

Expert Opinion

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General

The role of investigative molecular toxicology in early stage drug development

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Molecular toxicology, the application of molecular biology principles and technologies to preclinical safety assessment, represents a key tool for understanding mechanisms of toxicity and assessing the risks associated with specific toxicities. The application of gene expression markers to early stage preclinical safety assessment has the potential to impact pipelines in two main areas: lead optimisation and issue management. Lead optimisation focuses on deprioritising leads with significant, development-limiting toxicological liabilities while advancing those compounds with the greatest chance of successfully navigating the gauntlet of preclinical and clinical safety studies. Issue management utilises mechanistic toxicology studies to position non-development-limiting findings prior to the onset of Good Laboratory Practice studies in full development, and can help to identify and validate gene expression markers predictive of adverse events to avoid issues in second-generation projects. In this review, the authors describe the application of molecular toxicology to a standard pharmaceutical testing funnel, provide examples of the successful application of gene expression markers, and discuss the potential for future impact in several broad categories of clinically relevant toxicity.

Keywords: expression profiling, mechanistic toxicology, molecular biology

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1. Introduction

The cost of bringing new drugs to market, as well as the hurdles that must be overcome to address safety issues, are increasing. The result has been a decrease in productivity in the pharmaceutical industry despite an increase in most R&D budgets [1]. In part, this is due to past successes of the pharmaceutical industry. For many therapeutic indications, safer and more efficacious drugs have been developed in recent years [2,3], causing the bar to be raised for new competing therapeutics. On the other hand, new development-limiting safety issues, such as QT interval prolongation [4,5] that must be screened for prior to the first dose in humans, have come to the attention of the industry and regulators alike. These factors have resulted in continual increases in the cost of bringing new chemical entities to market. By one estimate, it costs as much as \$800 million to bring a drug to market [6], and only three in ten marketed drugs return the investment made in their development [7]. All of this is occurring against a backdrop of calls in the US for government-mandated price controls for drugs, and discussions of reducing the length of exclusivity provided for patented pharmaceuticals. The ability of the pharmaceutical industry to develop and deliver new pharmaceuticals in the future may appear bleak.

Among the strategies undertaken to address the growing hurdles to bringing new drugs to market, the application of investigative toxicology to early stage discovery projects holds great promise for increasing success in selecting the best compounds to move into full development, while deprioritising compounds with poor chances

of successful development earlier in the testing scheme [8]. Molecular toxicology holds the potential to be a key contributor to the investigative toxicology paradigm. Among those molecular technologies with applicability for early stage preclinical safety assessment are cDNA library screening, gene expression and cloning, expression analysis technologies and the various '-omic' technologies. The '-omics' technologies, including transcription profiling, proteomics and metabolomics, represent high-throughput approaches to identifying changes in mRNA, protein or metabolite profiles (respectively) in response to medical or environmental conditions or experimental perturbations. All three technologies have applicability to preclinical development and have been reviewed elsewhere [9-11]. Whereas proteomics and metabolomics have direct applicability in identifying non-invasive protein or metabolite biomarkers for both preclinical species and humans alike, gene expression markers are less likely to provide information without either sacrificing the preclinical animal model or performing often painful and intrusive biopsies. Upon sacrifice, existing clinical chemistry markers, haematological changes and histopathology evaluations already provide much information about a lesion. For these reasons, in order to be useful, gene expression markers must provide additional mechanistic information about a lesion, or they must be able to predict a lesion in advance of other methods.

In the past, toxicology has been largely an applied science, with minimal technology development taking place. Instead, there was a degree of art involved in interpreting clinical chemistry and physical data from in-life studies. Historically, drug discovery and safety assessment occurred in parallel, as pharmacologists performed *in vivo* studies in which both efficacy and toxicity end points were assessed. With the inception of high-throughput screening programmes to increase the number of potential drug candidates, efficacy and toxicity evaluations were largely separated into two phases; drug discovery and preclinical drug development [12]. Initially, this new bifurcated drug discovery/development paradigm involved discovery transferring a potent, biologically active lead compound with desirable physical chemical properties to the preclinical safety group, with little information regarding the compound's toxic liabilities. Typically, upon reaching preclinical development, compounds were run through a gauntlet of standardised drug safety studies of increasing duration in an effort to identify dose-limiting adverse events and maximally tolerated doses. A sufficiently negative outcome in any of those studies could kill a compound. As a result, an alarming number of compounds that entered preclinical development failed due to toxicities that could have been identified earlier in the compound's lifecycle. Within the preclinical group, decisions to stop the development of a compound or to move it forward were typically based on the therapeutic index, assessed by comparing the efficacious dose with the lowest toxic dose [12]. Toxicity was based upon pathology and/or well-established clinical chemistry markers, often with limited understanding of the precise molecular mechanisms of the observed

toxicity. However, retrospective evaluations indicate that studies using common preclinical toxicology species have only an ~70% true positive rate for predicting human toxicity [13]. As a result, it is not uncommon for a compound to be discontinued late in the development process after significant time and money have been expended during the compound's development. Molecular toxicology has the potential both to identify development-limiting toxicities early in the drug discovery process, and to understand and position non-development-limiting toxicities. In this review, the authors will describe how molecular toxicology can be applied to a standard pharmaceutical testing funnel in Section 2, then detail two examples of the successful application of gene expression markers to pharmaceutical development in Section 3. In Section 4, several broad categories of clinically relevant toxicity will be described, and the potential for the development and application of gene expression markers of toxicity will be discussed.

2. Application of molecular toxicology to the testing funnel

The application of investigative molecular toxicology to early stage preclinical safety assessment has the potential to impact drug discovery pipelines in two main areas: lead optimisation and issue management. A generalised pharmaceutical testing funnel is indicated in **Figure 1** [9]. As detailed above, discovery entails several stages, including target identification and validation, high-throughput screening and lead identification. Preclinical development typically begins with *in vitro* toxicology screening, followed by acute and repeat dose rodent and non-rodent toxicology studies. Compounds with promising pharmacological and safety profiles move forward to first-in-man studies, and then onto Phase I, II and III clinical trials. Research typically continues even after new products reach the market, as patient populations are significantly larger for marketed pharmaceuticals than can be achieved in highly controlled clinical trials. Although the three main stages of drug discovery/development in **Figure 1** appear distinct, there is significant overlap between them. For example, upon compiling sufficient safety data for a compound, it is common for compounds to be dosed short-term in humans before the longer duration preclinical studies have been performed. In addition, many pharmaceutical companies have begun to apply toxicity assays earlier in the testing funnel, in parallel with efficacy and potency assays. It is in this investigative toxicology realm that the application of molecular toxicology holds great promise.

Upon achieving proof-of-concept, that is, biological evidence that modulating the target causes a beneficial outcome in an animal model of disease, it becomes desirable to begin to apply toxicology assays to help prioritise lead compound classes. Among the earliest toxicology assays to be applied are relatively high-throughput *in vitro* assays that may predict general toxicity of a large group of compounds. With high-throughput comes increased error rates. As such, these assays are typically

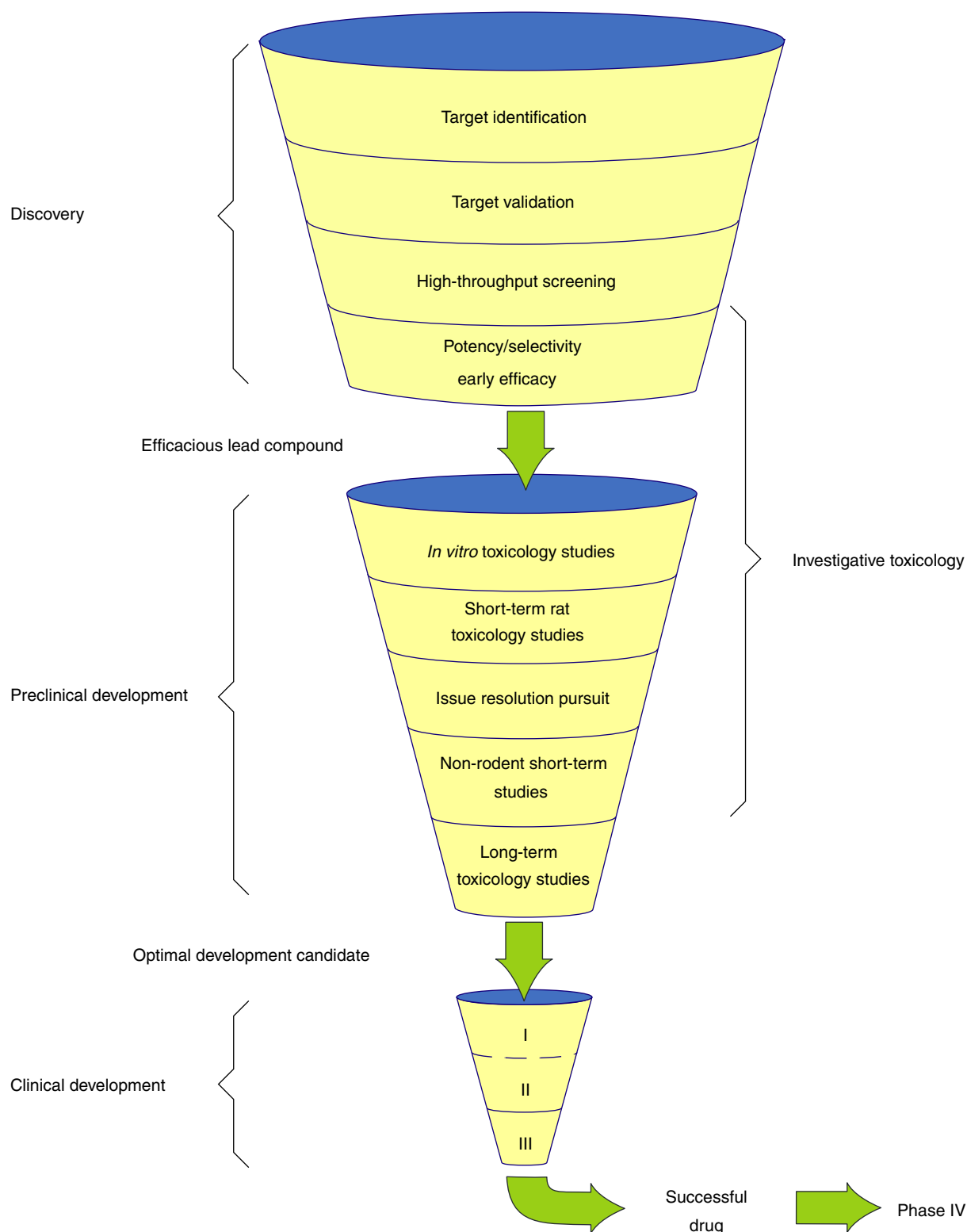


Figure 1. A prototypical pharmaceutical testing funnel. The drug discovery testing funnel represents a sequential screening process by which specific target criteria are either achieved, resulting in advancement to the next stage, or not achieved, resulting in removal of a compound from further consideration. Investigative toxicology seeks to bridge discovery and preclinical development, providing earlier insight into potential toxicological liabilities. Molecular toxicology affords numerous opportunities to impact the drug discovery process at the investigative toxicology level. Figure adapted from [9].

not used to kill compounds but rather to prioritise those compounds that ought to go into early short-term *in vivo* studies first. *In vitro* toxicology assay data are used along with efficacy, physical/chemical and metabolic stability data to identify the best compounds within the most promising chemical classes. In addition, *in vitro* toxicity assays may be used to help interpret *in vitro* efficacy data and provide some preliminary information about mechanisms of toxicity likely to be observed in *in vivo* studies. Often, the end points of such assays are molecular in nature. For example, primary rat hepatocytes have been used to predict Phase I and II metabolic enzyme induction [14]. *In vitro* screening strategies have also been expanded to include many other mechanistic categories, and transcription profiling has been applied in an effort to distinguish between multiple toxic mechanisms in *in vitro* systems [15,16].

Early *in vitro* toxicity assays may have poor correlations with *in vivo* toxicity when compared across large compound sets with significant chemical complexity. Nevertheless, once a body of data and a structure–activity relationship (SAR) are established for a given chemical structural class, these assays are often very predictive within chemical classes [17]. As the discovery chemistry effort is ramped up to synthesise new derivatives of the most promising chemical template, the *in vitro* assays are often particularly useful for delineating SAR. Although there are persistent concerns regarding functional differences between *in vivo* and *in vitro* systems, *in vitro* assays have a great deal to offer the drug development testing scheme. The degree to which an *in vitro* assay is predictive of *in vivo* outcomes can be assessed by more complete understanding of the *in vitro* system being applied. To this end, Baker *et al.* [18] performed transcription profiling on primary rat hepatocytes to understand the responses of toxicologically relevant mechanistic pathways that had previously been studied in whole tissues. These authors demonstrated general repression of Phase I metabolic enzymes, and regulation of several discrete classes of Phase II metabolic enzymes and cell cycle control genes [18]. Such exhaustive analyses may enable toxicologists to maximise the value of their *in vitro* assays while avoiding faulty interpretations based upon occasionally poor *in vivo*–*in vitro* concordance.

Upon identification of a single promising chemical template, *in vivo* toxicology studies are typically initiated. These assays begin with acute dose studies in which very high, supra-*efficacious* doses are used to identify adverse events and primary target organs. Quite often, the results of these early toxicology assays serve as warning bells rather than death knells, informing the discovery team of potential toxicity issues and triggering additional mechanistic studies aimed at understanding the toxicity and evaluating the risk. Molecular toxicology plays a key role in understanding mechanisms of toxicity and assessing the risks associated with specific kinds of toxicity. It is important to realise that molecular toxicology alone provides an insufficient understanding of toxicities observed in *in vitro* and *in vivo* studies. Rather, the results of mechanistic molecular toxicology studies, along with established clinical

chemistry, histopathology, and biochemical toxicology end points, as well as *in vivo* study observations, together make more accurate risk assessment possible. The decision about whether or not to move a compound forward is based first and foremost on therapeutic index, that is, the minimum dose at which toxicity appears divided by the dose at which efficacy is achieved [12]. If a sufficient therapeutic index can be achieved, the mechanism of the dose-limiting toxicity may be somewhat moot. However, in cases where the median efficacious dose and the dose at which toxicity occurs are near, the nature of the toxicity becomes relevant. As described above, preclinical toxicology species are only ~70% predictive of human clinical safety outcomes [13]. This is due, in large part, to the vast genetic and metabolic differences between humans and the common toxicology species. There exist many examples, such as those described in Section 3, of toxicities that are seen in one or more of the common preclinical toxicology species but are not relevant to human health. In such cases, understanding the molecular mechanisms behind an issue can help the toxicologist to move a promising lead forward despite an apparent lack of a therapeutic index in the preclinical toxicology study. Conversely, some mechanisms of toxicity that are relevant to human health cannot be adequately modelled in one or more toxicology species, due to species differences that result in non-toxic outcomes in the animal models. However, an understanding of the mechanisms involved may enable the toxicologist to develop surrogate molecular markers for use in *in vitro* or *in vivo* assays to predict human toxicity in spite of an absence of observable toxicity in animal models. Such mechanistic information may also inform decisions regarding which toxicology species to use. Finally, mechanistic understanding of toxicity may enable the development of predictive *in vitro* assays capable of screening out specific types of toxicity in future projects.

3. Examples of molecular toxicology 'success stories'

As described above, the decision to stop development of a promising lead in light of observed toxicity is based primarily upon therapeutic index. The occurrence of many common types of development-limiting toxicity can be accurately assessed using existing histopathology and clinical end points. Over years of pharmaceutical experience, many of the most common and clinically relevant types of toxicity have been identified, and accurate assays have been developed. One key application of molecular toxicology then is to 'save' compounds that might otherwise have been killed due to apparent toxicological liabilities. Several such examples can be found, two of which are described in detail below.

One type of toxicity that is seen in rats, but is completely irrelevant to human health, involves a specific mechanism of nongenotoxic thyroid carcinogenesis. Rats are the most common toxicology species, but they are unusually prone to developing thyroid tumours. In some cases, this may be due to

xenobiotic-mediated induction of the Phase II metabolism gene UDP-glucuronosyltransferase 2B1 (UGT2B1). This enzyme mediates conjugation and increased clearance of endogenous thyroxin in the liver, and its induction in rat liver correlates with an increased occurrence of thyroid tumours [19]. The mechanisms and pathogenesis of thyroid tumours are well understood both in man and in rodents [20]. A key difference between rats and humans is that the rat genome lacks a functional thyroid hormone-binding globulin (TBG) gene [21]. The lack of TBG, which has a binding affinity for thyroid hormones 3 – 5 orders of magnitude greater than albumin, results in a thyroxin half-life on the order of hours in rats, rather than several days in humans and non-human primates [22]. This makes the rat, which has a basal prevalence of thyroid tumours several orders of magnitude greater than humans, significantly more sensitive to compounds that induce UGT2B1 [23]. In contrast, neither moderate hyperthyroidism nor increased expression of UGT2B1 has been shown to cause significantly increased thyroid tumorigenicity in human populations. It has been suggested that a chemical compound would produce toxicity well before an increased risk of thyroid neoplasia in humans [22]. Thus, induction of a UGT2B1 message in a screening assay may predict for tumours in the 2-year rat carcinogenicity assay, but the issue could be managed in such a way that development may continue for the compound in question, in spite of the appearance of thyroid hyperplasia in short-term studies, resulting in thyroid tumours in the 2-year bioassay.

Another common mechanism of hepatic toxicity in rats that is largely irrelevant to humans, is seen with a class of compounds called peroxisome proliferators. Peroxisome proliferators are a large group of structurally diverse compounds that act via the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR)- α to regulate the expression of a number of lipid and xenobiotic metabolism genes. Among the receptor-mediated effects of peroxisome proliferators are hepatomegaly, increased fatty acid β -oxidation and an increase in the number of peroxisomes in responsive tissues [24,25]. Many peroxisome proliferators are also potent rodent hepatocarcinogens, although the fibrate class of PPAR- α -agonist drugs do not promote incidence of liver tumours in man [26]. Specifically, receptor density and basal expression levels of PPAR- α itself [27], as well as the response of specific peroxisome proliferator-regulated genes [28,29], are significantly lower in humans, non-human primates and dogs than in mice and rats. Induction of PPAR- α -responsive genes, such as the xenobiotic metabolism enzyme CYP4A1, represents a convenient way to assess PPAR- α agonism [30]. A hepatic lesion at or near an effective dose might end development of a compound. However, a hepatic lesion accompanied by CYP4A1 induction would predict a mechanism of rodent hepatotoxicity and carcinogenicity that is likely to be irrelevant to human health. Although PPAR- α agonism is the archetypal mechanism for CYP4A1 induction, other mechanisms may exist. For this reason, the occurrence of a hepatic lesion with

CYP4A1 induction should trigger additional mechanistic studies, using additional PPAR- α -responsive genes, to ensure that the toxicity was mediated by PPAR- α and therefore not relevant to human health. In addition, the appearance of tumours in the 2-year rodent carcinogenicity assay could proactively be shown to be irrelevant for human health by the same mechanistic studies, thereby avoiding the demise of a potentially useful compound.

4. Mechanisms of toxicity

The examples described above represent two particularly well-studied mechanisms of preclinical toxicity that are irrelevant to clinical safety. However, there exist many other mechanisms of toxicity that carry varying degrees of risk. One approach to identifying mechanistic detail in early *in vivo* studies is the application of boutique arrays, microarrays that contain a manageable number of elements that correspond to well characterised, toxicologically relevant genes [31,32]. Other applications of transcription profiling, proteomics and metabolomics provide mechanistic insight into specific mechanisms of toxicity [33-39], which may provide additional molecular biomarkers capable of being incorporated into new molecular assays within the preclinical testing scheme. However, there are many additional toxicities that may each be mediated by several distinct mechanisms [40]. In some cases, one or more of the mechanisms may be more or less relevant to clinical safety than the others, such as the example above of a mechanism of thyroid hyperplasia that is not relevant to human health. Several examples of clinically relevant toxicities that might be impacted preclinically using molecular toxicology are described in more detail below. In many cases, varying amounts of mechanistic detail are known.

4.1 Apoptosis and proliferation

There is a delicate balance between apoptosis, or programmed cell death, and proliferation within the cell. Apoptosis is a common response to intoxication by xenobiotic agents. In contrast to necrosis, apoptosis has the advantage of removing damaged cells without initiating a large inflammatory response. Concomitantly, cells neighbouring the damaged lesion are able to mount a proliferative response moving from quiescence into the G1-phase of the cell cycle. Apoptosis can be initiated by a number of mechanistic pathways, and there exist several complex mechanisms by which apoptosis can be inappropriately induced or blocked. For example, overexpression of the *BAX* gene induces apoptosis by initiating so-called mitochondrial permeability transitions [41]. Increased permeability of the mitochondrial outer membrane may release cytochrome c [42], which may trigger apoptosis. Activation of one of the TNF receptors, DNA damage, growth factor deprivation, and Fas ligand-receptor interactions may also signal apoptosis. Although apoptosis is highly evolutionarily conserved, species differences in one or more of the apoptotic signalling pathways may exist that render one species more or less susceptible to

some classes of toxicants. Whereas many of the molecular mechanisms of apoptosis have been clarified in recent years, Type II or autophagic programmed cell death is less well understood [43]. Given the complex nature of the molecular signalling pathways for apoptosis and autophagic programmed cell death, molecular toxicology may be particularly well suited to teasing out species differences when they exist. Typically, proliferation and sensitivity to apoptosis are tightly linked. However, high rates of cell proliferation tend to increase both the induced and spontaneous mutations in a tissue as well as 'fixing' mutations, as cell replication may precede DNA repair. Indeed, chemical carcinogenesis is likely to result from several failures, including the cell's inability to repair DNA and carry out apoptosis combined with its failure to terminate cell replication. Roberts *et al.* suggest that the capacity of nongenotoxic carcinogens to perturb both apoptotic and proliferative pathways explains the carcinogenicity of such compounds despite their lack of direct DNA-damaging effects [44,45]. Molecular toxicological approaches will be key to understanding the mechanism(s) by which these processes are controlled.

4.2 Cholestasis

Cholestasis is the failure of bile formation in the liver or the impaired secretion of bile into the gut. Hepatic bile formation has two main functions; absorption of dietary lipids such as cholesterol, and clearance of bile, bilirubin and hydrophobic xenobiotics. Cholestasis may be caused by several processes, such as repression of hepatic bile secretion, obstruction of the ductules or interlobular duct, or obstruction of extrahepatic bile ducts. As bile formation is a primary function of the liver, many distinct mechanisms of hepatic injury may cause cholestasis. As such, there exists a myriad of mechanisms by which xenobiotics may affect bile formation and/or secretion. In addition, genetic variability among patient populations in components of the relevant transport systems can lead to widely variable responses. The transport of bile salts, as well as bilirubin and xenobiotics, is mediated by numerous ATP-binding cassette (ABC) transporters. This may be transcriptionally regulated or the result of direct interactions with specific ABC proteins. For example, some compounds induce the expression of ABCs B1 and C1 (MDR-1 [multiple drug resistance protein-1] and MRP-1 [multi-drug resistance protein-1]), whereas it has been suggested that troglitazone and its primary metabolite directly inhibit the ABC transporter bile salt export pump [46]. The study of the ABC transporters and other relevant genes, along with the conditions that result from their absence, has begun to unlock the molecular mechanisms both in acquired human cholestatic syndromes and in xenobiotic-mediated cholestasis [47]. Some mechanisms involved in bile acid production and cholestasis have been shown to be species-dependent, providing an opportunity to use mechanism-specific molecular markers to position cholestasis observed in preclinical species. For example, transcriptional regulation of cytochrome 7 α -hydroxylase, the first and rate-limiting

enzyme in the classical bile acid synthesis pathway, has been shown to be different between rodents and primates [48].

4.3 Mitochondrial toxicity

As a result of the critical role mitochondria play in cellular processes such as fatty acid β -oxidation, oxidative phosphorylation to generate ATP, and calcium homeostasis, chemicals or drugs which directly or indirectly impair mitochondrial function often lead to toxicity. Mitochondrial toxicity may be manifested in a number of ways, depending on the molecular mechanisms involved. For example, several forms of steatosis (or fatty liver), as well as cytolytic hepatitis, may involve a dysfunction of mitochondrial fatty acid β -oxidation [49,50]. Steatosis is a common xenobiotic-induced pathology of the liver. Many molecular mechanisms may contribute to this abnormal accumulation of lipids in hepatocytes, including dysregulation of the oxidation of fatty acids as well as other alterations in lipid uptake, transport and secretion. Other forms of mitochondrial toxicity may result from inhibition of the citric acid cycle or mitochondrial respiration, damage to mitochondrial DNA, or mitochondrial membrane permeability transitions. Fialuridine, a nucleoside analogue drug developed to treat hepatitis B, suppresses mitochondrial DNA (mtDNA) synthesis causing hepatic failure, lactic acidosis and pancreatitis in patients, resulting in discontinuation of clinical trials [51]. Many anti-HIV drugs, and also nucleoside analogues that interfere with polymerase- γ , have been linked to similar mitochondrial toxic effects such as hepatic steatosis, lactic acidosis and lipodystrophy. Free radical damage, linked to superoxide and peroxynitrite, is also considered a major contributor to mitochondrial toxicity due to generalised oxidative damage, as well as an increased mutation rate for mitochondrial and nuclear genes. Induction of the mitochondrial permeability transition, and the resultant mitochondrial depolarisation and uncoupling, which can lead to necrosis or apoptosis, is another mechanism that may lead to severe liver damage [40]. The myriad types of mitochondrial toxicity may present opportunities for the application of molecular toxicology tools to understand better both the relevant mechanisms as well as the contributions of nuclear versus mitochondrial genes. Such an understanding should provide an opportunity to apply molecular approaches to predicting mechanisms and positioning their relevance to man.

4.4 Oxidative stress

Oxidative stress, the increased production of reactive oxygen species (ROS), is another process implicated in a host of toxicities. Biotransformation of xenobiotics that leads to the production of superoxide anions, hydrogen peroxide and highly reactive hydroxyl radicals, may lead to cell injury and death via several mechanisms such as: inducing lipid peroxidation, which may alter membrane fluidity, enzyme activity and membrane permeability and transport; inactivating cellular enzymes via direct oxidation of critical protein sulfhydryl or amino groups; depolymerising polysaccharides; and inducing DNA strand

breaks and chromosome damage [52]. CYP2E1-mediated ethanol metabolism provides a good example of liver injury enhanced by increased lipid peroxidation [40]. The extent of oxidative damage may also be affected by the availability of cellular antioxidants (GSH [glutathione], ascorbic acid, vitamin E) and antioxidant enzymes such as catalase, superoxide dismutase and glutathione S-transferases. The expression of genes encoding antioxidant enzymes is regulated by the transcription factor NF-E2-related factor-2 (Nfr-2) and other mechanisms [53,54]. Nfr-2 recognises a consensus sequence (an antioxidant response element [ARE]) in the promoter of Nfr-2-responsive genes. Numerous pharmaceutical compounds have been shown to regulate expression of ARE-containing genes [55]. Studies have demonstrated that the sequence context of the ARE and the affected cell type, as well as the compound itself, determine the degree of regulation of ARE-containing genes [56]. There is the possibility that species differences exist in the regulation of the genes and pathways related to antioxidants and Nfr-signalling. Molecular toxicological approaches will be able to tease out such differences should they exist, and may be applied to molecular screens to position findings related to compound-mediated oxidative stress.

4.5 Phospholipidosis

Phospholipidosis, the intracellular accumulation of phospholipids (PLs), is most commonly observed with cationic amphiphilic compounds [57,58]. PLs are major components of both plasma and intracellular membranes. The relative levels of the different types of PLs, as well as the length and degree of saturation of their fatty acid components, are highly tissue-dependent. However, relative PL levels and fatty acid composition in a particular organ across different species are often quite similar [59]. Phospholipidosis is mediated by one of at least two related mechanisms, either direct inhibition of enzymes involved in cell membrane PL maintenance, or accumulation of the compound within cell membranes. In many cases, these mechanisms may be closely related, as cationic amphiphilic agents that cause phospholipidosis may both accumulate in membranes and mimic endogenous ligands of PL metabolism as a function of their physical chemical properties. In either case, compounds that affect the structure and properties of cell membranes or that inhibit basal cell membrane maintenance, will likely induce PL metabolism genes. Phospholipidosis has been successfully modelled in primary rat and human hepatocytes [17]. Whether or not the induction of specific genes *in vitro* can be shown to predict phospholipidosis has yet to be determined, but may represent a useful application of molecular toxicology. Considering the reported conservation of tissue-specific PL profiles, rodent primary cells from multiple tissues ought to be suitable for modelling and predicting phospholipidosis.

4.6 Metabolism and clearance

A key issue in developing new drugs is metabolic stability and clearance. In some cases, compounds may induce the

expression of the very enzymes that metabolise and/or clear them from the body. In addition, induction of metabolic enzymes may cause an increased risk of drug–drug interactions or reduced efficacy. For example, it has been demonstrated that women taking both the nutritional supplement St John's Wort and ethinyl oestradiol for birth control, may have unusually low serum drug levels and can experience break-through bleeding due to induction of CYP3A4 by components of St John's Wort [60]. Autoinduction is typically inferred in drug metabolism studies from falling drug levels upon repeat dosing, and cytochrome P450 (CYP450) enzyme activity and inhibition studies, but could be readily screened for using transcriptional profiling methods. Similarly, xenobiotics may also cause increased clearance, by inducing one or more of the transporters that mediate their removal from the body [61]. The potential for the application of molecular toxicology to demonstrate induction of genes encoding metabolism and clearance functions could predict such effects earlier in the testing funnel. It is important to note that RNA does not always correlate with protein levels or enzymatic activity, although many xenobiotic metabolism and clearance proteins have been shown to be regulated transcriptionally [62]. As such, rapidly falling drug levels in preclinical species may be understood more quickly by the application of molecular toxicology. Metabolism-mediated toxicity is also a significant issue in the pharmaceutical industry. While many xenobiotics may not be directly toxic, reactive metabolites, often generated by Phase I metabolic enzymes, may cause toxicity. Acetaminophen (APAP) is one of the most extensively studied examples of metabolism-mediated toxicity [63]. The drug is initially metabolised by sulfation and glucuronidation. Upon saturation of these pathways by high doses of APAP, its metabolism to the electrophilic reactive intermediate N-acetyl-*p*-benzoquinoneimine (NAPQI) is mediated by CYP450s. NAPQI is purported to deplete hepatocellular GSH, which is involved in detoxifying and clearing NAPQI. Upon GSH depletion, the unprotected NAPQI exerts cytotoxic effects via protein binding, protein thiol oxidation and lipid peroxidation.

Although some of the relevant metabolic enzymes are not particularly inducible or are regulated post-transcriptionally, CYP3A, which metabolises 50 – 60% of all marketed pharmaceutical agents, is transcriptionally regulated. Whilst the correlation between induction, selectivity and specificity of rodent CYP3A1 and human CYP3A4 are not perfect, there is still partial agreement, and induction of rat CYP3A1 preclinically could trigger additional activity to assess induction of CYP3A4 in human primary hepatocytes. Just as CYP3A is involved in a significant proportion of human drug metabolism, so too is MDR-1 (P-glycoprotein), involved in the clearance of many marketed pharmaceutical agents. MDR-1 and other members of the extended ABC transporter gene family, such as other MDRs and MRPs, are expressed widely, and actively transport xenobiotics and their conjugates across membranes to facilitate clearance. Although the correlation

between rodents and humans is not exact [64], as with CYP3A, induction of MDR-1 and related proteins in early rodent studies could be used to trigger earlier evaluations of protein levels and activity in cultured human cells.

4.7 Nongenotoxic carcinogenicity

At present, the only way to reliably identify nongenotoxic carcinogens is in long-term repeat dose assays such as the 2-year rodent carcinogenicity assay. This assay is time consuming and expensive and requires large amounts of active pharmaceutical ingredient. As a result, it is run very late in the drug discovery and development paradigm. Although, in many cases, nongenotoxic carcinogenicity in the rodent may not be relevant for humans, a positive result in a 2-year carcinogenicity trial can increase the number of studies and the time required to register a compound. In addition, a black box warning of rodent carcinogenicity can be deleterious to the successful marketing of a pharmaceutical agent. It is therefore imperative that such findings are understood, and it may be desirable to screen out such effects early in a project's life. Induction of several metabolic enzymes in the rodent has been associated with an increased incidence of nongenotoxic carcinogenicity [65]. For this reason, screening compounds early in the testing funnel for the induction of selected rat metabolic enzymes such as CYP1A1, -2B1 and -4A1 [66], and UGT2B1 [19], may be a useful way of identifying and reducing the risk of nongenotoxic carcinogenicity.

Induction of some metabolic enzymes may presage tumour incidence in the 2-year rodent carcinogenicity assay as described above, although this fact alone is not adequate to justify the application of a molecular toxicology approach. Although enzyme induction occasionally occurs in the absence of or prior to the appearance of a hepatic lesion, in most cases, liver weight changes and liver pathology, which are already assessed at the conclusion of early non-GLP (Good Laboratory Practice) repeat dose *in vivo* studies, go hand-in-hand with enzyme induction. A comparable correlation has also been established between hepatocarcinogenicity and both liver weight changes and hepatomegaly in rodents [67,68]. Furthermore, due to the great cost of a 2-year rodent carcinogenicity assay, studies to accurately determine the capacity of any of these end points to predict rodent carcinogenicity are often not conducted. One might reasonably ask, 'What does this application of molecular toxicology add to the existing testing funnel?' In brief, this and other applications of molecular toxicology provide mechanistic insight into both rodent hepatotoxicity findings and rodent carcinogenesis. When applied along with pathology, pharmacokinetic and *in vivo* data, as well as an understanding of the commercial and competitive space of the particular discovery platform, this information has the potential to help teams select the best possible lead compound in the early stages of development.

4.8 Immune toxicity and idiosyncratic toxicity

Immunity is mediated by multiple interconnected regulatory pathways, providing ample opportunity for the application of

molecular biology tools. Jaeschke *et al.* [40] have described the mechanisms of neutrophil-induced injury leading to liver toxicity, which are illustrated in Figure 2. A xenobiotic, with or without metabolism, may cause toxicity resulting in the release of inflammatory mediators such as complement factors. Complement factors and cytokines activate liver neutrophils and Kupffer cells, causing increased ROS formation. Cytokines also activate expression of adhesion proteins on hepatocytes, which may result in adherence of primed neutrophils to hepatocytes, leading to neutrophil degranulation and release of proteases. Both ROS formation and protease release result in liver cell necrosis.

Alternatively, immune-mediated toxicity may result when biotransformation of xenobiotics leads to the formation of adducts with proteins. The anaesthetic, halothane, produces such adducts which leak out of damaged hepatocytes and can serve as antigens leading to antibody formation. Upon re-exposure, additional adducts are formed and, if present on hepatocyte plasma membranes, are vulnerable to antibody-mediated cell lysis [69]. Such immune-modulatory drug-protein adducts may be the cause of idiosyncratic toxicity, an adverse reaction to a drug that is observed in a very small fraction of a patient population, which is a major concern to the pharmaceutical industry and the medical community at large. At this time, it is not possible to predict idiosyncratic toxicity preclinically. However, the mechanism(s) of idiosyncratic toxicity has been a major focus of study in recent years. The so-called 'hapten hypothesis' of immunoallergic drug reactions has been put forward to delineate the sequelae leading to idiosyncratic toxicity [70]. In brief, this hypothesis implicates covalent conjugation of a reactive intermediate with an endogenous, relatively high molecular weight protein. The drug-protein conjugate initiates an immunological reaction resulting in tissue damage and hypersensitivity. It has also been suggested that an underlying endotoxaemia may augment toxic responses to xenobiotic agents, leading to drug hypersensitivity [71]. As detailed above, both metabolism-mediated toxicity and immune-related toxicity have genetic and molecular components, and molecular toxicology may help the understanding of idiosyncratic drug toxicity [72]. In time, it may become possible to develop molecular assays that predict the possibility of idiosyncratic toxicity preclinically.

4.9 Secondary pharmacology

A common source of drug-mediated toxicity may be attributed to secondary pharmacology. Occasionally (somewhat inaccurately) referred to as 'loss of selectivity,' secondary pharmacology refers to effects on a secondary target that may or may not be closely related to the primary target. Within the mammalian genome, there are many gene families which contain several members that are closely related at the sequence level, but have significantly different biological functions. Pharmaceutical agents must be frequently designed to target only a single member of a gene family. A prime example of this is the new class of cyclooxygenase (COX)-2-selective

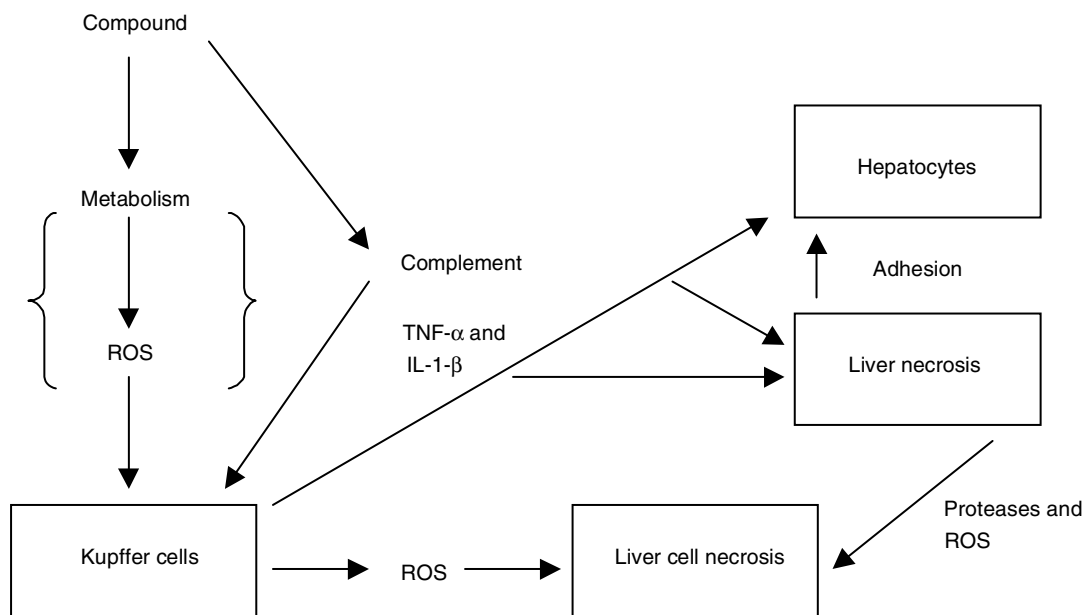


Figure 2. Molecular signalling in immune-related toxicity. A compound or metabolism-derived reactive intermediate may mediate immune toxicity by activating Kupffer cells, the resident macrophage in the liver, and by inducing complement factors. Upon activation, Kupffer cells produce pro-inflammatory cytokines that, like the complement factors, activate neutrophils, induce formation of ROS in Kupffer cells and neutrophils, and induce expression of adhesion molecules on hepatocytes and neutrophils. Upon receiving appropriate chemotactic signals, activated neutrophils may transigrate and adhere to hepatocytes, resulting in degranulation and production of proteases. Both ROS and protease activity cause hepatocyte necrosis. Figure adapted from Jaeschke H *et al.*: Mechanisms of hepatotoxicity. *Toxicol. Sci.* (2002) **65**(2):166-176, by permission of Oxford University Press.
ROS: Reactive oxygen species.

anti-inflammatory drugs that target prostaglandin G/H synthase-2, while sparing prostaglandin G/H synthase-1. The inducible COX-2 is regulated in response to pro-inflammatory signals and is involved in arthritis, pain and cancer, whereas the constitutively-expressed COX-1 is involved in the normal maintenance of the gastric lining, among other functions [73]. It has been demonstrated that inhibiting COX-2, while sparing COX-1, results in an inhibition of pro-inflammatory prostaglandin synthesis but does not result in the gastric ulceration seen with COX-1 inhibition [74]. Gastric ulcers are related to the primary pharmacology of COX-1 inhibitors such as aspirin but can be considered to be secondary pharmacological effects of COX-2-selective inhibitors, seen only at extremely high doses where specificity is lost.

Secondary pharmacology is often sorted out as part of the selectivity screens during the hit identification process in the early discovery phase of drug development. However, the function and expression pattern of all potential targets for a novel pharmaceutical agent cannot be known ahead of time, and, increasingly, the pharmaceutical industry is targeting members of more complex gene families. For example, matrix metalloproteinases (MMPs), which are involved in disease processes in several therapeutic areas including oncology, arthritis, cardiovascular disease and ophthalmology, have been targets of investigation at many pharmaceutical companies. Recently, membrane-bound MT1-MMP knockout

mice have been shown to exhibit connective tissue effects similar to those observed with the first MMP inhibitors to go into clinical trials [75]. For this reason, more selective MMP inhibitors have been sought, and screening assays that distinguish inhibition of individual MMPs have been developed [76]. However, the MMP gene family contains more than 20 members, many of which have been discovered only very recently, after the inception of pharmaceutical MMP inhibitor projects. Without foreknowledge of closely related proteins, reagents would not exist with which to develop specificity screens, making it impossible to screen out inhibition of such enzymes. However, it may be that inhibition of some such recently discovered (or unknown) enzymes might cause development-limiting toxicities that are only identified upon inception of preclinical safety studies. In such cases, a role of the molecular toxicologist will be to understand the pharmacology of inhibition of novel secondary targets.

With the recent completion of the human genome [77,78], the problem described above should become less of an issue. However, identifying all of the most closely related potential secondary targets is only part of the solution to secondary pharmacology. The tissue-specific expression pattern of closely related proteins is also important. One such example can be seen with kinases, which have recently come under scrutiny as pharmaceutical targets [79]. There are more than 500 kinases in the human genome, and their expression levels

in different tissues vary widely [80]. Even if numerous screens are in place in the discovery testing funnel to ensure selectivity for the specific target, expression levels in individual tissues may result in toxicity even with highly selective compounds. For example, a compound that exhibits 20-fold selectivity for its target versus a closely related kinase, might still exhibit secondary pharmacological effects in a tissue that expresses the related kinase at very high levels. If the second kinase is expressed at 20 times higher levels in one or more tissues, the selectivity for the target will essentially be lost in that tissue, which may result in unexpected toxicity. Thus, identification and expression levels are key to understanding secondary pharmacology. Molecular toxicology will have a significant impact in understanding such effects.

5. Conclusions

The drug development process continues to grow more expensive and more challenging. The application of investigative toxicology to the pharmaceutical discovery pipeline represents one promising approach to shortening timelines while delivering lead compounds to full development with better chances of success. The principles of molecular toxicology are a key component of the emerging investigative toxicology paradigm. A primary function of molecular toxicology is the identification and validation of molecular biomarkers capable of predicting toxicity in early, high-throughput *in vitro* assays, and the ability to provide mechanistic insight into dose-limiting toxicity. The ability to delineate the exact mechanism of toxicity holds the promise of differentiating species susceptibility and enabling the toxicologist to position findings that are not relevant to human health, thus potentially 'saving' a compound that might otherwise be de-prioritised due to the occurrence of previously unexplained toxicity in preclinical studies.

6. Expert opinion

This review focuses almost exclusively on gene expression analysis, a key activity of a molecular toxicology laboratory. Indeed, the development of molecular biomarkers that are capable of predicting toxicity and defining mechanisms of intoxication are primary functions of molecular toxicology. However, these functions need not be the only focus of molecular toxicology laboratories. Particularly in cases of secondary pharmacology, as detailed in Section 4, the full panoply of tools and techniques of molecular biology can be utilised to understand toxicity, and to either screen out the effects in back-up programmes or position the findings when the mechanisms can be shown to be irrelevant to human health. Transfections, cloning and expression, and the generation of transgenic and knockout models, along with gene expression profiling for the delineation of toxicity mechanisms, will impact the drug discovery and development

process and promise to deliver safer pharmaceutical candidates for full development.

Conversely, the power of expression profiling, particularly microarray-based transcription profiling, can also provide huge amounts of largely extraneous information that may be difficult or impossible to understand without significant additional experimental data. Full disclosure of such results may lead to erroneous interpretations in the hands of naive scientists or an uninformed public. For example, many pro-carcinogens present in cigarette smoke are metabolised into carcinogenic intermediates by xenobiotic metabolism enzymes, and induce the expression of those same enzymes [81,82]. It has also been shown that certain chemical components found in cruciferous vegetables have been reported to induce those same metabolic enzymes in humans [83]. The uninformed may draw the erroneous conclusion that consumption of cruciferous vegetables, like cigarette smoking, may present an increased cancer risk. In fact, a significant body of evidence suggests that the opposite is true; specifically, that the dietary induction of these metabolic enzymes has a protective, anticarcinogenic effect [84]. Although this example is overly simple and easily dismissed, this is only because the mechanisms have been studied and understood for many years. Similar errors of interpretation may occur if large transcription profiling data sets were generated for candidate pharmaceutical compounds, which would be subject to full disclosure.

Finally, the appeal of molecular toxicology is great, but the final value of any particular molecular marker will vary. The impact of molecular toxicology will not be in replacing existing methods of toxicological safety assessment. In fact, molecular toxicology will achieve the greatest impact when it is applied alongside existing methodologies, and only when experiments are carefully thought out. The ability to show toxicity *in vivo* using molecular biomarkers will only be useful when there are no existing means to identify the toxicity, or when additional mechanistic information can be provided by the molecular assay. For example, fatty liver in a preclinical study can be diagnosed immediately upon sacrifice. Harvesting tissues, isolating RNA and demonstrating induction of a molecular marker of steatosis will only be valuable if it provides additional mechanistic information or if it can be shown to precede the actual appearance of toxicity. Toxicogenomic results are only of value if they provide mechanistic insight into toxicity or if they identify gene expression changes that predict toxicology, and may be incorporated into the testing scheme as *in vitro* or early *in vivo* assays. In addition, the ability to predict in several hours a toxicity that results in observable pathology in 1 – 3 days may be of limited value, however intriguing the findings may be. Most preclinical testing funnels do not include studies of < 1 day in duration. In contrast, markers that can predict in 1- or 7-day studies effects seen only in extended duration studies have the potential to save significant time and resources, and would speed up the process of identifying the most promising candidates for development.

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