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Idiosyncratic toxicity: the role of toxicophores and bioactivation

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Drugs and chemicals can undergo enzyme-catalyzed bioactivation reactions within cellular systems, with the formation of reactive chemical species. These reactive metabolites can lead to thiol depletion, reversible protein modification (glutathionylation and nitration), further irreversible protein adduct formation and subsequent irreversible protein damage. The incorporation of potentially reactive chemical moieties – toxicophores – within new therapeutic agents should be limited. However, this cannot always be prevented, particularly when the structural feature responsible for toxicity is also responsible for the pharmacological efficacy. The identification and further knowledge of critical levels of thiol depletion and/or covalent modification of protein will aid in the development of new drugs. Importantly, the identification of drug–thiol conjugation should provide a warning of potential problems, yet not hinder the development of a potentially therapeutically useful drug.

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▼ The ability to predict the safety profile of new chemical entities will transform the drug development process. It is currently impossible to identify patients who are likely to experience an adverse drug reaction (ADR) from the chemical structure of the drug. Drug toxicity is a major complication of drug therapy and drug development [1,2]. Potentially fatal, but rare, idiosyncratic ADRs are usually only detected when the drug has been used in a large number of patients. Almost any body system may be adversely affected by drugs; however, common ADR's include hepatotoxicity, severe cutaneous reactions, anaphylaxis and blood dyscrasias [3]. Drug-induced liver injury has become the number one cause of acute liver failure, with most cases requiring a liver transplant and some resulting in death [4]. During the development of a new therapeutic chemical, drug manufacturers conduct pre-clinical toxicity tests with the aim of identifying potential tissue damage and cellular stress. The main focus of these tests is hepatocellular injury. Liver function testing is conducted on humans to hopefully detect possible adverse reactions, which might then prohibit further development. Aside from the distress caused to the individual and their family, such reactions are costly to the pharmaceutical manufacturers. In 1998, the pharmaceutical industry spent more than US$20 billion on drug discovery and development, with more than one-fifth of this total being put into screening assays and toxicity testing [5]. A thorough molecular knowledge concerning the early events in drug-induced toxicity is required to aid the decision-making processes during the development of new therapeutic agents.

The problem – reactive metabolites?

It is generally thought that reactive, electrophilic compounds, formed either from the parent drug (e.g. a reactive quinoneimine from paracetamol) or as a consequence of increased cellular production of reactive oxygen and/or nitrogen species (hydroxyl radical, superoxide and peroxynitrite) are responsible for initiating toxicity. Reactive metabolites can cause tissue damage by direct modification of cellular proteins, such as covalent binding of the drug to the protein, or oxidation of redox-sensitive thiol or amine groups that are contained within the protein. This can subsequently initiate apoptosis/necrosis, perturbation of the host's immune system (often referred to as drug hypersensitivity or drug allergy) or modification of DNA, leading to carcinogenicity or teratogenicity.

In 1998, the non-steroidal anti-inflammatory drug (NSAID) bromfenac was withdrawn after less than a year on the market. The FDA received 20 reports of serious hepatotoxicty, four of these individuals died of liver failure and eight required liver transplants [6]. One

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of the most important factors that can influence drug disposition is phase I and II drug biotransformations, leading to increased hydrophilicity and excretion or, in certain cases, bioactivation. Using bromfenac as an example, further chemical knowledge of the toxicophores contained within the molecule could have saved lives (Figure 1) [7]. The first toxicophore is the bromophenol moiety, similar to the model hepatotoxicant, bromobenzene, which induces liver injury through enzymatic activation to reactive, epoxide and quinone metabolites [8–10]. These reactive metabolites are able to elicit critical cellular damage by binding cellular proteins through sulphydryl modification, once the key detoxification pathway (i.e. glutathione (GSH) conjugation) has been overwhelmed [11–16].

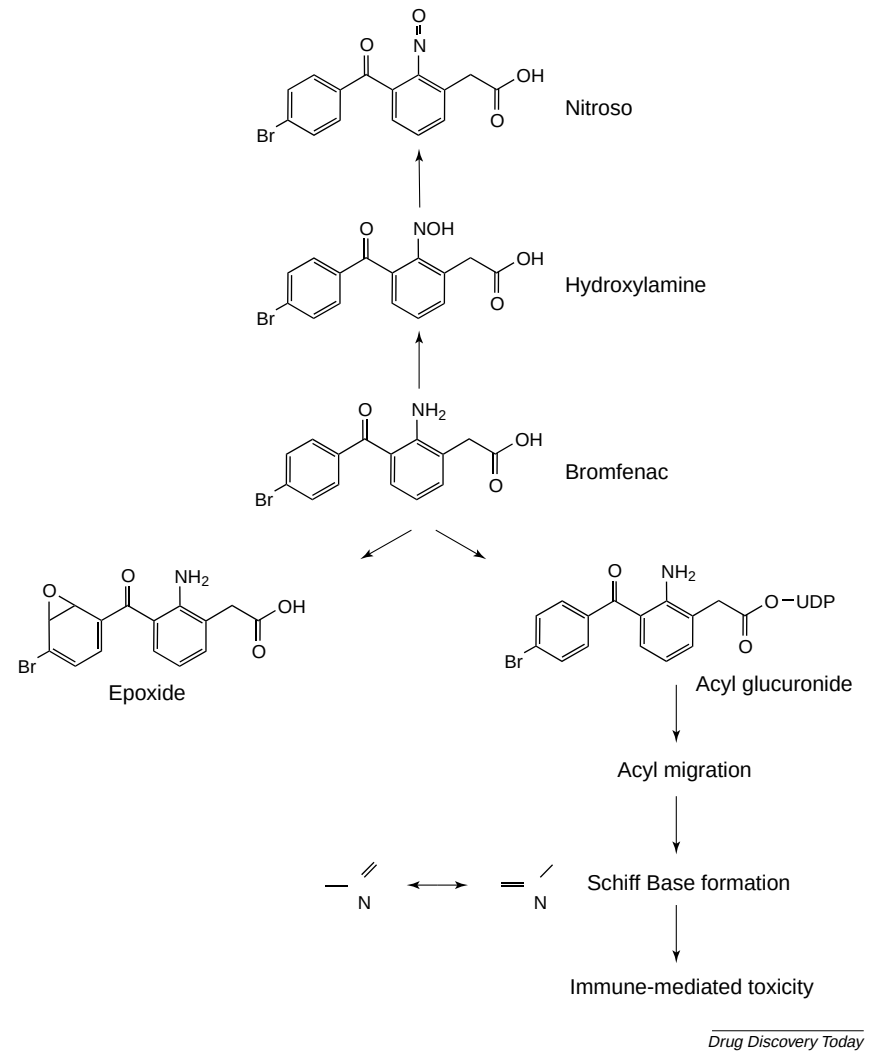

Figure 1. Potential reactive metabolites formed from bromfenac, an NSAID that was withdrawn from the market in 1998, due to reports of idiosyncratic hepatotoxicity.

The second toxicophore contained within bromfenac is the aniline ring [7], with the potential to form reactive nitroso compounds. The sulphonamides are a group of chemicals that contain an aniline ring structure. Sulfonamides, such as sulfamethoxazole, cause a variety of unpredictable drug reactions (fever, skin rashes, hepatitis, nephritis and blood dyscrasias) [17,18]. The identification of drug-specific T cells from peripheral blood and blister fluid provides convincing evidence that adverse events associated with administration of sulfamethoxazole require activation of the host's immune system [19,20]. According to the hapten hypothesis (reactive metabolite binds to endogenous protein, which is then recognized as a 'non-self' protein, and an immune response is initiated) and the danger hypothesis (reactive metabolite binds to protein and causes stress or damage, a subsequent 'danger' signal is released, combining to elicit an immune response [21]), immune activation is thought to involve drug bioactivation and covalent binding to protein. Sulfamethoxazole is not protein-reactive *per se*; hepatic cytochrome P450-mediated oxidation of the terminal amino group generates a pro-reactive hydroxylamine. This, in turn, can become (auto)oxidized yielding nitroso sulfamethoxazole, an electrophilic metabolite that is able to haptenate cysteine residues in proteins, including those on the surface of viable lymphocytes [22].

The third potential toxicophore contained within bromfenac is the arylacetic acid moiety, commonly contained within NSAIDs [7]. Although the chemical structures of NSAIDs differ considerably, many of them contain arylacetic acid, 2-arylpropionic acid or anthranilic acid derivatives [23]. The anti-inflammatory drug, diclofenac, has been associated with severe hepatic injury [24–26]. Most evidence suggests that phase II conjugation of diclofenac to form an acyl glucuronide is responsible for the toxicity [27]; but more recently, the acyl-CoA synthase-catalyzed formation of acyl-CoA thioesters has been proposed [23].

The diuretic, furosemide, can produce massive hepatic necrosis in mice, at a dose of 300 mg kg^{-1}, by a mechanism independent of its diuretic effect [28]. This has also been demonstrated in rats and hamsters at doses of 400 mg kg^{-1} [29]. However, there are no reports of liver necrosis in

humans. The combination of the therapeutic use of the drug, coupled with its high potency (typical dose is 20 mg per day) means that small doses produce suitable therapeutic effects. Furosemide can be converted by microsomal enzymes to a protein reactive metabolite, which binds covalently with hepatic macromolecules *in vivo* and *in vitro* [30]. Incubation with inhibitors of P450 enzymes, decreased the incidence and severity of furosemide-induced hepatic necrosis [28], suggesting that the toxic metabolite is the result of a P450 enzyme-mediated metabolic route. The hepatic lesion is reproducible in mice with at least eight other furan-containing compounds, including 2-acetylfuran and furan itself [28]. *In vitro* and *in vivo* radiolabelling experiments demonstrate that the entire furosemide molecule is covalently bound to protein and suggest metabolic activation of the furan ring, possibly through epoxidation [30]. Hepatic glutathione levels were not altered by furosemide administration to mice before a hepatotoxic response [28]. Furosemide has also been shown to have no effect on cytosolic or mitochondrial glutathione content in mice [31], and it has been reported that no glutathione depletion was seen in rat hepatocytes [32]. However, a reduction in cellular glutathione content has been reported in isolated mouse hepatocytes [33]. It is highly likely that a new chemical entity, with the same pre-clinical toxicity profile as furosemide, would be stopped in further development today, preventing the licensing of a highly useful drug with a low incidence of toxicity.

In certain cases, toxicophores may be linked with the mode of action of the drug. Removal of the terminal amino moiety of sulfamethoxazole will prevent bioactivation and might also prevent the direct interaction of sulfamethoxazole with T cells; however, because the mechanism of action of sulfamethoxazole competes with that of 3-aminobenzoic acid, which is essential for bacterial folic acid synthesis, it appears unlikely that a synthetic analogue of sulfamethoxazole, that retains efficacy but does not cause toxicity, will be developed.

The solution?

The first alert to potential toxicity should be the identification of potential toxicophores within the proposed candidate compound, by the medicinal chemist or the drug metabolist. Although this is not a sufficient reason for stopping drug development at this stage, it should serve as a warning. Obviously, prevention of the release of potentially toxic drugs on the market takes a high priority. However, with the growing use and stringency of toxicity screens, there is an increased chance of false positives, leading to the failure of a therapeutically useful new chemical entity [4].

The potential for toxicity of a particular compound depends on the specific disposition, efficacy and dose of the compound in question. The antipsychotic drug, clozapine, has been shown to be metabolically bioactivated by human liver and peripheral neutrophils to a toxic, protein-reactive nitrenium metabolite [34–37]. It is thought that this metabolite is primarily involved in the initiation of agranulocytosis that is associated with clozapine use [38]. Olanzepine, a neuroleptic with a similar structure to clozapine, also contains the potential to undergo nitrenium ion formation [39] but, to date, it has not been associated with a significant incidence of agranulocytosis. It has been suggested that a possible reason for the difference in toxicity between the two compounds with potentially similar mechanisms of bioactivation is the maximum daily dose. Olanzepine is given at ~10 mg per day, whereas clozapine is given up to ~900 mg per day [40]. The difference in dose might lead to a decreased mass of compound available for bioactivation to a reactive metabolite. This theory suggests a dose-dependent relationship between reactive metabolite formation and development of idiosyncratic toxicity. However, it has been demonstrated that the reactive metabolite formed from olanzepine is inherently less toxic to peripheral human neutrophils than the nitrenium ion that is derived from clozapine [41]. However, this potential dose-response relationship might become shifted during the duration of therapy. For example, the contraction of a viral infection or inflammatory condition, that is unrelated to the administered drug, could alter the metabolism of the drug or increase the sensitivity of cellular components, initiating toxicity. It has been demonstrated that an underlying viral infection in mice can potentiate the hepatotoxicity caused by N-acetyl-p-benzoquinoneimine, the reactive metabolite of paracetamol [42,43]. In this case, the observed toxicity is dose-dependent, not idiosyncratic, but viral-induced changes lead to increased susceptibility to hepatotoxicity. Additionally, elevated serum creatinine kinase was demonstrated in rats that were treated concurrently with the phenothiazine antipsychotic, chlorpromazine, and bacterial lipopolysaccharide, compared to rats that were treated with either compound alone [44]. The clinical use of chlorpromazine has resulted in idiosyncratic reactions that include hepatic cholestasis, neuroleptic malignant syndrome, and increased serum creatinine kinase activity, suggestive of rhabdomyolysis [44].

Detection of cell stress

Further knowledge of the molecular consequences of drug bioactivation, downstream from glutathione depletion and covalent binding, are required to ultimately define a drug and/or particular dose as toxic or non-toxic. Downstream

molecular pathways, such as transcription factor activation, gene and protein expression, and protein degradation, will give greater insight into the underlying mechanisms of toxicity. Transcription factors (proteins involved in cellular signalling and modulation of gene expression) are generally resident within the cytosol in an inactive form. Upon activation, often resulting from protein phosphorylation or modification of the redox state of the protein, nuclear localization occurs. Once inside the nucleus, the transcription factor is able to bind to its consensus sequence, usually located within the promoter region of gene targets for the particular transcription factor, thereby initiating gene expression [45–48]. Transcription factor activity can be regulated by thiol interactions at several levels. Binding of the transcription factor to DNA can be sensitive to either the thiol/disulphide redox status or to the presence of specific soluble thiols. Activation and translocation of the transcription factors to the nucleus might be oxidant sensitive, and finally, synthesis of the transcription factor or its subunits might, itself, be regulated by thiols [46,49–51].

Table 1. Comparison of Nrf2-mediated gene expression from toxicogenomic studies

Paracetamol	Bromobenzene
Epoxide hydrolase 1	Microsomal epoxide hydrolase
Aldehyde reductase	Aflatoxin B1 aldehyde reductase
NQO1	NADPH-cytochrome P450 oxidoreductase
GST Ya	GST Ya
GST Mu type 2	GST Mu type 2
γ-GCS heavy subunit	γ-GCS light subunit
Glutathione peroxidase	Glutathione peroxidase
HSP 40 and 86	Heme oxygenase gene
Glutathione reductase	Peroxiredoxin 1
Aldehyde oxidase	Ferritin-H subunit
Cyp 1A2	Proteasome subunit β type 3
26S proteasome	Proteasome subunit α type 1
28S proteasome	Proteasome subunit β type 5 precursor

Data taken from references [60–62] and Williams *et al.*, unpublished data, compared with Nrf2-mediated genes [52,59].
Abbreviations: Cyp, cytochrome P450; GCS, glutamyl cysteine synthetase; GST, glutathione S transferase.

Transcription factor regulation

The transcription factor nuclear factor erythroid-2 related factor (Nrf2) is currently the subject of intense investigation into chemotherapy and drug toxicity. Nrf2 nuclear localization has been shown to result in the expression of numerous defensive genes, such as NAD(P)H:quinone oxidoreducatse-1 (NQO1) and heat shock proteins (HSPs) [52]. It has also been confirmed that Nrf2 associates with a novel cytoplasmic protein, Kelch-like ECH-associated protein1 (Keap1), that directly negatively regulates the transactivation potential of Nrf2 [53]. It was observed that electrophilic agents prevent Nrf2 transactivation repression by Keap1, causing the recompartmentalization of Nrf2 to the nucleus [53]. Recent work has demonstrated that the repression by Keap1 appears to direct Nrf2 towards proteosome-mediated degradation under conditions of normal cellular homeostasis [54,55]. Initiation of the uncoupling of Keap1–Nrf2 is thought to involve oxidation of one or more reactive thiol groups within the Keap1 molecule [56]. It is clear that Nrf2 has an important role in the mobilization of intrinsic cellular defence mechanisms, and that the regulation of its activity is one of the primary sensors for cellular response to oxidative stress.

Chemopreventative agents, such as dithiolethione compounds (D3T, oltipraz) and sulphoraphane, induce the expression of phase II enzymes in various organs [52,53,57], and this gene expression is dependent on Nrf2 nuclear translocation [57,58]. The use of microarrays and Nrf2 knockout mice has provided an important insight into the genes that display Nrf2-mediated expression on D3T and sulphoraphane treatment [52,59]. A comparison of this investigation with toxicogenomic studies of hepatic gene expression in response to paracetamol and bromobenzene demonstrates the regulation of several Nrf2-mediated genes [60–62] (Table 1). The regulation of these genes indicates the initiation of early hepatocellular stress-reducing mechanisms. However, owing to the diverse nature of the initiating chemical species and the reactive metabolite formed from them, the ultimate pathological outcome and the molecular pathways leading to it might vary greatly. It is interesting to note that in proteomic investigations concerning paracetamol and bromobenzene, there appears to be little correlation between gene and protein expression [60,62]; but there was a greater correlation between microarray and proteomic data in rat liver after treatment with carbon tetrachloride [63]. Here, gene expression was determined after six hours and 24 hours, whereas protein expression was studied only at 24 hours after administration. In a comparison between gene (microarray) and

protein [2D electrophoresis (2DE)/MALDI-MS)] regulation, similar expression levels were found in 12 out of the 21 genes or proteins investigated [63]. Significant correlations between gene and protein expression, determined by microarray and proteomic methods are likely to be difficult to obtain, owing to the half-life of the mRNA and proteins concerned. Indeed, the possibility that increased mRNA expression might not lead to increased amounts of the proteins under consideration adds another level of complexity.

Downstream considerations

The majority of idiosyncratic drug reactions (IDRs) at initial challenge, require weeks, if not months, of chronic dosing, before they are apparent [21,64]. This is suggestive of an accumulation or chronic depletion mechanism. For example, accumulation of a drug–protein conjugate might eventually initiate a toxic or immune reaction when the concentration of conjugate reaches a specific level. Conversely, chronic low-level reactive metabolite formation could lead to lowered antioxidant levels and/or protein malfunction, which increases over time. But what is the nature of the underlying mechanism? Chronic administration of low doses of paracetamol to rats and mice over a period of 4–7 days provided protection against a subsequent hepatotoxic or lethal dose [65,66]. This protective effect has been attributed to several factors, including up-regulation of hepatic glucose-6-phosphate dehydrogenase and glutathione reductase [65], and down-regulation of CYP2E1 and 1A2 with increased glutathione and increased hepatocyte proliferation, leading to a diffuse distribution of covalent binding, and hence, enabling tissue repair and recovery [66].

In the context of down-regulation of enzymes that are responsible for drug bioactivation and up-regulation of defence systems, it is possible that the covalent modification of proteins might not lead to the development of toxicity. It has recently been demonstrated in rat liver that acrylonitrile can undergo selective Michael addition to only one of five cysteine residues within the enzyme carbonic anhydrase III. The true physiological function of carbonic anhydrase is unknown. One cysteine group in the enzyme (CYS 186) has been shown to undergo reversible S-thiolation with glutathione, which is proposed to offer protection to reactive cysteine residues in proteins from damage by irreversible oxidative reactions [67]. The ability of acrylonitrile to covalently modify protein varies with different tissues [68]; investigation of acrylonitrile adducts in blood suggest that they could be good biomarkers of body burden of chemicals, but above a certain point, the toxicity of acrylonitrile rises sharply [68]. This would indicate that covalent binding of non-critical protein or of protein at non-critical levels is of little consequence. When this threshold is passed, however, critical proteins become covalently modified or a specific level of protein modification is surpassed, leading to overt toxicity [68]. Koen and Hanzlik [69] have surveyed the literature regarding covalent modification of proteins, finding that 45 individual proteins have been identified, of which paracetamol binds to 31, halothane binds to nine and bromobenzene binds to 11 [69]. Only two proteins are common to all three agents: protein disulphide isomerase A1 and thiol–protein disulphide oxidoreductase [70], with two proteins being common between halothane and bromobenzene and two proteins between paracetamol and bromobenzene [69]. A study by Pohl's group demonstrated that after halothane administration to out-bred guinea pigs, there were higher levels of trifluoroacetyl-protein adducts in individuals with toxicity, in comparison to non-sensitive individuals [71], and it is suggested that the liver of the sensitive guinea pigs has an enhanced ability to metabolise halothane. Many of the adducted proteins (+30) were not identified and one could speculate that there might be further common proteins between halothane, paracetamol and bromobenzene, that are yet to be identified. Caution should be used when comparing common protein targets of reactive metabolites, as differing methods of drug–protein interaction have been used for detection, for example, anti-hapten antibody and radiolabelled drug. Also, toxic doses of paracetamol are used in adduct detection compared with non-toxic doses of halothane. The alkylation of these proteins might not have a significant functional consequence other than to 'mop-up' excess electrophiles. Regardless of the identification of critical proteins and/or levels of protein modification and glutathione depletion, this should not become a substitute for the discovery and use of safer chemical moieties, in addition to the limitation of toxicophores within new therapeutic agents.

The careful use of toxicophores within new chemical entities is one approach to minimize drug-induced toxicity. However, drugs containing toxicophores might be considered safer if the dose does not exceed 10 mg per day. It is important to fully elucidate the pathogenic mechanisms of dose-dependent toxins *in vitro* and in animals, to determine similarities with idiosyncratically toxic drugs. This could help to side-step the need for animal models of idiosyncratic toxicity, of which there are none currently recognized. Aside from toxicophores, another major factor influencing susceptibility to toxicity is the genotype of the patient. Mutations or SNP's (single nucleotide polymorphisms) that can be present in drug-metabolising enzymes, antioxidant defences and the immune system can radically alter an individual's response to a drug. The identification

of susceptible patient populations, based on genotyping assays, is crucial and the development of microarray-based genotyping assays looks set to transform this field [72]. From a patient and clinician perspective, it is imperative that both avenues of research are pursued, so that the administration of inherently less toxic drugs is coupled with identification of genetically susceptible patient groups.

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