Application of Cell-Based Assay Systems for the Early Screening of Human Drug Hepatotoxicity in the Discovery Phase of Drug Development

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Abstract

While drug toxicity (especially hepatotoxicity) is the most frequent reason cited for withdrawal of an approved drug, no simple solution exists to adequately predict such adverse events. Simple cytotoxicity assays in HepG2 cells are relatively insensitive to human hepatotoxic drugs in a retrospective analysis of marketed pharmaceuticals. In comparison, a panel of pre-lethal mechanistic cellular assays hold the promise to deliver a more sensitive approach to detect endpoint-specific drug toxicities. The panel of assays covered by this review includes steatosis, cholestasis, phospholipidosis, reactive intermediates, mitochondria membrane function, oxidative stress, and drug interactions. In addition, the use of metabolically competent cells or the introduction of major human hepatocytes in these \textit{in-vitro} studies allow a more complete picture of potential drug side effect. Since inter-individual therapeutic index (TI) may differ from patient to patient, the rational use of one or more of these cellular assay and targeted \textit{in-vivo} exposure data may allow pharmaceutical scientists to select drug candidates with a higher TI potential in the drug discovery phase.

Keywords: Cell-based system; Hepatotoxicity; Cytotoxicity, Mechanistic assay, Drug discovery.

Introduction

It is estimated that for the drug research that was initiated in 2001, and resulting in a planned drug approved in 2013, the average cost is US$ 1.9 billion (1). About half of this cost is the “time costs”, or “capitalizing out of-pocket costs to the point of marketing approval” (1). Therefore, tremendous amount of resources and time are wasted if a drug is withdrawn after launch due to toxicity reasons, not to mention the human suffering it may have caused to patients.

Drug-induced toxicity, or adverse drug reactions, have emerged as a major cause of post-market withdrawal of medications. During 1975–1999, 45 of previously approved drugs have acquired one or more black box warnings and 16 were withdrawn from the market for safety reasons (2). Of the 45 that acquired black box warnings, 10 were due to hepatic toxicity (22%). Of the 16 that were withdrawn from the market, 5 were due to hepatic toxicity (31%). These statistics make hepatic toxicity the largest root cause issue in this cohort of black box warnings and withdrawals (2). In addition to post-market attrition, toxicity is also a major reason of pre-launch attrition of drug candidates. It is thus highly desirable to devise a strategy to detect and therefore minimize such toxicity in the earlier phases of drug development.

A survey to examine the strengths and
weaknesses of animal studies to predict human toxicity indicated that current safety studies in animal models do not adequately predict human hepatic toxicities (3). In a recent study of predictivity of human drug toxicity by regulatory animal toxicity testing, 72% (167 of 230) of human toxicities were found to be associated with toxicity in animals (3). Of these, 8% were in rodents only, 28% were in non-rodents only, and 37% were in both. Correlation between animal and human toxicities was high for cardiovascular, hematologic, and gastrointestinal toxicities. However, among human toxicities, hypersensitivity, cutaneous reactions, and hepatotoxicity had the poorest correlation with regulatory animal toxicity tests (3).

Drug-induced hepatic injury is the most frequent reason cited for withdrawal of an approved drug and accounts for more than 50% of cases of acute liver failure (4). However, in only approximately half of new pharmaceuticals that produced hepatotoxicity in clinical drug development was there any concordance with animal toxicity studies (3). Occurrence of these adverse drug reactions is largely attributable to the imperfect extrapolatability of animal study findings to humans. Several possible reasons have been proposed to explain such animal/human discrepancy, including:

2. Animal species under well-controlled experimental settings do not predict the diversity of human patients living in heterogeneous conditions.
4. A sub-population of human patients, for reasons attributed to pharmacogenetic, environmental, and/or life-style susceptibility factors, are particularly sensitive to adverse drug reactions.

Given that there is a plethora of possible reasons for missing a toxic “signal” in current animal studies, it is unlikely that isolated and simple solutions exist to adequately predict human toxicity. Instead, a multitude of experimental and theoretical methods need to be explored and data combined to address this complex issue. This review will focus on the use of cell-based assay systems for the early screening of human drug toxicity in the discovery phase of drug development, especially of human hepatic toxicity. The rationale for the placement of these screens in the drug discovery phase is obvious: this is the phase when chemical choices and chemical modifications can still be made before significant development resources are committed to a particular new chemical entity (NCE).

The relevance of using cell-based systems as surrogate models to organ toxicity in-vivo is analogous to the well-accepted practice of screening for desired pharmacological effects. Therefore, all the caveats of the in-vitro systems would apply. Most importantly, while the in-vitro systems can identify a chemical’s potential to cause an effect (in this case, side effect), the local exposure levels of an NCE in-vivo would largely determine whether the chemical would ultimately be toxic to humans. The famous “Paracelsus doctrine” is just as relevant today as it was 500 years ago, the difference being today we should use “concentration” instead of “dose” to predict toxicity.

In addition to challenges in scalability between in-vitro and in-vivo exposure levels, the practice of human cellular toxicity also faces another challenge. That is, in the most extreme scenario, the mechanisms of drug toxicity to human organs could be as diverse as the mechanisms of human physiology. Even within a single cell type, e.g. hepatocytes, the mechanisms of chemical injury can be quite diverse. A recent review of hepatocyte toxicity explored at least 6 and 10 manifestations (see Table 1) of liver injury (4, 5, 6). In light of such complexity, how can one practically address so many potential possibilities of “chemical toxicity” within a finite amount of time in the drug discovery phase? A logical approach is to use a panel of better-characterized assays in parallel, in order to identify one or more chemical molecules with the best overall “therapeutic indices” in-vitro. However, no in-vitro systems today can mimic the potential complexities of the human body. Therefore, it is impractical to expect “perfect predictions” of in-vivo outcomes by any in-vitro systems. That said, some in-vitro systems have better predictivity than others and they are the focus of this review.
The utility and limitations of the conventional cytotoxicity assays in predicting human hepatic toxicity

Conventional cytotoxicity assays rely on measuring one or more cytotoxic indicators, including loss of membrane integrity or cytolysis (e.g. LDH release, membrane-impermeable DNA stain), apoptosis (e.g. activation of caspases), loss of critical macromolecules (e.g. ATP depletion, GSH depletion), anti-metabolic effects (e.g. tetrazolium salt assays, Alamar blue assay), or anti-proliferative effects (e.g. inhibition of DNA or protein synthesis).

In vitro, known hepatotoxic chemicals can be frequently identified and ranked by their 50% inhibitory effect concentration (μM) in cellular models, such as HepG2 cell line (a human hepatoma cell line), primary hepatocytes, cryopreserved hepatocytes or Liver beads, and WIF-B9 cell lines, by measures of any of the aforementioned cytotoxic indicators (7, 8). It is noteworthy that in terms of drug metabolizing activity, the rank order among various cellular models is: primary hepatocytes > cryopreserved hepatocytes > HepG2 cell line ~ WIF-B9 cell line. Liver beads are cryopreserved hepatocytes entrapped in alginate beads; WIF-B9 cells are hybrids of rat hepatoma (FaO) and WI38 human fibroblasts. Cytotoxicity is not detected in these cell lines for some hepatotoxins, such as ethionine, valproate, galactosamine, thioacetamide, diclofenac and isoniazide. This may be in part attributable to incomplete metabolic competence of these cellular systems (7).

Assessment of the predictivity of drug-induced human hepatotoxicity by cytotoxicity assays can be made by comparison with that of regulatory animal toxicity testing. If the cytotoxicity assay has similar predictivity, it should be considered of high value for safety assessment in drug discovery because of its much lower cost and much faster turn around time. For such assessments, drugs that have been marketed were classified into three categories according to the severity of human hepatotoxicity they produce: severe or “bin 1” (producing >1% frequency of increased serum ALT plus 2 of jaundice, >3 reports of liver failure or black box warning; 42 drugs); moderate or “bin 2” (producing 0.1–1% frequency of increased serum ALT plus jaundice or label of occurrence of adverse effect; 283 compounds); none to minimal or “bin 3” (<0.1% frequency of increased ALT, no clinical symptoms; 286 compounds). Table 1 compares the predictivity of various cytotoxicity assays applied to these drugs (8) and demonstrates that these assays have up to half the sensitivity (proportion of positives identified) of animal tests (4). These values may be substantially underestimated because they are based on retrospective assessment of concordance of test results with drugs that have already been marketed for usage in humans. Therefore, those discovery compounds that would have tested positive by in-vitro cytotoxicity tests and animal toxicity tests and would have caused severe human hepatotoxicity (or in “bin 1”), were already excluded in this retrospective analysis. Although the in-vitro assays were still relatively insensitive at detecting hepatotoxicity, when drugs were tested positive in these assays (e.g. greater than 50% effect at 30 μM in HepG2 cells), there was high probability of human toxicity (i.e. they had high specificity, see Table 1).

The predictivity of cell-based assays for in-vivo toxicity depends in part on the point in the sequence of pathogenic events that they target (Table 2). Assays that target late events in the process of cell injury, when the cell is near death, are more likely to miss toxicities that require chronic exposure or exert adverse but non-lethal effects. Many of the above cytotoxicity assays (e.g. LDH release, mitochondria dye reduction, cell rupture, membrane blebbing, DNA degradation and nuclear condensation) are for late-stage toxicity and cellular events associated with a lethal apoptotic or necrotic effect (9, 10). Such assays have low sensitivity (Table 1) for detection of adverse cellular effects and furthermore provide little mechanistic understanding of the toxicologic effects in humans. Conversely, assays that provide early assessment of specific toxicologic mechanisms in cells (Table 2) prior to the onset of the late stages of non-specific degeneration and apoptotic or necrotic death should theoretically have greater predictive power and extrapropolatability across models and species.

While these retrospective analysis support the value of in-vitro cytotoxicity screens to identify the “overtly” toxic compounds, it points
to the need of further refinement in the method to predict subtle or sub-lethal adverse events that account for the majority of side effect profiles of human pharmaceuticals.

Pre-lethal mechanistic assays

It is well recognized that even within the same organ, drug-induced toxicity can be caused by a variety of mechanisms. For example, at least six mechanisms that involve hepatocytes have been proposed to produce liver injury (4, 5, 6, 11, 12). Therefore, a variety of pre-lethal mechanistic assays have been used by researchers to study potential hepatic side effects of drugs, each addressing a specific mechanism (steatosis, cholestasis, phospholipidosis, reactive intermediates, mitochondria membrane function, oxidative stress, intracellular calcium homeostasis, etc.). These assays have the advantage to identify the “subtle” and potentially more clinically relevant lesions in a human or mammalian cell-based assay system. They also have the advantage to identify such lesions at much lower drug concentrations than the concentrations needed for gross or overt cytotoxicity (i.e. more sensitive). An ideal in-vitro assay is such that both the assay endpoints and drug concentrations needed to elicit those effects are more relevant to the human clinical observations. For the remaining of this article, we will review those mechanism-specific pre-lethal cellular assays that have generally been considered useful by multiple practitioners in this field.

This is not intended to be a comprehensive review of all of the available and applicable in-vitro assays that exist in the literature. For readers who are interested in earlier reviews of hepatotoxic mechanisms, drugs and experimental tools, please refer to classical text books such as Zimmerman’s Hepatotoxicity (13).

Steatosis assays

Steatosis, or accumulation of fatty acids (i.e. “fatty liver”), can be caused by alcohol, aspirin, tetracycline, amiodarone, valproic acid, and several antiviral nucleoside analogues, the most prominent of which is fialuridine. These adverse effects have led to the recall of diet hyaminoethoxyhexestrol (DEAEH), the abrupt interruption of clinical trials with fialuridine, essential abandonment of perhexiline, and therapeutic guidelines for the use of tetracycline and valproic acid (14).

One of the major mechanisms involved in steatosis is the inhibition of beta-oxidation of long-chain fatty acids, either by direct inhibition or indirect inhibition such as CoA sequestration or mitochondria DNA damage (15, 16). The resulting fatty acid accumulation can be detected and quantified by staining primary hepatocytes with neutral lipid stains such as Oil red O (17, 18).

In addition, DEAEH, amiodarone, and perhexiline also block the transfer of electrons in the respiratory chain, produce superoxide anion, and cause lipid peroxidation (19, 20). The aldehyde products of lipid peroxidation, 4-hydroxynonenal and malondialdehyde (MDA), are known activators of hepatic stellate cells, the principal collagen-producing cells within the liver. MDA is also known to stimulate inflammatory responses. Therefore, the combination of decreased beta-oxidation (resulting in lipid accumulation) and increased reactive oxygen generation (resulting in lipid peroxidation) represents an important mechanism of drug-induced steatohepatitis.

Both of these biochemical endpoints can be measured relatively rapidly using either hepatocytes (4, 5, 11, 21, 22) or isolated liver mitochondria (19, 23), thereby offering promising assay opportunities to study the potential steatohepatitic effect of drug candidates in drug discovery.

The difference between these two in-vitro systems (hepatocytes versus isolated mitochondria) is that isolated mitochondria does not have the complexity of the freshly isolated hepatocytes, which have both the barrier of membrane permeability and an active drug metabolizing system (e.g. phases I and II enzymes).

Cholestasis assays

Intrahepatic cholestasis, defined as impairment in bile formation and/or bile flow, is another common manifestation of drug-induced liver disease. In humans, intrahepatic cholestasis most often occurs as a side-effect of drug therapy and the clinical manifestation of this condition,
jaundice, has been estimated to account for hospitalization in 2–5% of the cases for the general population and approaches as much as 20% in the elderly (24). As the population ages and the occurrence of multiple drug therapy in geriatric patients increases, it is to be expected that jaundice and/or drug-induced intrahepatic cholestasis will become even more prevalent. Bile formation is dependant on the specific transporter proteins in hepatocytes. As expected, inhibition of important hepatobiliary transporters can result in cholestasis. The functions of these hepatobiliary transporters, including the bile salt export pump (BSEP), multidrug-resistance-3 (MDR3), multidrug-resistance-1 (MDR1), and multiresistanceprotein-2 (MRP2), have recently been elucidated (25).

BSEP transports bile salts, MRP2 transports divalent bile salts and bulky organic conjugates, while MDR3 transports phospholipids into the bile canalicular space. Inside bile canalicular spaces, the phospholipids form mixed micelles with the bile salts to minimize the cytotoxic effects of free bile salts on cholangiocytes (cells lining the bile duct). It is therefore expected that disruption/inhibition of the activity of these transporters can have toxic consequences. Indeed, human mutations in BSEP and MDR3 result in familial intrahepatic cholestasis types 2 and 3, respectively (26). In addition, women with heterozygosity for a nonsense mutation of the MDR3 transporter are susceptible to cholestasis of pregnancy as a result of the high circulating levels of estrogens (26). Recently, several chemicals that have caused intrahepatic cholestatic side effects in humans were found to inhibit BSEP at low micro molar concentrations. These drugs include cyclosporine A (CsA), rifamycin SV, rifampicin, glibenclamide, the cholestatic estrogen metabolite (estradiol-17-betaglucuronide) (27), taurolithocholate (28), troglitazone, troglitazone sulfate (29), bosentan and its metabolites (30). In these studies, inhibition of BSEP was successfully shown using isolated canalicular liver plasma membrane vesicles, or rat or human BSEP-expressing Sf9 cell vesicles. In another study, lithocholic acid was found to decrease the expression of BSEP through its farnesoid X receptor antagonist activity in both primary human hepatocytes and HepG2 cells (31). A much higher throughput cell-based assay for BSEP inhibition using a fluorescent substrate, dihydrofluorescein, was also reported (32). It is therefore possible to utilize these rapid in-vitro assays to study the inhibition of BSEP, in order to identify the intrinsic cholestatic property of new chemical entities. It is noteworthy that metabolites of the parent drug can be a more potent inhibitor of BSEP (e.g. (29)). Since the Sf9 insect cells or human cell lines do not readily express the major human drug metabolizing enzymes, human metabolites identified from the in-vitro microsomal or hepatocyte incubation will also need to be tested in these in-vitro models before a conclusion can be made about a drug candidate.

**Phospholipidosis assays**

Phospholipidosis, defined as the accumulation of excess phospholipids in cells, is often accompanied with various associated or coincidental toxicities, especially in the lung and also liver (33-35). Cationic amphiphilic drugs can often induce this phenomenon in-vivo. While phospholipidosis per se does not constitute frank toxicity (36), it is reportedly predictive of drug or metabolite accumulation in affected tissues (37), and as such, possibly associated with toxicities that may warrant further in-vivo investigation (e.g. longer term animal toxicity studies, reversibility and biomarker studies, etc.). In-vitro, the accumulation of phospholipids in cells can be monitored by staining cells with fluorescent phospholipid analogs such as NBD-PE (38) or NBD-PC (39, 40). Using this method, the phospholipidosis observed for a pharmaceutical lead was attributed to a cationic metabolite in primary human hepatocytes (38). Structural changes of the parent compound to alter the properties of its metabolite, diminished phospholipidosis both in-vitro and in-vivo (38). This example highlights the usefulness of this cell-based phospholipidosis assay in the lead optimization stage of drug discovery. Once again, it highlights the necessity to study a major human metabolite in the in-vitro systems to get a better concordance with the in-vivo outcome of a drug candidate.
Reactive metabolite assays
It is well known that many hepatotoxic agents can be metabolized to reactive metabolites that can either be detoxified or react with glutathione (GSH), enzymes, nucleic acids, lipids, or proteins (6, 41, 42, 43). These reactive intermediates are electrophilic metabolites or free radicals that are generated during the metabolism of a broad range of functional groups. Therefore, on a molar basis, they are likely to be more reactive to biological nucleophiles (peptides, proteins, nucleic acids, etc.) than their parent compound or non-electrophilic metabolites. However, in practice, since other metabolites are usually in molar excess over the electrophilic metabolites, it can be difficult to differentiate the toxic contribution of the parent and non-electrophilic metabolites versus the electrophilic metabolites vs. free radicals. With regard to covalent binding of electrophilic metabolites to proteins, it has been reported to correlate with cytotoxicity (44, 45), although exceptions were also reported (46). Reactive metabolite formation is also considered an important factor in immune-mediated idiosyncratic drug hypersensitivity (47). However, it remains to be seen whether all covalently modified proteins or only a subset of modified proteins or free radicals are capable of initiating an immune response (47). These uncertainties aside, there exist several rapid in-vitro methods to detect and measure the generation of such reactive intermediates. For example, a high-throughput assay for identifying pharmaceutical compounds that produce reactive metabolites have been developed (48). The method involves incubating drug candidates with a liver microsomal drug metabolizing enzyme system in the presence of glutathione and detecting glutathione conjugates via tandem mass spectrometry (48-50). Therefore, this method actually uses a native defense mechanism (i.e. GSH detoxification pathway) as a surrogate marker for the generation of the initial reactive metabolites. In cells, reduced form of glutathione is known to interact with electrophilic compounds/metabolites and free radicals to play a key role in the detoxification of such reactive molecules. Depletion of reduced form of glutathione was reported to be a marker of hepatotoxicity (e.g. (51)), suggesting its evaluation as an important functional readout in cellular toxicological studies. For example, monochlorobimane and chloromethyl fluorescein diacetate (CMFDA) have been successfully used to monitor cellular GSH levels by epifluorescence in isolated hepatocytes in-vitro (52, 53).

Mitochondria toxicity assays
Mitochondria are pivotal in the pathophysiology of apoptotic and necrotic cell death because of their central role in energy metabolism, calcium homeostasis, and activation of apoptosis. Accordingly, evaluation of their function should provide a sensitive indication of adverse cell effects. Recent advances in the development of micro fluorescent probe technology enable in-vitro monitoring of live cells for multiple cellular processes, including inner mitochondrial membrane potential (e.g. TMRM), intracellular free Ca (e.g. Fluo4), membrane permeability (e.g. TOTO3) and DNA, as well as cell number, size and morphology (54). Ref 55 demonstrates characteristic kinetics of changes of the former 3 parameters for 30–56 cells (mean±S.E.M.) as monitored on the Cellomics KSR (Kinetic Scan High Content Screening Reader) (55) after exposure of HepG2 cells in 96-well plates to dantrolene. Typically, mitochondrial dysfunction precedes calcium dyshomeostasis, which precedes membrane permeability increases. Occasionally, a drug may produce an apparent increase in mitochondrial membrane potential prior to its depolarizing and subsequent rise in intracellular Ca. Whether this is an artifact of transporter inhibition or due to hyperpolarization and adaptive response should be investigated.

Sensitization to drug-induced cytotoxicity can be enhanced for some drugs by co-addition of a chemical Stressor, such as oligomycin (56) (Fig. 1, reproduced from (57)), or by prolonged exposure (Fig. 2, reproduced from (57)) (57, 58).

Mitochondrial membrane potential was more sensitive than other cytotoxicity assays in a subset of fourteen drugs (Table 3). These drugs have been marketed, and as described before were classified into three categories according to the severity of human hepatotoxicity they
produce: severe or “bin 1” (producing >1% frequency of increased serum ALT plus 2 of jaundice, >3 reports of liver failure or black box warning); moderate or “bin 2” (producing 0.1–1% frequency of increased serum ALT plus jaundice or label of occurrence of adverse effect); none to minimal or “bin 3” (<0.1% frequency of increased ALT, no clinical symptoms). As described in Table 1 before, a positive in the cytotoxicity tests is defined by having a greater than 50% effect at 30 M in HepG2 cells in any of the cytotoxicity assays listed. Specificity (proportion of negatives identified) was as high as for other cytotoxicity assays. In this subset of compounds, the mitochondrial potential assay had at least double the sensitivity (proportion of negatives identified) of other cytotoxicity assays (comparing Table 3 with Table 1). However, a more thorough testing with large numbers of drugs is needed to accurately quantify its sensitivity.

A promising finding of high sensitivity of mitochondrial membrane potential assays is not surprising, especially in light of the current mechanistic understanding of drug-induced hepatotoxicity (16, 59). Many different mechanisms have been identified that indicate mitochondria as a key player in drug toxicity:

(a) Uncoupling by lipophilic and slightly acidic drugs that shuttle protons into the mitochondria such as sulfonamides;
(b) drugs that shuttle electrons from mitochondrial respiration to oxygen;
(c) redox cycling within the mitochondria by drugs such as quinines and nitroarenes;
(d) opening of the mitochondrial permeability transition pore by drugs producing oxidative stress or increasing ionized calcium, such as bile acids and thiol cross linkers;
(e) impairment of fatty acid oxidation such as by tetracyclines, glucocorticoids, non-steroidal anti-inflammatory drugs, and cationic amphiphilic drugs such as amiodarone and perhexiline;
(f) mitochondrial DNA synthesis inhibition such as the nucleoside reverse transcriptase inhibitors zidovudine, zalcitabine and didanosine that are used in treatment of HIV infection. As with other cytotoxicity assays, the current mitochondrial potential assay in HepG2 cell lines did not detect cytotoxicity of compounds needing metabolic activation for their toxicity (e.g. acetaminophen, diclofenac, isoniazide and valproate).

This is most likely due to the lack of metabolic competence of the HepG2 cell model. It is...
expected that such limitations can be overcome by the use of a metabolic competent cell line such as primary human hepatocytes.

**Oxidative stress assays**

Oxidative stress has been implicated in various degenerative diseases and in aging, and is an important mechanism of drug-induced toxicity (60). Credible implication of oxidative stress in the etiopathogenesis of a disease or toxicity requires demonstration of both increased production of oxidants or free radicals, and intracellular macromolecular change due to oxidative injury such as decreased glutathione, or cellular adaptation of antioxidant defense mechanisms.

The fluorometric dye, 2′,7′-dichlorodihydrofluorescein (H2DCF) can be used to detect oxidative stress induced by various free radical generators in cells grown in 96-well plates (61-65) for rapid throughput analyses. The diacetate form of H2DCF freely enters the cell and is hydrolyzed by intracellular esterases to liberate H2DCF, which then reacts with oxidizing species to produce the highly fluorescent DCF. Cell based screening for oxidative stress potential can be accomplished with the use of epifluorescent microscopy, fluorescent plate readers or flow cytometry. In Fig. 3, dose and time dependency of oxidant-induced oxidative stress is shown for human SH-SY5Y neuroblastoma cells. It is evident that diquat-induced oxidative stress, as measured by DCF fluorescence, increased in a dose-dependent and time-dependent manner over 60 min.

Cellular homeostasis depends on protection from oxidative damage by antioxidant systems that consist of networks of metabolites and enzymes of metabolic pathways. Small molecules such as glutathione, ascorbate, alpha-tocopherol, and the carotenoids act as rapid chemical traps or physical quenchers. Glutathione is also involved in detoxification and excretion pathways and is a cofactor for enzymatic reduction of peroxides. Key antioxidant system enzymes include:

(a) superoxide dismutase, which catalyses the one electron dismutation of superoxide into hydrogen peroxide and oxygen;

(b) catalase which catalyses the two-electron dismutation of hydrogen peroxide into oxygen and water;

(c) glutathione peroxidase which uses glutathione as a cofactor to detoxify hydrogen peroxide and lipoperoxides

(d) gamma-glutamyl cysteine synthetase which is the rate-limiting enzyme for glutathione synthesis;

(e) glutathione reductase which acts to reduce

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**Figure 3.** Diquate-induced increase in free radical production. Dose and time-dependency of diquat-induced oxidative stress in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells were seeded in 96 well plates, at 5×10^4/well, and grown overnight. The cells were loaded with 20 µM DCF (Molecular probes, Eugene, Oregon USA) for 45 min at 37°C in culture medium (Dulbecco’s modified eagle medium containing 1% fetal bovine serum and 100 IU/1ml streptomycin containing 2.5 mM probenecid). After DCF loading, cells were washed three times in Krebs Henseleit buffer containing 1% fetal bovine serum and 2.5 Mm PROBENECID (KHB). KHB (100 µl) was then added to the cells and the baseline fluorescence measured at 485 nm excitation and 535 nm emission. Test substances (e.g. diquat) were added in another 100 µl KHB and fluorescence was measured every 5 min for 60 min. The Y-axis represent mean relative fluorescence (RF) readings from four independent wells by the cellomics KSR (Adapted from ref. 66).

**Figure 4.** Key antioxidant system components (Adapted from ref. 66).
glutathione disulphide to glutathione using NADPH;
(f) nicotinamide adenine dinucleotide as a cofactor, and which is supplied by the pentose phosphate pathway at a rate determined by the rate-limiting enzyme;
(g) glucose-6-phosphate dehydrogenase. The interplay among these components is shown in Fig. 4. Up-regulation of a panel of antioxidant system enzymes can be assessed in rapid throughput assays using automated clinical chemistry analyzers and aliquots of cell lysate, as illustrated below. These assays measure maximal activity of enzymes under optimal conditions and are proportional to the amount of enzyme present. In Fig. 5, the mechanism by which diquat produces oxidative stress is indicated, namely shunting of electrons from cytochrome P450 to oxygen data demonstrate that diquat produces oxidative stress with cellular adaptation including moderate induction of glutathione production, increased glutathione reductase, and glucose-6-phosphate dehydrogenase, and up-regulation of enzymes detoxifying reactive oxygen species, superoxide dismutase and, especially, catalase. This contrasts for example with acetaminophen toxicity, where there is marked up-regulation of only the glutathione system (66).

**Drug interaction assays**

In the clinic, many drugs are metabolized by cytochrome P450s, mainly by CYP3A4, such as theophylline, erythromycin, terfenadine, astemizole, cisapride, and many more. When these drugs are co-administered with CYP3A4 inhibitors, such as ketoconazole, ritonavir and cimetidine, a drug concentration in plasma could increase and could cause toxicity, especially for drugs with narrow therapeutic indexes (67, 68). Therefore, these drug–drug interactions (DDIs) should be avoided. Numerous reviews are available regarding DDIs (e.g. ref. 69, 70). Human cell based systems can offer advantages over cell-free systems to the screening of drug–interactions. For example, the freshly isolated human hepatocytes encompass both permeability barriers and complete enzyme systems (71). The human hepatocyte system also offers the possibility to study drug-related regulations of P450 enzyme systems (72, 73). To overcome the limitation posed by fresh human tissue, cryopreserved human hepatocytes have been used to study metabolic stability and inhibitory drug–drug interactions (74). Cryopreserved cells from well-characterized but different donors can be pooled together in one study to mimic the “averaged” human liver enzymatic levels.

It is now well accepted that drug–drug

| Table 1. Predictivity of drug-induced human hepatotoxicity by citotoxicity assays and animal testing (4, 5, 6) |
|-----------------|-----|-----|-----|
| DNA synthesis  | 10  | 92  | 60  | 47  |
| Protein synthesis | 4  | 97  | 57  | 47  |
| Glutathione depletion | 19 | 85  | 60  | 48  |
| Superoxide induction | 1 | 97  | 29  | 46  |
| Caspase-3 induction | 5  | 95  | 54  | 47  |
| Membrane integrity | 2  | 99  | 67  | 47  |
| Cell viability | 10  | 92  | 59  | 47  |
| Cell viability or GSH or DNA syn | 25  | 83  | 62  | 49  |
| Regulatory animal toxicity tests | 52  | -   | -   | -   |
| Sensitivity (Se)/positive predictive value (PPV)/negative predictive value (NPV). Tests were applied to 42 drugs causing severe human hepatotoxicity, 283 drugs producing moderate human hepatotoxicity, and 286 drugs producing negligible human hepatotoxicity.
interactions can also occur at the level of membrane transporters. For example, inhibiting the \( P \)-glycoprotein (PgP) can be an important mechanism of drug–drug interactions \((75, 76)\). Because of its wide substrate specificity and tissue distribution, PgP plays a critical role in excreting drugs and their metabolites into the intestinal lumen, bile, urine, and in limiting their exposure to the brain and uterus \((75)\). Clinically important PgP mediated drug interactions have been reported and include: digoxin cardiotoxicity when co-administered with PgP inhibitors such as verapamil, quinidine, or cyclosporin A \((77)\); vincristine neurotoxicity when co-administered with CsA \((78)\); and loperamide neurotoxicity

Table 2. Pre-lethal indicators of cell stress and sequence of events and biomarkers \((9, 10)\)

<table>
<thead>
<tr>
<th>Glutathione depletion</th>
<th>Pre-apoptotic/necrotic stress signal transduction, e.g. nrf-2, AP-1, NF-kB transcription factor translocation</th>
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<tbody>
<tr>
<td></td>
<td>Cellular adaptation, e.g. antioxidant enzymes, mitochondrial membrane hyperpolarisation, mitochondrial biogenesis</td>
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<tr>
<td></td>
<td>Mild impairment of energy and calcium homeostasis</td>
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<td></td>
<td>Mild impairment of cell function, e.g. proliferation</td>
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<td></td>
<td>Autophagocytosis of abnormal intracellular organelles</td>
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<tr>
<td>Apoptotic cell death and caspase activation</td>
<td>Mitochondrial permeability transition with membrane depolarization, cytochrome c release, and mitochondrial swelling and fragmentation</td>
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<td></td>
<td>Activation of caspases cell death pathway</td>
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<tr>
<td></td>
<td>Cytoskeletal disruption with membrane blebbing</td>
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<tr>
<td></td>
<td>Marked impairment of energy homeostasis with volume contraction</td>
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<tr>
<td></td>
<td>Endonuclease fragmentation of DNA with chromatin and nuclear condensation</td>
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<tr>
<td></td>
<td>Phosphatidylserine exposure on cell surface</td>
</tr>
</tbody>
</table>

Necrotic cell death

Impaired mitochondrial reductive activity with decreased ATP concentration \(\text{e.g. MTT, Alamar blue}\)

Organelle and cell swelling and distortion

Cell lysis with intracellular enzyme release \(\text{e.g. LDH release}\)

Table 3. Drugs to assess human hepatotoxicity \((16, 59)\)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bin</th>
<th>Cytotoxicity test</th>
<th>Mitochondrial membrane potential</th>
<th>Therapeutic indication</th>
<th>Chemical class</th>
</tr>
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<tbody>
<tr>
<td>Diclofenac(^a)</td>
<td>1</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Anti-inflammatory</td>
<td>Anthranilic acid</td>
</tr>
<tr>
<td>Paracetamol(^b)</td>
<td>2</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Anti-inflammatory</td>
<td>Salicylates</td>
</tr>
<tr>
<td>Flufenamate</td>
<td>3</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Anti-inflammatory</td>
<td>Anthranilic acid</td>
</tr>
<tr>
<td>Isoniazid(^a)</td>
<td>1</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Antibacterial</td>
<td>Quinolones</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>3</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Antibacterial</td>
<td>Sulfonamide</td>
</tr>
<tr>
<td>Valproate(^a)</td>
<td>1</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Anticonvulsant</td>
<td>Organic acid</td>
</tr>
<tr>
<td>Primidone</td>
<td>3</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Anticonvulsant</td>
<td>Barbiturate</td>
</tr>
<tr>
<td>Zalcitabine</td>
<td>1</td>
<td>Pos.</td>
<td></td>
<td>Antiviral</td>
<td>Nucleoside analog</td>
</tr>
<tr>
<td>Zidovudine</td>
<td>2</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Antiviral</td>
<td>Nucleoside analog</td>
</tr>
<tr>
<td>Vidarabine</td>
<td>3</td>
<td>Pos., DNA</td>
<td>Neg.</td>
<td>Antiviral</td>
<td></td>
</tr>
<tr>
<td>Dantrolene</td>
<td>1</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Muscle relaxant</td>
<td>Hydantoin</td>
</tr>
<tr>
<td>Diquat</td>
<td>1</td>
<td>Neg.</td>
<td>Pos.</td>
<td>Herbicide</td>
<td>Bipyridyl</td>
</tr>
<tr>
<td>Zomepirae(^b)</td>
<td>3</td>
<td>Neg.</td>
<td>Pos.</td>
<td>NSAIIDs</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>astemizole(^b)</td>
<td>3</td>
<td>Viab, DNA, GSH</td>
<td>Pos.</td>
<td>Antihistaminic</td>
<td>Amidine</td>
</tr>
</tbody>
</table>

\(^a\) Drug needs to be metabolized to exert toxicity.

\(^b\) Drug also has other organ toxicities.
when co-administered with quinidine (79). As digoxin is one of the most frequently prescribed drugs to elderly patients, avoidance of potent PgP inhibitors minimizes the potential drug interaction with this common medication. Cells and cell lines that express human PgP have been used to study the drug interactions with this transporter. For example, using polarized cell lines expressing PgP, both transport and inhibition of PgP can be studied (80-82). High throughput assays to measure PgP inhibition using calcein fluorometric readout in whole cell systems have been reported (83-85). These assays have made it possible to screen for potent PgP inhibitors in drug discovery, therefore minimizing the potential to cause drug interaction with marketed PgP substrates such as digoxin and vincristine.

Summary

One of the most challenging research areas in toxicology today is to understand why individuals respond differently to drug therapy and to what extent that individual variability in genetics and non-genetic factors (e.g. inflammation, life-styles, etc.) is responsible for the observed differences in adverse reactions. While identifying a “safer patient” for each NCE by considering both genetic and non-genetic factors remains a lofty goal of the future, identifying a “safer chemical” in the drug discovery phase is a practical approach that one can implement today. Given that the individual TI response may be different both between the object and within the same object at different time depending on other patient factors such as low level of inflammation (86), it is probably more imperative to be able to identify NCEs with a high TI potential in the drug discovery phase. Human and mammalian cell-based assay systems will continue to play a key role in this endeavor.

The successful drug research program must integrate cellular pharmacology, cellular toxicology, and drug metabolism and disposition into study design, and conduct balanced in-vitro and in-vivo experiments to allow a higher probability of success in drug therapy and safety.

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