Managing the challenge of chemically reactive metabolites in drug development

B. Kevin Park, Alan Boobis‡, Stephen Clarke§, Chris E. P. Goldring*, David Jones ||, J. Gerry Kenna¶, Craig Lambert#, Hugh G. Laverty*, Dean J. Naisbitt*, Sidney Nelson**, Deborah A. Nicoll-Griffith‡‡, R. Scott Obach§§, Philip Routledge||||, Dennis A. Smith¶¶, Donald J. Tweedie##, Nico Vermeulen***, Dominic P. Williams*, Ian D. Wilson‡‡‡ and Thomas A. Baillie§§§*

Abstract | The normal metabolism of drugs can generate metabolites that have intrinsic chemical reactivity towards cellular molecules, and therefore have the potential to alter biological function and initiate serious adverse drug reactions. Here, we present an assessment of the current approaches used for the evaluation of chemically reactive metabolites. We also describe how these approaches are being used within the pharmaceutical industry to assess and minimize the potential of drug candidates to cause toxicity. At early stages of drug discovery, iteration between medicinal chemistry and drug metabolism can eliminate perceived reactive metabolite-mediated chemical liabilities without compromising pharmacological activity or the need for extensive safety evaluation beyond standard practices. In the future, reactive metabolite evaluation may also be useful during clinical development for improving clinical risk assessment and risk management. Currently, there remains a huge gap in our understanding of the basic mechanisms that underlie chemical stress-mediated adverse reactions in humans. This Review summarizes our views on this complex topic, and includes insights into practices considered by the pharmaceutical industry.

**MRC Centre for Drug Safety Science, Division of Molecular & Clinical Pharmacology, The Institute of Translational Medicine, University of Liverpool, Liverpool, L69 3GE, UK. Correspondence to B.K.P. or D.J.T*
e-mails: B.K.Park@liverpool.ac.uk; donald.tweedie@boehringer-ingelheim.com
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Adverse drug reactions (ADRs) are a major complication of drug therapy and an impediment to drug development and clinical use after marketing. As a consequence of evidence of toxicity, 16 out of 548 (2.9%) new chemical entities that were approved for the US market between 1975 and 1999 were subsequently withdrawn from the market, and 56 out of 548 (10.2%) acquired a black box warning[1]. Excessive dose, drug accumulation and/or the formation of chemically reactive metabolites (CRMs) have been implicated in many off-target (including idiosyncratic) ADRs.

The organ that is most frequently affected by CRM-mediated ADRs is the liver. Drug-induced liver injury accounts for more than half the cases of acute liver failure in the United States, and acetaminophen is responsible for 80% of drug-associated cases of liver failure[2]. Acetaminophen-induced hepatotoxicity is generally predictable from our understanding of its metabolism; however, many other drugs cause idiosyncratic drug-induced liver injury, which, although rare and unpredictable, can cause significant morbidity and mortality. Studies with model compounds and drugs — such as acetaminophen — have helped to define the roles that chemical stress and drug bioactivation have in the various biological outcomes that may be triggered by CRMs. These include effects on transcription factors and/or signalling protein-adaptation (cell defence), apoptosis, necrosis, inflammation and activation of the innate and adaptive immune systems[3].

In addition to their role in drug-induced liver injury, CRMs have been implicated in a number of off-target ADRs in humans; these ADRs have the clinical hallmarks of hypersensitivity reactions and may affect various systems in addition to the liver, skin and formed

elements of blood and kidney; they may also present as generalized hypersensitivity or anaphylaxis. Such reactions are usually rare and are not evident in animal species, but they can be serious and even fatal in humans[4,5], and may lead to the withdrawal of otherwise effective therapeutic agents. The fear of such reactions occurring at the post-approval stage — when such problems typically first become evident — is a major impediment to drug development. At present, during preclinical drug evaluation there are no accepted methods for the identification of drugs that may cause hypersensitivity or idiosyncratic drug reactions in humans[5].

The first step towards developing such methodology has been to tackle the source of chemical insult by testing molecules for their propensity to form CRMs. The chemical basis of drug bioactivation can usually be rationalized and synthetic strategies can be put in place to prevent such bioactivation, usually without substantial loss of primary pharmacology. However, there is no simple correlation between drug bioactivation *in vitro* and ADRs in the clinic. Such a chemical approach is clearly limited by the fact that not all drugs that can undergo bioactivation by human drug-metabolizing enzymes are associated with ADRs in the clinic, and drug bioactivation is not always a mandatory step in drug toxicity. In particular, although it is clear that CRMs differ in terms of their electrophilicity, intracellular targets, stress signalling, detoxication pathways and immunological recognition of the protein adducts that they give rise to, very little is known about the relationship between these chemical factors and the mechanisms that underlie clinical ADRs. Studies on how the chemistry of a molecule contributes to toxicity (in preclinical species and in humans) appear to be a key area for future research. A new approach is required to address this important issue, and should be based on integrated informatics and encompass chemoinformatics, systems biology and clinical informatics. This will require data mining and data sharing in order to develop a mechanistic understanding that can inform both the chemist and the clinician. The same is true for another important factor, namely the total functional *in vivo* exposure of the organism to CRMs.

The purpose of this Review is to evaluate what is understood about the formation of CRMs in the context of drug safety science and how this information affects the drug development process. In particular, we address the question of how the drug metabolism scientist might be able to inform medicinal chemists, toxicologists and clinicians about the safety risks posed by the detection of a CRM at various stages in the discovery and development of new medicines.

Experimental methods for the detection of CRMs

The two conventional methods for the experimental detection of drug bioactivation are shown in BOX 1.

Formation of CRMs within a cell poses an undesirable chemical liability. However, it must be stressed that although experiments that evaluate covalent binding (CB) and thioether adduct formation can provide valuable information on the chemistry and metabolic fate of a molecule, they cannot be used to predict biological and toxicological consequences *in vivo*. This is because there is no simple relationship between universal or total CB to liver microsomes or hepatocyte proteins *in vitro* and clinical ADR risk. Identification of critical cellular proteins and non-critical cellular proteins is underway to explore how this relates to ADR mechanisms and risk.

Throughout preclinical drug discovery and development, assessment of CB or thioether adduct formation resides firmly in the realm of drug metabolism. The drug metabolism scientist can inform the medicinal chemist about this characteristic in the context of defining the general drug metabolism and pharmacokinetics (DMPK) profile of the compound with respect to the 'ideal' DMPK properties that take into account absorption, routes and rate of drug metabolism, enzyme induction, enzyme inhibition, transporters, and drug interactions.

The first interaction between the drug metabolism scientist and the chemist is to define the chemistry of bioactivation. If this does not involve the target pharmacophore, and is related to a defined metabolic toxicophore in model toxins, then the desired solution is usually straightforward; that is, eliminate the chemical liability by alternative chemical synthesis, and thereby avoid the need for risk assessment beyond standard drug safety work packages and further internal debate on this topic for the remainder of the development programme. If the chemical mechanism of bioactivation involves part of the essential pharmacophore, the issue becomes more complex, as it may be necessary to proceed with the candidate that strikes the best possible balance between efficacy and diminished bioactivation liability (for the purposes of this discussion, the term toxicophore refers to specific chemical substituents that are either known or are suspected to be metabolized into reactive intermediates).

Author addresses

‡Department of Medicine, Imperial College London, South Kensington Campus, London SW7 2AZ, UK.
§GlaxoSmithKline, Drug Metabolism and Pharmacokinetics, Ware, Hertfordshire SG12 0DP, UK.
‖Medicines and Healthcare Products Regulatory Agency, 151 Buckingham Palace Road, London SW1W 9SZ, UK.
¶Safety Assessment UK, AstraZeneca, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK.
#Clinical Pharmacology and DMPK (Charnwood) AstraZeneca R&D Charnwood, Loughborough LE11 0RH, UK.
**University of Washington, Department of Medicinal Chemistry, Box 357610, Seattle, Washington 98195-7610, USA.
‡‡Merck Research Laboratories,126 East Lincoln Ave, Rahway, New Jersey 07065, USA.
§§Pharmacokinetics, Dynamics and Metabolism, Pfizer, Groton, Connecticut 06340, USA.
‖‖Department of Pharmacology, Therapeutics and Toxicology, Wales College of Medicine, Heath Park, Cardiff CF14 4XN, UK.
¶¶Pharmacokinetics, Dynamics, and Metabolism, Pfizer, Sandwich, Kent CT13 9NJ, UK.
##Boehringer Ingelheim Pharmaceuticals, 900 Ridgebury Road, PO Box 368, Ridgefield, Connecticut 06877, USA.
***Division of Molecular Toxicology, Leiden/Amsterdam Center for Drug Research, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.
‡‡‡Clinical Pharmacology and DMPK, AstraZeneca, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK.
§§§Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Washington 98195, USA.

Box 1 | **Detection of bioactivation**

Measurement of a drug that becomes irreversibly bound to a protein, in either *in vitro* or animal studies

The tacit assumption here is that irreversible binding equates to covalent binding (CB), although this has only been proven by mass spectrometry in a few recent studies[37–39]. This type of study requires a radiolabelled compound, which is not usually available at the early stages of drug discovery. However, the synthesis of tritium-labelled compounds (as opposed to ^{14}C-labelled analogues) is often relatively straightforward, and tracers that are labelled at specific sites with tritium can prove to be very valuable for preliminary studies on metabolic profiles and CB. Nevertheless, in terms of cost-effectiveness during preclinical evaluation, radiolabelling is the most expensive option and is therefore often reserved for late-stage preclinical candidates.

Mass spectrometric detection of thioether adducts and/or conjugates

Mass spectrometric detection of thioether adducts and/or conjugates (for example, reduced glutathione (GSH) conjugates) can be accomplished using *in vitro* incubations with hepatic microsomes, and cellular, animal and human studies. Such studies detect stable GSH conjugates that can be inferred to have been produced via chemically reactive metabolites (CRMs). As they do not require radiolabelled compounds, they can be used throughout drug discovery to identify and minimize bioactivation potential. GSH trapping and measurement of the neutral loss of 129 Da by positive ion electrospray–tandem mass spectrometry (MS) provides a generic qualitative end point. Modifications to this experimental paradigm using analogues of GSH that bear a fluorescent tag (for example, dansylated GSH) and microsomal systems can provide semi-quantitative estimates of thioether adduct formation[40]. In addition, *in vitro* incubations can be conducted in the presence of glutathione *S*-transferases to trap CRMs that escape spontaneous chemically reactive nucleophiles.

It is important to recognize that measurements of CB and thioether adduct formation use very different end points, and serve different purposes. Thus, CB may be used as a measure of that fraction of CRM formation that escapes capture by low molecular mass cellular nucleophiles (for example, GSH), and thus represents the potential cellular burden of a reactive species that has not been 'detoxified'. By contrast, GSH conjugates arise from the successful interception of these short-lived intermediates, and provide valuable (albeit indirect) structural information on the identities of the CRMs themselves.

One generic limitation of GSH-trapping experiments is that they cannot detect all types of CRMs. Some of the GSH adducts are unstable and GSH is known to have a limited trapping efficiency towards hard electrophiles. The latter CRMs will react more readily with lysine and histidine residues in proteins and/or with hard nucleophilic sites in membranes or DNA. These CRMs can be trapped by hard nucleophiles such as the cyanide anion (CN^-). For example, a new bifunctional trapping agent that contains both a cysteine residue and a lysine residue has been developed for the simultaneous screening of hard and soft electrophiles[41].

Drug bioactivation can be detected in humans by the measurement of thioether conjugates and certain stable metabolites in plasma and urine — for example, by measuring dihydrodiols that are formed from arene oxide intermediates. These are products of bioinactivation, and are therefore biomarkers for a CRM-associated hazard, but they cannot directly be used for assessing the risk of toxicity. However, urinary mercapturic acids have been used successfully for the assessment of human occupational exposure to (primarily industrial) electrophilic chemicals[19].

Variations in MS technology allow more sophisticated analysis of reactive metabolites and provide rapid detection and high-throughput capability for all classes of GSH adducts, even at low levels. For instance, rapid analysis of reactive metabolites with a linear ion trap mass spectrometer is possible. In this approach, an isotope pattern-dependent scanning method is applied to the data acquisition of GSH-trapped reactive metabolites. Subsequently recorded full-scan MS and tandem MS/MS data sets are processed by data mining techniques such as neutral loss filtering and product ion filtering[42]. MS approaches may limit the sample throughput if very high-throughput assays are sought. To overcome these limitations, fluorescence-based GSH-conjugated 96-well plate assays have been developed. The preparation of these plates utilizes oxidized GSH (GSSG), which is conjugated onto a cyanogen bromide ($CNBr^-$)-activated pHEMA (poly(2-hydroxyethyl methacrylate)) surface. The conjugated GSH is regenerated before use by reduction with D,L-dithiothreitol (DTT)[43].

Like any other biotransformation process, CRM formation requires a quantitative context. In animal studies, this can be measured easily by radiometric analysis, in which bioactivation simply equates to tissue CB plus products of bioinactivation, such as thioether conjugates, which is expressed as a percentage of the dose administered. This gives an indication of the overall chemical insult. In humans, we are limited to analysis of products of bioinactivation and have no direct measure of chemical insult, or response, in target tissues such as the liver or the immune system. However, in principle, appropriate cell-based systems — such as human or humanized hepatocytes — may be used as a bridge between animal models and humans to assess the potential scale of chemical insult in patients at pharmacologically relevant concentrations, as the first step in risk assessment. Currently, a key capability gap is the lack of well-validated cell-based approaches that can be used for this purpose.

What have we learned about structural alerts?

Just as past and present events can inform us about — but not predict — the future, chemical substructures that are intrinsically chemically reactive or associated with the formation of CRMs can inform us of the potential for drug-induced toxicities. Substructures that are most frequently associated with severe CRM-mediated toxicities — which have led to either drug withdrawal or black box

warnings — have been discussed previously[6–10] and are summarized in BOX 2. Of these structural alerts, the following have been most frequently associated with severe toxicities: anilines and anilides; arylacetic and arylpropionic acids; hydrazines and hydrazides; thiophenes; nitroaromatics; and structures that either contain or form α,β-unsaturated enal and/or enone-like structures, including quinones and quinone methides.

However, the presence of chemical substructures that may form CRMs cannot in itself predict the type, severity or incidence of ADRs that may arise. Numerous drugs contain substructures that form CRMs that may cause toxic effects, yet they remain on the market and are widely used because of favourable benefit–risk considerations; for example, anti-infective agents such as the aniline sulphonamides and aniline sulphones.

In some cases, the fraction of a drug dose that is metabolized to a CRM is inconsequential because of extensive clearance via other routes of metabolism. For example, atorvastatin (Lipitor; Pfizer) is cleared primarily through either oxidation of the phenyl ring and subsequent glucuronidation or sulphation of the resulting phenols, or through glucuronidation of the carboxylic acid, which leaves its anilide ring intact; the anilide ring has the potential to form CRMs[11]. Raloxifene (Evista; Eli Lilly and Company) is cleared primarily by glucuronidation and sulphation of the phenolic $^{-}$OH groups, therefore fewer reactive quinone or quinone methide metabolites are formed through oxidative metabolism[12].

In other cases, Phase I metabolism at metabolic 'soft spots' in structures directs metabolism away from positions that could form CRMs. This phenomenon is well illustrated by comparing the non-steroidal anti-inflammatory drugs (NSAIDs) sudoxicam and meloxicam (Mobicox; Boehringer Ingelheim), in which the only structural difference is the presence of a methyl group on the 2-position of the thiazole ring in meloxicam. Sudoxicam was withdrawn from clinical development owing to hepatotoxicity, whereas meloxicam is a therapeutically valuable anti-inflammatory agent. Meloxicam is primarily excreted in humans as the 2-carboxylic acid metabolite[13], whereas sudoxicam is instead metabolized by the opening of the oxidative ring to form a thiourea that can be further oxidized into a reactive *S*-oxide, which is likely to be responsible for its toxicity[14].

Thus, CRM mitigation strategies should not rely on structural alerts alone, as metabolism and other considerations provide additional valuable information that can be used in a 'weight of evidence' approach for risk assessment and risk management. The fact that certain classes of drugs, such as the artemisinin antimalarials and the thienopyridine antithrombotics, rely on CRMs for pharmacological action underscores the concept that bioactivation *per se* need not equate to a toxicological liability.

As newer, more complex drug structures are introduced, additional structural alerts may surface. Collaborative efforts between drug metabolism scientists in academia, industry and government are needed to develop databases that relate chemical space to potential toxicity risk. These databases should be annotated rigorously, and should be easily interrogated and updated (possibly using intelligent design systems). Such databases could serve an important informative function in the early drug discovery process.

The physiological response to bioactivation

It is important to bear in mind that the most common chemical outcome of bioactivation is bioinactivation, for example, through conjugation of a CRM with glutathione (GSH) or via another detoxication system. In this respect, we need to be aware that certain CRMs react spontaneously with GSH, whereas others require catalysis by glutathione-*S*-transferases. This requirement may be concentration-dependent; for example, the reactive acetaminophen metabolite *N*-acetyl-*p*-benzoquinone imine may react spontaneously with GSH at physiological concentrations but it requires a transferase at low concentrations of the endogenous nucleophile[15]. A summary of the initial physiological response to bioactivation is given in BOX 3.

Toxicological implications of drug bioactivation

Experiments that define the relationship between drug bioactivation and drug metabolism in humans are rare. A good example of this relationship is the demonstration, in volunteers and patients, that inhibition of the formation of the toxic hydroxylamine metabolite of dapsone was accompanied by a parallel reduction in haemotoxicity[16]. To our knowledge, no such experiment had previously been undertaken to establish the relationship between thioether excretion and an ADR in humans. Although GSH adducts and/or their thioether decomposition products that are measured *in vivo* represent short-term exposure to CRMs, protein adducts reflect the internal exposure of cells to CRMs *in vivo*, which is more relevant for risk assessment purposes.

Novel approaches for the prediction of the hepatotoxic potential of drug candidates in humans — which involve *in vitro* studies with human hepatocytes that take into consideration estimates of clinical dose and the 'body burden' of CRMs — have shown particular promise and appear to be worthy of further development[17,18]. CB *in vivo* is likely to be more informative of the *in vivo* safety risk than CB *in vitro*, as it is better at reflecting semi-chronic and chronic exposure to electrophiles[19].

However, it must be stressed that to date, no one has proposed an absolute level of *in vivo* CB that is toxic, or indeed levels of CB that are safe; it must also be stressed that *in vivo* CB studies that are undertaken in animals are of uncertain direct relevance to *in vivo* CB (and toxicity risk) in humans. In addition, comparative CB studies that have been undertaken with acetaminophen and its relatively non-hepatotoxic regioisomer 3′-hydroxyacetanilide have shown that equivalent levels of CB to proteins *per se* are not sufficient to induce toxicity[11,20]. As such, next to dosimetry and quantitative considerations, identifying many — if not all — toxicologically relevant protein targets of CRMs is an important challenge to a better understanding of the links between CB to critical proteins and organ toxicity[12].

Box 2 | **Structural alerts**

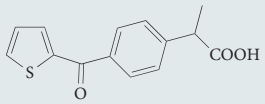
The general principle of structural alerts has evolved from a consideration of genotoxic carcinogens and the fundamental concepts of chemical carcinogenesis. In such circumstances, identification of a hazard without consideration of risk can be sufficient for decision-making in drug discovery and drug development, as genotoxicity that arises from covalent modification of DNA is considered by regulatory agencies to not exhibit a threshold of toxicity. This concept does not translate to non-genotoxic chemically reactive metabolite (CRM)-mediated toxicities, as:

- The chemistry and biochemistry of 'soft' CRMs imply a threshold for toxicity
- Toxicities and clinical adverse drug reactions (ADRs) that are caused by CRMs exhibit clear dose dependence and dose thresholds *in vivo*; this is well-documented for stereotypic hepatotoxins such as acetaminophen, which are only toxic at high doses in animal models that overwhelm metabolic detoxication and biological compensation mechanisms, and is supported by the observation that in general, idiosyncratic ADRs occur more frequently with drugs that are given at doses exceeding 10mg per day than with drugs that are given at less than 10 mg per day
- Although many articles contain extensive lists of metabolic structural alerts for toxicity, for just about every example cited it is possible to give an example of a drug that is not only safe but is also an essential part of the physician's armamentarium

It is therefore imperative that risk assessment takes place in order to eliminate both false negatives and false positives, and that concepts and tools are developed to do this in a reproducible and regulated manner.

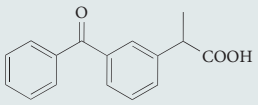
Nonetheless, it may be wise to remove at-risk structures in the design and discovery phase to avoid the possible marketing of drugs such as bromfenac that contained three structural alerts (aniline, arylacetic acid and a bromophenyl ring)[44–46]. This drug was withdrawn from the market because of the frequency of developing liver injury in patients who were treated systemically with the drug for longer than the approved duration of 10 days[47]. Subsequently, it has been safely reintroduced for topical (that is, low-dose) ophthalmic use, which further emphasizes the importance of dose/exposure as a safety risk co-factor[48]. Two examples that demonstrate how some structures in drugs can be replaced with analogous structures that are less toxic are presented in the figures.

a



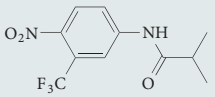
Suprofen

b



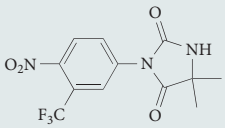
Ketoprofen

c



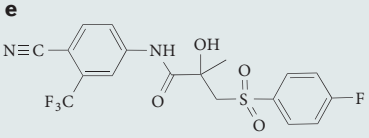
Flutamide

d



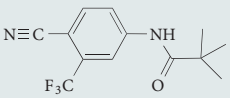
Nilutamide

e



Bicalutamide

f



Cyano analogue

The first example, suprofen (**a**), is a non-steroidal anti-inflammatory drug (NSAID) that was withdrawn from the market because of a relatively high incidence of severe flank pain associated with kidney injury[49]. Ketoprofen (**b**) is a close structural analogue of suprofen wherein the thiophene ring in suprofen has been replaced with the less toxicophoric phenyl ring. Ketoprofen is a significantly safer NSAID that has not been associated with any organ toxicities.

The second examples, flutamide (**c**) and nilutamide (**d**), are androgen receptor antagonists that are used in the treatment of prostate cancer. Both are labelled with black box warnings owing to a relatively high incidence of liver and lung injury associated with their use[50,51]. Bicalutamide (**e**) is an analogue of nilutamide in which the toxicophoric nitrophenyl substructure has been replaced by a cyanophenyl group. Bicalutamide is a safer drug to treat prostate cancer than either flutamide or nilutamide[52]. Furthermore, studies with a close structural analogue of flutamide (cyano analogue; **f**) — in which the only change to the structure is a replacement of the nitro group with a cyano group — have shown that the cyano analogue is significantly less cytotoxic than flutamide, yet it is more potent as an androgen receptor antagonist[53].

Carboxylic acid drugs provide a particular conundrum with respect to drug bioactivation. The carboxylic acid is an important pharmacophore and is a substructure that the medicinal chemist can introduce into a drug series in order to minimize drug–drug interactions with cytochrome P450 enzymes and improve solubility. Drugs that contain the carboxylate function — most notably NSAIDs — have been associated with a range of idiosyncratic ADRs, which include liver and other organ toxicities as well as immune-mediated reactions, and have resulted in numerous drug withdrawals[54–56]. Metabolic bioactivation of carboxylic acid functional groups can occur, and is catalysed by microsomal UDP-glucuronosyltransferases (UGTs) and by microsomal or mitochondrial acyl CoA synthetases. Metabolism via acyl CoA synthetases results in the formation of acyl CoA thioesters, which for many drugs are markedly more reactive than the corresponding acyl glucuronides[57]. UGT-dependent metabolism results in the formation of acyl glucuronides.

For some drugs, these metabolites are chemically unstable in aqueous solution at physiological or alkaline pH, and may interact with nucleophilic sites on proteins to form stable protein adducts[54]. The aqueous stabilities (and hence *in vitro* protein reactivities) of acyl glucuronides vary markedly between drugs, and an apparent correlation between short aqueous half-life at pH 7.4, covalent binding to protein and propensity of the parent drug to cause idiosyncratic ADRs in humans has been observed, which suggests that protein adduct formation may play a part in the toxicities of these drugs[56,58]. However, many drugs that contain a carboxylic acid also contain another structural alert for bioactivation (for example, suprofen) and it is currently unclear which substructure represents the chemical liability.

There is a concern that an overly stringent use of a CRM screen can pose an unnecessary impediment to the discovery and development of new medicines and might, in the limiting situation, lead to the loss of new drugs that could have a significant impact on the treatment of human diseases. The evidence that CRMs are responsible for serious ADRs in humans is based almost entirely on drug metabolism studies that have shown a retrospective association between the formation of CRMs and clinical toxicity. Supportive experimental evidence is based primarily on a very limited number of studies that have demonstrated the presence of drug-metabolite-specific antibodies or T cell-mediated responses[4,5]. However, once again these have been primarily qualitative associations. The clearest association between the extent of CRM formation, the level of CB to protein (*in vitro* and *in vivo*) and the clinical ADR risk is provided by volatile anaesthetics[13] (discussed further below). For other drug classes, there is no simple quantitative relationship between the formation of a CRM, CB to a protein in *in vitro* test systems and clinical toxicity. Therefore, it is inappropriate to place reliance on preclinical evaluation of CRM formation as a simple generic index of the extent of drug safety risk.

Box 3 | **Physiological response to bioactivation**

Many species had evolved sophisticated and efficient cell defence systems for chemically reactive species long before drugs that also pose a chemical hazard were manufactured. Following exposure to acetaminophen or other xenobiotics, an array of transcription factors and signalling proteins have been implicated in sensing and potentially adapting to chemical stress with associated endogenous perturbation, and they include the following: transcription factor AP-1 (REF. 59), NF-κB[60], nuclear factor (erythroid-derived 2)-like 1 (NFE2L1; also known as NRF1)[61], NFE2L2 (also known as NRF2)[62], NFE2L3 (also known as NRF3)[63], signal transducer and activator of transcription 3 (STAT3)[36], heat shock factor protein 1 (HSF1)[35], pregnane X receptor[64], TWIST1 and E2A[34], redox factor 1 (REF. 65), tumour suppressor protein p53 (REF. 66), activating transcription factor 4 (REF. 67), CCAAT/enhancer-binding protein 1α[68] and the mitogen-activated protein kinases[69,70].

Although some proteins may be more important than others in determining the physiological response to stress, and there will be a degree of chemical specificity in driving this response, it is clear that no single pathway acts as the ultimate sole determinant of adaptation[7]. An important emerging theme in the wider cell signalling field is that functional interactions occur between many of these transcription factors, and experimental approaches for mapping these changes in a more holistic manner are emerging. Nevertheless, the induction of several key transcription factor pathways, such as NRF2, is clearly an important mechanism for adaptation to chemical toxicity, and through reactivity of key cysteine residues in the inhibitor of NRF2 — KEAP1 — this pathway can sense chemical danger and orchestrate cell defence[71–74]. Importantly, nuclear translocation of NRF2 occurs at non-toxic doses of acetaminophen and at time points before the manifestation of overt toxicity[75]. An important question that remains to be resolved is whether electrophilic metabolites that are potent inducers of this pathway are safer (that is, less toxic) than electrophilic metabolites that are non-inducers.

Ultimately, it is important to define mechanism-based and translational biomarkers that will help us to understand the basic biology that determines how impending stress is sensed. Ideally, these biomarkers should be applicable both in the clinic and in the laboratory for bridging purposes. In order to inform drug discovery processes, diagnostic biomarkers are required that will indicate the occurrence of chemical stress and discriminate between protein modification and oxidative stress after exposure to new chemical entities. The experimental evidence for adaptation might be utilized as an early indicator of potential toxicity, and markers of this may become valuable for preclinical toxicity testing and in the clinic.

This aspect of CRM formation has been discussed recently by Obach *et al.*[14], who have shown that quantification of CB to microsomal protein *in vitro* alone cannot be used to distinguish between hepatotoxic and non-hepatotoxic drugs. Comparative studies have shown that hepatocytes offer major advantages over liver microsomes, which is not surprising as they represent a more physiological system[17,21]. When it is considered together with the total dose administered and the fraction of the drug that is predicted to proceed through the metabolic pathway to form the reactive intermediate, CB data generated in hepatocytes offers the best delineation between hepatotoxic and non-hepatotoxic drugs, although some overlap in this classification remains[17,21]. However, as yet, there is no means of standardizing human hepatocytes with respect to quality, viability and reproducibility of metabolic performance. Such problems are compounded by inherent human variation caused by genetic and other host factors. There is, therefore, an urgent requirement to develop model systems that have a reproducible and efficient metabolic capacity in a physiologically relevant cell, in order to relate the chemistry of drug bioactivation to the primary biological responses in the human liver.

Drug metabolism and drug hypersensitivity

The most feared consequence of the discovery of a CRM at the earliest stages in drug development is that the only detectable biological outcome might be a severe life-threatening, idiosyncratic, hypersensitivity reaction that will only become apparent at a very late stage in drug development or in clinical use post-approval. Our understanding of the chemical basis of drug hypersensitivity is limited by the absence of validated animal models. Thus, *ex vivo* studies that are undertaken using lymphocytes from hypersensitive patients (that is, patients with an immune-mediated adverse reaction) represent the only relevant system to study cellular, molecular and chemical mechanisms. These studies confirm that hapten-specific T cells are present in hypersensitive patients and that T cell responses are directed against the drug metabolite-conjugated protein. Evidence linking drug metabolism and drug hypersensitivity is given in BOX 4.

The recently described strong association between expression of specific human leukocyte antigen (*HLA*) alleles and susceptibility to different forms of drug hypersensitivity represents a fundamental breakthrough in our understanding of drug hypersensitivity. The introduction of a HLA-B*5701 screen for patients with HIV who are commencing abacavir (Ziagen; GlaxoSmithKline) therapy has effectively prevented the adverse reaction to this drug[22,23]. Abacavir presentation to $CD8^+$ T cells is restricted to HLA-B*5701, and is not mediated by other closely related HLA types[23]. These data elegantly demonstrate that abacavir ligates a single immunological receptor to stimulate T cells; however, to date, the nature of the drug antigen (parent drug or metabolite), the drug–receptor binding interaction (covalent or non-covalent) and the involvement of protein processing have not been fully defined.

Box 4 | **Drug metabolism and drug hypersensitivity**

The perceived relationship between drug metabolism and drug hypersensitivity is based on two pieces of evidence:

- The obligatory role of covalent modification of protein as the basis of the haptenicity, antigenicity and immunogenicity of β-lactam antibiotics
- Numerous retrospective drug metabolism studies that have shown that many drugs that cause severe adverse drug reactions (ADRs), which are clearly immune-mediated, can undergo metabolic activation to form products that bind covalently to proteins, and thus have the potential to function as haptens, antigens and immunogens

It is possible to summarize our understanding of the role of chemically reactive metabolites (CRMs) in drug hypersensitivity with reference to three model drug allergens, discussed below.

Sulphamethoxazole

Both the parent drug and synthetic metabolites have been shown to interact directly with major histocompatibility complex (MHC) and T cell receptors with sufficient affinity to stimulate blood and skin-derived T cells[76–78]. Furthermore, in recent studies it has been shown that antigen presenting cells metabolize sulphamethoxazole; subsequent irreversible binding to endogenous proteins stimulates dendritic cell co-stimulatory signalling[79] and generates a functional T cell antigen[80]. Enhanced metabolism in immune cells might represent an important mechanism to explain the increased incidence of ADRs seen in patients with diseases such as cystic fibrosis and AIDS.

Halothane

The inhaled anaesthetic halothane stimulates an immune response that results in hepatitis in susceptible individuals. This reaction is mediated by a CRM (trifluoroacetyl chloride) that binds covalently to lysine residues on proteins[81]. Anti-immunoglobulin G antibodies directed against the CRM of halothane and microsomal proteins have been detected in patient sera[82]. More recently, drug metabolite-specific T cells have been described in murine models of halothane-induced tissue injury[83,84]. The role of bioactivation in the toxicity of halothane is perhaps best illustrated by a global consideration of the relationship between the *in vivo* metabolism of halocarbon-based general anaesthetics and the observed incidence of ADRs in humans. The incidence of anaesthetic-induced immune-mediated hepatitis declines as the extent of metabolism decreases[81].

Diclofenac

Covalent modification of proteins by reactive acyl glucuronides that might cause idiosyncratic ADRs in humans has been suggested to occur via drug hapten-induced activation of specific immune responses. Drug-specific T cell responses have been observed in patients who have experienced ADRs caused by diclofenac and other carboxylic acid-containing non-steroidal anti-inflammatory drugs (NSAIDs)[85]. Furthermore, antibodies targeted to diclofenac-modified liver protein adducts have been detected in sera from patients who have been treated with diclofenac[86]. For diclofenac and several other carboxylic acid-containing NSAIDs, it has been found that acyl glucuronide formation *in vivo* mediates selective covalent modification of several proteins located on the apical (bile canalicular) domain of the hepatocyte plasma membrane[56,58]. This might help to explain why many carboxylic acid-containing NSAIDs cause cholestatic liver injury in humans.

In the case of diclofenac, hepatic protein adduct formation requires functional expression of the biliary transporter ATP-binding cassette sub-family C member 2 (ABCC2, also known as MRP2), which mediates efflux of diclofenac-acyl glucuronide from hepatocytes into bile and has been implicated in intestinal toxicity[56,58]. Moreover, genotyping studies have demonstrated an association between UDP-glucuronosyltransferase 2B7, cytochrome P450 2C8 and ABCC2 genotypes, and susceptibility to diclofenac-induced liver injury in patients, which provides further support for an association between formation and cellular accumulation of reactive acyl glucuronide metabolites and the mechanism of toxicity[87]. Such studies illustrate how a complex interplay between host variables in drug metabolism and the immune response may combine to elicit a drug hypersensitivity reaction.

Management of CRMs during drug development

Given the issues associated with the detection of a CRM, the key question is: what relevance should be given to such reactive species during drug development? There is general agreement on several points:

- Drug bioactivation, in a generic sense, can confer no biological advantage, with the exception of certain classes of drugs that depend on the covalent modification of specific proteins for their pharmacological effect, such as β-lactam antibiotics, proton pump inhibitors and thienopyridine antiplatelet drugs.
- The simplest solution is to eliminate a chemical liability at the drug discovery phase (avoidance strategy) either by appropriate candidate selection (chemicals without this liability) or by early chemical modification. Thereafter, any decision to progress a drug with a 'bioactivation liability' is dependent on the weight of evidence and the process is very much dependent on the specific case.
- At present, there is no consensus on a preclinical strategy to investigate potential safety hazard and risk posed by bioactivation of a particular drug in humans.
- Only studies in humans can currently be used to discover mechanisms of serious idiosyncratic adverse reactions (ADRs), and determine 'cause and effect' with respect to bioactivation in humans and clinical outcome.
- Until the previous two points have been achieved for a number of paradigm compounds, it is not possible to perform an adequate risk assessment with respect to drug bioactivation.

Are currently used CRM decision trees relevant to the challenges posed in drug development? Within many pharmaceutical companies, all compounds are screened for their ability to form CRMs and go through a series of tests. Over time, 'decision trees' have evolved to help rationalize decision-making based on data generated. The identification of a CRM and the characterization of its behaviour may be enough to halt the development of a new compound.

The paper by Evans *et al.*[24] was a landmark publication for drug metabolism as it was the first paper that attempted to address CRM formation from a quantitative perspective for risk assessment. It has not been universally accepted because its implementation could reduce the overall rate of drug development and because of the uncertain relationship between CRM detection and level of safety risk. Nevertheless, it has provoked a much-needed debate, which has shifted the science from speculation based on qualitative data to hypothesis-driven experiments that demand robust quantitative end points. It has to be accepted that any decision tree needs to be based on incomplete science and that there may be insufficient predictivity of any given piece of data to be able to make a binary decision.

In FIG. 1 we provide a theoretical working relationship between chemistry and drug metabolism. Ideally, there should be two outcomes with respect to the management of a CRM problem: first, elimination of the chemical liability, and second, assessment of the total chemical insult. The interpretation of the second outcome requires a database of the following: drugs in current use that are deemed 'safe'; drugs in clinical use with an established frequency of ADRs that permit access to relevant patient cohorts; and drugs that have been withdrawn because of an ADR. In constructing such a training set we need to be mindful of the changing use of substructures in drug discovery. We need to use drugs with defined clinical toxicities as reagents to explore the unknown biology involved in serious ADRs.

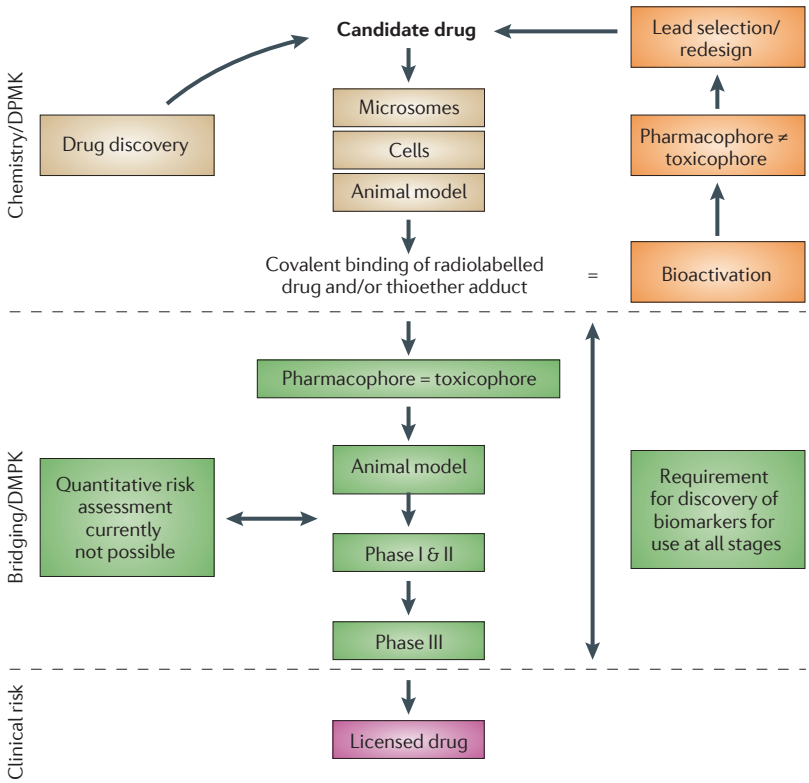


Figure 1 | **An ideal working relationship between chemistry and drug metabolism.** During drug design, if clear separation exists between the pharmacophore and the toxicophore, this allows assessment of total chemical insult and structural redesign before lead selection. If the pharmacophore incorporates the toxicophore, a more complex model emerges in which risk assessment is required from non-clinical through to the post-licensing stage. DMPK, drug metabolism and pharmacokinetics.

Addressing CRM-related risk in drug research. There are no definitive rules by which binary decisions can be made using CRM data as a new compound progresses through the research and development process. Owing to the broad range of toxicities that are presumed to be elicited by CRMs and their highly variable frequencies and severities, as well as the poor correlation between idiosyncratic ADRs in humans and the toxicity end points that are detected *in vivo* in laboratory animals, investigators are faced with the prospect of using CRM data to make decisions that have varying degrees of uncertainty. It should also be emphasized that, in general, data evolve through the research and development process. As investigational compounds progress, the assays increase in sophistication, predictivity and cost. However, as has been discussed, owing to the lack of a direct link between the formation of a CRM in preclinical studies and the ability to predict a clinical ADR, considerable uncertainty can remain throughout the discovery and development process.

Decision trees can be useful not just to aid in decision-making around complex issues, but also to highlight points of contention and areas for further scrutiny and research, especially for those topics possessing input factors that have a greater degree of uncertainty, such as CRM-related issues. In FIG. 2, examples of schemes are provided, which show various approaches that can be used to address CRM formation at different stages in drug development. It is important to understand that with the uncertainties and gaps in knowledge that such schemes immediately identify, it is impossible to develop a single decision tree that could be universally adopted.

CRM-related decisions based on minimal data (avoidance strategy). During early stages of the drug research process, there can be hundreds to thousands of compounds synthesized for any given disease target and this number of compounds precludes gathering extensive data sets on which decisions can be based for each compound. However, over the past several years, trends have developed for fundamental drug and pharmaceutical properties that can be leveraged in an 'avoidance strategy' for CRMs, shown in FIG. 2a. Key to any avoidance strategy is a parallel, iterative, medicinal chemistry effort to synthesize new analogues, or even to select an alternative chemical scaffold upon which to optimize the pharmacological and pharmaceutical properties and eliminate the potential for generation of CRMs altogether.

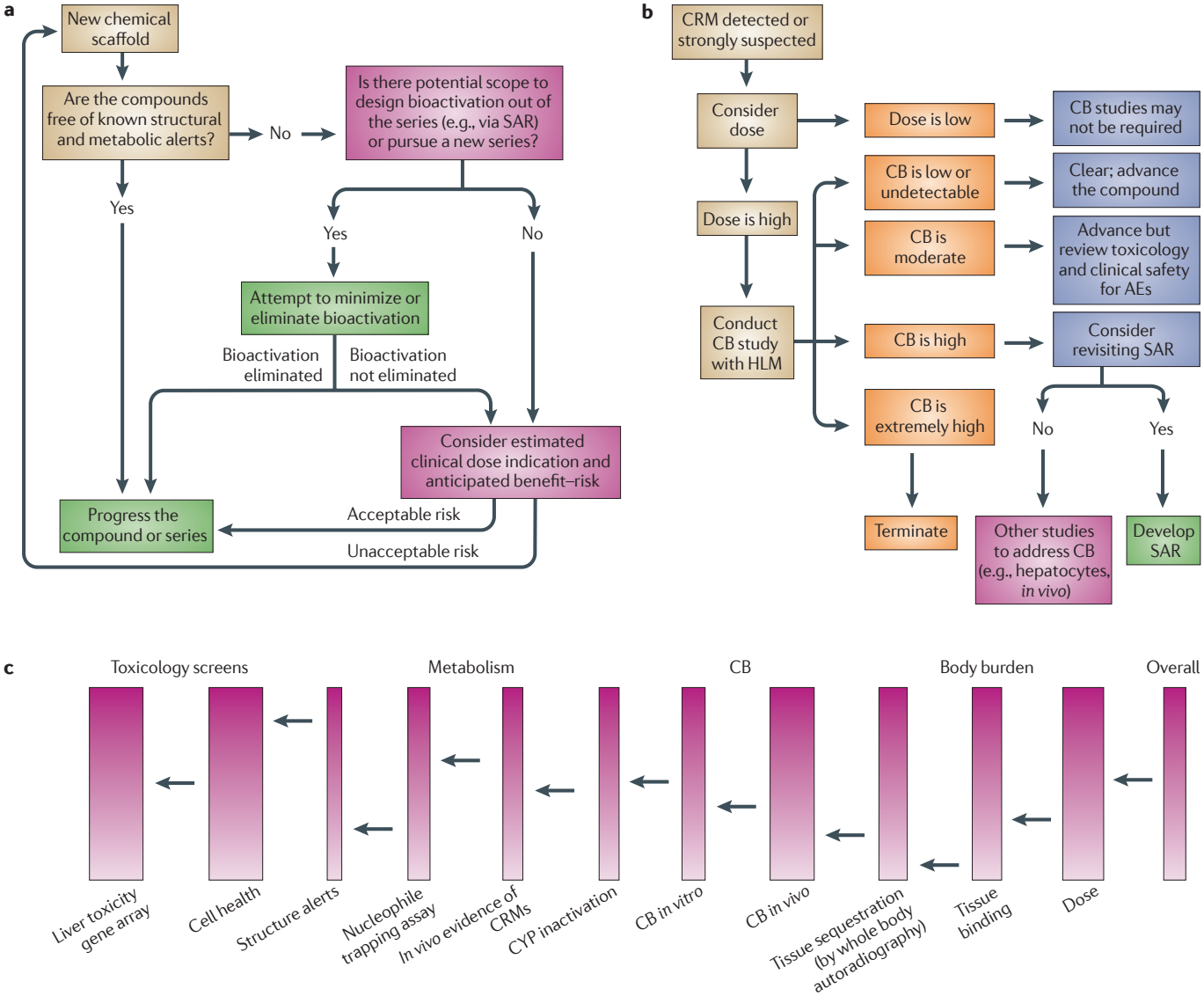


Figure 2 | **Examples of possible decision scheme structures for handling bioactivation information in various stages of drug research.** Utilizing information on reactive drug metabolites in decision-making regarding the potential for low-incidence toxicity is challenging and there is no single established decision pathway that can be followed in all cases. A few examples of decision schematics are illustrated, which depend on the phase of drug research and the input information that is available. In all cases, such decision schemes are predicated on important criteria regarding not only the technical aspects but also other considerations for any given drug research programme[89]. It is important to understand that these three decision schemes are presented as examples only. There is no universally applicable scheme that can handle this complex, multifactorial and sometimes nebulous issue. The links between experiments that are carried out to study reactive metabolites and toxicity outcomes in humans have not been well defined. Individual researchers and research organizations need to develop their own decision criteria and many of these will need to be customized for individual drug research projects. They will range from a view that reactive metabolites pose no threat to views that are very conservative and restrictive, depending on the risk-aversiveness of individual research organizations. **a** | Reactive metabolite data in early research during drug design and optimization. The 'avoidance strategy' can be applied early in the drug research phase when medicinal chemistry efforts on a project are still active. Compound design can be targeted to avoid reactive metabolite generation, provided that the offending bioactivated substituent can be designed out while still maintaining acceptable pharmacological properties. Other considerations include the anticipated daily dose and the urgency of the clinical need for a new treatment. **b** | Covalent binding (CB) data in late preclinical or early clinical research. In contrast to the scheme illustrated in part **a**, there is no option to redesign the compound, and CB data must be used in decision-making, for example, in deciding whether the compound should be further progressed in the absence of an extensive clinical safety database. Total dose, indication and existing preclinical toxicology data are taken into consideration in this decision scheme. **c** | Weight-of-evidence approaches in late preclinical or clinical phases of research consider multiple data end points together with data on reactive metabolite formation. As with the scheme illustrated in part **b**, this would only be appropriate in a drug development setting in which there is no longer an option to redesign the candidate compound. In a weight-of-evidence approach, multiple data sets that have some relationship to clinical safety are simultaneously considered. The output data for each parameter is considered with the positioning of the arrow as an indicator of the extent of 'concern' (the higher the arrow, the greater the issue), and the width of the bars depends on the relative importance of each end point in relation to the potential for toxicity. The sum total of these data are considered in decision-making. However, at this time, there is no specific formula that can be applied to objectively categorize compounds using this approach. AE, adverse events; CRM, chemically reactive metabolite; CYP, cytochrome P450; HLM, human liver microsomes; SAR, structure–activity relationship.

There are several basic assumptions that are required in the application of an avoidance strategy. First, it is assumed that there is a cause–effect relationship between the generation of CRMs and toxicity; this assumption is based on a large body of literature and is therefore reasonable. Second, it is assumed the total daily dose of a compound has a bearing on toxicities that are elicited via CRMs[25,26]. Third, knowledge of chemical structures that have been previously shown to be involved in the generation of CRMs is leveraged as part of the decision-making process. Consequently, there is a list of structural moieties that should be avoided in drug design, if possible[27]. Finally, *in vitro* CRM data is assumed to be useful for indicating the possibility of CRM formation *in vivo*.

The first step in the avoidance strategy is to ask whether the structure contains any known alerts for substituents that are frequently associated with the generation of CRMs. If so, can active medicinal chemistry efforts replace the substituent containing the potential liability with an alternative substituent that is not associated with the generation of CRMs? If, however, the structural motif that is associated with CRM generation is essential to the pharmacophore for the pharmacological target, then both *in vitro* and *in vivo* assays can be used to determine whether the compound will generate CRMs. It is possible, of course, that a structural alert may be present in a molecule but does not undergo bioactivation. The ability to confidently predict the sites of metabolism of new chemical entities *in silico* has not yet evolved to a satisfactory level, so the potential to generate CRMs must be determined experimentally. It is possible to develop structure–activity relationships to avoid the generation of CRMs and to direct metabolism to sites on the molecule that are not associated with reactivity. A further advantage of generating experimental data is that it minimizes the likelihood that important structural alerts will be overlooked during the chemical design phase. If CRMs are not detected, the safety and risk assessment can focus on potential causes of toxicity other than bioactivation.

If CRMs are detected or strongly suspected, and they cannot be eliminated using available chemical options, the next key question is: what is the anticipated dose range of the compound that is required for clinical efficacy? Although this estimation cannot be precise at an early stage of drug research, a number of contributory factors can be considered. General experience, acquired in the field of pharmacology in the past century, can be used to suggest whether a compound will be a low-dose (that is, <10 mg per day), mid-dose (~100 mg per day) or high-dose (>1,000 mg per day) drug. The intrinsic potency of a compound can determine this dose range, along with whether the compound will be highly bound in the plasma, readily penetrate biological membranes, be rapidly metabolized, and so on. If the compound is anticipated to be given at a low dose daily — <10mg per day — it is likely that these more potent drugs will be associated with a lower incidence of idiosyncratic ADRs; consequently, concerns regarding CRM-mediated toxicity will be diminished[28].

An additional important consideration, even at early stages of drug discovery, is the clinical indication. For example, the level of risk that is considered to be acceptable for a molecule or a chemical series is likely to be markedly higher if the aim is to treat a life-threatening condition for which there is an unmet clinical need than if the objective is to treat a chronic non-debilitating condition for which alternative therapies are already available.

CRM-related decisions based on CB and other data. Although the above avoidance strategy is feasible in the early drug design stage of research, as the number of individual compounds in any one research project is narrowed down to those possessing superior properties (that is, potency, efficacy, pharmaceutics, and so on), a greater amount of information can be gathered on individual compounds. This is especially true as an individual compound is progressed into the development phase. The schemes outlined in FIG. 2b;c are illustrative of different approaches to address CRM formation at later time points in the research and development process. The most relevant timeframe for conducting CB studies is during or before lead selection, when the information can be applied to develop structure–activity relationships to minimize metabolic activation. This was one of the key principles of the target criterion proposed by Evans *et al.*[24] for CB. In addition, this is a good example of rationalizing a decision point to stimulate a discussion regarding specific drug candidates rather than advocating a strict go/no-go decision point. An additional value of formulating a decision matrix is that future evaluation can provide indices of success of the process (see below).

The most frequently utilized quantitative method of measuring CRM formation is the determination of metabolism-dependent CB of radiolabelled compound to protein, typically human liver microsomal protein[29] (BOX 1). A scheme based on *in vitro* CB data is shown in FIG. 2b. Many pharmaceutical companies will code the decision points on a flow scheme as: go, no issues; caution, some issues; stop or go slow, some major issues; and definitive no-go (not just for this property but overriding other properties to halt development). Ascribing appropriate values for stopping development based on *in vitro* CB data will also depend on the medical need for the new therapy, how risk-averse a research organization decides to be, and the breadth of the chemical space that is available to avoid this issue while maintaining pharmacological potency (that is, going back into earlier research and selecting or designing an alternative candidate compound).

A decision also needs to be made regarding whether to conduct additional studies to provide some context to the possible level of risk. For example, hepatocytes provide a more complete metabolic process than hepatic microsomes[17,21] by offering both competing metabolic clearance routes and protective mechanisms (for example, through conjugation with GSH), and they could be used in a follow-up experiment to more accurately place *in vitro* CB data into the context of the potential for toxicity.

The use of *in vivo* surrogate animal models can provide valuable insights into the extent of CRM formation through CB, typically to liver and plasma proteins, although limitations of cross-species extrapolation must be considered. The fundamental question when interpreting CRM data is as follows: in the absence of a clearly defined relationship between CRM formation and detection and ADR risk in patients *in vivo*, how can CRM data be used to decide whether or not to progress an individual compound? There is clearly an arbitrary component to this decision when it is taken in the absence of data on toxicological outcome. This difficulty intensifies when this concept is presented to drug project teams and managers, to convince them to recommend spending considerable additional resources or even terminating the compound. Conversely, if it is believed that CRMs are inherently undesirable, a good decision point must be identified to terminate a compound based on sound experimental evidence.

FIGURE 2c illustrates a scheme providing a weight-of-evidence approach. Here, several factors are considered simultaneously, all of which may be related to the potential for ADRs arising from CRMs, but to varying degrees. As this approach requires substantial input of data, its most appropriate use is to support decision-making before entry into the development phase or during development, when resources can be used to undertake detailed profiling of any given compound. By considering the relative importance of the various criteria (for example, *in vitro* CB data, metabolic routes, animal tissue distribution data, *in vitro* safety data, and so on) no single data set overly influences the final decision. It is recognized that at this time there is no defined, data-driven manner in which to assign relative weights in a quantitative manner. However, knowledge gained within an organization using such data sets, across an entire portfolio of development compounds, could lead to a greater understanding of the type of profile that is associated with those compounds that have CRM-related safety issues. It may be the case that, across the entire pharmaceutical industry, a sufficiently robust data set could be gathered to develop reasonably reliable weight-of-evidence criteria for decision-making regarding compounds with CRM issues.

However, it is imperative for CRM management that these schemes are used with a great deal of caution, that they are continually challenged with emerging data and science, and that we avoid using them in an overly strict fashion. With these caveats in mind, FIG. 2 shows examples of schemes that have been constructed to address different aspects of dealing with CRMs. These range from a simple avoidance approach in early research to the later stage examples in which additional characteristics of the project also need to be considered. It is important to understand that with CRMs, the uncertainties and gaps in knowledge at this time preclude the development of any single scheme that can be universally adopted.

Experience gained from previous decision trees. A paper that was published in 2004 by Evans and colleagues[24] included a decision tree that proposed a target for CB of <50 pmol per mg protein. This pioneering work (reviewed in REF. 30) offered a definitive proposal for a very difficult and controversial scientific topic and provided a database that was unique to Merck. Taking advantage of this information, an internal review of CB studies was conducted at Merck in 2009. This internal review indicated that CB issues were present in 25% of lead optimization programmes and that, on average, 6 months were required for project teams to redesign a compound with abrogated metabolic activation while retaining pharmacological potency and other desirable drug-like qualities.

A comparison of ~100 Merck drug candidates indicated that there was no correlation between incidence of liver toxicity observed *in vivo* in preclinical safety studies and level of CB (measured as pmol bound per mg protein, either *in vitro* or *in vivo*; TABLE 1). For this assessment, outcomes such as elevated alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase or histopathology findings were considered to be positive, whereas hepatomegaly, phospholipidosis and vacuolization were considered to be negative as bioactivation was deemed unlikely to be the causative factor. For all ranges of CB observed, all groups gave a

Table 1 | **Merck development candidates: CB data and liver findings of concern from rat studies***

Study	Observed pmol‡	*n*	Indication of toxicity (%)§	
			Positive	Negative
In vitro (LM)	<50	54	17	83
	50–200	33	15	85
	>200‖	13	0	100
In vitro (LM + GSH)	<50	51	17	83
	50–220	16	14	86
In vivo	<50	78	14	86
	50–150	9	22	78

CB, covalent binding; GSH, glutathione. *(D.A.N.-G., unpublished observations). ‡pmol refers to pmol equivalent per (mg protein at 1 hour) for *in vitro* studies and pmol equivalent per mg protein for *in vivo* investigations. §Toxicity findings were defined as outlined in the section entitled 'Experience gained from previous decision trees'. ‖Out of the 13 compounds in this group, 12 exhibited values ranging from 204 pmol per mg protein to 621 pmol per mg protein (mean = 374), whereas one compound gave a value of 1,680 pmol per mg protein, using covalent-binding methodology described in REF. 24 and REF. 29. LM, liver microsomes.

findings incidence in the range of 0–22%. However, it should be noted that relatively few compounds with CB considerably in excess of 200 pmol equivalent per mg protein *in vitro* and none *in vivo* were represented in the analysis, as these were not progressed to preclinical safety evaluation; this is recognized as a limitation of the analysis.

Based on these findings, consideration was given to increase the target CB value that was considered to be indicative of potential safety risk to above 50 pmol per mg protein. However, no clear scientific evidence could be found to define an alternative target value. CB studies are still conducted at Merck, but not on a routine basis for all programmes. These studies are reserved for use in an issue-driven fashion for problem solving in the event that toxicity findings are repeatedly observed with analogues, and in which bioactivation is suspected to be the causative factor. Lead optimization teams are encouraged to avoid structural alerts where possible[8,27], to note the formation of GSH adducts in *in vitro* and *in vivo* experiments, using techniques such as high resolution mass spectroscopy[31], and to strive to reduce time-dependent inhibition (TDI) of cytochrome P450 enzymes, as mechanisms for TDI are frequently related to CRM formation[32,33]. Finally, consistent with concepts described above, project teams are encouraged to improve potency and pharmacokinetic properties in order to reduce the predicted efficacious dose and reduce total body burden[17].

This retrospective study by the Merck group is consistent with the findings of assessments by other pharmaceutical companies[34–36]. These studies demonstrated a lack of simple correlation between measurements of CB and the risk of hepatotoxicity when values for CB were relatively low and viewed in isolation. Nevertheless, when it was considered in the context of additional indices of bioactivation (for example, the propensity for GSH-conjugate formation, projected clinical dose, body burden of CRMs, presence of structural alerts, TDI, and so on), CB was found to be a valuable parameter that aided in the assessment of overall toxicological risk. The lack of an overt correlation between CB and preclinical liver toxicity of candidate pharmaceuticals is perhaps not surprising, given the multidimensional nature of drug-induced liver injury. Also, it should be recognized that measurements of gross CB, as they are presently conducted, represent a very crude tool in assessing the exposure of tissues to biological CRMs. A critical question will be: which components of the proteome represent the key targets for reactive drug metabolites that trigger a toxicological response? Ongoing studies that utilize the technique of mass spectrometry are likely to provide the basis for such research.

Implications for drug regulation

It appears that the formation of a CRM has never presented itself as an issue for the UK regulatory authorities. Therefore, there are no regulatory guidelines to deal with detection of drug bioactivation, or to assess the safety of drugs associated with the metabolic process. Overall, the reason for the lack of regulatory guidelines worldwide for dealing with CRMs is that our level of scientific understanding of the toxicological consequences of bioactivation remains poorly developed, and is insufficient to warrant regulation.

Examining excreta allows the determination of the proportion of metabolites that are derived from primary clearance pathways and thereby validates *in vitro* enzymology, which predicts contributing factors to inter-patient variability (for example, polymorphisms and drug–drug interactions). Excreted metabolite studies are the most likely source of information on the formation of CRMs. They are identified as stable downstream products (such as GSH conjugates) of the CRM.

If humans produce unique metabolites, then evaluation of the risk that these pose may involve their synthesis and administration to experimental animals.

Box 5 | **Academic approaches for the investigation of CRMs**

It has been recognized that there are multiple, parallel, biological outcomes with respect to the formation of even the simplest models of chemically reactive metabolites (CRMs). Tissue damage can be both a multistep and multicellular process. It was therefore anticipated that to fully understand the mechanism of a model hepatotoxin — such as acetaminophen — and of other well-established causes of drug-induced liver injury (DILI) in humans (for example, with isoniazid and nevirapine), which occur at frequencies that are amenable to prospective investigation, a systems-pharmacology approach is needed, which integrates:

- Drug metabolism, including exposure biomarkers for CRMs (for example, glutathione (GSH) conjugates, related thiol metabolites and protein adducts)
- Conventional safety end points *in vivo* in preclinical species
- *In vitro* toxicity data obtained in relevant cell model systems (for example, hepatocytes)
- Use of metabolomics, transcriptomics, proteomics and other profiling technologies to profile biological responses at the tissue and cellular level
- Mechanism-based blood-borne biomarkers for chemical stress responses, apoptosis and necrosis, inflammation, and innate and adaptive immune responses
- Novel biomarkers that can be used to bridge between *in vitro* systems, animal models and human studies

With respect to biomarkers for drug hypersensitivity, the MRC Centre for Drug Safety Science has developed an integrated genetic–proteomic–cellular–phenotype approach to investigate mechanisms of toxicity using clinical cohorts of patients on established drugs. It is important that problems with new drugs are fed into this system immediately in order to capture potentially new mechanisms as they happen — that is, before the drug is withdrawn and patients are no longer accessible. The development of a systems pharmacology approach to assess CRM-mediated toxicity will be complex, and can only be realized through the formation of consortia (involving academia and industry) that can collectively provide the necessary expertise, technology and resources. The outcome of such work must be linked to development of *in vitro* systems that are relevant to humans and can be used in the pharmaceutical company setting.

It is now appreciated that adverse drug reactions are a sum of the function of the chemistry of the molecule (f_1) and a function of the biology of the patient (f_2) (see equation).

$$\text{Frequency/severity of adverse drug reaction} = f_1\{\text{Chemistry of drug}\} + f_2\{\text{Biology of individual}\}$$

In the limiting situation in which f_2 is greater than f_1, the reaction is, by definition, idiosyncratic. We require established consortia for the retrospective analysis of drugs that have failed in late development, which can be used as reagents to define f_1 and f_2. The outcome of this type of work should be directed towards further refinement of the aforementioned *in vitro* screening systems and towards providing predictive test systems for personalized medicines.

Box 6 | **Advances in identification of key protein adducts**

Major advances in proteomics technology (for example, two-dimensional SDS–PAGE separation techniques combined with autoradiography, mass spectrometry and/or immunoblotting techniques) have allowed for a more precise identification of protein adducts. In 2007, more than 120 individual target proteins of drugs and/or other xenobiotics were identified[39]. A web-accessible Target Protein Database was subsequently created for the storage of such information and to facilitate the identification of common protein alkylation patterns of different drugs and/or toxicants. The number of identified target proteins in this database is rapidly expanding — at the end of 2008 this number was increased to 268. For example, 32 target hepatic proteins of acetaminophen, 32 target lung proteins of BHT (2,6-di-tert-butyl-4-hydroxytoluene), 17 target proteins of naphthalene and more than 46 target proteins of bromobenzene have currently been identified[38,88].

Recent technological developments in protein targets (for example, shotgun proteomics) and in protein functional assays should allow the study of specific biological consequences of protein adduction and the outcomes from these studies are eagerly awaited, although this will be a significant technical challenge. A relevant paradigm to exemplify this challenge is the cell-protective nuclear factor (erythroid-derived 2)-like 2 (NFE2L2; also known as NRF2) pathway and its regulation by its inhibitor: the protein KEAP1. There are currently no means available to measure precisely how, and indeed if, the modifications that are typically measured among the cysteine residues in KEAP1 following exposure to chemically reactive metabolites are translated into biological effects. Therefore, although there is strong evidence to suggest that NRF2-activating molecules directly modify cysteine residues in KEAP1 *in vitro* and in living cells, it has yet to be demonstrated unequivocally that modification of KEAP1 triggers the activation of NRF2 *in vivo*.

However, it may be both impractical and irrelevant to synthesize and dose experimental animals with most CRMs, because of their inherent chemical instability and their deleterious effects on systems that are distal to their physiologically relevant site of formation. One exception could be acyl glucuronides. However, it was agreed by participants from both industry and academic institutions that no one has yet provided direct evidence for the toxicity caused by this class of CRMs, despite the fact that they are referred to collectively in the recent US Food and Drug Administration guidance document as "toxic molecules". Clearly, relevant model systems need to be invented to provide the data required to allow evidence-based decision-making. Academic approaches for the investigation of CRMs are described in BOX 5.

Conclusions and identification of knowledge gaps

Recent developments in molecular toxicology have provided new technologies and methodologies that have greatly improved our fundamental understanding of the role of drug metabolism in ADRs. However, much remains to be done to further our understanding of basic mechanisms, and to enable the translation of the knowledge and methods to the prediction of drug safety. It is clear that CRMs still pose many unsolved problems and paradoxes, namely:

- CB should be regarded as a marker of bioactivation and not toxicity and therefore must be discussed in the context of drug metabolism and chemical structure
- *In vitro* studies of drug metabolism cannot predict risk in either experimental animals or humans, and can only identify potential hazards
- Not all idiosyncratic drug toxicity involves either the immune system or CRMs; therefore, chemical modification to remove this metabolic liability is not a universal panacea for ADRs
- It is not possible to dismiss the potential hazard associated with drug bioactivation based on current science
- If the potential liability associated with a particular bioactivation pathway is removed without evaluation of toxicity, we will never be able to measure the success of the process; continued investment is required in approaches and methodologies that can evaluate the contribution of bioactivation to toxicity
- There is a need to inform not only those involved in the drug development process but also those involved in drug regulation, as well as the public, that preclinical scientists do not yet have the tools to define, let alone predict, the role of CRMs in serious ADRs

Therefore, there is a need for more basic research on model CRMs; methods that reflect *in vivo* exposure to CRMs (that is, both recent exposure (for example, using GSH conjugates and related thioether compounds) and previous exposure (for example, using protein adducts)); and drugs that are associated with ADRs for which there is access to patient material. There is also a need for such drug safety science research to be better directed towards informing both the chemist and the clinician about risk and hazard identification.

Although recent developments in molecular toxicology have increased our understanding of how drug metabolism may contribute to drug toxicity, we are still some way from being able to predict toxicity based on chemical structure alone. Not all drugs that can undergo bioactivation by human metabolizing enzymes are associated with ADRs in the clinic, and drug bioactivation may not always be a mandatory step in drug toxicity. Experiments that elucidate the chemistry of molecules are not sufficient to predict the biological outcome. We therefore need to be able to identify the proteins that are chemically modified and understand whether they have a critical or non-critical role (BOX 6). Generating this information will require concerted efforts in the screening of cellular proteins that are exposed to CRMs from compounds that form metabolites and that may or may not have toxicity associated with them.

It will be important to generate these data in physiologically relevant models. Currently, the models are not ideal and efforts must be focused on understanding the results from *in vitro* screening systems and determining

whether they reflect exposure in patients. For example, do the CRM–protein adducts and protein changes that are observed in liver microsomes reflect what is happening in humans? Should we be moving away from microsomes and towards human hepatocytes? Even when suitable *in vitro* systems are available, they will clearly not exactly replicate the DMPK experience that a compound undergoes in patients; therefore, a lot more effort must be placed in obtaining relevant samples from patients who have been exposed to new compounds, especially those patients who present with toxicities. By characterizing the changes observed in patients, we will begin to generate the quality of data that we require to unravel the mechanism by which a given CRM impinges on protein function and is ultimately linked to toxicity.

The evidence to date is that although the assays that are currently used allow us to determine chemical hazard, the data generated is not of sufficient quality or quantity to form a decision tree that can enable robust decision-making on whether or not a compound that forms CRMs should progress through drug development. Currently, we are only in a position to produce a risk assessment plan upon which decisions can be made, and it is clear that we need a much better definition of the additional data that are required to do this more effectively. The ability to assess risk will be augmented by the generation of more clinically relevant data and a thorough reassessment of the value and limitations of the tools currently used. In this context, better biomarkers of toxicity will become as important as biomarkers of efficacy in the development of future drug candidates.

Ultimately, a completely new approach is required to develop a mechanistic understanding and inform not only the medicinal chemist but also both non-clinical and clinical drug safety clinicians of the potential risks associated with CRM formation. Central to this approach will be an integrated informatics approach that encompasses chemoinformatics, systems biology and clinical informatics.

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Competing interests statement

The authors declare competing financial interests: see Web version for details.

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