants equal caution, especially in vulnerable populations.

2.9.4 Relevance to Current Research

Chloramphenicol's well-defined mitochondrial target, dose-dependent toxicity, and persistence in essential clinical use make it a strategic model drug for evaluating antioxidant interventions. In your thesis, its selection enables mechanistic exploration of antioxidant-mediated protection across both in vitro and in vivo models, aligning directly with objectives such as:

- Quantifying mitochondrial bioenergetic deficits and ROS overproduction.
- Assessing gene expression changes in mitochondrial biogenesis and antioxidant defense pathways.
- Determining the hepatoprotective efficacy of Astaxanthin and Quercetin in mitigating chloramphenicol-induced oxidative and bioenergetic stress.

2.10 Experimental Models for Mitochondrial DILI

The accurate assessment of mitochondrial dysfunction in drug-induced liver injury (DILI) requires robust in vitro and in vivo experimental models that can reproduce the metabolic, biochemical, and pathological hallmarks observed in humans. These models allow mechanistic investigations, safety screening, and evaluation of potential protective interventions such as antioxidants.

2.10.1 In Vitro Models

- **HepG2 Cells**

HepG2, a human hepatocellular carcinoma cell line, is widely used for hepatotoxicity studies because of its well-characterized metabolic profile and reproducibility [152]. However, under normal glucose culture conditions, HepG2 cells rely heavily on glycolysis for ATP production, which can mask mitochondrial toxicities. To overcome this limitation, cells are adapted to galactose-containing media, which forces reliance on oxidative phosphorylation (OxPhos) for ATP generation [153]. This modification enhances the sensitivity of the system to detect mitochondrial toxicants such as chloramphenicol.

Key Advantages:

Human origin, relevant metabolic pathways.

Amenable to high-throughput screening.

Compatible with multi-parametric readouts (ATP, ROS, mitochondrial membrane potential).

Limitations:

Cancer-derived cells may differ from normal hepatocytes in drug metabolism.

Lower cytochrome P450 activity compared to primary hepatocytes [154].

1. Primary Human Hepatocytes (PHH)

PHHs represent the gold standard for in vitro hepatotoxicity studies due to their preserved drug-metabolizing enzyme profile and mitochondrial function [155]. They are particularly useful for confirming findings from HepG2 or other immortalized cell lines. However, their limited availability, variability between donors, and rapid loss of phenotype in culture pose challenges.

2. Other In Vitro Systems

2.1 HepaRG Cells – Hepatic progenitor-derived cell line capable of differentiating into hepatocyte-like and biliary-like cells, maintaining drug-metabolizing activity and mitochondrial function [156].

2.2 Patient-Derived iPSC Hepatocytes – Offer a personalized medicine approach by capturing genetic susceptibility to mitochondrial DILI [157].

2.3 3D Liver Spheroids – More physiologically relevant architecture and improved long-term viability for chronic exposure studies [158].

3. Key Assays for Mitochondrial Function

- ATP Measurement (e.g., CellTiter-Glo®) – Indicator of cellular bioenergetic status.
- ROS Quantification (e.g., ROS-Glo™ H O Assay) – Measures oxidative stress burden.
- Mitochondrial Membrane Potential (ΔΨm) – Assessed via fluorescent dyes such as JC-1 or TMRE.
- Oxygen Consumption Rate (OCR) – Measured using Seahorse XF Analyzer to assess OxPhos capacity.
- Gene Expression Profiling – Targets mitochondrial biogenesis (*TFAM, NRF1*), antioxidant defense (*SOD2*), and energy uncoupling (*UCP2*).

2.10.2 In Vivo Models

1. Rodent Models (Wistar Rats, Mice)

Rodents remain the most widely used in vivo systems for evaluating mitochondrial DILI. Wistar rats, in particular, are preferred for their well-characterized physiology, ease of handling, and reproducible responses to mitochondrial toxicants [159].

In chloramphenicol-induced DILI models:

- Drug is administered intraperitoneally or orally at defined doses for a specific duration (e.g., 14 days).
- Blood samples are analyzed for serum markers (ALT, AST, ALP).
- Liver tissue is examined for oxidative stress markers (GSH, NO), lipid peroxidation (MDA), and histopathology.

Strengths of Rodent Models

- Ability to capture systemic effects of mitochondrial toxicity.
- Tissue-level assessments (biochemistry, histology, electron microscopy).
- Feasibility for combination therapy studies.

Limitations:

- Species differences in drug metabolism and mitochondrial physiology.
- Ethical and cost considerations.

2.11 Translational Considerations

The combination of galactose-adapted HepG2 cells and Wistar rat in vivo studies allows for mechanistic insights that are translatable to human pathophysiology. The in vitro system permits high-throughput, mechanistic dissection of mitochondrial effects, while in vivo models validate these findings in an integrated organismal context. This dual approach aligns with modern regulatory expectations for mechanistic safety evaluation [160].

2.12 Antioxidant Therapy for Mitochondrial Dysfunction in DILI

Mitochondrial dysfunction in drug-induced liver injury (DILI) is frequently accompanied by excessive production of reactive oxygen species (ROS) and depletion of cellular antioxidant

defenses. Oxidative stress disrupts mitochondrial membranes, damages mitochondrial DNA (mtDNA), and inactivates critical enzymes, creating a self-perpetuating cycle of injury [161]. Therapeutic strategies aimed at reducing oxidative stress, stabilizing mitochondrial function, and promoting mitochondrial biogenesis have therefore emerged as promising interventions. Natural antioxidants, particularly Astaxanthin and Quercetin, have shown considerable potential in mitigating mitochondrial injury in preclinical studies.

2.12.1 Rationale for Antioxidant Therapy

ROS overproduction in DILI arises primarily from the electron transport chain (ETC) during oxidative phosphorylation. Drugs that impair ETC components (e.g., chloramphenicol inhibiting mitochondrial ribosomal protein synthesis) cause electron leakage from complexes I and III, producing superoxide anions. These anions are rapidly converted to hydrogen peroxide by superoxide dismutase 2 (SOD2), but in excess, hydrogen peroxide forms hydroxyl radicals that damage lipids, proteins, and nucleic acids [164].
Antioxidants act through multiple mechanisms:

- Direct ROS scavenging.
- Chelation of transition metals to prevent hydroxyl radical generation.
- Upregulation of endogenous antioxidant enzymes (*SOD2*, *GPx*, *CAT*).
- Stabilization of mitochondrial membranes to prevent permeability transition pore (mPTP) opening.
- Enhancement of mitochondrial biogenesis via transcriptional regulators such as NRF1 and TFAM.

2.12.2 Astaxanthin (AXN)

1. Structure and Properties

Astaxanthin is a xanthophyll carotenoid derived from marine organisms such as microalgae (*Haematococcus pluvialis*), krill, and salmon [163]. Its polyene chain and polar terminal groups enable integration into lipid bilayers, allowing it to stabilize membranes and quench ROS at both the membrane surface and the lipid core [164].

Mechanisms of Action

- Potent scavenger of singlet oxygen and peroxyl radicals.
- Inhibits lipid peroxidation in mitochondrial and cellular membranes.
- Preserves mitochondrial membrane potential (ΔΨm) under oxidative stress conditions.
- Activates Nrf2-mediated transcription of antioxidant response genes.
- Enhances mitochondrial biogenesis through upregulation of *PGC-1α* and *NRF1* [165].

2. Evidence in Liver Injury Models

Preclinical studies demonstrate that AXN reduces serum ALT and AST levels, decreases hepatic MDA content, and restores GSH levels in rodent models of hepatotoxicity induced by ethanol, CCl , and acetaminophen [166]. In mitochondrial-specific injury models, AXN preserves ATP content and reduces mitochondrial swelling [167].

2.12.3 Quercetin (QRN)

1. Structure and Properties

Quercetin is a polyphenolic flavonoid abundant in onions, apples, and berries. Its multiple hydroxyl groups confer strong radical-scavenging activity [149].

Mechanisms of Action

- Direct neutralization of ROS and reactive nitrogen species (RNS).
- Inhibition of NADPH oxidase activity, reducing ROS generation at the source.
- Activation of AMPK signaling, which promotes mitochondrial biogenesis and fatty acid oxidation.
- Induction of phase II detoxification enzymes via Nrf2 pathway activation.
- Inhibition of pro-inflammatory cytokines (TNF-α, IL-6) that exacerbate mitochondrial injury [150].

2. Evidence in Liver Injury Models

In rodent models of CCl and acetaminophen hepatotoxicity, QRN reduces ALT/AST elevations, inhibits lipid peroxidation, and preserves mitochondrial function [151]. Studies using

mitochondrial inhibitors demonstrate that QRN maintains mitochondrial membrane potential and reduces apoptosis by inhibiting cytochrome c release [152].

Combination Therapy Potential

Combining AXN and QRN may yield additive or synergistic benefits by targeting multiple mechanisms of mitochondrial protection.

- AXN's lipid membrane stabilization complements QRN's signaling pathway modulation.
- Both compounds upregulate mitochondrial biogenesis regulators (*PGC-1α*, *NRF1*, *TFAM*).
- Preliminary in vitro studies suggest combined treatment offers greater preservation of ATP levels and ΔΨm compared to either compound alone [153].

- **Relevance to Current Study**

The selection of AXN and QRN for the present research was based on:

- Proven efficacy in oxidative stress and mitochondrial injury models.
- Distinct but complementary mechanisms of mitochondrial protection.
- Favorable safety profiles with potential for translational applications.

This study aims to quantify their protective effects against chloramphenicol-induced mitochondrial toxicity in **galactose-adapted HepG2 cells** and **Wistar rats**, evaluating biochemical, molecular, and histological endpoints.

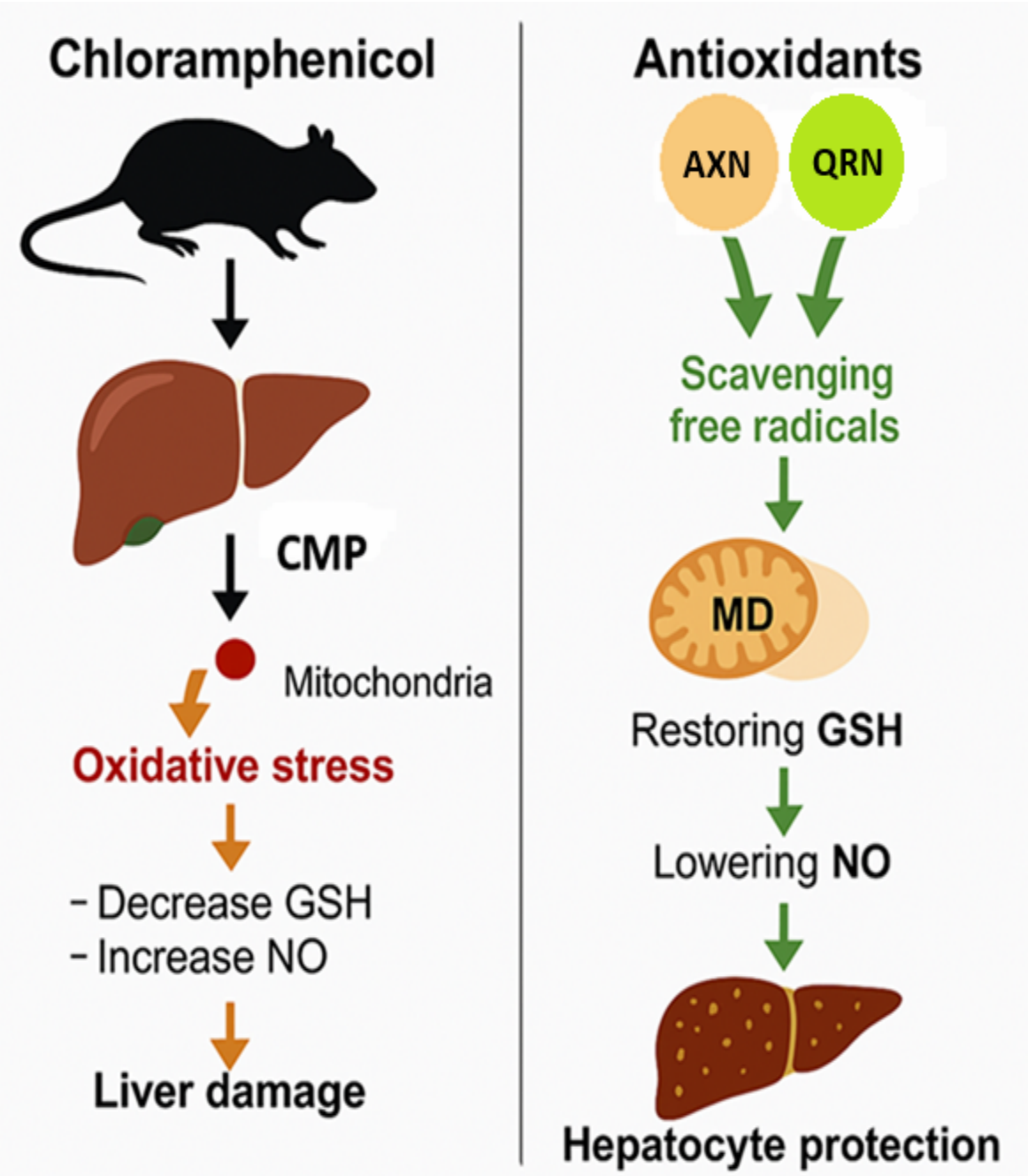

Figure 5: Schematic of antioxidant-mediated protection against chloramphenicol (CMP) induced liver injury in Wistar rats.

2.13 Biomarkers for Mitochondrial DILI

Early and accurate detection of mitochondrial dysfunction in drug-induced liver injury (DILI) is essential for both **preclinical safety screening** and **clinical management**. Conventional liver function tests (e.g., ALT, AST) are non-specific and often fail to detect injury at an early stage. In the context of mitochondrial DILI, specialized **mechanism-based biomarkers** can provide

deeper insight into pathogenesis, aid regulatory decision-making, and guide therapeutic interventions [90].

2.13.1 Serum Biomarkers

1. Glutamate Dehydrogenase (GLDH)

GLDH is a mitochondrial matrix enzyme released into the circulation upon mitochondrial membrane damage. Unlike ALT and AST, GLDH is highly specific to mitochondrial injury and is not influenced by muscle injury [154].

- ***Clinical utility*:** Differentiates mitochondrial hepatotoxicity from other types of DILI.
- ***Regulatory interest*:** FDA and EMA recommend monitoring GLDH in early-phase trials for drugs with mitochondrial liability [155].

2. Fibroblast Growth Factor 21 (FGF21)

FGF21 is a metabolic stress hormone produced in the liver and other tissues in response to mitochondrial dysfunction. Elevated plasma FGF21 levels correlate with impaired oxidative phosphorylation [156].

- ***Advantages*:** Sensitive to mitochondrial stress even before overt hepatocyte death.
- ***Limitations*:** Not liver-specific; also elevated in muscle disorders and systemic metabolic stress.

3. Circulating mtDNA

Mitochondrial DNA fragments released into the bloodstream act as damage-associated molecular patterns (DAMPs), activating innate immune responses and exacerbating inflammation [157].

- ***Advantages*:** Mechanistic relevance to mitochondrial injury.
- ***Limitations*:** Requires careful normalization to nuclear DNA levels to avoid false positives.

2.13.2 Molecular and Cellular Biomarkers

1. Gene Expression Profiles

Alterations in genes regulating mitochondrial biogenesis (*PGC-1α*, *NRF1*, *TFAM*), oxidative stress defense (*SOD2*), and apoptosis (*BAX*, *BCL-2*) can indicate mitochondrial dysfunction before biochemical markers are altered [158].

Application: Can be measured in liver biopsy samples, in vitro systems, or circulating cells.

2. Oxidative Stress Markers

Malondialdehyde (MDA): End-product of lipid peroxidation, elevated in mitochondrial ROS-mediated injury [159].

8-Hydroxy-2′-deoxyguanosine (8-OHdG): Marker of oxidative DNA damage, including mtDNA damage [160].

3. Functional Biomarkers

- **Oxygen Consumption Rate (OCR):**

  Measured in isolated hepatocytes or platelets, OCR provides a direct assessment of oxidative phosphorylation capacity [161].

  ***Advantages*:** Real-time, mechanistic data.
  ***Limitations*:** Requires specialized equipment (e.g., Seahorse XF Analyzer).

- **ATP Levels**

Total cellular ATP depletion is a hallmark of mitochondrial injury, measurable in both in vitro and in vivo systems [162].

4. Imaging Biomarkers

Magnetic Resonance Spectroscopy (MRS) can detect hepatic ATP and phosphocreatine levels, providing a non-invasive window into mitochondrial bioenergetics [163]. Although primarily a research tool, its translational potential is increasing with advancements in clinical imaging technologies.

2.13.3 Relevance to Current Study

In the present research:

- GLDH was not directly measured but could serve as a confirmatory biomarker in future work.
- GSH and NO were chosen as oxidative stress markers in the in vivo model, reflecting antioxidant defense status and nitrosative stress, respectively.
- Gene expression analysis in galactose-adapted HepG2 cells targeted *SOD2*, *NRF1*, *SURF1*, *TFAM*, and *UCP2*, all of which are relevant to mitochondrial function and stress adaptation.

  This integrative biomarker approach strengthens mechanistic interpretation and aligns with current regulatory recommendations for mitochondrial safety assessment.

2.14 Regulatory and Translational Perspectives

The growing recognition of mitochondrial dysfunction as a central mechanism in drug-induced liver injury (DILI) has significant implications for **drug safety regulation**, **clinical practice**, and **therapeutic innovation**. Your study's integration of mechanistic in vitro assays, in vivo validation, and antioxidant intervention aligns closely with emerging regulatory priorities and translational research strategies.

2.14.1 Regulatory Context

Regulatory agencies such as the **U.S. Food and Drug Administration (FDA)** and the **European Medicines Agency (EMA)** increasingly emphasize **mechanistic toxicology data** in drug development. Drugs suspected of mitochondrial liability are expected to undergo targeted mitochondrial safety evaluations, including:

- **In vitro mitochondrial function assays** in relevant cell types (e.g., HepG2 in galactose media, primary hepatocytes).
- **Biomarker integration** in preclinical and early-phase clinical studies (e.g., GLDH, FGF21, mtDNA levels).

- **Risk–benefit justification** for drugs that remain essential despite mitochondrial toxicity risks [164].

Examples of regulatory actions include:

- Withdrawal of **troglitazone** (antidiabetic) and **trovafloxacin** (antibiotic) due to mitochondrial toxicity-mediated hepatotoxicity.
- Black box warnings for **valproic acid** (antiepileptic) and **linezolid** (antibiotic) highlighting mitochondrial-related adverse effects.

2.14.2 Translational Relevance of Antioxidant Interventions

Study's dual focus on **Astaxanthin (AXN)** and **Quercetin (QRN)** addresses a critical regulatory and clinical challenge: how to **mitigate mitochondrial toxicity without compromising therapeutic efficacy**. From a translational standpoint:

- These antioxidants have favorable safety profiles and are already available as nutraceuticals in several jurisdictions.
- Their mechanisms membrane stabilization, ROS scavenging, and promotion of mitochondrial biogenesis are mechanistically aligned with the pathogenesis of mitochondrial DILI [165].
- Combining them with essential but toxic drugs like **chloramphenicol** may enable continued clinical use while reducing adverse hepatic outcomes.

2.14.3 Preclinical-to-Clinical Translation

The in vitro galactose-adapted HepG2 model provides mechanistic clarity and predictive value for mitochondrial toxicants, while the Wistar rat model offers systemic validation, including oxidative stress markers and histopathology. This combined approach:

- Satisfies regulatory expectations for tiered testing strategies.
- Facilitates selection of biomarkers that could be deployed in early-phase human trials.
- Provides data supporting risk mitigation strategies for clinical drug use.

For clinical translation:

- Dose-ranging studies of antioxidants in human volunteers could establish safe and effective co-treatment regimens.
- Biomarker-guided monitoring (GLDH, mtDNA, oxidative stress panels) could serve as early warning systems in clinical use.

2.14.4 Implications for Drug Safety Policy

Given the regulatory precedent of drug withdrawals due to mitochondrial toxicity, your findings suggest a **risk minimization pathway** for high-need drugs:

1. **Identify mitochondrial liability** during preclinical development.
2. **Introduce protective co-therapies** validated in robust models.
3. **Incorporate mechanistic biomarkers** into post-marketing surveillance.

Such a framework could be particularly impactful in resource-limited settings, where drugs like chloramphenicol remain indispensable despite known toxicity risks [166].

2.14.5 Future Perspectives

- **Clinical Trials**: Conduct phase I–II trials to evaluate antioxidant co-therapy safety and efficacy with chloramphenicol in high-risk patient populations.
- **Expanded Antioxidant Panels**: Explore combinations with other mitochondrial-protective compounds (e.g., coenzyme Q10, α-lipoic acid).
- **Regulatory Guidance Development**: Work with health agencies to develop formal guidance for antioxidant-based risk mitigation in mitochondrial DILI.
- **Precision Medicine Approaches**: Use patient-derived hepatocytes or iPSC models to personalize therapy based on genetic susceptibility to mitochondrial injury

Drug-induced liver injury (DILI) remains a major clinical and regulatory concern, representing a leading cause of drug development attrition and post-marketing drug withdrawals. Within its diverse mechanisms, **mitochondrial dysfunction (MD)** has emerged as a central and well-characterized pathway, responsible for impairing hepatocellular energy production, increasing reactive oxygen species (ROS) generation, and triggering apoptotic or necrotic cell death.

Numerous therapeutic classes including antibiotics, antivirals, antiepileptics, NSAIDs, and anticancer agents have been implicated in mitochondrial hepatotoxicity. Regulatory agencies such as the **U.S. FDA** and **EMA** have responded with drug withdrawals, black box warnings, and increased preclinical screening expectations. Among these drugs, **chloramphenicol** stands out for its **dual profile**: it remains indispensable in certain global healthcare settings but has a well-defined mitochondrial toxicity mechanism involving inhibition of the mitochondrial 55S ribosome and subsequent oxidative phosphorylation impairment.

Experimental approaches to studying mitochondrial DILI employ **tiered *in vitro* and *in vivo* models**.

- ***In vitro***: Galactose-adapted HepG2 cells enhance detection sensitivity for mitochondrial toxicants by forcing oxidative phosphorylation reliance.
- ***In vivo***: Wistar rat models reproduce systemic features of mitochondrial hepatotoxicity, enabling assessment of biochemical markers (e.g., GSH, NO) and histopathological changes.

The integration of these models facilitates translational relevance and aligns with regulatory recommendations for mechanistic toxicology studies.

Emerging evidence underscores the **therapeutic potential of natural antioxidants** in mitigating mitochondrial DILI.

- **Astaxanthin (AXN):** A xanthophyll carotenoid with potent ROS scavenging ability, membrane stabilization properties, and mitochondrial biogenesis-enhancing effects.
- **Quercetin (QRN):** A polyphenolic flavonoid with strong antioxidant, anti-inflammatory, and mitochondrial regulatory actions.
  When used individually or in combination, these compounds demonstrate the ability to preserve mitochondrial membrane potential, maintain ATP production, and attenuate oxidative stress in preclinical models.

Biomarkers such as **GLDH**, **FGF21**, and **circulating mtDNA**, alongside functional readouts like oxygen consumption rate and ATP levels, provide mechanistic insight and are increasingly recognized by regulators for preclinical and clinical applications.

From a regulatory perspective, the integration of mechanistic insights, predictive biomarkers, and protective interventions can inform **risk mitigation strategies** for drugs with essential therapeutic value but known mitochondrial liabilities. The approach taken in this thesis

combining mechanistic in vitro analysis, in vivo validation, and antioxidant-based intervention exemplifies the type of translational framework needed to bridge laboratory findings with clinical practice and policy.

Overall, the literature underscores that targeted antioxidant therapy holds promise as an adjunct strategy for preventing or reducing mitochondrial DILI, particularly for indispensable but hepatotoxic drugs such as chloramphenicol. By addressing this gap, the present research not only advances mechanistic understanding but also supports the development of clinically relevant interventions with significant global health implications.