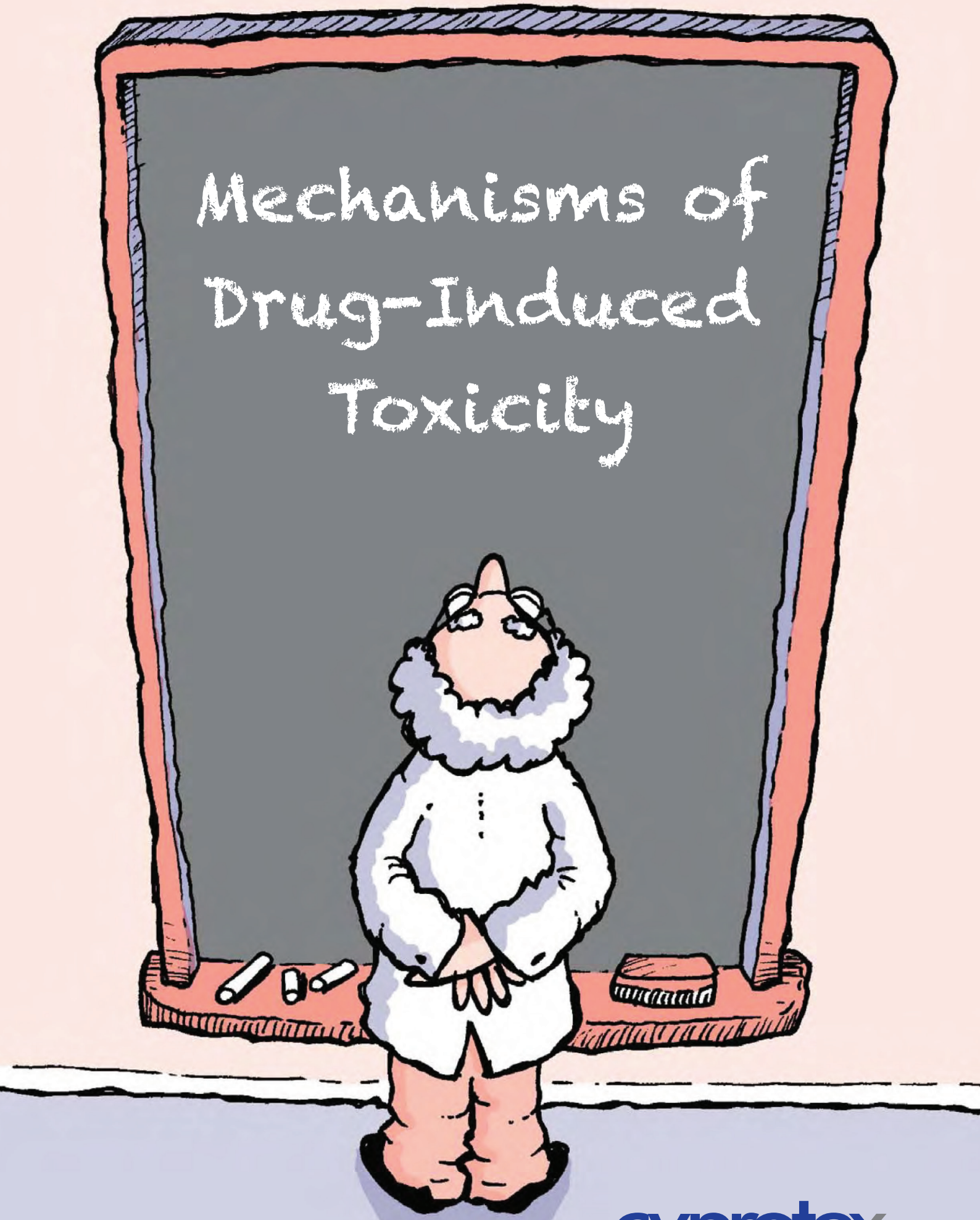
Mechanisms of Drug-Induced Toxicity



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Contents

Chapter 1: Introduction

1.1. The Impact of Drug-Induced Toxicity

Drug induced toxicity remains a key contributor to late stage attrition of drugs. Companies are still relying on preclinical safety testing to determine liability, which clearly is too late and often cannot detect certain types of toxicity due to species differences in physiology and drug metabolism compared to humans. Occasionally safety issues only become apparent during clinical trials, or even post launch. Once a therapeutic has reached the market, these toxicities can result in black-box warnings, lawsuits, or withdrawal of approved drugs from the market. Between 1975 and 1999, 16 out of 548 (2.9%) marketed drugs were withdrawn from the US market because of toxicity issues, and 56 out of 548 (10.2%) acquired a black box warning[1].

Adverse drug reactions associated with marketed drugs are a major cause of hospitalisation. One UK study estimated that 6.5% of admissions were related to an adverse drug reaction, which led to a median bed stay of eight days and accounted for 4% of the hospital bed capacity. Although the overall fatality rate was 0.15% of all patients admitted[2], in reality, this figure could be much higher, especially in the elderly, where multiple therapies are given simultaneously and adverse effects may be mistakenly attributed to age-related medical conditions.

These statistics have obviously caused considerable economic burden on both the healthcare system as well as the pharmaceutical industry and have led to a greater focus on early stage safety testing with increased resource being placed on addressing this problem. The advent of new *in vitro* cellular models (e.g., stem cell derived models) and techniques (e.g., high content imaging and high throughput microelectrode array) are helping to advance this field. The liver and the heart have been shown to be the main targets for drug-induced adverse effects with these effects often only manifesting themselves during clinical trials or post approval (table 1.1.)[3]. For this reason, cardiotoxicity and hepatotoxicity continue to be the main area of investigation and focus.

Table 1.1. Evidence, prevalence and occurrence of safety liabilities relating to the cardiovascular and hepatic systems[3].

Phase	Non-clinical	Phase I	Phase I-III	Phase III/ post-approval	Post-approval	Post-approval	Post-approval
Information	Causes of attrition	Serious ADRs	Causes of attrition	ADRs on label	Serious ADRs	Withdrawal from sale	Withdrawal from sale
Source	Car (2006)	Sibille et al. (1998)	Olson et al. (2000)	BioPrint® (2006)	Budnitz et al. (2006)	Fung et al., (2001)	Stevens & Baker (2009)
Sample size	88 CDs stopped	1,015 subjects	82 CDs stopped	1,138 drugs	21,298 patients	121 drugs	47 drugs
Cardiovascular	27%	9%	21%	36%	15%	9%	45%
Hepatotoxicity	8%	7%	21%	13%	0%	26%	32%

CD = candidate drug

0% 1-10% 10-20% >20%

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The mechanisms of toxicology are complex comprising of a multitude of different and often interlinking pathways resulting in manifestation of a particular toxic outcome. This guide has been prepared in an attempt to simplify some of the toxicological processes involved and focuses mainly on the *in vitro* approaches to understand drug-induced toxicity. It is important to note that the mechanisms of drug-induced toxicity are vast and this guide focuses predominantly on the most common mechanisms.

1.2. References

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Chapter 2: Mitochondrial Toxicity

2.1. Background

Mitochondria are membrane-bound organelles with an outer and inner membrane. The inner membrane is highly convoluted, forming folds known as cristae. It is on these cristae that most of the cell's supply of ATP (adenosine triphosphate) is generated which is the primary energy source for the cell.

The number of mitochondria in a cell varies depending on tissue type. Many cells only have one mitochondrion whereas others can contain several thousand[1]. Although most of a cell's DNA is contained in the cell nucleus, the mitochondrion has its own independent genome (mtDNA)[1] which encodes proteins involved in oxidative phosphorylation. As well as being known as the 'powerhouse of the cell', mitochondria have involvement in apoptosis, calcium signalling, regulation of cellular metabolism and proliferation and synthesis of haem and steroids[2].

2.2. Mechanisms of Mitochondrial Toxicity

Five protein complexes (Complexes I, II, III, IV and V) are located within the inner mitochondrial membrane which are involved in oxidative phosphorylation and ATP production. These complexes are involved in the following tasks[1];

- **Complex I (NADH ubiquinone oxidoreductase) and Complex II (succinate dehydrogenase)**
  During the oxidation of substrates, NAD+ (nicotinamide adenine dinucleotide) is reduced which donates electrons to Complex I and FAD (flavin adenine dinucleotide), a prosthetic group within Complex II.

- **Complex III (ubiquinol cytochrome c oxidoreductase)**
  Ubiquinone (an electron carrier) transfers electrons from Complex I and II to Complex III.

- **Complex IV (cytochrome c oxidase)**
  Cytochrome c then shuttles electrons from Complex III to Complex IV. Oxygen reacts with electrons donated by Complex IV and protons from the mitochondrial matrix to form water.

- **Complex V (ATP synthase)**
  The electron transfer process via Complex I, III and IV also results in protons being pumped from the mitochondrial matrix into the inter-membrane space. This is known as the proton motive force and consists of a pH gradient and a mitochondrial membrane potential. Complex V uses the energy contained in the proton motive force to synthesis ATP from adenosine diphosphate (ADP) and inorganic phosphate. The entire process of ATP synthesis coupled to electron transport is known as oxidative phosphorylation.

Drug induced mitochondrial toxicity can be a consequence of several mechanisms[1,3].

2.2.1. Inhibition of Protein Complexes

Certain drugs are able to inhibit Complex I (e.g., haloperidol, clofibrate, troglitazone, metformin and flutamide), Complex II/III (e.g., cyclophosphamide and ciglitazone), Complex IV (e.g., troglitazone and tamoxifen) or Complex V (e.g., troglitazone) and so impair mitochondrial ATP synthesis.

2.2.2. Uncoupling of Electron Transport from ATP Synthesis

Certain drugs may uncouple electron transport from ATP synthesis (e.g., some NSAIDs and sulphonamides) by shuttling protons across the inner mitochondrial membrane or by forming channels or simply disrupting the lipid bilayer.

2.2.3. Inhibition of Mitochondrial Membrane Transporters

Inhibition of transporters such as the adenine nucleotide translocator can cause substrate depletion and reduced ATP production.

2.2.4. Inhibition of Krebs Cycle Enzymes and Fatty Acid Metabolism

Mitochondrial dysfunction can result from inhibition of enzymes involved in the Krebs cycle (e.g., fluoroacetate) or fatty acid β-oxidation (e.g., tetracyclins and steroid hormones) resulting in depletion of substrates.

2.2.5. Inhibition of mtDNA Replication and mtDNA-encoded Protein Synthesis

Some drugs (e.g., nucleoside reverse transcriptase inhibitors) are able to inhibit mtDNA replication or mtDNA-encoded protein synthesis.

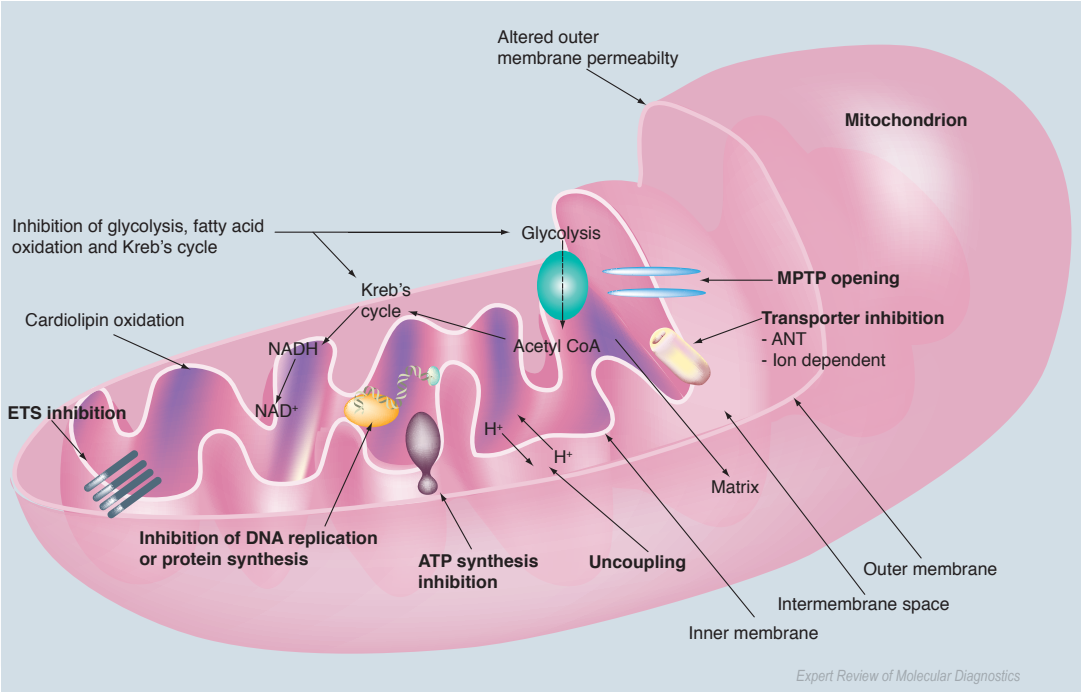
2.2.6. Oxidative Stress

Redox cycling (e.g., quinones, nitroarenes), depletion of antioxidants such as glutathione or reactive oxygen species (e.g., acetaminophen) can lead to activation of cell death signalling, induction of mitochondrial membrane permeabilisation, cardiolipin oxidation and apoptosis.

2.2.7. Mitochondrial Permeability Transition Pore

Some drugs (e.g., some anticancer drugs) can induce irreversible opening of the pore leading to influx of water and osmotic swelling.

Figure 2.1. Illustration of the mechanisms of drug-induced mitochondrial toxicity[3].



Dykens JA *et al.*, Strategies to reduce late-stage drug attrition due to mitochondrial toxicity., **7(2)** ©2007, Informa Healthcare. Reproduced with permission of Informa Healthcare.

2.3. Common *In Vitro* Methods for Assessing Mitochondrial Toxicity

The various mechanisms of drug-induced mitochondrial toxicity cannot be detected using a single assay and therefore multiple assays are recommended.

2.3.1. ATP Levels in Cells Grown in Glucose- and Galactose-Containing Medium

Many cell lines developed for use *in vitro* are metabolically adapted for growth under hypoxic and anaerobic conditions using high glucose media and derive most of their energy from glycolysis rather than mitochondrial oxidative phosphorylation (a process termed the Crabtree effect). This reduces the cells susceptibility to mitochondrial toxicants[4]. Circumventing the Crabtree effect by replacing glucose with galactose in the cell media increases the reliance of the cells on mitochondrial oxidative phosphorylation to obtain ATP. By comparing the toxic effects of different drugs in the glucose and galactose media, it is possible to detect mitochondrial impairment and identify if this is a primary effect or secondary to other cytotoxic mechanisms[4].

2.3.2. Mitochondrial Membrane Potential

A number of fluorescent dyes (e.g., tetramethylrhodamine methyl ester (TMRM), JC-1, rhodamine 123 and Mitotracker® Red) are available to measure mitochondrial membrane potential with the majority being cations that distribute to the mitochondrial matrix as a function of the Nernst equation[5] due to membrane potential differences (inside negative) and the imposed concentration gradients across the membrane. Although mitochondrial membrane potential assessment is a useful assessment of mitochondrial function, it cannot distinguish compounds which inhibit mitochondrial respiration from those that uncouple electron transport from ATP synthesis.

2.3.3. Mitochondrial Mass

Mitochondrial mass (number of mitochondria per cell) can be another measure of mitochondrial toxicity. An increase in mitochondrial mass is thought to occur as a consequence of an adaptive response by the cell to increase energy production. Early stage mitochondrial damage can also manifest itself as a decrease in mitochondrial mass in the cell. Mitotracker® Green FM and 10-N-nonyl-acridine orange chloride (NAO) dyes have the advantage that they preferentially target mitochondria regardless of mitochondrial membrane potential making them suitable tools for determining mitochondrial mass[6,7].

2.3.4. Oxygen Consumption and Extracellular Acidification

Oxygen consumption can be monitored using fluorescent based oxygen sensors. There are commercially available systems (developed by Luxcel Biosciences Ltd and Seahorse Bioscience) which can measure oxygen consumption as well as probes for monitoring extracellular acidification rate and hence glycotic rate in cells[8,9,10].

2.3.5. Inhibition of Protein Complexes involved in Oxidative Phosphorylation

Mitosciences Inc (now acquired by Abcam plc) have developed an immunocapture system for the individual protein complexes (I, II, III, IV and V) in oxidative phosphorylation. Inhibition of each complex activity in the presence of test compounds can be assessed using this technique[11].

2.3.6. mtDNA-encoded Protein Levels in Cells

Certain compounds can impair mtDNA replication (e.g., nucleoside reverse transcriptase inhibitors) or mtDNA-encoded protein synthesis (e.g., some antibiotics). Often these effects cannot be picked up in 24-72 hour cytotoxicity assays due to the fact that both mtDNA and the 13 proteins they encode have low turnover rates. A high content imaging approach has been developed which detects changes in mtDNA-encoded protein levels in eukaryotic cells[12].

2.3.7. Metabolomics

There is growing interest in metabolomics to assess drug-induced mitochondrial dysfunction. In an approach known as SIDMAP (stable isotope-based dynamic metabolic profiling), non-radioactive stable tracers (e.g., [1,2-$^{13}C_2$]-D-glucose and [U-$^{13}C_{18}$]-stearic acid) can be added to culture media of cells and the distribution of the tracer into multiple metabolites produced by processes such as the lactate production, Krebs cycle, and fatty acid oxidation can be monitored by GC-MS[13].

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Chapter 3: Reactive Oxygen Species and Oxidative Stress

3.1. Background

Reactive oxygen species (ROS) are derived from molecular oxygen and can be classified into two groups; radicals (chemical species with one unpaired electron) (e.g., nitric oxide, superoxide ion, hydroxyl, peroxyl and alkoxy radicals) and non-radicals (e.g., hypochlorous acid, hydrogen peroxide, organic peroxides, aldehydes and ozones). ROS accept electrons from antioxidants (or reducing agents). The balance between ROS and antioxidants is tightly regulated and important for maintaining vital cellular and biochemical functions. If the balance tips towards an excess of ROS over antioxidants then this is known as oxidative stress and could lead to macromolecular damage and/or disruption of redox signalling and control[1,2]. Although an excess of ROS and oxidative stress can be detrimental to the cell, ROS also have beneficial roles in gene activation, cellular growth, defence against bacteria and viruses (e.g. the killing response of immune cells) and dilation of blood vessels. Therefore, a tip in the balance to an excess of antioxidants can lead to a process known as reductive stress which can also be detrimental to cellular processes[1]. It is important to note that the topic of reductive stress is not covered in this review.

Sources of ROS can be exogenous (e.g., through γ irradiation, UV irradiation, ultrasound, food, drugs, pollutants and other chemicals) and endogenous (e.g., through white blood cells, direct and indirect ROS producing enzymes such as nitric oxide (NO) synthase and xanthine oxidase respectively, mitochondria and disease)[1].

3.2. Mechanisms of Reactive Oxygen Species and Oxidative Stress Mediated Toxicity

The targets of oxidative stress include;

- **Lipid membranes** - via lipid peroxidation and damage to cellular membranes[1].
- **DNA** - via modification of DNA bases, single and double strand breaks, loss of purines, damage to deoxyribose sugar, DNA-protein linkage and damage to the DNA repair process[1].
- **Proteins** - via peroxidation, damage to specific amino acid residues, changes to tertiary structure, degradation and fragmentation leading to loss of enzyme activity, altered cellular functions and interference in membrane potential[1].
- **Modulation of Kinase Signalling** - Multiple kinase pathways are affected by ROS and these pathways detect oxidative stress and transduce signals to initiate a cellular response. MAP kinases play a key role in this process leading to pro-apoptotic signalling through activation of JNK and p38. Activation of kinase signalling pathways such as PI3K/Akt can also occur to promote cell survival[3].

The mechanisms by which drugs cause oxidative stress are varied. Examples are shown in table 3.1.

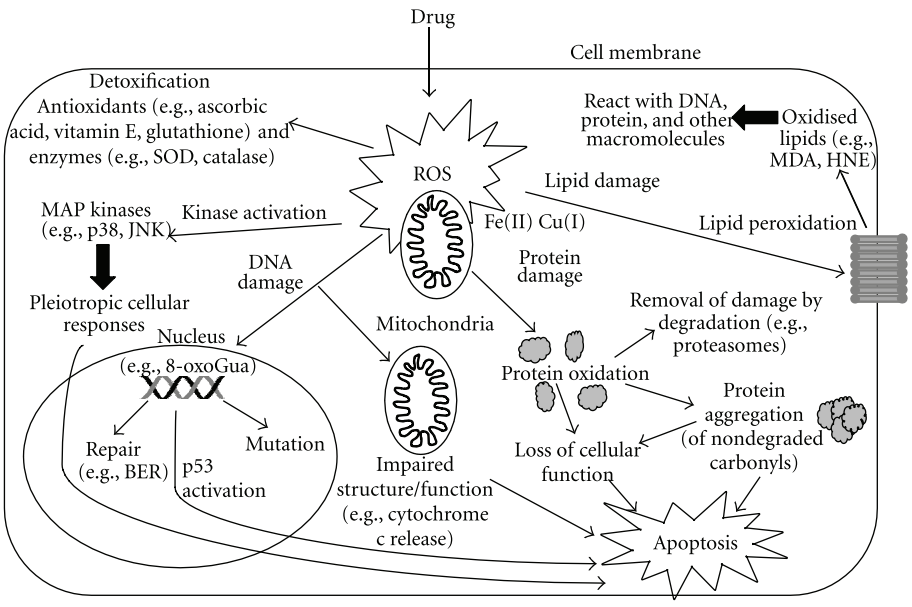
Table 3.1. Examples of mechanisms of drug-induced oxidative stress.

Drug	Toxicity related to Oxidative Stress	Mechanism
Doxorubicin	Cardiotoxicity	ROS formation resulting in lipid peroxidation, mitochondrial dysfunction, altered calcium effects, DNA damage, accumulation of p53 and activation of proapoptotic signalling cascades.
Cisplatin	Nephrotoxicity and Ototoxicity	ROS formation and subsequent lipid peroxidation, mitochondrial perturbations, decrease in antioxidants and apoptosis.
Azidothymidine (AZT)	Skeletal Myopathy and Cardiotoxicity	ROS formation leading to mitochondrial dysfunction and ultimately apoptosis and cell death.
Diclofenac	Nephrotoxicity and Hepatotoxicity	ROS formation resulting in lipid peroxidation, mitochondrial dysfunction, DNA damage, altered calcium effects and apoptosis.
Chlorpromazine	Dermal Toxicity	Photoactivation of chlorpromazine in response to UVA/B irradiation can generate ROS which react locally in the skin to cause toxicity.

The cell has certain defence mechanisms against oxidative stress. These include;

- **Antioxidant enzymes** such as superoxide dismutase (SOD), catalase and peroxidase.
- **Low molecular weight antioxidant scavengers** which are either endogenous such as glutathione (GSH), melatonin, histidine dipeptides or exogenous through diet such as ascorbic acid, tocopherols and carotenoids.

Figure 3.1. The main effects of drug-induced oxidative stress in cells[3].



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3.3. Common *In Vitro* Methods for Detecting Reactive Oxygen Species and Oxidative Stress

There are three main approaches which can be taken to assess oxidative stress as detailed below[1,4,5];

3.3.1. Direct Measurement of ROS

Although this is the preferred method, many ROS are highly unstable and difficult to measure directly which limits the use of this approach. These investigations are usually performed by electron spin resonance for relatively stable radicals. Alternatively, spin trapping methods can be used to isolate the radical where the radical reacts with a trapping molecule (e.g., C-nitroso or nitrones) to form stable nitroxide radical adducts[3].

3.3.2. Direct Measurement of Damage to Biomolecules (e.g., DNA, Proteins, Lipids)

These techniques can include:

- A common method for assessing DNA damage associated with ROS is detection of 8-OHdG (8-oxo-7,8-dihydroguanine) DNA adducts and base modifications using LC-MS or GC-MS analysis or the Comet assay. Phospho H2AX is another common marker of oxidative stress and DNA damage which is often measured using high content imaging techniques. Chemically induced oxidative stress can also be measured by incubating cells with DHE (dihydroethidium). In the presence of ROS, non-fluorescent DHE is oxidised to fluorescent ethidium which then intercalates with DNA and can be quantified by high content imaging.

- To assess lipid peroxidation, one of the most common methods is to measure the natural bi-product, malondialdehyde (MDA), by reacting with thiobarbituric acid to generate an MDA-TBA adduct which can easily be quantified colorimetrically or fluorometrically. Alternatively, other bi-products of lipid peroxidation such as 4-hydroxynonenal (4-HNE) and F_2-isoprostanes can also be measured.

- Protein oxidative damage typically results from ROS reacting with amino acid residues in proteins which can be detected using the carbonyl assay. Most of the assays for detection of protein CO groups involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl (DNP) hydrazone product. This then can be detected either by spectrophotometric, ELISA or Western blot analysis.

3.3.3. Detection of Antioxidant Levels

Glutathione is an antioxidant which acts as a protective defence mechanism against oxidative stress. Analysis of both reduced glutathione (GSH) and glutathione disulphide (GSSG) levels are useful indicators of oxidative stress, and can be measured by luciferin-based luminescent assays, spectrophotometric assays, and fluorometric assays some of which are linked to HPLC. High content screening approaches using dyes which react with reduced thiols are also becoming popular (e.g., ThiolTracker™ Violet). Other antioxidants such as ascorbate, urate, α-tocopherol and protein sulphydyls can be measured individually. A method known as TRAP (total peroxyl radical-trapping antioxidant parameter), which represents the total antioxidant capacity, is also sometimes used.

3.4. References

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Chapter 4: Reactive Metabolites

4.1. Background

Reactive metabolite mediated toxicity can either be dose dependent (e.g., acetaminophen) where the toxicity is usually predictable, or idiosyncratic (e.g., sulphamethoxazole, halothane and diclofenac) where the toxicity is unpredictable, dose independent and not evident in animal preclinical testing. Although idiosyncratic reactions are relatively rare and not always related to reactive metabolites, they can be serious and even fatal in humans and may only be discovered post-approval of the drug[1].

Due to the fact that the liver is the main organ involved in drug metabolism, chemically reactive metabolites tend to target the liver most frequently in terms of their toxic effects. A publication by Walgren *et al.*, (2005)[2] illustrates the significance of reactive metabolite formation in drug-induced hepatotoxicity. Of 21 drugs, which have either been withdrawn from the US market due to hepatotoxicity or have a black box warning for hepatotoxicity, 62% of cases have evidence for reactive metabolite formation (figure 4.1.). In addition to reactive metabolite-mediated hepatotoxicity, clinical hallmarks of hypersensitivity are often observed which can affect other organs and systems such as the skin and blood[1].

4.2. Mechanisms of Reactive Metabolite Mediated Toxicity

Drug metabolism usually acts to detoxify drugs and facilitate their elimination from the body. Occasionally, certain drugs undergo bioactivation to a reactive metabolite which, if not adequately detoxified, covalently binds to biological macromolecules (e.g., protein or DNA) and can subsequently cause drug-induced toxicity[3]. In most instances, the precise mechanisms by which chemically reactive metabolites result in toxicity are not well established. However, in the case of acetaminophen, effects on transcription factors and signalling mechanisms, apoptosis, necrosis, inflammation and activation of the innate and adaptive immune systems have been implicated[2].

Occasionally with idiosyncratic reactions, effects on the immune system and clinical signs of drug hypersensitivity are observed. These often manifest themselves as fever, rash and eosinophilia and exhibit a prompt immune-mediated response on re-challenge with the drug[2].

The hapten carrier hypothesis has been proposed as a mechanism by which reactive metabolites cause drug hypersensitivity reactions. This hypothesis is based on the fact that small molecular weight compounds are generally not immunogenic by themselves but need to be coupled to a larger molecule (e.g., proteins) to produce an immune response. Reactive metabolites are capable of irreversibly binding to proteins or other cellular macromolecules and therefore can potentially act as haptens. These altered proteins are recognised as foreign by the body and induce hapten-specific antigen presentation by T cells. Subsequent antibody production targets the sites in the protein which are modified by the hapten. Occasionally hapten-protein formation may result in altered protein proteolysis and the generation of cryptic antigens may induce autoimmunity (response against the carrier protein itself)[2].

It is important to note that the immunopathological response can only occur in the presence of a second signal (commonly called the 'danger signal') which involves modulation of cytokine release. The alteration of the cytokine balance may be a consequence of direct cellular injury by the drug or its metabolite, inhibition of key enzymes, changes in RNA/DNA/protein synthesis, decreased thiol levels or other forms of cell stress such as existing viral or bacterial infections. In fact the hepatotoxicity of some compounds (e.g., halothane, clozapine, chlorpromazine, and ranitidine) has been shown to be enhanced in the presence of low dose lipopolysaccaride (LPS)[2].

Direct metabolism-independent T cell stimulation has been proposed as an alternative mechanism to the hapten hypothesis. This suggests that activated T cells are capable of directly recognising low molecular weight drugs bound non-covalently to MHC (major histocompatibility complex) on the surface of antigen presenting cells without any requirement for metabolism or hapten protein formation[2].

One important observation has been made in the case of abacavir where it is suggested that the drug (or its metabolite) binds to a single immunological receptor to stimulate T cells. The nature of the interaction and the mechanisms involved have not been elucidated but it has been shown that the expression of a specific human leukocyte antigen (HLA) allele is associated with susceptibility to different forms of hypersensitivity and, through genetic testing, adverse reactions to abacavir in HIV patients have effectively been prevented[1].

Figure 4.1. Drugs associated with idiosyncratic hepatotoxicity which are known to form reactive metabolites[2].

Drugs Withdrawn for Hepatotoxicity:	Drugs with Black Box Warnings for Hepatotoxicity:	Drugs with Warning for Hepatotoxicity:	Miscellaneous Hepatotoxic Drugs:
5 of 6 have reactive metabolites **Benoxaprofen** **Iproniazid** **Nefazodone** **Tienilic acid** **Troglitazone**	*8 of 15 have reactive metabolites* **Dacarbazine,** **Dantrolene** **Felbamate** **Flutamide** **Gemtuzumab** **Isoniazid** **Ketoconazole** **Valproic acid**	**Acetaminophen** **Carbamazepine** **Clozapine** **Diclofenac** **Disulfiram** **Halothane** **Leflunomide** **Methyldopa** **Rifampin** **Tacrine** **Tamoxifen** **Terbinafine** **Ticlopidine** **Zileuton**	**Alpidem** **Amineptine** **Amodiaquine** **Dihydralazine** **Ibufenac** **Isaxonine** **Pirprofen**

4.3. Common *In Vitro* Methods for assessing Reactive Metabolites

Structural alerts, or toxicophores, can be used as a simple and easy guide to detect potential reactive metabolites. Anilines and anilides, arylacetic and arylpropionic acids, hydrazines and hydrazides, thiophenes, nitroaromatics and structures containing or forming α,β-unsaturated enal and/or enone-like structures are often frequently associated with severe toxicities. Despite being a useful guide, structural alerts alone should not determine reactive metabolite mitigation strategies but should act as a flag for further investigation with consideration of other qualifying factors[1].

Several *in vitro* and *in vivo* methods are available for detecting reactive metabolite formation. Recent studies have shown the benefit of combining these data with other *in vitro* toxicity data for the prediction of clinical hepatotoxicity[3,4].

4.3.1. Trapping and Characterising Reactive Metabolites

Reactive metabolites can be broadly classified into electrophiles ('hard' or 'soft') and radicals (containing an unpaired electron)[5].

Electrophilic reactive metabolites, formed in the presence of a drug metabolising system, are often able to form adducts with small molecule trapping agents. These adducts can easily be characterised by LC-MS/MS and can provide indirect information on the structure of the reactive species from which they are formed, enabling chemical intervention strategies to be devised.

Although no single trapping agent can act as a universal surrogate, several common trapping agents are routinely used including glutathione or gluthatione ethyl ester (trap quinoneimines, nitrenium ions, arene oxides, epoxides, quinones, imine methides and Michael acceptors), potassium cyanide (traps iminium ions) and methoxylamine and semicarbazide (traps aldehydes)[5]. Glutathione tends to be one of the most widely used trapping agent as it works well for a range of soft electrophiles, however, one disadvantage is that it has a low trapping efficiency for hard electrophiles. A number of reportedly more specific and sensitive methods have been published in recent years. These include use of γ-glutamylcysteinlysine (γ-GSK) which can trap both hard and soft electophiles simultaneously, use of glutathione ethyl ester and use of stable isotope labelled glutathione[6,7,8].

For detection of radicals, electron spin resonance is the standard technique. If the radical is particularly unstable then spin trapping agents (e.g., C-nitroso or nitrones which form stable nitroxide radical adducts with free adducts) can be used to isolate the radical[5].

4.3.2. Covalent Binding to Proteins

One known characteristic of reactive metabolites is that they irreversibly bind to cellular macromolecules. Assessment of covalent binding of the reactive metabolite to proteins is a common technique, often used at a later stage of drug development due to the fact that radiolabelled compound is required. The method can be performed *in vitro* using liver microsomes or hepatocytes, or *in vivo* (typically in the rat)[9].

An original threshold of 50 pmol equivalent bound per mg total liver protein was proposed by Evans *et al* (2004)[9]. Below this level (in both *in vitro* and *in vivo* studies) being a signal to advance the compound. If the covalent binding is above this threshold then further assessments may be required and other qualifying factors may need to be taken into consideration (e.g., therapeutic area, clinical dose, duration of therapy and other factors)[5]. More recent studies have proposed alternate thresholds for covalent binding with the relevance of the data dependent on other end-points being flagged[3,4]. In a study by Thompson *et al.*, (2012)[3], covalent binding burden was estimated by determining the covalent binding of radiolabelled compound to human hepatocytes and factoring in the maximum daily dose and the fraction of metabolism leading to covalent binding. Combining the covalent binding burden with a number of other *in vitro* assays (viability in THLE and THLE-CYP3A4 cells, mitochondrial toxicity assessment and BSEP/Mrp2 inhibition) enabled an integrated *in vitro* hazard score to be determined. Another study by Sakatis *et al.*, (2012)[4] described an approach where daily dose, glutathione trapping and cytochrome P450 time dependent inhibition data were combined with covalent binding data to predict potential hepatotoxicity.

4.3.3. Time Dependent Cytochrome P450 Inhibition

Formation of reactive metabolites often occurs through cytochrome P450-mediated bioactivation. If the metabolite is particularly reactive then it can bind irreversibly to the active site of the enzyme resulting in loss of enzyme activity – a process known as mechanism based inhibition. As the interaction is irreversible, the inactivated enzyme must be re-synthesised before activity is restored[5].

Time dependent cytochrome P450 inhibition studies are used to identify these types of interaction where enhanced inhibition is observed if the test compound is pre-incubated with the metabolising system prior to the addition of a specific cytochrome P450 probe substrate. A positive result in this assay indicates the formation of reactive metabolites which covalently bind to cytochrome P450 enzymes[5].

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Chapter 5: Cell Cycle Mediated Toxicity

5.1. Background

The primary function of the cell cycle is to duplicate DNA in the chromosomes and then segregate the copies precisely into two genetically identical daughter cells.

There are 4 main sequential phases:

G1 Phase (first growth phase) – The G_1 phase is part of the interphase stage and represents a gap stage which allows cells time to grow and to prepare for DNA synthesis. Under certain conditions, cells in G_1, before commitment to DNA replication, can enter a resting stage called G_0 phase where the cells do not proliferate.

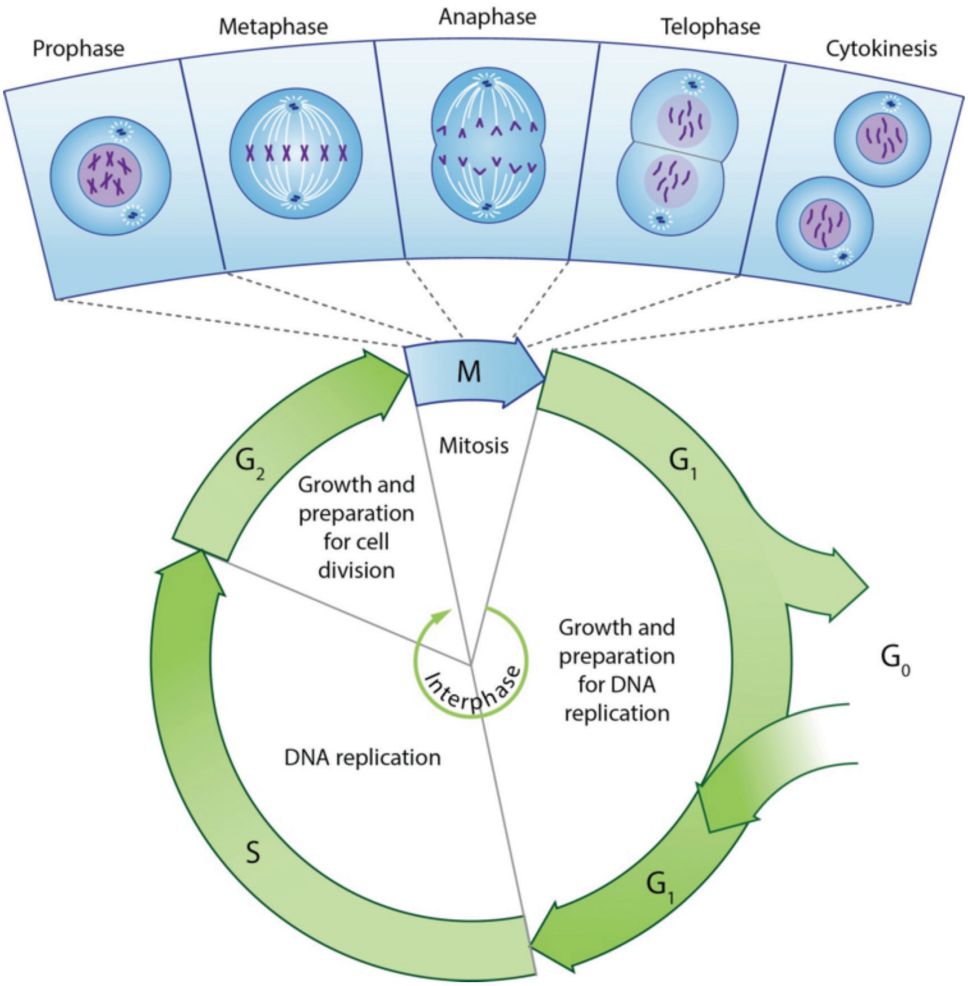
S Phase (synthesis phase) – The S phase is part of the interphase stage. DNA duplication occurs during this phase.

G_2 Phase (second growth phase) – The G_2 phase is part of the interphase stage and represents a gap stage which allows cells time to grow and prepare for mitosis.

M Phase (mitosis phase) – The M phase is when chromosome segregation and cell division occur. The M phase is subdivided into several processes;

- *Prophase* - Chromatin in the nucleus begins to condense. Centrioles begin moving to opposite ends of the cell.
- *Metaphase* - Spindle fibres align the chromosomes along the middle of the cell nucleus.
- *Anaphase* – The spindle fibres shorten and the paired chromosomes separate and move to opposite sides of the cell.
- *Telophase* - Chromatids arrive at opposite poles of cell, and new membranes form around the daughter nuclei. The spindle fibres disperse.
- *Cytokinesis* - Cytokinesis results when the cytoplasm of the cell is pinched into two daughter cells.

Figure 5.1. Illustration of the main phases of the cell cycle.



5.2. Mechanisms of Cell Cycle Mediated Toxicity

Progress through the cell cycle is monitored at the following key checkpoints.

- **G_1 (Restriction)-S Checkpoint** – decision point on whether the cell should divide, delay division, or enter a resting stage.
- **S Phase (Replication) Checkpoint** – checkpoint determines progression through S-phase.
- **G_2-M Checkpoint** – decision point on whether to trigger the start of the M-phase.
- **Metaphase (Spindle Assembly) Checkpoint** – checkpoint ensures chromosome alignment and spindle integrity before proceeding through mitosis.

These checkpoints have 2 main functions;

- To ensure that each stage of the cell cycle is completed before progression into the next stage.
- To detect and respond to DNA damage by delaying or arresting DNA replication and mitosis pending repair of the damage[1].

The control of the cell cycle through these checkpoints is based on highly complex signalling pathways, which are not covered in detail in this chapter. However, central to the regulation of these processes are the cyclin-dependent kinases (cdks) and the cyclin proteins. Both phosphorylation state of the cdks and binding to the cyclin proteins is a necessity for activation of the cdks. Individual cyclins are expressed at specific stages of the cell cycle ensuring that cdks can only be activated at particular times during the cell cycle. For example; in G_1 phase, D-type cyclins activate cdk4 and/or cdk6; in late G_1 phase, cyclin E activates cdk2; in S phase, cyclin A activates cdk2; in M phase, cyclin B1 activates cdk[1,2,3,4,5,6].

Due to the complex signalling pathways, which can result in activation (proliferation) or inhibition (arrest) of the cell cycle, the mechanisms by which drugs can act are varied and may exert beneficial effects in the case of cancer therapeutics or detrimental effects, often as a consequence of DNA damage/genotoxicity.

Examples of drugs affecting the cell cycle include;

- Etoposide, which inhibits DNA topoisomerase II, and has its greatest effect at the G_2-M checkpoint[7].
- Vinblastine, which is a microtubule inhibitor, and arrests the cells in mitosis[8].
- The antimetabolite, 5-fluorouracil inhibits thymidylate synthase, which blocks synthesis of the nucleoside thymidine, and so affects DNA synthesis at the S phase.

5.3. Common *In Vitro* Methods for Assessing Cell Cycle Effects

Effects on the cell cycle can be detrimental (in the case of unwanted DNA damage and/or potential induction of apoptosis) or beneficial (in the case of cancer therapeutics). Understanding the precise stage of the cell cycle affected is important in identifying the mechanism of action/toxicity.

Single time point measurements can be used to measure the percentage of cells in G_1 vs S vs G_2/M but not provide information on cell cycle kinetics. Other methods such as time lapse measurements of cell populations synchronised in the cycle, or the use of agents to arrest at a specific point in the cell cycle can be used to investigate the kinetics of progression through the cell cycle. For example, vinblastine or colcemide can be used to arrest in mitosis, and the rate of cell entrance into mitosis can be estimated from the cumulative increase in the percentage of mitotic cells as a function of time of the arrest.

A number of markers are available for evaluating cell cycle effects as detailed below;

5.3.1. DNA Content

Fluorescent dyes are available (e.g., Hoescht, DAPI and propidium iodide) which bind to DNA in the nucleus and can be used as a quantitative measure of cellular DNA content. Using this technique in proliferating cells, it is possible to identify the cell distribution during the various phases of the cell cycle due to changes in DNA content. As the G_2 and M have identical DNA content it is difficult to discriminate between these phases, however, it is possible to distinguish between G_1 vs S vs G_2/M (G_2/M has twice as much DNA as G_1 cells). By co-staining with other markers of the cell cycle (e.g., by monitoring individual cyclin expression in addition to DNA content), it is possible to better discriminate between the phases of the cell cycle.

5.3.2. Tritiated Thymidine, BrdU (Bromodeoxyuridine) or Click-iT® EdU Incorporation

Some of the most commonly used cell proliferation methods are based on the incorporation and measurement of nucleoside analogues in newly synthesised DNA during S phase. Non-radiolabelled methods such as the BrdU and the Click-iT® EdU assays tend to have superseded more traditional radioactive methods such as the tritiated thymidine assay. BrdU is used to label DNA and then following DNA denaturation, anti-BrdU antibodies are detected by immunofluorescence. Click-iT® EdU is the most recently introduced method. EdU is incorporated into new synthesized DNA and is detected using a labelled fluorescent azide probe and a click chemistry reaction based on copper catalysis. It therefore avoids use of antibodies and any denaturation steps[9]. These methods detect cell proliferation by identifying cells which have progressed through the S phase of the cell cycle by quantifying the extent of incorporation of the nucleoside analogues in the DNA.

5.3.3. Ki-67

Ki-67 is present in all cycling cells but is not present in quiescent cells (G_0). The Ki-67 staining pattern is variable depending on the phase of the cycle. In the G_1/S/G_2 phases it is punctate (visible spots inside the nucleus), whereas in late G_2/M it appears throughout the entire nucleus. The assay uses a highly specific antibody against Ki-67 which is detected through immunofluorescence[10]. Ki-67 acts as a general marker of cell proliferation.

5.3.4. PCNA

PCNA (proliferating cell nuclear antigen) is a protein which is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle. It helps in the binding of DNA polymerase delta to DNA, and assists in DNA repair as well as general DNA synthesis. Levels of PCNA increase in the nucleus during the G_1-phase, peaks at the S-phase, and declines during G_2/M-phases of the cell cycle[11]. Like Ki-67, PCNA acts as a marker of cell proliferation.

5.3.5. Phospho Histone H3

Phospho-histone H3(Ser 10) is expressed in the nuclei of cells during M-phase (mitosis) and so it commonly used as a measure of the mitotic index (% cells in M phase). Detection is typically through immunofluorescence[12]. This is particularly useful for detection of drugs which disrupt microtubules and so arrest the cell cycle in the M phase[13].

5.3.6. Cyclins and Cdk

Antibodies against cyclins A, B1, D1, D3 and E and cdk1 and 2 have been developed. These proteins are exclusively expressed in cycling cells, and in the case of cyclins are only expressed in particular regions of the cell cycle[2]. If DNA content is monitored also, it is possible to discriminate between cells having the same DNA content but residing in difference phases of the cell cycle based in differences in specific cyclin levels. For example, G_2 and M cells can be distinguished based on differences in cyclin A content. G_0 cells lacking expression of cyclin D or cyclin E can also be distinguished from G_1 cells which are cyclin D and/or cyclin E positive[13].

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Chapter 6: Apoptosis

6.1. Background

Apoptosis is the process of programmed cell death. It can be a beneficial process (e.g., during embryonic development and as a natural process to remove unwanted cells) or a detrimental process (e.g., if suppressed in the case of autoimmune diseases, cancer or inflammatory disease). The morphology of apoptotic cells is characterised by;

- Cell shrinkage and rounding (losing contact to neighbouring cells) caused by breakdown of the proteinaceous cytoskeleton by caspases.
- Nuclear chromatin condensation into compact patches against the nuclear envelope.
- DNA fragmentation.
- Membrane blebbing.
- Cytoplasm appears dense and organelles appear tightly packed.
- Cell eventually breaks apart into several vesicles (apoptotic bodies) which are then engulfed by phagocytoic cells and removed before contents spill out onto surrounding cells and cause damage (this is in contrast to necrosis where cell contents are released in an uncontrolled manner and result in damage of surrounding cells and a strong inflammatory response)[1].

The morphological changes observed are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA as well as the cleavage of a multitude of specific protein substrates[2].

It is important to note that cell death can also occur through other processes such as necrosis and autophagy. These processes are not covered in detail in this booklet but are briefly described below:

- Necrosis is an unordered, unplanned process which results in cell swelling and organelle disruption, and ultimately premature death of the cells and tissues. The necrotic cells usually lyse causing release of the contents of the cells which can lead to inflammation.
- Autophagy is a form of programmed cell death with different pathways and mediators from apoptosis. The purpose of autophagy is to maintain the balance between the manufacture of cellular components and the breakdown of damaged or unnecessary organelles or other cellular constituents. The lysosomes play a major role in this process.

6.2. Mechanisms Involved in Apoptosis

The process of apoptosis is complex and is controlled by a diverse range of extracellular (e.g., toxins, hormones, growth factors, nitric oxide or cytokines) or intracellular (often as a consequence of cell stress caused by activation of nuclear receptors or increased intracellular calcium concentrations) signals. These signals may positively (i.e., activate) or negatively (i.e., inhibit) regulate apoptosis. After a cell receives stimulus, it usually undergoes organised degradation of cellular organelles by activated proteolytic caspases. Activation of upstream (initiator) caspases such as caspase 2, 8, 9 and 10 by pro-apoptotic signals leads to proteolytic activation of the downstream or effector caspases 3, 6 and 7[1].

Two major signalling pathways which lead to apoptosis have been identified[3]. There is evidence to suggest that these pathways are linked with the molecules in one pathway having the ability to influence the other[4]. These pathways are described below:

6.2.1. Mitochondrial Mediated (Intrinsic Pathway)

Mitochondria play a key role in apoptosis. In response to excessive levels of cell stress, the mitochondria can initiate or enhance cell death signalling. Following these cell stress stimuli, the fate of the cell is under the control of the protein family, Bcl-2, including the pro-apoptotic family members Bax and Bak, which are regulated by a number of anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1) and pro-apoptotic (BH3-only) family protein members. Once activated, and if not suppressed, Bax and Bak are capable of inducing mitochondrial outer membrane permeabilisation[4].

Mitochondrial outer membrane permeabilisation results in pro-apoptotic factors being released and crossing the outer membrane to induce apoptosis by caspase activation and DNA fragmentation[5]. These pro-apoptotic factors include;

- **Cytochrome c** – binds to apoptotic protease activating factor 1 (Apaf-1) and dATP which initiates apoptosome complex and activates caspase 9 and triggers caspase cascade.
- **Apoptosis inducing factor (AIF)** – translocates to the nucleus where it results in chromatin condensation and DNA fragmentation via caspase independent mechanisms.
- **Endonuclease G (EndoG)** – translocates to the nucleus where it can result in DNA fragmentation.
- **Omi/HtrA2** – contributes to apoptosis by cleaving IAP (inhibitor of apoptosis proteins) and cytoskeletal proteins.
- **Smac/DIABLO** – promotes caspase activation by binding to and sequestering IAP.

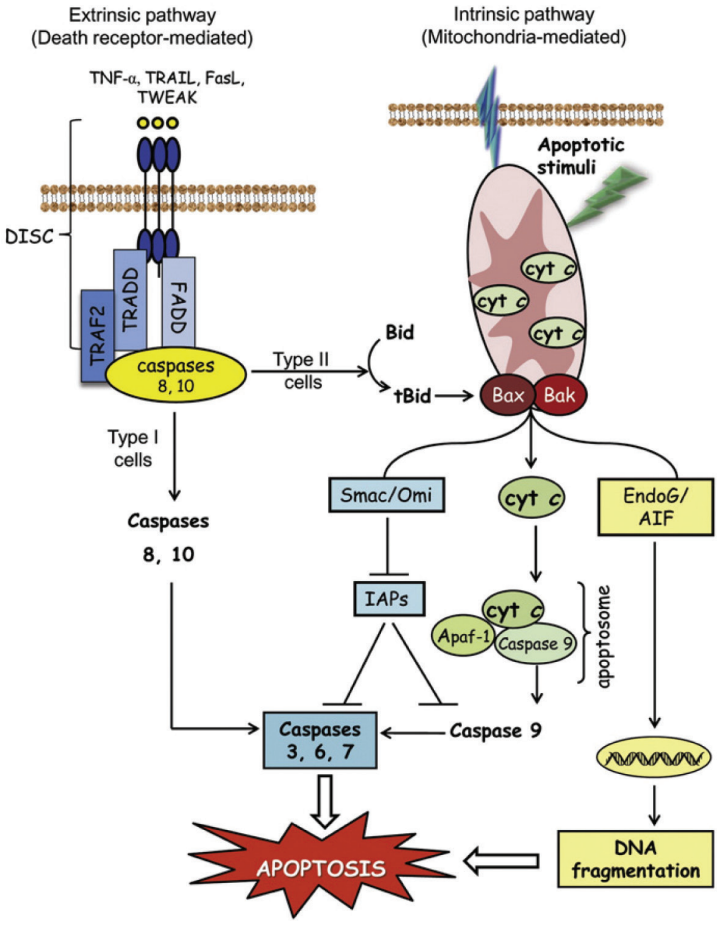
Excessive calcium influx into the mitochondrial matrix can result in formation of a complex consisting of cyclophilin D, the voltage-dependent anion channel (VDAC) and the adenine nucleotide transporter (ANT). This complex forms the mitochondrial permeability transition pore (MPTP) which spans the inner and outer membrane allowing water and other molecules to enter the mitochondria, resulting in swelling and rupture of the membranes and release of mitochondrial pro-apoptotic factors[5].

6.2.2. Receptor Mediated (Extrinsic Pathway)

The extrinsic or death receptor-mediated pathway is initiated by the engagement of ligands (e.g., FasL, TNF-α, TRAIL, and TWEAK) with surface receptors leading to formation of the death-inducing signalling complex, and activation of initiator caspases 8 and/or 10. In type I cells (mitochondrial independent), caspase 8 activates effector caspases 3, 6 or 7, while in type II cells (mitochondrial dependent), caspase 8 cleaves pro-apoptotic Bid and leads to activation of Bax and/or Bak which in turn induce mitochondrial outer membrane permeabilisation[3].

The two signalling mechanisms described above provide a relatively simplified overview of apoptosis which is controlled by a highly complex array of different stimuli and signalling pathways, many of which are not covered in this summary.

Figure 6.1. Simplified schematic diagram of the key signalling pathways involved in apoptosis[3].



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6.3. Common *In Vitro* Methods for Assessing Apoptosis

6.3.1. Caspase Activation

Caspase activation plays a major role in the execution of apoptosis. Various kits are available for detecting the activity of key caspase proteins typically using specific substrates, dyes and/or antibodies.

6.3.2. Mitochondrial Assessment

Mitochondria are known to play an important role in apoptosis.

- **Mitochondrial Membrane Potential** - Disruption of the mitochondrial transmembrane potential is one of the earliest intracellular events that occur following initiation of apoptosis. Various fluorescent dyes (e.g., TMRM, JC-1, rhodamine 123 and Mitotracker® Red) are available which accumulate in mitochondria when the membrane potential is functioning normally.

- **Mitochondrial Permeability Transition Pore (MPTP)** - The mitochondrial permeability transition pore is a nonspecific channel which appears to be involved in the release of mitochondrial components during cell death. Assays are available which utilise a fluorescent dye and quencher. In healthy cells, the dye, but not the quencher, enters the mitochondria and then accumulates. On mitochondrial pore opening the dye exits the mitochondria and is quenched in the cytoplasm.

6.3.3. Pro-Apoptotic and Anti-Apoptotic Proteins

Various antibodies are available to detect specific pro-apoptotic and anti-apoptotic proteins. These include cytochrome c, the Bcl-2 proteins, IAP, Smac/DIABLO and many others.

6.3.4. Nuclear Morphology/Nuclear Condensation

Dyes are available (e.g., Hoescht and DAPI) which bind to DNA in the nucleus and enable morphological changes in the nucleus to be observed.

6.3.5. Annexin V

During apoptosis, the characteristic cell surface phospholipid asymmetry is disrupted. This leads to the exposure of phosphatidylserine on the outer leaflet of the cell membrane. In the presence of calcium, Annexin V preferentially binds phosphatidylserine and, when conjugated to a dye or fluorescent molecule, can be used as an indicator of apoptosis.

6.3.6. TUNEL Assay

This assay detects DNA strand breaks of apoptotic cells. 3′hydroxyl ends are exposed during DNA fragmentation and can be labelled (e.g., using BrdUTP).

6.3.7. Actin Cytoskeleton

During apoptosis, changes in actin cytoskeleton are occasionally affected. This can be detected by measuring increases in F-actin by labelling with phalloidin, an F-actin specific stain.

6.4. References

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Chapter 7: Genotoxic and Non-Genotoxic Carcinogens

7.1. Background

Carcinogens are classified according to their mode of action as genotoxic or non-genotoxic carcinogens.

- **Genotoxic carcinogens** – direct interaction of the chemical (or its metabolite) with DNA and/or the cellular apparatus that regulates the fidelity of the genome, such as the spindle apparatus and topoisomerase enzymes[1].
- **Non-genotoxic carcinogens** – act via a non DNA damaging mechanism in which a wide variety of cellular processes may be involved. The diversity of modes of action of non-genotoxic carcinogens, the tissue and species specificity, and the absence of genotoxicity makes predicting their carcinogenic potential extremely challenging[2].

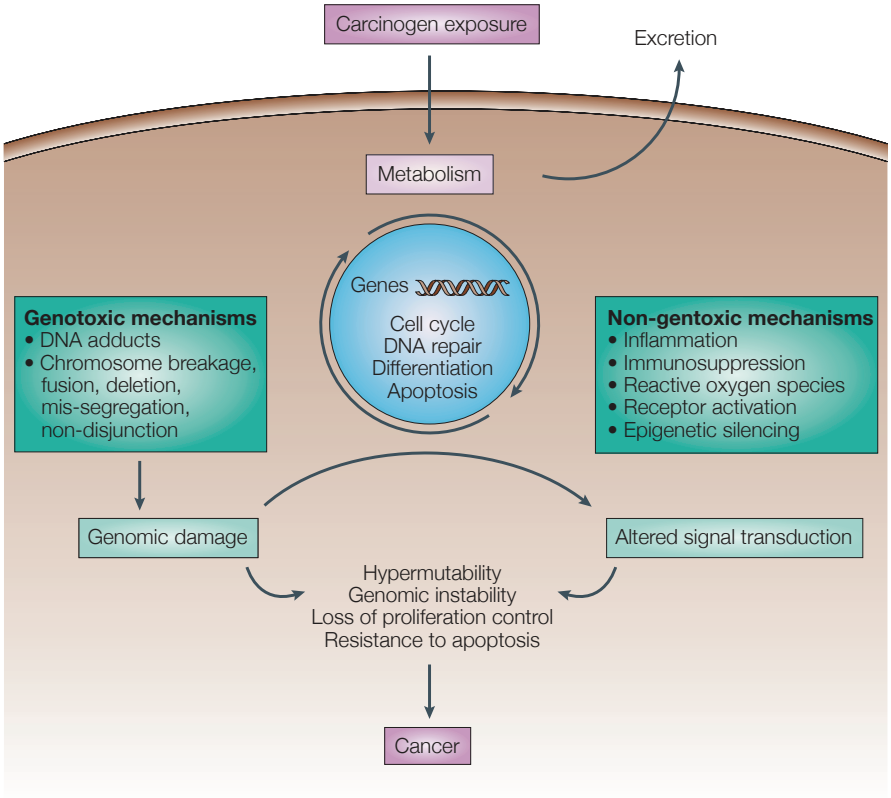
7.2. Mechanisms of Genotoxic and Non-Genotoxic Carcinogens

The main mechanisms by which genotoxic and non genotoxic compounds exert their effects are illustrated in table 7.1. and figure 7.1.

Table 7.1. Main genotoxic and non-genotoxic mechanisms of carcinogenicity[2,3].

Genotoxicity Mechanisms		Description
Gene Mutations (often caused by DNA adducts)	Point Mutations	A simple change in one base of the gene sequence.
	Frameshift Mutations	One or more bases are either inserted or deleted.
Chromosome Aberrations	Chromosome Number	Numerical change in part of the chromosome set (aneuploidy) or a numerical change in the whole set of chromosomes (polyploidy).
	Chromosome Structure (clastogenicity)	Structural change in chromosome caused by breakage, deletion or rearrangement.
Non-Genotoxicity Mechanisms		**Description**
Receptor-Mediated Endocrine Modifiers		Endocrine modifiers can act by binding to receptors such as the estrogen receptor (e.g., estradiol), the progesterone receptor (e.g., progestins), the aryl hydrocarbon receptor (e.g., TCDD) or the thyroid receptor (e.g., 'non-dioxin-like' polychlorinated biphenyl). Activation of these receptors can then result in changes in signalling transduction pathways which ultimately can affect cellular processes such as proliferation, differentiation, cell motility, apoptosis and drug metabolising enzyme expression.
Non-Receptor Mediated Endocrine Modifiers		Several drugs/chemicals (e.g., 6-propyl-2 thiouracil, phenobarbital, chlordane, polybrominated biphenyls) can effect metabolism of thyroid hormones and are thought to induce tumour formation through this mechanism.
Tumour Promoters		Many non-genotoxic carcinogens have tumour promoting activity but often other mechanisms (e.g., oxidative stress, cytotoxicity or regenerative hyperplasia) are required for cancer to develop.
Immunosuppressants		Immunosuppressants (e.g., cyclosporine and phenytoin) can play an important role in tumour induction through effects on cytokine levels and immune cell activity.
Tissue-Specific Toxicity and Inflammatory Responses		Induction of tissue specific toxicity resulting in inflammation and regenerative hyperplasia is thought to be an important mode of action for some non-genotoxic carcinogens. Metals such as arsenic and beryllium are thought to act through this mechanism.
Inhibition of Gap-Junction Intercellular Communications (GJIC)		Communication between the cells is important in maintaining homeostasis by controlling cell differentiation and proliferation. Many non-genotoxic carcinogens inhibit or reduce GJIC (e.g., chlordane, TCDD).

Figure 7.1. Overview of the mechanisms of genotoxic and non-genotoxic carcinogenesis[3].



In the cell, carcinogens (or their metabolic products) can act through either genotoxic or non genotoxic mechanisms. Together, these genotoxic and non-genotoxic mechanisms can alter signal-transduction pathways that finally result in hypermutability, genomic instability, loss of proliferation control, and resistance to apoptosis - some of the characteristic features of cancer cells.

7.3. Common *In Vitro* Methods for Assessing Genotoxic and Non-Genotoxic Carcinogens

7.3.1. Genotoxic Carcinogens

The regulations for genotoxicity testing of pharmaceuticals are covered under the ICH Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use. S2 (R1) Issued Nov 2011[4]. This guidance recommends assessing one of 2 options;

Option 1;

i. Gene mutation test in bacteria (e.g., Ames test).

ii. Cytogenetic test for chromosomal damage (the *in vitro* micronucleus test, the *in vitro* chromosome aberration test or the mouse lymphoma assay).

iii. *In vivo* test for genotoxicity, generally for chromosomal damage using rodent haematopoietic cells (usually micronuclei or chromosome aberrations in metaphase cells).

Option 2;

i. Gene mutation test in bacteria (e.g., Ames test).

ii. *In vivo* test for genotoxicity using 2 different tissues types, generally rodent haematopoietic cells and a second *in vivo* assay (typically to assess DNA strand breakages in liver).

Many other non-regulatory methods are available, and are often used as early screening tools. The most commonly used regulatory and non-regulatory tests for detecting genotoxic carcinogens are detailed in table 7.2.

Table 7.2. Common *in vitro* methods for detecting genotoxic carcinogens.

Method	Description	ICH Regulatory Approved
Bacterial Reverse Mutation Test (Ames Test)[4,5]	The test uses strains of the *Salmonella typhimurium* or *E.Coli* bacteria which carry a defective (mutant) gene that renders them unable to synthesise the amino acid histidine. The Ames test investigates the potential of the test compound to result in a back mutation that causes the gene to regain its function and grow in a histidine-free medium. The test detects point and frameshift mutations.	Yes
***In Vitro* Mammalian Cell Micronucleus Test**[4,6]	During cell division, if a chromosome or a fragment of a chromosome is not incorporated into one of the daughter nuclei, typically as a consequence of genetic damage, it may form its own "micronucleus". The *in vitro* micronucleus test detects both aneugens (compounds which cause an abnormal number of chromosomes) and clastogens (compounds which cause breaks in chromosomes, leading to sections of the chromosome being deleted, added, or rearranged). The assay uses cytochalasin B to block cytokinesis and therefore accumulates dividing cells at the binucleated stage where the formation of micronuclei can easily be observed using staining techniques.	Yes
***In Vitro* Mammalian Chromosomal Aberration Test**[4,7]	This test detects the presence of chromosome aberrations microscopically after the cells have been arrested in the metaphase.	Yes
Mouse Lymphoma L5178Y Cell Tk (thymidine kinase) gene mutation assay (MLA)[4,8]	The test has the potential to detect mutagenic and clastogenic events at the thymidine kinase (tk) locus of L5178Y mouse lymphoma tk (+/-) cells by measuring resistance to the lethal nucleoside analogue triflurothymidine (TFT).	Yes
***In Vitro* Sister Chromatid Exchange Assay in Mammalian Cells**[9]	The exchange of genetic material between two identical sister chromatids. This is efficiently induced by mutagens that form DNA adducts or that interfere with DNA replication.	No
***In Vitro* Comet**[10]	DNA fragmentation is detectable using electrophoresis exhibiting a comet-like appearance with the head consisting of intact DNA and the tail consisting of damaged DNA.	No
Balb/c 3T3 and Syrian Hamster Embryo (SHE) Cell Transformation Assays[11]	The method is highly sensitive for detecting both genotoxic and non genotoxic carcinogens, and is based on the principle that carcinogenic compounds cause an increase in morphologically transformed colonies which are characterised by disorganised growth patterns.	No
Cellular Biomarkers	Phosphorylation of biomarkers such as p53 (tumour suppressor protein) and H2AX histone[12] are common high throughput methods for detecting DNA damage.	No
Reporter Gene Systems	GreenScreen HC™ uses a human-derived, p53 competent cell line to host a patented green fluorescent protein (GFP) reporter system which exploits the proper regulation of the GADD45a gene as a marker of genotoxicity[13]. The Vitotox™ test is a bacterial genotoxicity assay which can detect DNA damage as light emission changes depending on SOS DNA repair induction[14]. The RadarScreen assay is a RAD54 promoter-linked beta-galactosidase reporter assay in yeast[14].	No

7.3.2. Non-Genotoxic Carcinogens

Due to the fact that non genotoxic carcinogenesis often occurs through a number of different modes of action, identification and characterisation can be difficult. For this reason, at present, there are very few *in vitro* methods for detecting non genotoxic carcinogens and none have been validated for regulatory purposes (table 7.3)[2].

Table 7.3. *In vitro* methods for detecting non genotoxic carcinogens.

Method	Description	Regulatory Approved
Balb/c 3T3 and Syrian Hamster Embryo (SHE) Cell Transformation Assays[11]	The method is highly sensitivity for detecting both genotoxic and non genotoxic carcinogens, and is based on the principle that carcinogenic compounds cause an increase in morphologically transformed colonies which are characterised by disorganised growth patterns.	No
Inhibition of Gap-Junction Intercellular Communication (GJIC)[2]	A number of preliminary methods have been suggested to assess inhibition of GJIC. Although promising results have been obtained, further validation is required, and it only represents a single mechanism of potential non-genotoxic carcinogenesis.	No

Changes in gene expression using toxicogenomic approaches (microarray and / or qPCR technology) may show future promise in detecting non-genotoxic carcinogens but their utility requires further exploration[2].

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Chapter 8: Phospholipidosis

8.1. Background

Phospholipidosis is a lysosomal storage disorder characterised by excessive accumulation of intracellular phospholipids in tissues, such as the liver, kidney, brain and lung[1].The morphological hallmark of phospholipidosis is the presence of lamellar bodies (also called lysosomal inclusion bodies or myeloid bodies)[2] as illustrated in figure 8.1. It is well established that a large number of cationic amphiphilic drugs (CADs) have the potential to induce phospholipidosis. These include antibacterials, antipsychotics, antidepressants, antianginals, antimalarials, antiarrhythmics, and cholesterol-lowering agents[1].

Organs affected by phospholipidosis often exhibit inflammatory reactions and histopathological changes such as macrophagic infiltration or fibrosis[2]. If adverse effects are observed, then the process is often reversible on withdrawal of the drug[3].

8.2. Mechanisms of Drug Induced Phospholipidosis

Despite considerable research, the mechanisms of drug-induced phospholipidosis are still not fully understood. The mechanisms are thought to vary for different drugs and thus the composition of accumulated phospholipids can also differ. Individual drugs can, therefore, manifest themselves in different ways especially in terms of the severity of the adverse effects, and organ/tissue/species specificity[3]. For this reason predicting clinical phospholipidosis effects can be challenging.

The primary organelle associated with drug-induced phospholipidosis is the lysosome and this tends to be where the CADs accumulate. The weak basic properties of these drugs result in them becoming protonated after entering the low pH of the lysosome. The ionised form of the drug then becomes trapped within the lysosomal compartment as it is unable to pass through the phospholipid bilayer. Drugs that are lysosomotropic (accumulate in lysosomes) tend to possess a clogP > 2 and a basic pKa between 6.5 and 11[4].

The primary mechanisms proposed tend to be direct effects of the lysosomotropic compounds on the lysosomal enzymes;

8.2.1. Inhibition of Phospholipidases

Lysosomal phospholipidases (e.g., phospholipidase A1 (PLA1), calcium dependent and independent phospholipidase A2 and calcium dependent phospholipidase C) play a major role in the degradation of cellular membranes[2]. It is believed that these enzymes may be directly inhibited by drugs and so lead to an increase in phospholipid levels within the cell.

8.2.2. Phospholipid Binding

It has been proposed that CADs can bind directly to phospholipids to form drug-phospholipid complexes that cannot be broken down by phospholipidases[1]. These complexes can accumulate and are stored in the form of lysosomal lamellar bodies.

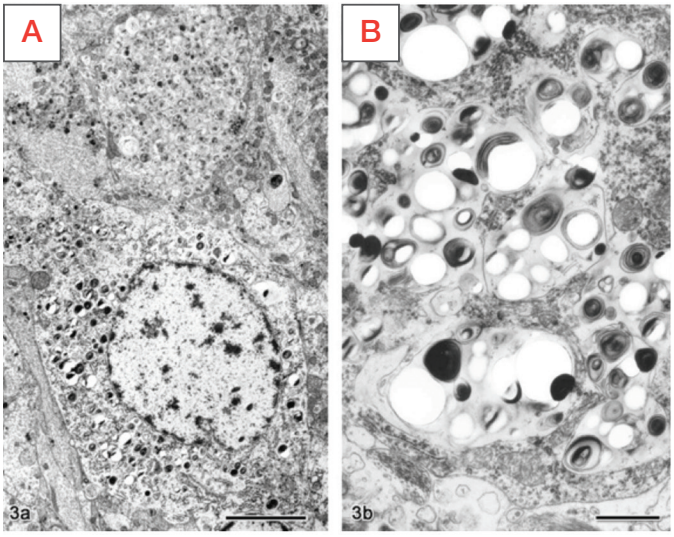
8.2.3. Inhibition of Autophagy

Autophagy is a homeostatic process by which old or damaged intracellular components are degraded. This process occurs by these components becoming enveloped in double membrane vesicles (autophagosomes) which fuse with lysosomes to form autophagolysosomes. Lysosomal acid hydrolases subsequently degrade the contents of the autophagolysosomes. It has been proposed that lysosomotropic compounds can increase the pH in the lysosome thereby impairing lysosomal acid hydrolases and so inhibit the degradation of the autophagolysosomes. It has been suggested that chloroquine may act by this mechanism[4].

A number of indirect mechanisms have also been hypothesised. For example, toxicogenomic studies have investigated gene expression changes following CAD dosing. These implicate up-regulation of genes involved in phospholipid and cholesterol biosynthesis and down-regulation of genes involved in lysosomal enzyme transport and lysosomal phospholipidase activity[5]. These indirect mechanisms have not been extensively studied and as such are not yet fully proven.

Figure 8.1. Representative transmission electron photomicrographs of neurons following treatment with posaconazole, a drug which can cause phospholipidosis[6].

Figure A is a low magnification (bar = 5 µm) of an enlarged neuron with abundant cytoplasmic vacuoles; many of the vacuoles contain electron dense figures associated with phospholipid accumulation. **Figure B** is a high magnification (bar = 1 µm) of the neuronal cytoplasmic vacuoles. The vacuoles are clear, variably sized lysosomes that contain electron-dense, laminated whorls engorged with phospholipid known as lamella bodies.



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8.3. Common *In Vitro* Methods for Assessing Phospholipidosis

8.3.1. Transmission Electron Microscopy

Due to the high level of magnification, transmission electron microscopy is the gold standard method for identifying, and confirming the presence of, phospholipidosis. This technique can detect membranous lamellar inclusions, concentric multilamellar bodies, myeloid bodies, and other similar structures, all of which are characteristic of phospholipidosis. However, performing electron microscopy is expensive and the technique is not suitable for high throughput screening.

8.3.2. Uptake of Fluorescent Phospholipid Probes

Fluorescent phospholipid probes such as NBD-PE and LipidTOX™ accumulate in lysosomes when phospholipidosis is induced[7]. Detection is typically by high content imaging.

8.3.3. Competition Methods to Identify Lysosomotropic Compounds

LysoTracker® Red is a fluorophore linked to a weak base which readily enters acidic organelles such as lysosomes. In the presence of lysosomotropic compounds, competition between the compound and the LysoTracker® dye occurs, resulting in a pH-independent decrease in the fluorescence signal of the dye[4]. High content imaging can be used to detect lysosomal accumulation.

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Chapter 9: Steatosis

9.1. Background

Hepatic steatosis (fatty liver) refers to an intracellular accumulation of lipids and the formation of lipid droplets in the cytoplasm of hepatocytes which is associated with enlargement of the liver. If inflammation accompanies steatosis then this is known as non-alcoholic steatohepatitis (NASH). Steatosis is covered under the term of non-alcoholic fatty liver disease (NAFLD) along with the progressive stages of NASH and fatty liver-associated fibrosis and cirrhosis[1].

9.2. Mechanisms of Drug-Induced Steatosis

NAFLD is a multifactorial disorder which is often linked to hypertriglyceridemia, obesity and insulin resistance[1]. However, hepatic steatosis can also be drug-induced with a number of mechanisms proposed.

9.2.1. Impaired Mitochondrial Function

With respect to drug-induced steatosis, inhibition of mitochondrial fatty acid oxidation through impaired mitochondrial function is thought to be the most common cause of intracellular lipid accumulation[1]. Several drugs (e.g., amiodarone, perhexiline, tamoxifen, NRTIs and glucocorticoids) can induce steatosis via a number of different mechanisms described below:

- Direct inhibition of mitochondrial fatty acid oxidation enzymes[2].
- Decreased levels of mitochondrial fatty acid oxidation cofactors by affecting generation of coenzyme A and /or L-carnitine esters[2].
- Inhibition of the mitochondrial respiratory chain reducing levels of FAD and FAD+ required for fatty acid oxidation enzymatic reactions[2].
- Reduction of mtDNA levels resulting in impairment of the mitochondrial respiratory chain[2].

9.2.2. Impaired Hepatic VLDL Synthesis and Secretion

When liver function is normal, excess free fatty acids are esterified to triglycerides which are eventually secreted in the plasma as VLDLs (very low density lipoproteins)[3]. Drug-induced impairment of this process is thought to be caused by inhibition of the microsomal triglyceride transfer protein[2]. Amiodarone, perhexiline and tetracycline are thought to act by this mechanism[2].

9.2.3. Direct Activation of Transcription Factors involved in Hepatic Lipogenesis

Certain drugs can stimulate hepatic lipid synthesis (e.g., interferon-α, glucocorticoids, tamoxifen, troglitazone and nifedipine). Although the mechanism for this is not fully defined, it is thought this could occur through activation of lipogenic transcription factors (e.g., SREBP-1c, ChREBP, PXR and PPARγ)[2].

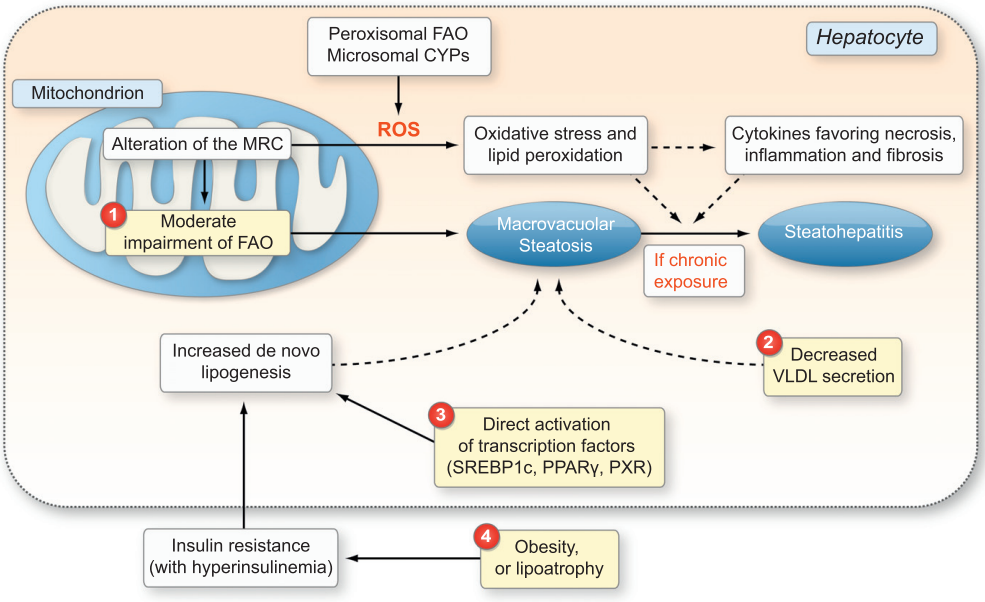
9.2.4. Insulin Resistance

Impaired insulin signalling can result in insulin resistance (where normal amounts of the hormone are no longer sufficient to facilitate transport of glucose into the cell). A decrease in insulin sensitivity can cause biochemical changes in the liver such as impaired VLDL synthesis and decreased lipid oxidation, ultimately leading to hepatic lipid accumulation and steatosis[3]. Drugs which indirectly affect insulin resistance often act through appetite stimulation resulting in obesity (e.g., glucocorticoids and some antipsychotic drugs such as clozapine, olanzapine, chlorpromazine and risperidone)[2].

It is often difficult to define the exact mechanism by which drugs induce steatosis as this is usually multifactorial and several of the individual factors may be interlinked as illustrated in figure 9.1.

Progression of steatosis into steatohepatitis can involve the production of reactive oxygen species, oxidative stress and cytokine induced liver injury[1]. Although alteration of the mitochondrial respiratory chain can produce ROS, peroxisomal fatty acid oxidation and microsomal cytochrome P450 enzymes may also be responsible[2].

Figure 9.1. Mechanisms of drug-induced steatosis and steatohepatitis[2].



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9.3. Common *In Vitro* Methods for Assessing Steatosis

9.3.1. Lipid Droplet Detection

Probes such as LipidTOX™ neutral lipid stain, Oil Red-O and Bodipy 493/503 have a high affinity for neutral lipid droplets and so allow for detection of lipid accumulation in the cells[4]. Detection is often performed using high content imaging.

9.3.2. Transmission Electron Microscopy

Transmission electron microscopy can be used to visualise steatosis in cells.

9.4. References

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Chapter 10: Cholestasis

10.1. Background

Cholestatic liver damage is typically characterised by increased levels of alkaline phosphatase, γ-glutamyl-transpeptidase and conjugated bilirubin in serum[1]. Often chronic drug-induced cholestasis will resolve itself on withdrawal of the offending drug, however, in some cases, if there is significant loss of the interlobular bile ducts then this can lead to chronic liver disease that may progress to liver failure[2].

Cholestasis can be due to either a failure to secrete bile (intrahepatic cholestasis) or to a mechanical obstruction of the bile ducts (extrahepatic cholestasis). Each of these processes can be categorised into several groups[2] as detailed in the table 10.1.

10.2. Mechanisms of Drug Induced Cholestasis

In many cases, the manifestation of drug-induced cholestasis is thought to be a consequence of several mechanisms and may in part be exacerbated by underlying genetic predisposition or medical conditions. The individual types of cholestasis are also thought to be linked to different drug-induced mechanisms.

10.2.1. Transporter Interactions

Pure cholestasis (without hepatitis) tends to be a consequence of interference with basolateral (sinusoidal) hepatic uptake, intracellular transport or canalicular transport of bile. Basolateral transporters (e.g., NTCP, OATP1B1, 1B3 and 2B1, OCT1, OAT2) are important for the hepatic uptake of drugs from the blood[1]. Canalicular transporters (e.g., MDR1 (P-gp), MDR3, MRP2, BSEP) are important for the elimination of drugs and bile acids across the canalicular membrane into the bile[1].

Currently, three of the most important transporters for evaluation of cholestasis prediction tend to be BSEP, MDR3 and MRP2 due to the fact that mutations in the genes for these transporters have been implicated in severe hereditary cholestatic-like disorders[3]. For drug-induced effects, it is thought, however, that in many cases, additional transporters or mechanisms may also play a role in the manifestation of cholestasis.

- BSEP mediates the biliary secretion of bile acids and dysfunction of BSEP can lead to intrahepatic accumulation of bile acids leading to toxicity[4]. Several papers have indicated that BSEP inhibition by drugs is a major factor in drug-induced cholestasis, with cyclosporine A and bosentan being examples[5,6].

- MDR3 regulates the biliary secretion of phospholipids including phosphatidylcholine which forms biliary mixed micelles with bile acids – a process which protects the hepatocytes from the detergent effects of bile acids. Itraconazole, a drug which can cause cholestasis has been shown to inhibit MDR3 at clinically relevant concentrations[4].

- MRP2 secretes glutathione and glutathione conjugates into bile. Glutathione provides an important osmotic force that drives the bile acid-independent bile flow. Reduction in the expression of MRP2 and subsequent bile flow effects are thought to be one mechanism by which ethinyl estadiol can induce cholestasis[7].

As well as general inhibition of transporters it is thought that nuclear receptors and STAT (a member of the signal transducers and activators of transcription) may also have a role in influencing transcriptional regulation of transporter proteins and post-translational regulation is mediated by classical second messengers[3]. These effects may also influence bile acid transport.

10.2.2. Immune Mediated Effects

Cholestasis with hepatitis typically has an immunoallergic mechanism and often results from hypersensitivity reactions. This may occur through the drug (or via formation of reactive metabolites) covalently binding directly to liver-specific proteins. Chlorpromazine is an example of one drug which can lead to cholestatic hepatitis through a hypersensitivity reaction[8]. Ductular forms of drug-induced injury such as vanishing bile duct syndrome are also believed to be a result of a drug (or drug metabolite) triggering an immune response against biliary epithelium[1].

10.2.3. Cholelithiasis

The mechanisms of drug-induced gallstone formation (cholelithiasis) are often not fully understood but are thought to include gall bladder stasis (e.g., in the case of octreotide) and/or enhanced secretion of cholesterol in bile (e.g., in the case of estrogens)[9].

Table 10.1. Classification of drug-induced cholestasis[2].

Types of Drug-Induced Cholestasis		Pathological Manifestation
Acute intrahepatic drug-induced cholestasis	Cholestasis without hepatitis (pure simple, canalicular or bland cholestasis).	Cholestasis with minimal or no hepatic parenchymal involvement.
	Cholestasis with hepatitis (hepatocanalicular hepatitis or mixed cholestatic hepatitis).	Characterised by hepatic parenchymal inflammation and hepatic injury and necrosis.
	Cholestasis with bile duct injury (ductular, cholangiolar, or cholangiolytic cholestasis).	Bile duct injury with minimal involvement of parenchymal liver cell injury.
Chronic intrahepatic drug-induced cholestasis	Mild non-specific bile duct injury.	Mild bile duct epithelial disarray.
	Vanishing bile duct syndrome (VBDS).	Loss of interlobular or septal bile ducts.
	Primary sclerosing cholangitis-like.	Inflammation and subsequent obstruction of bile ducts.
Extrahepatic drug-induced cholestasis	Cholelithiasis.	The formation of gallstones.
	Primary sclerosing cholangitis-like.	Inflammation and subsequent obstruction of bile ducts.

10.3. Common *In Vitro* Methods for Assessing Cholestasis

10.3.1. Transporter interactions

Most *in vitro* methods for determining potential cholestatic effects focus on evaluating transporter interactions. A number of models exist including sandwich-cultured hepatocytes, vesicles and cell based assays. A publication by Thompson *et al.*, (2012)[10] has proposed a combined screening approach where BSEP and Mrp2 inhibition data are combined with covalent binding burden and a number of other *in vitro* assays for the prediction of idiosyncratic adverse drug reactions (IADRs).

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Chapter 11: Cardiac Ion Channel Effects and Hypertrophy

11.1. Background

Cardiotoxicity is one of the leading causes of toxicity-related drug attrition. Initial physiological effects of drugs often manifest themselves as ECG changes and cardiac arrhythmias which can occur as a consequence of intracellular calcium concentrations and other biochemical alterations leading to miscommunication and misconduction of electricity between cells. Cardiomyopathy often occurs in the later stages of cardiac dysfunction with cardiac hypertrophy a common observation which can lead to heart failure[1].

11.2. Mechanisms of Cardiotoxicity

11.2.1. Ion Channel Effects

The human ether-a-go-go related gene (hERG) encodes the inward rectifying voltage gated potassium channel in the heart (I_{Kr}) which is involved in cardiac repolarisation. Inhibition of the hERG current causes QT interval prolongation resulting in potentially fatal ventricular tachyarrhythmia called *Torsade de Pointes*. A number of drugs have been identified which prolong the QT interval[2]. One of the most notorious hERG blockers is the antihistamine, terfenadine, which was withdrawn from the market in the 1990's.

Although blocking the hERG channel is considered to be the most serious ion channel effect, other major ion channels (e.g., Nav1.5, Cav1.2, Kv1.5, KvLQT1/minK, Kir2.1) may also play a role in drug-induced adverse cardiac effects.

11.2.2. Cardiac Hypertrophy

Cardiac hypertrophy is defined as an increase in heart muscle mass which reflects a remodelling of the myocardium in response to stress. This usually occurs as a consequence of hypertrophy of myocytes (i.e., enlargement of existing cells without an increase in the number of cells)[3].

During the initial stages of the development of hypertrophy, several key observations manifest themselves. These may include alterations in ionic homeostasis such as changes in calcium concentration, impaired fatty acid oxidation, aberrations in energy metabolism with changes in ATP levels and an increase in reactive oxygen species (ROS) and oxidative stress. Mitochondria appear to play an important role in these initial effects and the manifestation of cardiac hypertrophy[1].

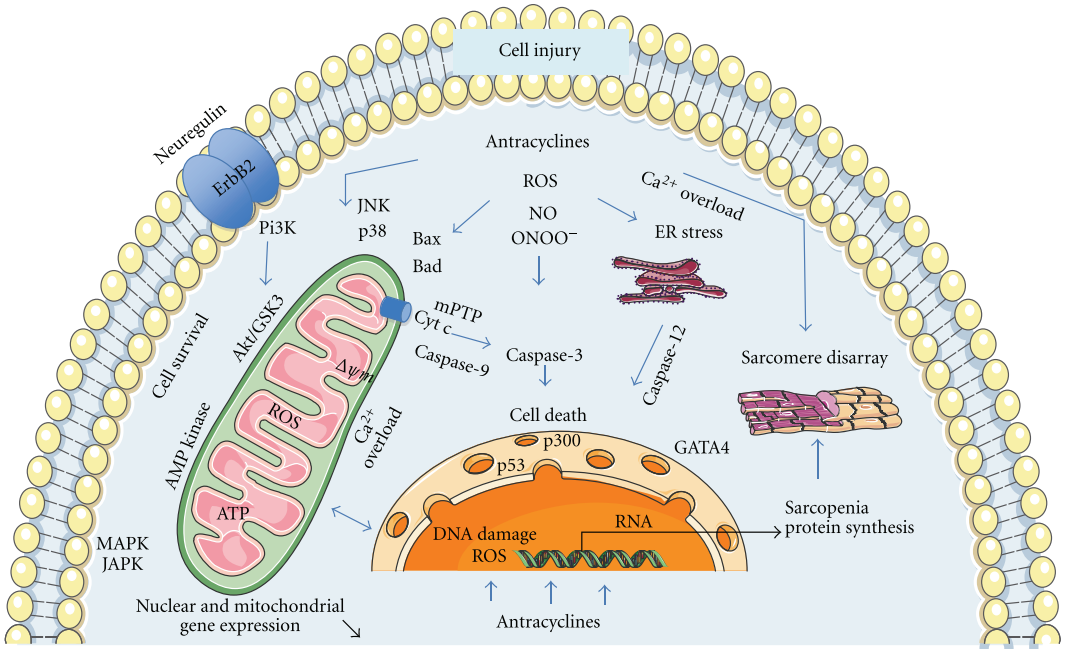
Mitochondria are a major component of the heart muscle occupying up to 50% of the cytoplasmic volume of the myocytes. It has been shown that impaired respiratory function of cardiomyocyte mitochondria leads to heart failure. Mitochondria are thought to play a role in cardiac remodelling and also in the activation of apoptosis pathways and necrosis[3].

Hypertrophy develops through modulation of transcription factors and signalling pathways which result in an increase in cell size and protein synthesis, enhanced sarcomeric organisation and an upregulation of fetal cardiac genes (e.g., ß-MHC and ß-skeletal actin). In the short term this is adaptive to account for increased energy demand on the heart but longer term this becomes detrimental which can lead to cardiac remodelling (changes in the structure and function of the ventricular myocardium), cardiac dysfunction and heart failure[1].

Several compensatory mechanisms are activated during the progression of hypertrophy to heart failure including the sympathetic nerve system and the renin-angiotensin system. These compensatory systems alter expression of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) expression, and/or TNF-alpha which influence cardiac remodelling and cell death[1].

Cardiomyopathy and congestive heart failure have been reported with the anthracyclines, the effects of which are usually dose-dependent, irreversible and cumulative[4]. The mechanisms involved in antracycline-induced cardiomyocyte injury are illustrated in figure 11.1.

Figure 11.1. Potential signalling pathways involved in anthracycline-induced cardiomyocyte injury[4].



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11.3. Common *In Vitro* Methods for Assessing Cardiac Ion Channel Effects and Cardiac Hypertrophy

11.3.1. Single Ion Channel Effects

Various cell lines are now available which express the most common ion channels of interest (e.g., hERG channel). Manual or automated patch clamp techniques are the most robust methods for assessing the electrophysiology of the cells and the effects of drugs. For early stage screening, the dofetilide competitive binding assay assesses potential hERG channel binding using membranes prepared from HEK 293 cells stably transfected with hERG K^+ channels.

11.3.2. Whole Cell Ion Channel Effects

Recent advances in microelectrode array (MEA) and stem cell derived cardiomyocytes now allow high throughput 'ECG-equivalent' electrophysiology measurements (beat rate, fast sodium amplitude, field potential duration and conduction velocity) to be determined and allow drug-induced effects to be monitored. This technology allows more physiologically relevant assessments to be achieved where all the ion channels are present in a single cell. Monitoring impedance to assess the contractility of stem cell derived cardiomyocytes is another recently introduced technology. Impedance methods can only monitor beat rate and so are less flexible in the amount of information generated.

11.3.3. Cardiac Hypertrophy

Very few *in vitro* models exist for determining cardiac hypertrophy. Most current models tend to focus on biomarkers of cardiac hypertrophy (e.g., the naturietic peptides BNP or ANP) or cardiac damage (e.g., cardiac troponin T, cardiac troponin I and heart fatty acid binding protein)[5]. *In vitro* 3D cluster models using stem cells have also been proposed for hypertrophy with an increase in cluster size monitored in response to drug exposure using high content imaging[6].

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Chapter 12: Future Outlook

12.1. Current Challenges within the Industry

In many cases, the approach to toxicology testing within the Pharmaceutical Industry has been conservative and slow to adapt, relying on later stage preclinical animal testing to identify potential liability. The success observed in bringing ADME testing earlier within the drug discovery process has been dramatic and has helped to reduce attrition rates in pharmacokinetics and bioavailability from approximately 40% in 1991 to less than 10% in the year 2000[1]. During this period, the rates of attrition associated with toxicology and clinical safety increased significantly. This led to the realisation that testing strategies in the field of toxicology needed to change to address this concern.

One of the major barriers to bringing toxicology testing earlier in the drug discovery process has been the lack of accurate *in vitro* models to predict clinical safety. Despite this, *in vitro* models for hERG liability, genotoxicity and drug-drug interactions are well established with several assays now required by regulatory authorities. However, predicting other mechanisms of toxicity can be challenging, especially in the case of hepatotoxicity where effects can be idiosyncratic and only present in a relatively small percentage of the population. Often, the reasons for these idiosyncratic effects are unknown but may be influenced by numerous factors such as age, organ impairment, genetic polymorphisms, immune status and/or interactions from polypharmacy or food/dietary supplements.

12.2. New *In Vitro* Models

There is a strong push within the industry for new cellular models and improved techniques for predicting toxicity.

Stem cells are showing promise especially in the field of cardiotoxicity where the cells have the potential to spontaneously beat, and new technologies such as high throughput microelectrode array (MEA) have been developed to monitor whole cell electrophysiology. It is likely that iPS-cell derived disease models may advance our knowledge and understanding further within specific patient populations.

3D cellular models are now becoming more widely available. These models more closely mimic the physiology of tissues and organs compared to 2D cellular models and retain viability over long time periods. Being able to reproduce long term repeat dose studies is an obvious advantage of these 3D models.

High content imaging is another important technological advance in the toxicology field. The technique uses fluorescent dyes or fluorescently labelled antibodies to visualise and track multiple toxicologically relevant processes within the cell. The development of more powerful instruments with greater magnification capability and more rapid image capture is likely to enhance this technology further and enable these techniques to keep in line with more sophisticated cellular models.

Integrated panels of *in vitro* assays which are predictive of clinical toxicology are showing value especially in more challenging areas such as hepatotoxicity and idiosyncratic adverse drug reactions[2,3]. Furthermore, combining ADME data and PBPK modelling with *in vitro* toxicology data can be a powerful approach in predicting exposure (and ultimately safety) of drugs in various populations or situations. As the sophistication of these PBPK models improve, it is likely that their utility in exposure and safety predictions will increase.

12.3. Summary

Toxicology research is advancing rapidly, with the introduction of a number of new and promising *in vitro* and *in silico* technologies for predicting clinical safety. Our ability to relate the *in vitro* data to the clinical situation is an important goal, and will test out the validity of these new technologies. A number of initiatives have been established to investigate and research alternative *in vitro* methods in the field of toxicology (e.g., ECVAM, NC3Rs, REACH, EPA ToxCast and FDA Tox Working Group). The success of these initiatives is particularly apparent in the case of the cosmetics industry where acceptable *in vitro* alternatives are now available, leading to a ban on animal toxicity testing for new cosmetics in Europe. Although preclinical animal testing within the pharmaceutical industry is expected to be a regulatory requirement for some time to come, an expansion in the battery of regulatory *in vitro* tests is likely to occur as protocols and data interpretation become more well-defined.

12.4. References

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