

Chapter 1: Literature review

1.1. Introduction

A significant advance in the field of early medicine was Paracelsus' recognition that all compounds have the capacity to be poisonous depending upon dosage. This has made toxicity testing a necessary and critical industry practice to identify and define safety thresholds for all new potential chemotherapeutics (Niles *et al.*, 2009). As there are a number of ways in which a substance can induce toxicity in an organism, there are a number of toxicities that can be tested for with any given substance. Within the scope of life sciences, toxicity generally refers to cellular toxicity that may result from a number of adverse alterations at a sub-cellular level. This type of toxicity is usually related to an organ or tissue type such as the kidney, brain, heart and liver. Apart from direct xenobiotic cytotoxicity, cardiotoxicity may also be the result of alterations in contractility or ventricular arrhythmias (Gwathmey *et al.*, 2009; Mandenius *et al.*, 2011). Of these, drug-induced ventricular arrhythmias have received the most attention of late, especially from regulatory authorities (Mandenius *et al.*, 2011). The reason for this is QT-interval prolongation, which is associated with the potentially fatal arrhythmia known as *torsades de pointes* (Liu *et al.*, 2011).

Other types of toxicity that are not due to cytotoxicity but rather perturbed signalling between systems within an organism as a whole include developmental toxicity, endocrine toxicity and allergenicity. In addition to these, there is also genotoxicity, which is related to genetic mutations and cancer. Collectively, the afore-mentioned serves to demonstrate the fragility of homeostasis and how easily it can be disrupted by the introduction of xenobiotics.

1.2. Safety evaluation

Considerable time, effort and expenses are spent on safety evaluation of pharmaceuticals in development. Pharmaceutical safety evaluation includes pre-clinical testing, clinical safety assessment and post-marketing safety evaluation. Pre-clinical testing involves both *in vitro* and *in vivo* safety assessment. The usual sequence of testing involves: *in vitro*, *in vivo* animal testing (rodent + non-rodent), clinical safety evaluation (Phase I - III trials) and finally post-marketing safety evaluation (adverse event reporting etc.). At the one end of the spectrum, clinical and post-marketing safety evaluations provide 'true' toxicity data that may be difficult to explain, whereas *in vitro* testing that may lack predictive power provides very specific (mechanistic) toxicological information. This illustrates why all of the aforementioned areas of safety evaluation are necessary to provide comprehensive information regarding the safety of a particular compound.

In vitro toxicology simply describes a field of endeavour that applies technologies inclusive of isolated organs, isolated tissues, cell culture, biochemistry, and chemistry to the study of toxic or adverse reactions to xenobiotics. This type of safety assessment is used principally for two reasons: 1) to generate more comprehensive toxicological profiles of a compound and 2) for screening purposes. *In vitro* methods are invaluable in providing mechanistic information on toxicological findings for both in experimental animals and in humans and they are also of potential use for studying local or tissue and target specific effects (Eisenbrand *et al.*, 2002). Cell-based approaches to toxicity testing generally involves cytotoxicity testing using one of the many assays that are prevalent in literature such as the neutral red uptake, dimethylthiazol diphenyltetrazolium (MTT), sulforhodamine B and lactate dehydrogenase (LDH) leakage assays, to name a few. As with any assay, each have advantages and disadvantages but all of them are relatively easy and fast to perform.

One area of toxicology in particular for which whole cell cultures are required is cardiotoxicity, the reason being that intact cells are necessary for researchers to study and detect xenobiotic-induced arrhythmias. Evaluating cardiotoxicity *in vitro* is made difficult for a number of reasons. There is significant interspecies variation and the lack of predictive

power prevents data derived from animal models being directly extrapolated to humans. This is further complicated by the fact that human cardiomyocytes are very difficult to obtain and highly differentiated with extremely low proliferative capacity and rapid loss of phenotypical characteristics in culture. Apart from the normal cytotoxicity testing, screening assays in cardiotoxicity also include electrophysiological measurements of action potentials of the cultured cells, from which the potential of a compound to induce arrhythmias is deduced (Mandenius *et al.*, 2011).

Cytotoxicity testing has proven to be accurate in predicting lethal human blood concentrations (Ekwall *et al.*, 1998). Realising that cytotoxicity testing alone has limited potential in detecting some subtle types of toxicity, researchers have developed cell-based strategies that examine more than one endpoint (Flynn and Ferguson, 2008; Wu *et al.*, 2009). Flynn and Ferguson (2008) tested the predictive power of their method and found good correlation with *in vivo* data, which is promising for this type of "expanded" cytotoxicity testing. However, some of the parameters were not very specific with regards to the endpoint that was examined. For example, rhodamine 123 was used to assess both mitochondrial effects and P-glycoprotein function in the same cell sample. The question arises that should a change be detected in this sample, would that be the result of mitochondria or P-glycoprotein function being affected? Although the advantages of a multiparametric cell-based model are obvious, minor oversights, such as the example given, and limited scalability have restricted the use of these techniques for screening purposes.

Apart from cytotoxicity, *in vitro* testing is also capable of examining the effects that xenobiotics may have on specific biochemical / signalling pathways at a sub-cellular level, which may be a difficult proposition *in vivo*, especially when human tissues are involved. Recently, commercially available molecular toxicology screening technology has made it possible to screen xenobiotics for affects they may have on up to 45 molecular pathways in microplate format (Panas *et al.*, 2011). This type of advancement is responsible for the growing significance of *in vitro* testing in safety assessment. Furthermore, because this type of testing is likely to be scalable to high-throughput screening, it may greatly enhance current understanding of molecular toxicology provided that enough classes of toxic compounds are screened and data deposited in appropriate databases.

Databases form a fundamental part of another technique that has gained popularity in toxicology over the last decade or two - *in silico* computational toxicology. Quantitative structure-activity relationship (QSAR) models generate equations by statistically identifying molecular descriptors and/or sub-structural molecular attributes that are correlated with toxicity (Kruhlak *et al.*, 2007). This type of toxicity prediction is especially useful when no previous toxicological information is available with regards to a particular molecule or to gather some initial information while proper toxicity testing is underway. In the pharmaceutical industry, computational toxicology is used as a sentinel tool for the early assessment of the toxicological potential of candidate molecules in lead selection and drug discovery (Pearl *et al.*, 2001).

1.3. The burden of hepatotoxicity on the pharmaceutical industry

More than 600 drugs have been associated with hepatotoxicity. The clinical picture is diverse, even for the same drug when given to different patients. The manifestations range from mild, asymptomatic changes in serum transaminases, which occur at a relatively high frequency with a number of drugs, to fulminant hepatic failure, which although rare, is potentially life threatening and may necessitate a liver transplant (Park *et al.*, 2005).

From a pharmaceutical viewpoint toxicology plays a major role during early drug development, from the initial pre-clinical development of lead compounds through to the late clinical evaluation (phase III trials). Between 1992 and 2002, 43%, 25%, and 35% of drugs undergoing Phase I, II, and III studies, respectively, were terminated due to the development of mostly hepatic injury (Schuster *et al.*, 2005). Also, the most common cause of drug attrition is drug-induced liver injury and between 1992 and 2002, 27% of market withdrawals in the United States and the European Union were due to hepatotoxicity (Lasser *et al.*, 2002; Schuster *et al.*, 2005). The pharmaceutical industry spends more than US\$ 20 billion on drug discovery and development per year, with approximately one fifth of this amount is being used in initial screening assays and toxicity testing. To alleviate this financial burden, knowledge concerning the early events in drug-induced toxicity is required

to aid in the decision-making processes during the development of new therapeutic agents (Hakimelahi and Kodarahmi, 2005). It is desirable to detect toxicity as early as possible, preferably during the pre-clinical phase, which will aid lead optimisation and subsequently increase the successful output of the entire process. Boosting the output from drug development programs is a necessity as there is a global need for faster drug development and nowhere is this more urgent than in the pharmaceutical field of antibiotics. Resistance to currently available antibiotics is rising and the arsenal of drugs seems to be shrinking as the development of novel antibiotics is not able to keep up with the increasing rate of microbial resistance (Livermore, 2004; Finch and Hunter, 2006; Baiden *et al.*, 2010). Market withdrawals due to hepatotoxicity of antibiotics like Temafloxacin and Trovafloxacin (Peters, 2005) are only contributing to the problem.

Toxicity testing during preclinical drug development is intended to identify target tissues and then assess potential risks prior to introduction of new molecular entities into the human population. The standard regimen is testing at different doses in at least two species of animals, one rodent (rats or mice) and one non-rodent (dogs, nonhuman primates, minipigs or rabbits) for at least two weeks of repeated dosing. Although experience has shown that this regimen “works” most of the time, in many cases hepatotoxicity is detected later in animal toxicity studies or clinical trials (Ballet, 1997). However, animal testing is financially burdensome and because hundreds of compounds are synthesized each year, the cost of animal testing, which may exceed several million dollars to test a single substance for safety assessment, needs to be reduced (Davila *et al.*, 1998).

1.4. Hepatotoxicity

The clinical patterns of hepatotoxicity are hepatocellular damage, cholestasis, a mixed hepatocellular and cholestatic injury, and steatosis (Abboud and Kaplowitz, 2007). Clinical signs relate back to the underlying molecular mechanisms, which can be invoked in hepatocyte injury induced by a xenobiotic itself or by any of its metabolites, leading to cell death by necrosis or apoptosis.

The critical synthetic, metabolic, and detoxifying functions of the liver partly explain its unique vulnerability to injury by xenobiotics (Hardisty and Brix, 2005; Gomez-Lechon *et al.*, 2010a). Liver injury can manifest in various ways, with the two main types of injury being cytotoxicity and cholestasis. The former refers to the death of hepatocytes following loss of cellular homeostasis mainly due to damage to cellular organelles such as mitochondria. After the ingestion of sufficient amounts of an intrinsic hepatotoxin the afore-mentioned damage on a cellular level may then lead to liver injuries such as zonal hepatocellular necrosis (Tsui, 2003). Cholestatic injury involves reduced formation or secretion of bile. Most cases of drug-induced cholestatic injury result from a drug- or metabolite-mediated inhibition of hepatobiliary transporters located in the plasma membranes of hepatocytes (Pauli-Magnus and Meier, 2006).

Intrinsic hepatotoxins exert toxic effects in all individuals in a predictable, dose-related manner either directly (active intrinsic hepatotoxins) or following biotransformation (latent intrinsic hepatotoxins)(Castell *et al.*, 1997). Examples of direct intrinsic hepatotoxins include: carbon tetrachloride (cytotoxic) and paraquat (cholestatic), whereas latent intrinsic hepatotoxins include: acetaminophen (cytotoxic) and steroids (cholestatic) (Tsui, 2003).

Unpredictable compound-induced liver damage is termed idiosyncratic hepatotoxicity and is not dose related. This type of liver injury is mostly attributed to host hypersensitivity and/or aberrant metabolism. It is usually a result of host genotype that results in either toxic metabolites or prolonged host exposure due to slow drug/xenobiotic metabolism (Tsui, 2003; Pauli-Magnus and Meier, 2006). Idiosyncratic reactions, which are qualitatively different from a drug's pharmacologic activity, are often immunologically mediated and have a host-dependent component (Peters, 2005). Symptoms may take weeks or months to manifest and is often characteristic of hypersensitivity-type reactions including eosinophilia and rash, such as seen with an amoxicillin-clavulinate hepatotoxic reaction. Idiosyncratic liver injury has been reported for the drugs Isoniazid and Troglitazone (Peters, 2005).

1.5. Molecular mechanisms of hepatotoxicity

1.5.1. Metabolic activation

The biotransformation of lipophilic compounds into hydrophilic derivatives, that are more readily excreted, is one of the physiological roles of the liver (Park *et al.*, 2005). There are two phases of metabolism. Phase I metabolism involves the cytochrome P450 enzymes (CYP), which are responsible for the functionalisation of xenobiotic compounds (Iyer and Sinz, 1999). This is accomplished by the introduction of polar and reactive groups into the specific compound through various reactions which include hydroxylation, oxygenation, dealkylation and epoxidation (Guengerich, 2001). Phase I metabolism is aimed at preparing the compound for phase II metabolism by making the molecule more polar and chemically reactive. Phase II metabolism is focused on the conjugation of the phase I metabolites to polar, charged molecules such as glutathione, sulphate, glycine and glucuronic acid. These reactions are catalysed by transferases like glutathione-S-transferase and render the phase I metabolite more polar and therefore biologically inactive as it cannot easily cross cell membranes. This polarity also aids in the elimination of the compound from the body through renal excretion (Jakoby and Ziegler, 1990).

Although xenobiotic metabolism is aimed at detoxification, some xenobiotics can undergo biotransformation to metabolites that have intrinsic chemical reactivity towards cellular macromolecules. A number of enzymes, and in particular CYPs, can generate reactive metabolites, even from inert substances (Ioannides and Lewis, 2004; Park *et al.*, 2005). The identity of the susceptible cellular macromolecules, and the physiological consequence of its covalent modification, will dictate the resulting toxicological response. Toxic responses may include carcinogenicity, cell death or hypersensitivity (Figure 1.1).

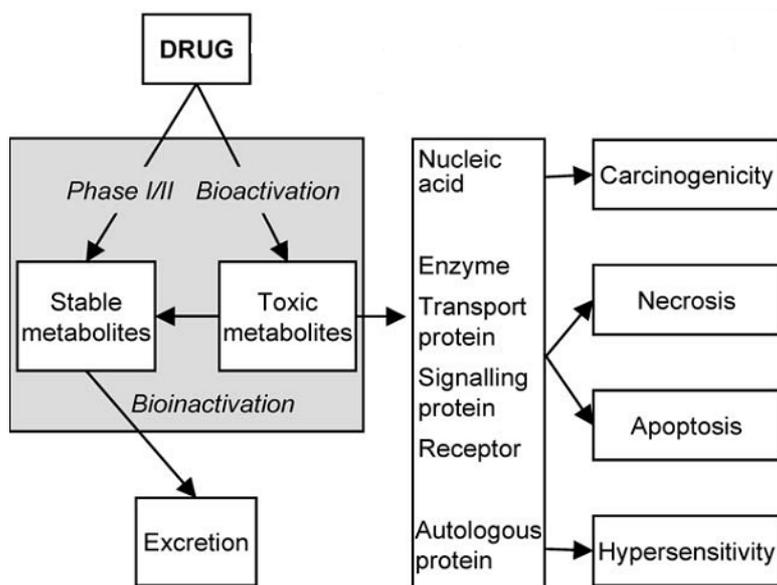


Figure 1.1. The relationship between drug metabolism and toxicity. Toxicity may occur through accumulation of parent drug or via metabolic activation, through formation of a chemically reactive metabolite, which, if not detoxified, can effect covalent modification of biological macromolecules. The identity of the target macromolecule and the functional consequence of its modification will dictate the resulting toxicological response (Park *et al.*, 2005).

A classic example of hepatotoxicity as a result of metabolic activation is acetaminophen (paracetamol) toxicity. At therapeutic doses, acetaminophen is well tolerated. However, at higher doses it can induce acute hepatotoxicity. This is due to the formation of the reactive metabolite *N*-acetyl-*p*-benzoquinone-imine, which depletes cellular antioxidants (glutathione) and results in cell death by necrosis (James *et al.*, 2003; Marzullo, 2005). Troglitazone, an antidiabetic drug, was withdrawn from the market due to idiosyncratic hepatotoxicity. It was found that metabolic activation of Troglitazone in patient populations with specific gene polymorphisms was responsible for the idiosyncratic hepatotoxicity of the drug (Ikeda, 2011).

Hepatotoxicity due to metabolic activation of drugs is prevalent enough that metabolite profiling in hepatic preparations from experimental species and humans has become a routine task which has been incorporated into drug discovery and development processes. Furthermore, the data constitutes an important section in regulatory filing to eliminate

hepatotoxic metabolites at an early stage of drug development (Kalgutkar and Soglia, 2005; Park *et al.*, 2005).

1.5.2. Mitochondrial toxicity

Mitochondria are present in almost all eukaryotic cells, their chief function being energy production for the benefit of the cell. Malfunctioning mitochondria present a hazard to any cell, not only due to depletion of energy stores but also because of disturbed signalling pathways (Wallace and Starkov, 2000). Mitochondria generate energy by actively producing an electrochemical proton gradient across the inner membrane of the mitochondrion, termed the mitochondrial membrane potential. The backflow of protons down the electrochemical gradient, from the intermembrane space into the mitochondrial cytosol is used to drive the enzyme ATP synthase and thus the synthesis of molecules that provide cellular energy.

Xenobiotics can affect hepatic mitochondria either directly or indirectly. Direct interaction may occur in one of two ways: either by inhibiting the production of the required electrochemical gradient or through its dissipation (Wallace and Starkov, 2000). Changes in this electrochemical gradient will manifest as either mitochondrial hyperpolarisation (steeper gradient) or depolarisation (loss of gradient). Examples of xenobiotics that affect mitochondria directly are diclofenac (Masubuchi *et al.*, 2002) and phenobarbital (Santos *et al.*, 2008). Diclofenac has been shown to inhibit enzymes involved in the mitochondrial electron transport chain, causing depolarisation of the mitochondrial inner membrane by acting as an uncoupler (Moreno-Sanchez *et al.*, 1999).

Xenobiotics can also affect mitochondria indirectly through cytosolic Ca^{2+} signalling. A number of researchers have established that mitochondria act as intracellular ' Ca^{2+} buffers', which eliminates excess cytosolic Ca^{2+} through active transport via a ruthenium red-sensitive uniporter (Parekh, 2003; Duchen, 2004). Furthermore, it has been demonstrated that mitochondrial Ca^{2+} concentrations influence mitochondrial respiration rate by directly modulating the activity of the various enzymes involved in the electron transport chain

(Brini, 2003). The latter implies that any xenobiotic affecting intracellular Ca^{2+} concentrations/signalling may possibly disturb mitochondrial respiration and thus adversely affect a cells energy homeostasis.

In recent years, considerable attention has been paid to mitochondria regarding both apoptotic and necrotic cell death because of the role of mitochondria in electrolyte homeostasis in cells (e.g. acting as ' Ca^{2+} buffers') and the myriad of proteins present inside the mitochondrial cytosol, one of which is cytochrome C (Cyt C). Disturbances in cellular electrolyte balance may lead to cellular swelling and necrotic death, whereas release of cyt C into the cytosol may trigger a signalling cascade leading to apoptotic death through caspase activation (Silva and Couthino, 2010).

1.5.3. Oxidative stress

Any atom/molecule with an unpaired valence electron(s) is referred to as a free radical. The widely used term reactive oxygen species (ROS) refers not only to oxygen-centred free radicals but also to other non-radical but reactive oxygen-centred molecules (Halliwell and Gutteridge, 2007). Under certain conditions the production of ROS is enhanced and/or the levels or activity of endogenous cellular antioxidant defences are reduced. The resulting state, which is characterized by a disturbance in the balance between ROS production, ROS removal, and repair of damaged complex molecules, is called oxidative stress (Cederbaum *et al.*, 2009). Oxidative stress has been implicated as a major cause of cellular injury in a variety of clinical abnormalities including cancer, neurological diseases, arthritis, and hepatitis and is also thought to accelerate the aging process (Droge, 2002; Wang and Weinman, 2006).

The major types of ROS in cells are superoxide anion ($\bullet\text{O}_2^-$), singlet oxygen, hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet\text{OH}$). The nitrogen-centred equivalents of ROS are referred to as reactive nitrogen species or RNS, which include molecules such as nitric oxide and peroxynitrite. The $\bullet\text{OH}$ radical is potentially the most reactive of the afore-mentioned

molecules because of its very strong oxidising potential (Powers and Jackson, 2006). This high reactivity also makes it likely to react very close to its site of generation causing DNA damage, initiating lipid peroxidation or causing protein residue modification (Zhang *et al.*, 2010). In order to generate $\bullet\text{OH}$ in a biological environment, H_2O_2 has to react with transition metals such as Fe^{2+} or Cu^{2+} (Zhao *et al.*, 2005) in the Fenton reaction where:



H_2O_2 is generated from nearly all sources of oxidative stress and has the ability to diffuse freely in and out of cells and tissues (Barbouti *et al.*, 2002). Therefore, one way of probing for potentially damaging ROS generation would be to determine H_2O_2 generation, which is required for the formation of $\bullet\text{OH}$ in biological systems (Powers and Jackson, 2006).

Two major sources of ROS in cells are the mitochondria and CYPs. The main type of ROS produced by mitochondria is $\bullet\text{O}_2^-$. The major contributor of mitochondrial $\bullet\text{O}_2^-$ is the respiratory complex III, where it is believed that O_2 present within the lipid bilayer of the inner mitochondrial membrane is reduced by the ubisemiquinone radical to form $\bullet\text{O}_2^-$. A portion of the $\bullet\text{O}_2^-$ produced is then converted into H_2O_2 by mitochondrial superoxide dismutase (SOD) (Jezek and Hlavata, 2005). With regards to CYPs, ROS generation is the result of uncoupling of the redox cycle between the haem active site and nicotinamide adenine dinucleotide phosphate (NADPH), which causes the release of $\bullet\text{O}_2^-$ and H_2O_2 . This type of event is highly substrate-specific and has been associated with tight binding of the substrate to the specific CYP catalytic site (Schlezingner *et al.*, 2006).

To protect against the adverse effects of ROS, cells possess a complex machinery of antioxidant compounds and enzymes, which include glutathione (GSH), SOD, catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) (Polaniak *et al.*, 2010). Figure 1.2 depicts the interaction between the various components of this machinery.

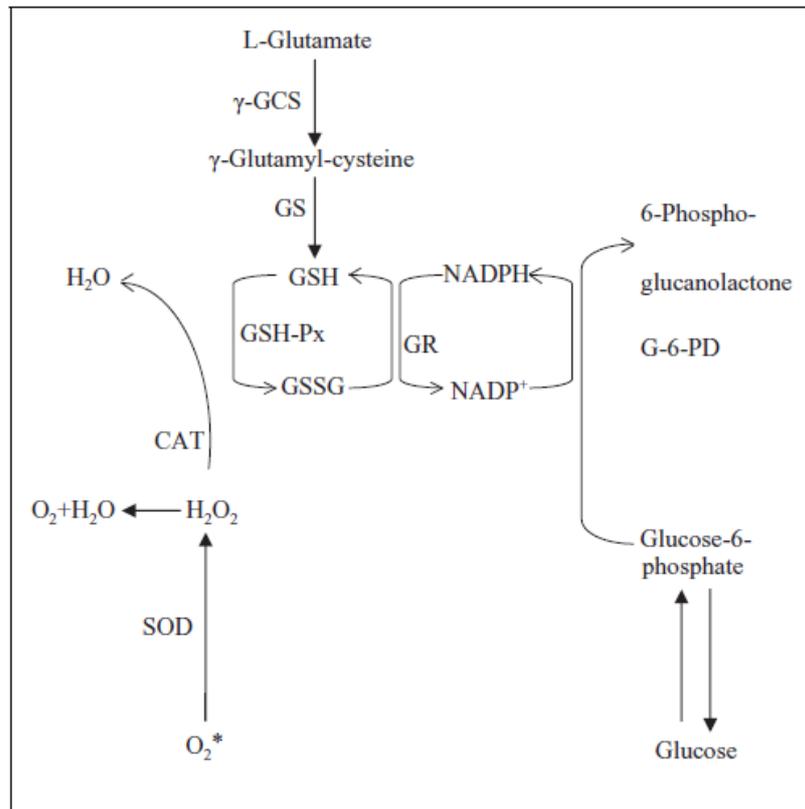


Figure 1.2. Cooperation between various antioxidant enzymes and cofactors. SOD – superoxide dismutase, CAT – catalase, GSH – glutathione, GSSG – glutathione disulfide, GSH-Px – glutathione peroxidase, GR – glutathione reductase, γ-GCS – γ-glutamylcysteine synthase, GS – glutamine synthase, G-6-PD – glucose-6-phosphate dehydrogenase (Polaniak *et al.*, 2010).

Glutathione (GSH), a tripeptide, is the most abundant non-protein thiol in cells (Meister and Anderson, 1983). It can counteract a state of oxidative stress in a number of ways:

- It can react directly with a radical by donating a hydrogen cation, thus eliminating the unpaired valence electron and rendering the free radical harmless (Yu, 1994)
- It serves as a substrate for GSH-Px to eliminate generated H₂O₂ (Meister and Anderson, 1983)
- It has the ability to reduce other oxidised antioxidant molecules such as vitamins E and C, replenishing their activity (Powers and Jackson, 2006)

Apart from excessive free radical generation, oxidative stress may also be induced by xenobiotics or their metabolites that diminish the cellular antioxidant capacity. This is done by inhibiting enzymes involved in generating endogenous antioxidant molecules (like GR) or depleting these endogenous antioxidant molecules (e.g. GSH), or other molecules required to generate endogenous antioxidant molecules (e.g. NADPH) (Figure 1.2). An example of a xenobiotic that is able to elicit this type of response is valproic acid, which is known to deplete cellular GSH content (Kiang *et al.*, 2011).

1.6. Need for improved cell-based hepatotoxicity screening

In light of the fact that hepatotoxicity is a major cause of drug attrition and drug development requires toxicity testing, evaluation of potential hepatotoxic effects represents a crucial step in the development of new drugs.

Human *in vitro* cellular models are valuable tools in understanding the molecular and cellular processes of drug-induced liver injury (Gomez-Lechon *et al.*, 2010a). The Multicentre Evaluation of In vitro Cytotoxicity program (MEIC) conducted a study in 29 different laboratories, where 50 reference chemicals were tested using 61 different *in vitro* cytotoxicity tests. The study concluded that *in vitro* cytotoxicity testing predicted the acute, lethal human blood concentrations just as well as mouse and rat LD₅₀s (Ekwall *et al.*, 1998). This demonstrates the potential of *in vitro* toxicity testing for reducing not only animal testing but also for reduction of post-commercialisation drug attrition due to hepatotoxicity. Furthermore, *in vitro* testing has the advantage over *in vivo* testing in being faster, more cost-effective and requiring less test material. These advantages show the vital and growing role *in vitro* testing is playing during early drug development, especially in light of the three R's of animal testing: replacement, reduction and refinement.

Gomez-Lechon *et al.* (2010a) stated: "*Current cytotoxicity assessments have been limited by their inability to measure multiple mechanistic parameters that capture a wide spectrum of potential cytopathological changes. By examining the effects on a hepatocyte-specific*

metabolism, it is possible to discover whether relevant hepatic functions are altered by the presence of a xenobiotic. However by addressing this issue, the development of robust in vitro-based multiparametric screening assays covering a wider spectrum of key effects will heighten the predictive capacity for human hepatotoxicity, and accelerate the drug development process". There is thus clearly a need for the development of a multiparametric hepatotoxicity screening model that assesses a wider spectrum of toxicity towards hepatic cells. The screening of multiple toxicity parameters should better reflect human *in vivo* hepatotoxicity and provide earlier safety assessment.

1.7. Model hepatotoxin

Dichlorodiphenyl trichloroethane (DDT) was originally synthesized in 1874 and put into use as an insecticide only in 1939. It was initially used by allied forces in the Second World War as vector control against malaria and was subsequently made available for commercial use in 1945 (World Health Organisation, 1979). DDT was the most extensively used insecticide across the world until the 1960's and has been credited with the eradication of malaria from the USA and Europe (Attaran and Maharaj, 2001).

In order to be eliminated from the body, DDT has to be dechlorinated and oxidised by CYPs to intermediate metabolites such as dichlorodiphenyl dichloroethylene (DDE) and dichlorodiphenyl dichloroethane (DDD). DDE is a non-biodegradable and a highly fat-soluble molecule that accumulates in the adipose tissue of the organism. DDD on the other hand is biodegradable and with further metabolism it is converted into bis(*p*-chlorophenyl)acetic acid (DDA), a water soluble molecule that is excreted in bile, faeces and urine (Klaassen *et al.*, 2001) (Figure 1.3).

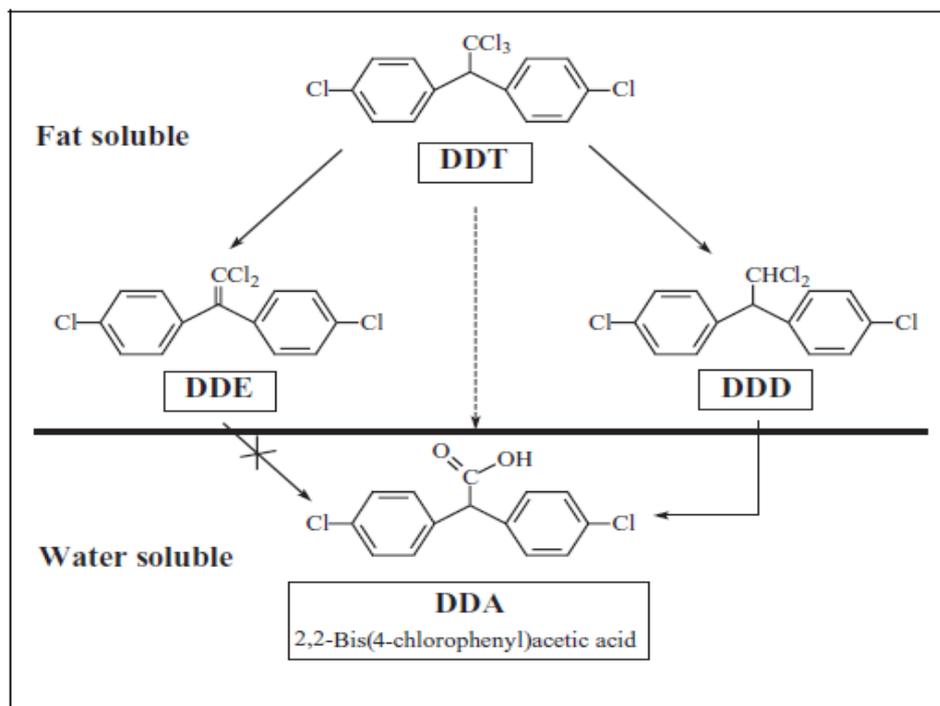


Figure 1.3. Illustration of the two routes of metabolism of DDT to produce either DDE or DDD, showing the structure of each of the test compounds utilised in this study (Chen *et al.*, 2009).

Studies have shown that DDT exposure increases the microsomal content and activity of CYP 2B1 and CYP 3A2 in liver tissue by enzyme induction (Klaunig and Ruch, 1987; Flodstrom *et al.*, 1990; Harada *et al.*, 2006). DDT partially exerts its anti-androgenic effects on the body by up-regulating the activity of, amongst others, these two enzymes (You *et al.*, 1999; Harada *et al.*, 2006). DDT has also been linked to increased oxidative stress by depleting cellular glutathione (GSH) content *in vitro* (Dehn *et al.*, 2005). It has been speculated that the depletion of GSH by DDT may be due to disruption of GSH regulation in the cell (Dehn *et al.*, 2005) and that there is a possible interaction between CYP induction and reduced GSH levels, which may lead to an increase in the generation of ROS (Bagchi *et al.*, 1993; Bagchi *et al.*, 1995) and depletion of cellular GSH stores. One of the major DDT metabolites, DDE, has also been shown to increase CYP activity, specifically the activities of CYP 2A1, CYP 2B1, CYP 2C11 and CYP 3A1 (You *et al.*, 1999).

Animal studies in rats have demonstrated that a single DDT dosage of 24 mg/kg body weight significantly increases relative liver weight (RLW) although a single dosage of 12 mg/kg body weight has no effect on RLW, (Kostka *et al.*, 1996). However, repeated dosages of 12 mg/kg

body weight do increase RLW (Kostka *et al.*, 1999). Histological examination revealed a high prevalence of cytoplasmic vacuolization and inflammatory infiltration, which is attributed to centrilobular zonal necrosis whereas an increase in RLW is attributed to a regenerative liver response. Zonal necrosis was found to up-regulate both mitoses and DNA synthesis resulting in hepatomegaly. It is well known that cell proliferation plays a crucial role in the initiation of carcinogenesis in the liver (Butterworth *et al.*, 1992) and DDT has therefore been regarded as a hepatocarcinogen (Kostka *et al.*, 1999; Sukata *et al.*, 2002). DDT-induced liver toxicity is well documented.

DDT follows a similar mechanism of hepatotoxicity as some well-known hepatotoxic drugs in that biotransformation of DDT yields two major metabolites, DDE and DDD, which are also known hepatotoxins. Acetaminophen has long been established as a hepatotoxin at excessive dosages and also yields toxic metabolites after biotransformation (Ray *et al.*, 1999; Ray *et al.*, 2001). Another possible route of toxicity is that the process of DDT biotransformation may produce non-drug related reactive molecules that could result in hepatotoxicity, similar in mechanism to that of Isoniazid (Weisiger, 2010). The aforementioned similarities indicate that DDT was a suitable candidate for inducing toxicity in the present study. In addition, DDT as well as its two metabolites, DDE and DDD, are all commercially available as standards.

1.8. Hepatoprotective agent

N-acetylcysteine (NAC) is a thiol that is used in the clinical setting to treat drug toxicity and disease-associated oxidative stress. It is probably best known for its use during acetaminophen overdose but has also been used in the treatment of HIV/AIDS, cystic fibrosis and chronic obstructive pulmonary disease (Smilkstein *et al.*, 1991; Atkuri *et al.*, 2007). NAC is also used to alleviate some of the adverse effects associated with anti-cancer therapies such as doxorubicin, cyclophosphamide and phosphamide (De Flora *et al.*, 2001). The reason that NAC is used to counter a state of oxidative stress is two-fold. Not only is NAC able to scavenge free radicals itself by donating a hydrogen cation, but it is also able to replenish and maintain intracellular levels of glutathione by providing the necessary cysteine

for GSH synthesis (De Flora *et al.*, 2001; Carageorgiou *et al.*, 2004; Oh and Lima, 2006; Atkuri *et al.*, 2007).

In the present study NAC, was utilised to assess how a widely used hepatoprotectant may affect the toxicity parameters measured during the study. Due to its antioxidant activities (Wang *et al.*, 2000), authors have described NAC as being able to inhibit oxidative stress (Park *et al.*, 2002; Reliene *et al.*, 2004). Since DDT has been speculated to deplete intracellular GSH (Bagchi *et al.*, 1993; Bagchi *et al.*, 1995) it was postulated that NAC may be able to alleviate DDT-induced toxicity.

1.9. Research topic

Literature highlights the significance of hepatotoxicity in both drug development and attrition, which directs current research in this field towards early, accurate detection of hepatotoxicity, preferably during the pre-clinical stages of drug development. Literature also demonstrates how hepatotoxicity may come about through a number of different mechanisms. The diversity of processes that may lead to hepatotoxicity complicates cell-based *in vitro* detection using an assay that evaluates only a single endpoint. For this reason, there exists room for the development of a cell-based *in vitro* technique that assesses multiple endpoints in an attempt to detect subtle hepatotoxicity that an assay evaluating a single endpoint (such as the conventional cytotoxicity assay) may fail to detect.

1.10. Aims of the study

- I. Develop an *in vitro* procedure capable of mechanistically profiling the effects of a model hepatotoxin on hepatocytes in culture by evaluating several different aspects of cellular function using a number of simultaneous *in vitro* assays on a single 96 well microplate.
- II. To determine whether the model can detect hepatoprotective effects of NAC on hepatocytes exposed to a known hepatotoxin.

1.11. Study objectives

- I. Assess a number of intracellular assays that test different aspects of toxin responses thereby allowing mechanistic profiling of the effect of a toxic compound on cultured hepatocytes.
- II. Optimisation of the assays for compatibility with the proposed microplate assay method.
- III. Stagger the hepatotoxicity assays in such a way that it allows simultaneous multiplexing of the selected assays using a single 96 well microplate.
- IV. Determine whether the method provides acceptable sensitivity and mechanistic data when testing a known hepatotoxin.
- V. Assess whether the developed assay method can detect the hepatoprotective effect on cultured hepatocytes pre-loaded with a known protective agent prior to hepatotoxin exposure.