Improving the decision-making process in structural modification of drug candidates: reducing toxicity

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The rule of three, relating to activity–exposure–toxicity, presents the single most difficult challenge in the design and advancement of drug candidates to the development stage. Absorption, distribution, metabolism and excretion (ADME) studies are widely used in drug discovery to optimize this balance of properties necessary to convert lead compounds into drugs that are both safe and effective for human patients. Idiosyncratic drug reactions (IDRs; referred to as type B reactions, which are mainly caused by reactive metabolites) are one type of adverse drug reaction that is important to human health and safety. This review highlights the strategies for the decision-making process involving substructures that, when found in drugs, can form reactive metabolites and are involved in toxicities in humans; the tools used to reduce IDRs are also discussed. Several examples are included to show how toxicity studies have influenced and guided drug design. Investigations of reactive intermediate formation in subcellular fractions with the use of radiolabeled reagents are also discussed.

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▼ There is no doubt as to the value of predicting the potential toxicity of compounds as early as possible in the drug discovery process. Pharmaceutical companies are coming under increasing pressure to prove the long-term safety of their products more precisely, and to provide more data on them. Failure to address these concerns successfully causes companies to withdraw drugs from the market; this not only presents human health consequences, but also has a negative economic and public-relations impact on the pharmaceutical industry. Because of human health-safety concerns, a long list of drugs have been withdrawn from the market, the most recent example being Merck pulling Vioxx off the market after finding it doubled risks of heart attack and stroke after 18 months' use. Failures in the drug discovery process account for a substantial part of the cost of drug development because many compounds are discarded after substantial investment owing to unforeseen toxicological effects.

One of the major challenges in drug discovery is to accurately predict which new drugs will be associated with a significant incidence of IDRs. An important consideration is that any screening process will produce false results, both positive and negative, and our goal must be to reduce or eliminate these, which will in turn lead to improved drug safety. The potential for IDRs can be minimized by appropriate structural modifications to the drug candidate in early discovery. Until recently, most of the optimization effort was directed toward the potency of the leads against the target of interest, and selectivity among subtypes, whereas the equally important aspects of toxicities were left to be addressed at the end of a potency optimization cycle, sometimes disqualifying the obtained candidates [1–8]. The ability to produce this information on potential toxicity early in the discovery phase has become increasingly important as a basis for judging whether a drug candidate merits further development. The effort to reduce IDRs during drug discovery has mostly been a combination of empirical methods and trial and error. This has led to an accrued interest in applying substructure analysis to the problem of toxicological predication, which is a process aimed at identifying the structural features that confer toxicological properties to molecules. One approach to this dilemma would be to thoroughly explore the structure requirements for *in vitro* pharmacological activity through combinatorial synthesis and identify the key structural features

1359-6446/04/$ – see front matter PII: S1359-6446(04)03297-0

that are essential for activity for each chemical series, and then attempt to improve the metabolism and toxicity properties through structure modifications to the regions of the molecule that have little or no impact on the desired activity. Thus one can maintain an optimal level of potency while introducing structural features that will improve the metabolism and toxicity characteristics.

Herein, we will discuss strategies to deal with reactive metabolites, and tools used to reduce them during the drug discovery process. Also, some of the chemical substructures that form reactive metabolites and are involved in toxicities in humans are discussed. Several examples from literature are included to show how toxicity studies have influenced drug design and strategies and have helped to facilitate improvements. Improved screening of reactive intermediate formation in subcellular fractions with the use of radiolabeled reagents is also discussed.

Reducing toxicity

One of the most important keys to successful drug design and development is a process of finding the right combination of multiple properties such as activity, toxicity and exposure. It is very important first to determine, and then to optimize, the exposure–activity–toxicity relationships or the rule of three for drug candidates, and thus their suitability for advancement to development. Toxicity problems, especially those which may only occur under unusual or idiosyncratic conditions during the late stages of drug development, are one of the most devastating surprises for pharmaceutical companies [9–13]. Variations in human drug metabolizing enzymes can produce subtle evidence of potential toxicity, or none at all, during pre-clinical safety studies. Such problems are also unlikely to show up in all but the largest clinical trials, but if the side effects are serious, can result in product withdrawal. There are indications that some substructures found in drugs can form reactive metabolites that are involved in toxicities in humans. These substructures include arylacetic and arylpropionic acids, aryl hydroxamic acids, oximes, anilines, anilides, hydrazines, hydrazides, hydantoins, quinones, quinone methides, nitroaromatics, heteroaromatics, halogenated hydrocarbons, some halogenated aromatics, chemical groups that can be oxidized to acroleins, and medium-chain fatty acids. Reactive metabolites are unstable, and are intermediates to more stable metabolites. Table 1 shows several examples of drugs that undergo metabolic activation and cause adverse reaction in humans, which have been withdrawn from the market or restricted in use with toxicity warnings. Clearly, a drug candidate that does, or might, metabolize to such substructures would increase the risk of failure or withdrawal.

Considerations

To illustrate how to reduce or eliminate reactive metabolites, consider drug-induced adverse reactions, particularly idiosyncratic drug reactions (IDRs; referred to as type B reactions). Such reactions are a major issue because, given current techniques and approaches, they often go undetected until late in the process, or even after the drug has been released onto the market, and the consequences are disastrous for all concerned. IDRs mediated through a reactive metabolite can be associated with several mechanisms [10–13]. Currently there is no general approach or 'one-size-fits-all' screen that addresses idiosyncratic reactions, because of the difficulties in understanding the mechanisms of these reactions and in accurately predicting clinical results. Some of the strategies under consideration to improve our understanding of these mechanisms and to develop safer drugs include:

- Avoidance or flagging of chemical functional groups that are known to cause toxicity during drug design (e.g. aromatic and hydroxy amines, phenols, epoxides, acyl halides, acyl glucuronides, thiopenes, furans, fatty acid-like compounds, hydroxylated metabolites and quinines).
- Development of suitable *in vitro* and *in vivo* systems to elucidate the role of short-lived, potentially toxic metabolites in the pathogenesis of idiosyncratic toxicity.
- Identification of chemical functional groups that are associated with low or no toxicity, and development of more metabolically stable drugs to potentially avoid metabolic interactions.

Although there is no evidence that safer drugs will be identified by addressing the following questions in the drug discovery/development stages, it is considered that drugs without these potential liabilities may have a better safety profile:

- Does the candidate have the potential to form reactive metabolites based on chemical 'structure-alerts'?
- Does the compound form reactive metabolites in liver, blood or skin tissue or cells?
- Is the binding of the drug > 50 pmol/mg of microsomal protein?
- Does the candidate form reactive intermediates that are able to 'travel' and react covalently with other tissues?
- What proteins are affected and what are the effects of the modified proteins?
- Which genes are affected? Could these affected genes generate a potential IDR?
- What cellular functions are affected by the reactive intermediate?
- Bioaccumulation in liver?
- Glutathione depletion?
- Drug-drug interactions?
- Ames and/or micronucleus positive responses?

It remains to be determined whether the answers to these questions will allow the pharmaceutical industry to reduce or eliminate IDRs. Because the major organs involved in IDRs are the liver, skin and bone marrow, preclinical approaches should consider these target organs to study the effects of reactive intermediates. Thus, a sound strategy should evaluate three major elements: reactive intermediate characterization, covalent binding and biological impact and/or function.

Key tools and strategies to improve drug safety

Many tools are in the exploratory stage, encompassing computational approaches and experimental assays to predict the formation of reactive metabolites *in vivo* and their role in IDRs [9]. For example, during *in vitro* testing, if significant protein binding is found, an attempt is made to discover the chemical basis for the binding so that it can be designed out of the structure. Another potential technique is to trap reactive intermediates by incubating them in human liver microsomes. To evaluate the biological effects of reactive intermediates initially, methods for cytotoxicity testing including tests for cell viability, membrane integrity, protein synthesis, DNA synthesis, glutathione [GSH] level, apoptosis, free radical production, lipid peroxidation, enzyme inhibition and other enzymatic activities due to oxidative stress are being developed for early drug discovery. Results suggest that these types of screens can be used to differentiate known toxicants from relatively safe drugs. Table 2 presents some of the current tools; below we will summarize the advantages and disadvantages of the current tools and relate them to the problems facing those in drug discovery and development, and attempt to synthesize these tools and strategies into an alternative strategy.

In silico screens as filtering process

A promising new trend in early drug discovery is the collaboration between medicinal and computational chemists and drug metabolism scientists seeking to identify and eliminate potential toxic chemical groups. Structure–activity relationships are well-known as a means to identify several toxic end points. One way to improve screening for such potentially toxic compounds would be to increase the use of *in silico* testing methods at earlier stages in drug discovery [2,4,14,15]. Several programs such as TOPKAT, CASE/MULTI-CASE, DEREK, HazardExpert and OncoLogic are commercially available for the prediction of mutagenicity and carcinogenicity based on chemical structure. These available commercial systems for mutagenicity and/or carcinogenicity prediction differ in their specifics, yet most fall into two major categories. One is automated approaches that rely on the use of statistics for extracting correlations between structure and activity. The others are knowledge-based expert systems that rely on a set of programmed rules distilled from available knowledge and human expert judgment. The advantages of *in silico* techniques include time and money savings, reduced use of laboratory animals, and the ability to rapidly screen large numbers of structures even before synthesis occurs. This high capacity improves prioritization in early discovery for toxicology testing and highlighting toxophores for easy identification. Because it might be difficult to avoid some of these functionalities in the design of new compounds, in some cases their presence should be considered as a 'structural-alert' for the drug metabolism scientist. In turn, studies can be conducted at an earlier stage to determine whether the compound in question undergoes metabolism at the site to generate a potentially toxic intermediate. However, *in silico* methods still cannot completely replace conventional toxicity testing. These systems are not designed or validated to screen for potential IDRs but could be useful as a first step in eliminating compounds with other potential toxic effects.

Trapping reactive intermediates

Direct detection of reactive intermediates has been proven difficult both *in vitro* and *in vivo*. The basic methods to trap reactive intermediates have been well established and recently have received interest for screening in a high-throughput manner in drug discovery [16]. For example, early methods to trap electrophiles have been designed to form glutathione (GSH) adducts, cyanide derivatives, nucleotide adducts, glucuronides, and albumin adducts. It is important to recognize that a compound may form one or several reactive intermediates. The difficulty arises in trying to assess which reactive intermediate could be responsible for certain toxic effects. For example, valproic acid, tamoxifen, and tolmetin might have competitive pathways for reactive metabolism. On the other hand, reactive intermediates might interact covalently and noncovalently with biomacromolecules as part of their potential mechanism for toxic reactions [17,18].

Electrophiles

Reactive electrophile screenings generated from *in vitro* experiments in several tissues (e.g. liver microsomes) have been designed to react with GSH and subsequent analysis of the GSH adducts by LC-MS [16]. Several types of reactive metabolites (epoxide, arene oxide, quinone, quinone imine, quinone methide, iminoquinone methide, nitroso, nitrenium ion, nitro reduction, nitro radical, iminium ion, free radical, *S*-oxidation, Michael acceptor, *S*-oxide, aliphatic aldehyde, or hydrolysis/acetylation) can be trapped in the

Table 1. Examples of chemical structures activating to produce toxic metabolites

Chemical class	Bio-transformation	Toxic metabolite	Compound		Biological effects	Refs
			Name	Clinical use		
Quinone	Oxidation	Quinone-type	Tacrine	Alzheimer's disease	Hepatic toxicity	[47]
			Troglitazone	Treat Type II diabetes	Hepatic toxicity	[48,49]
			Minocycline	Antibiotics	Hepatic toxicity Lupus-like syndrome	[50]
			Acetaminophen	Analgesic agent	Hepatic toxicity	[51]
			Aminosalicylic acid	Inflammatory bowel disease	Lupus-like syndrome Pancreatic toxicity Hepatic toxicity Renal toxicity	[52]
			Amodiaquine	Treat malaria	Hepatic toxicity Agranulocytosis	[53]
			Phenytoin	Anticonvulsant	Drug-induced hypersensitivity Teratogenicity	[54]
			Carbamazepine	Anticonvulsant	Teratogenicity	[55]
			Vesnarinone	Phosphodiesterase r inhibitor	Agranulocytosis	[56]
			Prinomide	Antiinflammatory	Agranulocytosis	[57]
			Estrogens	NSAID*	Breast cancer Uterine cancer	[58]
			Tamoxifen	NSAID	Endometrial cancer	[59]
			Fluperlapine	Antipsychotic agent	Agranulocytosis	[60]
Aryl nitro	Reduction	Nitroso	Tolcapone	Parkinson's disease	Liver toxicity	[61]
			Chloramphenicol	Antibiotic	Aplastic anemia Bone marrow toxicity	[62]
			Dantrolene	Muscle relaxant	Liver toxicity	[63]
			Nimesulide	COX 2 inhibitors	Liver toxicity	[64]
Nitrogen-containing aromatic	Oxidation	Nitrenium ion Free radical	Clozapine	Antipsychotic agent	Agranulocytosis Liver toxicity Myocarditis	[65,66]
			Aminopyrine	Painkiller	Agranulocytosis CNS toxicity	[67]
			Dipyrone	Painkiller	Agranulocytosis	[68]

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presence of either GSH or an equimolar mixture of *N*-acetyl-cysteine and *N*-acetyl-lysine. This method is relatively simple and rapid, and has been implemented for HTS to identify reactive metabolites generated by bioactivation with Phase I and Phase II enzymes [16,20–23]. A preliminary evaluation of this method was conducted using 20 commercially available compounds with known toxicological profiles in liver microsomes at substrate concentrations of 10 μM [16]. The results indicated that the method is unlikely to produce false negative response because relatively safe compounds did not generate GSH conjugates, whereas eight of the ten compounds that are known to generate

Table 1. (continued)

Chemical class	Bio-transformation	Toxic metabolite	Compound		Biological effects	Refs
			Name	Clinical use		
Aryl amines	Oxidation to hydroxylamine	Nitroso	Sulfamethoxazole	Antibacterial agent	Hepatotoxicity Agranulocytosis Lupus-like syndrome Skin rashes	[69]
			Dapsone	Antiparasitic	Agranulocytosis Flu-like syndrome Hemolytic anemia Methemoglobinemia	[70]
			Procainamide	Cardiac antiarrhythmic	Lupus-erythematosis Agranulocytosis Fever	[71]
			Nomifensine	Antidepressant	Hemolytic anemia Allergic reactions	[72]
			Sulfasalazine	Ulcerative colitis	Abnormal liver function Decreased blood counts Allergic reactions	[73]
			Aminoglutethimide	Breast cancer	Skin rashes Fever Agranulocytosis Thrombocytopenia Liver toxicity	[74]
Michael acceptors	Hydrolysis oxidation	Aldehyde Co-A conjugate	Felbamate	Anticonvulsant	Aplastic anemia Liver toxicity	[75,76]
			Terbinafine	Antifungal agent	Bone marrow toxicity Liver toxicity Skin rashes	[77]
			Valproic acid	Anticonvulsant	Liver toxicity	[78]
			Mianserin	Antidepressant	Agranulocytosis	[79]
			Leflunomide	Inflammatory arthritis	Liver toxicity Agranulocytosis	[80]
Carboxylic acids	Glucuronidation	Acyl glucuronides	Diclofenac	NSAID	Liver toxicity Agranulocytosis	[81]
			Zomepirac	NSAID	Liver toxicity	[82]
			Ibufenac	NSAID	Liver toxicity	[83]
			Bromfenac	NSAID	Liver toxicity	[84,85]
			Benoxaprofen	NSAID	Liver toxicity	[86]
			Indomethacine	NSAID	Bone marrow toxicity	[87]

* Non-steroidal anti-inflammatory drugs

Table 2. Approaches to help to screen for reactive metabolites

Stage	Approaches	Comments
Presynthesis of compound	*In silico* methods/drug design/data mining.	Flag potentially toxic compounds. Alert to conduct toxicity evolution earlier.
Early drug discovery	Microsomal/CYP time-dependent inactivation. Reactive intermediate screens in human liver.	*In vitro* studies in subcellular fractions or purified enzymes.
Late drug discovery	Covalent binding. Oxidative stress. Hapten characterization. Reactive intermediate comparison (human versus preclinical species).	Cellular/subcellular/enzymatic or preliminary *in vivo* studies. Is the binding > 50 pmol/mg microsomal protein?
Preclinical development	Animal models/biomarkers. Proteomics/metabolomics/genomics. Reactive intermediate exposure in animal models. Prediction of species differences in exposure of reactive intermediates.	Available animal models have limited prediction rates of IDRs, but new tools may improve predictability.
Clinical development	Biomarkers/proteomics/genomics/metabolomics. Reactive intermediate exposure when considered relevant.	Hapten identification. mRNA. Immunotoxicity. Reactive metabolites (in blood, skin and liver).

reactive metabolites resulted in positive responses. The two compounds that are known to generate reactive metabolites, but did not produce positive responses in the current assay, were valproic acid and phenytoin. The reason for this was that the formation of the valproic acid reactive metabolite (2,4-dieneVPA) requires P450-oxidation and β-oxidation catalyzed by a mitochondrial coenzyme A-dependent process, rather than microsomal enzymes [20–23]. The reactive metabolite of phenytoin could be a free radical instead of an epoxide which cannot form a stable GSH conjugate [24,26]. This method was further evaluated using 43 compounds as positive controls and 16 compounds as negative controls. The results indicated that 40 of 43 compounds tested gave positive results as expected from the literature. Those producing false negatives were felbamate, trimethoprim and sulfamethoxazole. The potential reason for this was a relatively low *in vitro* concentration used in the assay (100 μM). On the other hand, all 16 of the compounds used as negative controls were found to be negative as expected, indicating that this method could be useful in determining the potential for a compound to form reactive intermediates. Although feasible for HTS, the strategy has limited applications to trap the most stable GSH adducts. Those unstable GSH adducts can spontaneously regenerate GSH and the reactive metabolite. A highly reactive metabolite can react with an active site residue of the enzyme that forms it and thus might not be trapped by GSH. If the reactive metabolite is a free radical, it will most likely abstract a hydrogen atom from GSH rather than react with it. Alternative trapping reagents can be used for more reactive intermediates [27].

Free radicals

Techniques for trapping free radicals are well established. Free radicals resulting from reduction and oxidation can be trapped with spin-trapping reagents [27–29]. For example, a method for trapping free radicals was used on hydrazine analogs and their derivatives which are responsible for hemolytic and hepatotoxic events, presumably via the alkyl or aryl free radicals that oxidize essential cysteinyl residues in proteins or covalently react with biomacromolecules [30]. Another example is the bioactivation of phenytoin to a free radical species, proposed to be mediated by COX-1, that can be trapped with the spin trapping agent α-phenyl-*N*-t-butyl-nitrone (PNB) *in vitro* in embryo cell cultures [24–27]. The techniques to trap free radicals need further evaluation to develop high throughput methodology and their role in IDRs.

Iminium ion

Trapping iminium ions with cyanide is also a well-known technique [30]. The technique can be implemented as a screen for the detection of reactive iminium intermediates by trapping them with radiolabeled cyanide. With (S)-nicotine

as the reference compound, the extent of radiolabeled cyanide incorporated into the test compounds can be quantified. If compounds have higher cyanide incorporation than (S)-nicotine, it is considered indicative of the formation of iminium intermediates. However, the iminium ions can also form GSH adducts depending on their relative stability. For example, U-89843 incubated in liver microsomes formed *N*-acetylcysteinyl and GSH adducts in NADPH-supplemented rat liver microsomes supporting a bioactivation pathway potentially involving an iminium intermediate [31,32]. The reactive metabolites of DMP 406 and mianserin both reacted with a range of nucleophiles, but in many cases the reaction was reversible. The best nucleophile for trapping these reactive metabolites was cyanide [30]. These results suggest the potential need for a combination of trapping methods to evaluate the formation of reactive intermediates.

Reactive oxygen species

It has been proposed that some IDRs might be the result of oxidative stress – increases in the intracellular levels of reactive oxygen species (ROS) [33–36]. Generally, ROS are generated as byproducts from cellular metabolism, primarily in the mitochondria. When the production of ROS exceeds the antioxidant capacity of the cell, cellular macromolecules such as lipids, proteins and DNA can be damaged. To prevent oxidative stress under normal physiological conditions, these free radicals are neutralized by an elaborate antioxidant defense system consisting of enzymes (e.g. catalase, superoxide dismutase and GSH peroxidase), and numerous non-enzymatic antioxidants (e.g. vitamins A, E and C, GSH, ubiquinone and flavonoids) [37–39]. GSH-associated metabolism is a major mechanism for cellular protection against agents or reactive intermediates. It has been reported that several mechanisms could contribute to cell death associated with oxidative stress, which can be summarized in three steps [40]. The first stage is the recognition of stress by sensitive protein(s), (e.g. the depletion of GSH), mitochondrial damage, inactivation of critical cellular functions, activation of transcription factors, defense gene expression, protein expression, protein function, and release of pro-inflammatory cytokinase. SThe second stage is the subsequent activation of cellular defenses through Phase II metabolism enzymes [e.g. uridine diphosphate-glucuronyltransferase (UDP-GT), GSH-related enzymes (e.g. glutathione S-transferase (GST)], heat shock proteins (e.g. Hsp72), antioxidants and cell cycle inhibitors. At stage three, the cells of tolerant individuals are able to dynamically protect themselves from continued stress, but the cells of susceptible individuals might not be able to do so, leading to premature apoptotic death. Some strategies of antioxidative defence are: the transition metals can be inactivated by chelating proteins (e.g. ferritin); and ROS can be reduced enzymatically (e.g. by the glutathione peroxidase) or non-enzymatically by antioxidants (e.g. by vitamin E, vitamin C and glutathione).

Biological markers

Biological markers (biomarkers) can be measured and quantified, providing useful information for a wide range of clinical and pre-clinical uses [41–43]. Some potential examples of biological parameters that can be measured include: concentration of specific enzyme(s) and/or specific hormones; specific gene phenotype distribution in a population; the presence of biological substances that are useful as indicators for health and physiology related assessments such as disease risk, psychiatric disorders, environmental exposure and its effects; disease diagnosis; metabolic processes; substance abuse; pregnancy; cell-line development; and epidemiologic studies. These and other parameters can be used to identify a toxic effect in an individual organism and can be used to extrapolate between species. Biomarkers can serve to confirm diagnoses, monitor treatment effects or disease progression, and predict clinical results.

Biomarkers are clearly indicated as having important roles in drug development for several situations, such as their ability to provide a rational basis for selection of lead compounds, as a help in determining the ability to work toward qualification and use as a surrogate endpoint. Changes in a biomarker can provide useful indicators for pathophysiology, which in turn are important in identifying a suitable therapeutic target. For example, the association of elevated serum cholesterol levels with an increased incidence of coronary heart disease provides an underlying rationale for developing drugs that lower cholesterol by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase. Thus, total cholesterol is a good example of a clinical biomarker that has been qualified for use as a surrogate endpoint [44,45].

Biomarkers are also important to the preclinical assessment of the potential benefits and harmful effects of a new drug candidate. Screening tests in animals using biomarkers, such as reduction of blood pressure, provide important demonstration that a compound is able to produce the intended therapeutic activity in patients. By measuring blood levels during adverse events, such as seizures, in animal toxicology studies can help guide the design of dose-escalation studies in humans and serve as a surrogate for preventing or reducing the likelihood of similar adverse events in humans.

Biomarkers for potential toxicity play an equally important role. For example, a drug found to prolong the QT

interval in animals might warn of potential cardiovascular risk in subsequent clinical studies. Also, biomarkers could be glutathione conjugates and/or glutathionylated or oxidized proteins detectable both *in vitro* and *in vivo*. Biomarkers of oxidative stress involving gene/protein expression at least require a whole-cell system. It is highly unlikely that a single compound/gene/protein/function will be an effective biomarker; however, combinations of these might prove more successful. Several potential biomarkers of oxidative stress can be monitored to evaluate the potential toxic effects of drugs. Drugs that undergo redox cycling or form free radicals can generate toxic effects through oxidative stress without forming covalent adducts with biomolecules. This suggests that certain groups of compounds can be screened out in the drug design. The potential advantage of an oxidative stress biomarker is that it can be monitored during *in vitro* and/or *in vivo* studies.

Pharmacokinetic–pharmacodynamic studies using biomarkers can be particularly useful; for example, one such study showed good correlation between the hypotensive effects of an antiarrhythmic drug in dogs and humans. One shortcoming is that most biomarkers, used singly, are unlikely to capture all the effects of a drug, and thereby fulfill the most stringent criterion for a surrogate endpoint, although by using several of them in combination, it is more likely, and desirable, to produce evidence which is consistent enough to point in a particular direction.

Promising strategies

Because thousands of compounds must be screened in a typical pharmaceutical industry setting, it is almost impossible, and very expensive, to have all of them radiolabeled. Using radiolabeled reagents such as GSH and KCN is easier and more cost-effective to obtain an accurate measurement of adduct formation [46]. The reactive intermediates might be formed in other subcellular fractions such as liver S9, mitochondria, and cytosol, and thus, screening for reactive intermediates in these fractions will minimize false negatives. As an example, valproic acid forms reactive metabolites in subcellular mitochondria, as explained above. We have compared *in vitro* cellular and subcellular models for identifying drug candidates with the potential to produce reactive metabolites. For purposes of evaluating these models, six known compounds currently in the clinic with dose limitations due to hepatotoxicity were selected. Labetalol, Acetaminophen, Niacin, Iproniazid, 8-Hydroxyquinoline, and Isoniazid were incubated separately with human liver mitochondria, S9, microsomes, cytosol and hepatocytes. LC-QTOF-MS methods were used to elucidate metabolic profiles of selected compounds. The Phase II metabolites were in good agreement with previously published results. Without supplementation of reagents in subcellular fractions, Phase II metabolites were not detected. Hepatocytes also formed GSH conjugates but the relative abundance was lower, indicating that the best model to identify potential reactive metabolites involves the subcelluar fractions [46]. Using the results for Acetaminophen, for example, when reactive intermediates are produced, they can be trapped by GSH, whereas in the case of Labetalol no reactive intermediates were produced and subsequently no GSH adducts were detected. Studies should be done to improve our knowledge of, and hence techniques used for, screening with the use of radiolabeled reagents, which would give an accurate measurement of the amount of reactive intermediates [46]. Also, reactive intermediate formation in subcellular fractions should be investigated, which will aid in reducing false results [46].

Conclusions

We have discussed strategies and tools to enable researchers in the pharmaceutical industry to design drugs that are safer and more robust for humans. Structural information on metabolites is a great help in enhancing as well as streamlining the process of developing new drug candidates, which in turn has great value in several important aspects of drug discovery and development. By improving our ability to identify helpful and harmful metabolites, suggestions for structural modifications will optimize the likelihood that other compounds in the series are more successful. *In silico* and *in vitro* techniques are available to screen compounds for key ADME characteristics, which, when applied within a rational strategy, can make a major contribution to the design and selection of successful drug candidates. Screening for reactive intermediates with the use of radiolabeled reagents as has been discussed, which would give an accurate measurement of the amount of reactive intermediates.

These efforts will be aided by continued improvement and expansion of cooperation and dialogue among the disciplines involved in the entire process, especially between medicinal chemistry and drug metabolism to develop strategies to facilitate better and more-focused decision making throughout the drug discovery process. It is worth stating that drug safety should be further investigated by pharmaceutical companies to conduct post-marketing studies, which are required by the FDA when a safety question arises during the pre-approval period.

References

1 Meyboom, R.H.B. *et al.* (2000) An ABC of Drug-Related Problems. *Drug Saf.* 22, 415–423
2 Greene, N. *et al.* (2002) Computer system s for the prediction of toxicity: an update. *Adv. Drug Deliv. Rev.* 54, 417–431

3 Pirmohamed, M. *et al.* (1998) Adverse drug reactions. *BMJ* 316, 1295–1298
4 Richard, A.M. *et al.* (1998) Structure-based methods for predicting mutagenicity and carcinogenicity: are we there yet? *Mutat. Res.* 400, 493–507
5 Lasser, K.E. *et al.* (2002) Timing of new black box warnings and withdrawals for prescription medications. *J. Am. Med. Assoc.* 287, 2215–2220
6 Bakke, O.M. *et al.* (1995) Drug safety discontinuations in the United Kingdom, the United States and Spain from 1974 through 1993: A regulatory perspective. *Clin. Pharmacol. Ther.* 58, 108–117
7 Tiller, P.R. *et al.* (2002) Liquid chromatography/tandem mass spectrometric quantification with metabolite screening as a strategy to enhance the early drug discovery process. *Rapid Commun. Mass Spectrom.* 16, 1225–1231
8 Uetrecht, J.P. *et al.* (2002) Screening for the potential of a drug candidate to cause idiosyncratic drug reactions. *Drug Discov. Today* 5, 832–837
9 Nassar, A.F. *et al.* (2004) Strategies for Dealing with Reactive Intermediates in Drug Discovery and Development. *Curr. Opin. Drug Discov. Dev.* 7, 126–136
10 Matzinger, P. *et al.* (2002) The danger model: A renewed sense of self. *Science* 296, 301–305
11 Li, A.P. *et al.* (2002) A review of the common properties of drugs with idiosyncratic hepatotoxicity and the "multiple determinant hypothesis" for the manifestation of idiosyncratic drug toxicity. *Chem. Biol. Interact.* 142, 7–23
12 Mattocks, A.R. *et al.* (1990) Trapping of short-lived electrophilic metabolites of pyrrolizidine alkaloids escaping from perfused rat liver. *Toxicol. Lett.* 54, 93–99
13 Mehta, J.R. *et al.* (1982) Trapping of DNA-reactive metabolites of therapeutic or carcinogenic agents by carbon-14-labeled synthetic polynucleotides. *Cancer Res.* 42, 2996–2999
14 Judson, P.N. *et al.* (2003) A Comprehensive Approach to Argumentation. *J. Chem. Inf. Comput. Sci.* 43, 1356–1363
15 Judson, P.N. *et al.* (2003) Using Argumentation for Absolute Reasoning about the Potential Toxicity of Chemicals. *J. Chem. Inf. Comput. Sci.* 43, 1364–1370
16 Chen, W.G. *et al.* (2001). Reactive metabolite screen for reducing candidate attrition in drug discovery. In *Biological Reactive Intermediates VI. Chemical and Biological Mechanisms in Susceptibility to and Prevention of Environmental Diseases*. (Dansetter, P.M. *et al.* eds), pp. 521–524, Kluwer Academic/Plenum Press: New York
17 Evans, D.C. (2004) Drug-Protein Adducts: An Industry Perspective on Minimizing the Potential for Drug Bioactivation in Drug Discovery and Development. *Chem. Res. Toxicol.* 17, 3–16
18 Burkhart, C. *et al.* (2002) Non-covalent presentation of sulfamethoxazole to human CD4+ T cells is independent of distinct human leucocyte antigen-bound peptides. *Clin. Exp. Allergy* 32, 1635–1643
19 Steghens, J.P. *et al.* (2003) Fast liquid chromatography-mass spectrometry glutathione measurement in whole blood: micromolar GSSG is a sample preparation artifact. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 798, 343–349
20 Soglia, J.R. *et al.* (2004) The development of a higher throughput reactive intermediate screening assay incorporating micro-bore liquid chromatography-micro-electrospray ionization-tandem mass spectrometry and glutathione ethyl ester as an in vitro conjugating agent. *J. Pharm. Biomed. Anal.* 36, 105–116
21 Tang, W. *et al.* (1996) Characterization of thiol-conjugated metabolites of 2-propylpent-4-enoic acid (4-ene VPA), a toxic metabolite of valproic acid, by electrospray tandem mass spectrometry. *J. Mass Spectrom.* 8, 926–936
22 Tang, W. *et al.* (1997) A comparative investigation of 2-propyl-4-pentenoic acid (4-ene VPA) and its alpha-fluorinated analogue: phase II metabolism and pharmacokinetics. *Drug Metab. Dispos.* 25, 219–227
23 Kassahun, K. *et al.* (1993) In vivo formation of the thiol conjugates of reactive metabolites of 4-ene VPA and its analog 4-pentenoic acid. *Drug Metab. Dispos.* 21, 1098–1106
24 Liu, L. *et al.* (1994) In vivo phenytoin-initiated oxidative damage to proteins and lipids in murine maternal hepatic and embryonic tissue organelles: potential molecular targets of chemical teratogenesis. *Toxicol. Appl. Pharmacol.* 125, 247–255
25 Munns, A.J. *et al.* (1997) Bioactivation of phenytoin by human cytochrome P450: characterization of the mechanism and targets of covalent adduct formation. *Chem. Res. Toxicol.* 10, 1049–1058
26 Cuttle, L. *et al.* (2000) Phenytoin metabolism by human cytochrome P450: involvement of P450 3A and 2C forms in secondary metabolism and drug-protein adduct formation. *Drug Metab. Dispos.* 28, 945–950
27 Timmins, G.S. (1999) Trapping of free radicals with direct in vivo EPR detection: a comparison of 5,5-dimethyl-1-pyrroline-N-oxide and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide as spin traps for HO* and SO4. *Free Radic. Biol. Med.* 27, 329–333
28 Pritsos, C.A. *et al.* (1985) Use of high-performance liquid chromatography to detect hydroxyl and superoxide radicals generated from mitomycin C. *Anal. Biochem.* 150, 294–299
29 Sanders, S.P. *et al.* (2000) Measurements of free radicals in isolated, ischemic lungs and lung mitochondria. *Lung* 178, 105–118
30 Gorrod, J.W. (1991) Trapping of reactive intermediates by incorporation of 14C-sodium cyanide during microsomal oxidation. *Adv. Exp. Med. Biol.* 283, 657–664
31 Zhao, Z. *et al.* (1996) Bioactivation of 6,7-dimethyl-2,4-di-1-pyrrolidinyl-7H-pyrrolo[2,3-d]pyrimidine (U-89843) to reactive intermediates that bind covalently to macromolecules and produce genotoxicity. *Chem. Res. Toxicol.* 9, 1230–1239
32 Zhao, Z. *et al.* (1996) In vitro and in vivo biotransformation of 6,7-dimethyl-2,4-di-1-pyrrolidinyl-7H-pyrrolo[2,3-D]pyrimidine (U-89843) in the rat. *Drug Metab. Dispos.* 24, 187–198
33 Uetrecht, J.P. *et al.* (1989) Idiosyncratic drug reactions: possible role of reactive metabolites generated by leukocytes. *Pharm. Res.* 6, 265–273
34 Masubuchi, Y. *et al.* (2002) Role of mitochondrial permeability transition in diclofenac-induced hepatocyte injury in rats. *Hepatology* 35, 544–551
35 Masubuchi, Y. *et al.* (2000) Possible mechanism of hepatocyte injury induced by diphenylamine and its structurally related nonsteroidal anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.* 292, 982–987
36 Takeyama, N. *et al.* (1993) Oxidative damage to mitochondria is mediated by the Ca(2+)-dependent inner-membrane permeability transition. *Biochem. J.* 294, 719–725
37 Miyamoto, Y. *et al.* (2003) Oxidative stress caused by inactivation of glutathione peroxidase and adaptive responses. *Biol. Chem.* 384, 567–574
38 Nikulina, M.A. *et al.* (2000) Glutathione depletion inhibits IL-1 beta-stimulated nitric oxide production by reducing inducible nitric oxide synthase gene expression. *Cytokine* 12, 1391–1394
39 Yin, J.H. *et al.* (2001) Inducible nitric oxide synthase neutralizes carbamoylating potential of 1,3-bis(2-chloroethyl)-1-nitrosourea in c6 glioma cells. *J. Pharmacol. Exp. Ther.* 297, 308–315
40 O'Donovan, D.J. *et al.* (2000) Mitochondrial glutathione and oxidative stress: implications for pulmonary oxygen toxicity in premature infants. *Mol. Genet. Metab.* 71, 352–358
41 Ilyin, S.E. *et al.* (2004) Biomarker discovery and validation: technologies and integrative approaches. *Trends Biotechnol.* 22, 411–416
42 Lesko, L.J. and Atkinson, A.J., Jr (2001) Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies. *Annu. Rev. Pharmacol. Toxicol.* 41, 347–366
43 Colburn, W.A. (2003) Biomarkers in drug discovery and development: from target identification through drug marketing. *J. Clin. Pharmacol.* 43, 329–341
44 Schonbeck U et al (2004) Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? Circulation 109 (21 Suppl 1) II18-26
45 Cziraky, M. *et al.* (1998) Clinical positioning of HMG-CoA reductase inhibitors in lipid management protocols. *Pharmacoeconomics* 14(Suppl 3), 29–38
46 Nassar, A.E. et al. (2004) Detecting and minimizing reactive intermediates in R&D. *Current Drug Discovery,* July issue 20–25
47 Madden, S. *et al.* (1993) An investigation into the formation of stable,

protein-reactive and cytotoxic metabolites from tacrine in vitro. Studies with human and rat liver microsomes. *Biochem. Pharmacol.* 46, 13–20
48 Kassahun, K. *et al.* (2001) Studies on the metabolism of troglitazone to reactive intermediates in vitro and in vivo. Evidence for novel biotransformation pathways involving quinone methide formation and thiazolidinedione ring scission. *Chem. Res. Toxicol.* 14, 62–70
49 Tettey, J.N. *et al.* (2001) Enzyme-induction dependent bioactivation of troglitazone and troglitazone quinone in vivo. *Chem. Res. Toxicol.* 14, 965–974
50 Shapiro, L.E. *et al.* (1997) Comparative safety of tetracycline, minocycline, and doxycycline. *Arch. Dermatol.* 133, 1224–1230
51 Isaacson, J. *et al.* (1999) Index of suspicion. Case 3. Acetaminophen overdose. *Pediatr. Rev.* 20, 309–313, 312–313
52 Liu, Z.C. *et al.* (1995) Oxidation of 5-aminosalicylic acid by hypochlorous acid to a reactive iminoquinone. Possible role in the treatment of inflammatory bowel diseases. *Drug Metab. Dispos.* 23, 246–250
53 Ruscoe, J.E. *et al.* (1995) The effect of chemical substitution on the metabolic activation, metabolic detoxication, and pharmacological activity of amodiaquine in the mouse. *J. Pharmacol. Exp. Ther.* 273, 393–404
54 Munns, A.J. *et al.* (1997) Bioactivation of phenytoin by human cytochrome P450: characterization of the mechanism and targets of covalent adduct formation. Chem Res Toxicol. *Chem. Res. Toxicol.* 10, 1049–1058
55 Ju, C. *et al.* (1999) Detection of 2-hydroxyiminostilbene in the urine of patients taking carbamazepine and its oxidation to a reactive iminoquinone intermediate. *J. Pharmacol. Exp. Ther.* 288, 51–56
56 Uetrecht, J.P. *et al.* (1994) Metabolism of vesnarinone by activated neutrophils: implications for vesnarinone-induced agranulocytosis. *J. Pharmacol. Exp. Ther.* 270, 865–872
57 Parrish, D.D. *et al.* (1997) Activation of CGS 12094 (prinomide metabolite) to 1,4-benzoquinone by myeloperoxidase: implications for human idiosyncratic agranulocytosis. *Fundam. Appl. Toxicol.* 35, 197–204
58 Mohsin, S.K. *et al.* Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study. *Mod. Pathol.* (in press)
59 Fan, P.W. *et al.* (2001) Bioactivation of tamoxifen to metabolite E quinone methide: reaction with glutathione and DNA. *Drug Metab. Dispos.* 29, 891–896
60 Mann, K. *et al.* (1987) Differential effects of a new dibenzo-epine neuroleptic compared with haloperidol. Results of an open and crossover study. *Pharmacopsychiatry* 20, 155–159
61 Borges, N. *et al.* (2000) Tolcapone-related liver dysfunction: implications for use in Parkinson's disease therapy. *Drug Saf.* 26, 743–747
62 Yunis, A.A. *et al.* (1980) Chloramphenicol toxicity: pathogenetic mechanisms and the role of the p-NO2 in aplastic anemia. *Clin. Toxicol.* 17, 359–373
63 Utili, R. *et al.* (1977) Dantrolene-associated hepatic injury. Incidence and character. J. *Gastroenterology* 72, 610–616
64 Merlani, G. *et al.* (2001) Fatal hepatoxicity secondary to nimesulide. *Eur. J. Clin. Pharmacol.* 57, 321–326
65 Idanpaan-Heikkila, J. *et al.* (1977) Agranulocytosis during treatment with chlozapine. *Eur. J. Clin. Pharmacol.* 11, 193–198
66 Haack, M.J. *et al.* (2003) Toxic rise of clozapine plasma concentrations in relation to inflammation. *Eur. Neuropsychopharmacol.* 13, 381–385
67 Uetrecht, J.P. *et al.* (1995) Oxidation of aminopyrine by hypochlorite to a reactive dication: possible implications for aminopyrine-induced agranulocytosis. *Chem. Res. Toxicol.* 8, 226–233
68 Sabbaga, J. *et al.* (1993) Acute agranulocytosis after prolonged high-dose usage of intravenous dipyrone—a different mechanism of dipyrone toxicity? *Ann. Hematol.* 66, 153–155
69 Cribb, A.E. *et al.* (1992) Sulfamethoxazole is metabolized to the hydroxylamine in humans. *Clin. Pharmacol. Ther.* 51, 522–526
70 Coleman, M.D. *et al.* (1994) Reduction of dapsone hydroxylamine to dapsone during methaemoglobin formation in human erythrocytes in vitro. IV: Implications for the development of agranulocytosis. *Biochem. Pharmacol.* 48, 1349–1354
71 Woosley, R.L. *et al.* (1978) Effect of acetylator phenotype on the rate at which procainamide induces antinuclear antibodies and the lupus syndrome. *N. Engl. J. Med.* 298, 1157–1159
72 Salama, A. *et al.* (1983) The role of metabolite-specific antibodies in nomifensine-dependent immune hemolytic anemia. *N. Engl. J. Med.* 313, 469–474
73 Senturk, T. *et al.* (1997) Seizures and hepatotoxicity following sulphasalazine administration. *Rheumatol. Int.* 17, 75–77
74 Stuart-Harris, R.C. *et al.* (1984) Aminoglutethimide in the treatment of advanced breast cancer. *Cancer Treat. Rep.* 11, 189–204
75 Thompson, C.D. *et al.* (1996) Synthesis and in vitro reactivity of 3-carbamoyl-2-phenylpropionaldehyde and 2-phenylpropenal: putative reactive metabolites of felbamate. *Chem. Res. Toxicol.* 9, 1225–1229
76 Dieckhaus, C.M. *et al.* (2002) Mechanisms of idiosyncratic drug reactions: the case of felbamate. *Chem. Biol. Interact.* 142, 99–117
77 Gupta, A.K. *et al.* (1998) Severe neutropenia associated with oral terbinafine therapy. *J. Am. Acad. Dermatol.* 38, 765–767
78 Grillo, M.P. *et al.* (2001) Effect of alpha-fluorination of valproic acid on valproyl-S-acyl-CoA formation in vivo in rats. *Drug Metab. Dispos.* 29, 1210–1215
79 Inman, W.H. *et al.* (1988) Blood disorders and suicide in patients taking mianserin or amitriptyline. *Lancet* 2, 90–92
80 Jardine, D.L. (2002) Hodgkin's disease following methotrexate therapy for rheumatoid arthritis. *N. Z. Med. J.* 115, 293–294
81 Ware, J.A. *et al.* (1998) Immunochemical detection and identification of protein adducts of diclofenac in the small intestine of rats: possible role in allergic reactions. *Chem. Res. Toxicol.* 11, 164–171
82 Bailey, M.J. *et al.* (1996) Chemical and immunochemical comparison of protein adduct formation of four carboxylate drugs in rat liver and plasma. *Chem. Res. Toxicol.* 9, 659–666
83 Prescott, L.F. *et al.* (1986) Effects of non-narcotic analgesics on the liver. *Drugs* 4, 129–147
84 Skjodt, N.M. *et al.* (1999) Clinical pharmacokinetics and pharmacodynamics of bromfenac. *Clin. Pharmacokinet.* 36, 399–408
85 Moses, P.L. *et al.* (1999) Severe hepatotoxicity associated with bromfenac sodium. *Am. J. Gastroenterol.* 94, 1393–1396
86 Halsey, J.P. *et al.* (1982) Benoxaprofen: side-effect profile in 300 patients. *BMJ* 284, 1365–1368
87 Godessart, N. *et al.* (1999) Role of COX-2 inhibition on the formation and healing of gastric ulcers induced by indomethacin in the rat. *Adv. Exp. Med. Biol.* 469, 157–163