22 Bioactivation and Reactive Metabolite Assays

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22.1 INTRODUCTION: FORMATION OF REACTIVE METABOLITE AND DRUG-INDUCED ADVERSE EFFECTS

Drug molecules are typically subject to biotransformation, that is, metabolism, once administered. Drug metabolism usually serves as a defense/detoxification mechanism, converting a drug molecule to hydrophilic metabolites, followed by removal of the latter from the circulation through urinary and/or biliary (via fecal) excretion [1,2]. However, drug metabolism sometimes becomes a process of "metabolic activation" or "bioactivation," generating chemically reactive species such as α,β-unsaturated carbonyls, epoxides, isocyanates and isothiocyanates, quinones, quinone imines, and quinone methides. These metabolites are electrophiles capable of modifying proteins or nucleic acids, forming covalent chemical bonds with amino acid (e.g., cysteine, tyrosine, and lysine) residues or nucleoside base (e.g., adenine and guanine) components (Fig. 22.1a). The underlying mechanisms of these chemical reactions are often nucleophilic addition and substitution in nature. Structurally altered proteins and nucleic acids could lead to perturbed cellular functions, and consequently, drug-treatment-related AEs. Drug-metabolizing enzymes that catalyze the formation of reactive metabolites include cytochrome P450s (P450s), peroxidases, amine oxidase, and flavin-containing monooxygenase. Because these enzymes are proteins, they are sometimes prone to structural modification by reactive metabolites formed within their respective active sites, resulting in the so-called suicidal enzyme inactivation. For P450s, enzyme inactivation could also occur through heme alkylation or heme-iron

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
Edited by Alexander V. Lyubimov.

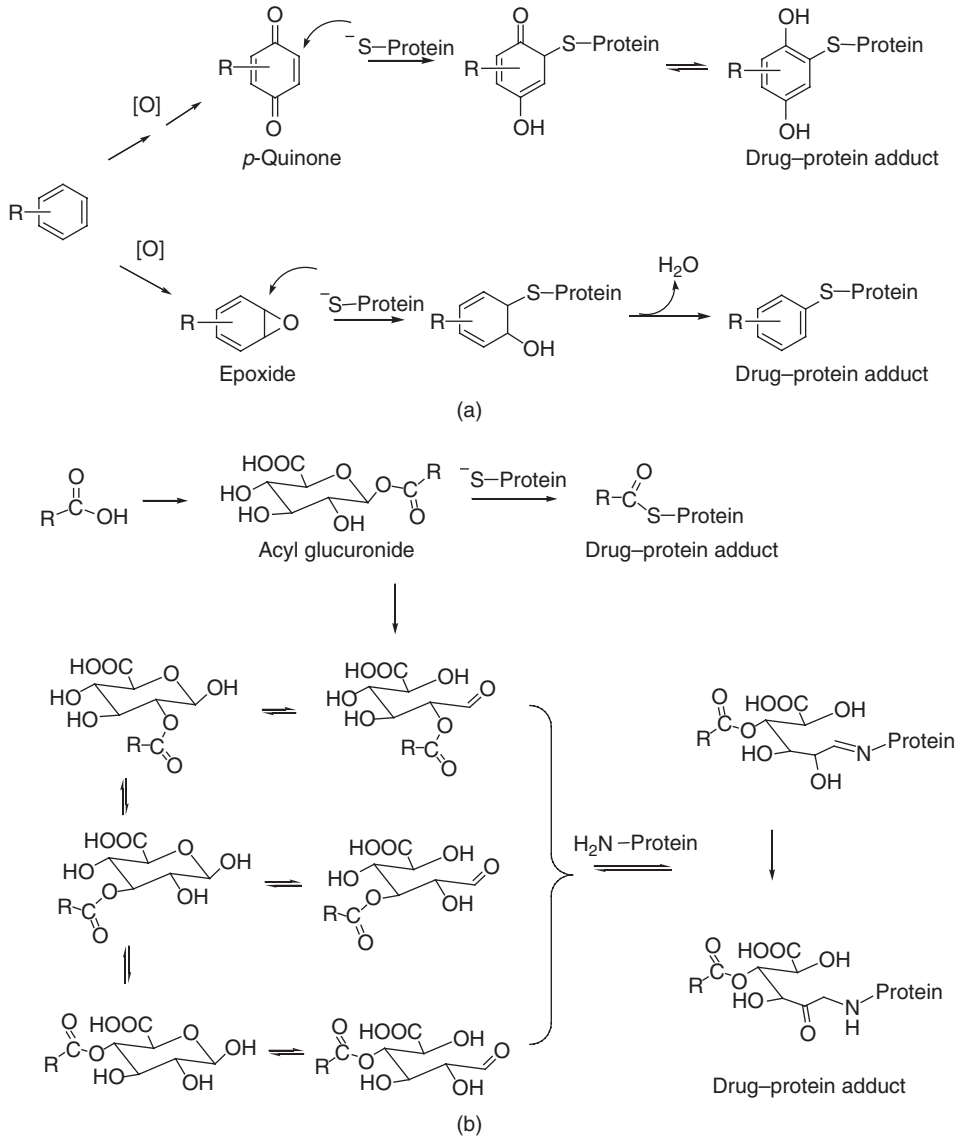

Figure 22.1 (a,b) Schematic representation of bioactivation and subsequent modification of proteins.

chelation. A covalently modified drug-metabolizing enzyme has the potential to cause drug treatment-related AEs not only by invoking a direct cytotoxic effect but also via drug-drug interactions due to diminished enzyme activity.

Parent drugs and their metabolites containing a carboxylic acid, alcohol, phenol, or amino functional group may undergo glucuronidation, sulfation, or acetylation. These biotransformation reactions occur in the presence of UDP-glucuronosyltransferases (UGT), sulfotransferases (SULTs), and *N*-acetyltransferases, respectively. Acyl glucuronides have been regarded as reactive species, in that they may acylate proteins through two different pathways, one of which is via displacement of the glucuronic

acid moiety by a cysteine, tyrosine, or lysine residue. The other mechanism involves migration of the aglycone at the pyranose ring, followed by scission of the hemiacetal ether bond to afford an aldehyde that reacts with a lysine residue to form an imine species, further intramolecular rearrangement of which yields a stable 1-amino-2-keto protein adduct (Fig. 22.1b). Acetylation and sulfation also mediate the formation of reactive metabolites, with acetate or sulfuric acid serving as a leaving group. The resulting cation species are capable of electrophilic addition to proteins or nucleic acids.

Drug molecules and metabolic products are eliminated from the circulation via biliary and/or urinary excretion, both of which in many cases are facilitated by active transporters such as bile salt export pump (BSEP), multidrug resistance-associated proteins (MRPs), and multidrug resistance proteins (MDRs). For those transporter substrates that are chemically reactive (e.g., acyl glucuronides or reversible GSH adducts), they could react directly with the transporter proteins or modify proteins/nucleic acids localized in a distal tissue compartment following migration assisted by transporters. Because active transporters serve to regulate the disposition of both xenobiotic (e.g., food, drug, and environmental chemicals) and endogenous substances (e.g., bile acids, cholesterols, and lipids), covalent modification of transporter proteins has the potential to disrupt transporter functions and produce drug treatment-related AEs resulting from excessive accumulation of cytotoxins.

Although circumstantial in nature, bioactivation appears particularly relevant to drug-induced hepatotoxicity on the basis that drug metabolism usually takes place in liver and reactive metabolites could readily alkylate/acylate hepatic proteins upon their formation *in situ*. This proposed scenario of liver injury is consistent with the observation that ~50% of clinical acute liver failure cases are likely drug treatment related [3]. In this context, mitochondria may either be a target or serve as a mediator for hepatotoxins, as the organelle not only produces ATP and other essentials to support cell survival but also regulates the programmed cell death process, namely, apoptosis. Insult from reactive metabolites would therefore start with structural modification of mitochondrial proteins and/or mitochondrial DNA (mtDNA), triggering pathogenesis such as inhibition of fatty acid oxidation, perturbation of cellular calcium homeostasis, oxidative stress, formation of peroxynitrite that induces membrane permeability transition, and collapse of mitochondrial membrane potential [4,5]. An alternative mechanism of drug-induced hepatotoxicity involves immune responses invoked by the formation of drug–protein adducts, which activate antigen-presenting Kupffer and Ito cells and produce T cells and antibodies that are cytotoxic to hepatocytes [6,7]. This type of liver injury usually exhibits a delayed onset of clinical symptoms on initial exposure to the offending drug. However, the AEs are to occur rapidly in response to rechallenge with either the implicated agent or a drug whose metabolism produces a structurally similar reactive species. Such a characteristic appears to represent a hallmark of immune-mediated AEs resulting from drug treatment. Injured hepatocytes undergo apoptosis if mitochondria remain functional and generate ATP. Necrotic cell death takes place when the damage to mitochondria is overwhelming and the organelles cease providing energy.

In addition to covalent modification of proteins and/or nucleic acids, reactive metabolites can form thioether or thioester with the cysteine residue of GSH (Fig. 22.2), a tripeptide consisting of L-γ-glutamyl-L-cysteinyl-glycine (Glu-Cys-Gly).

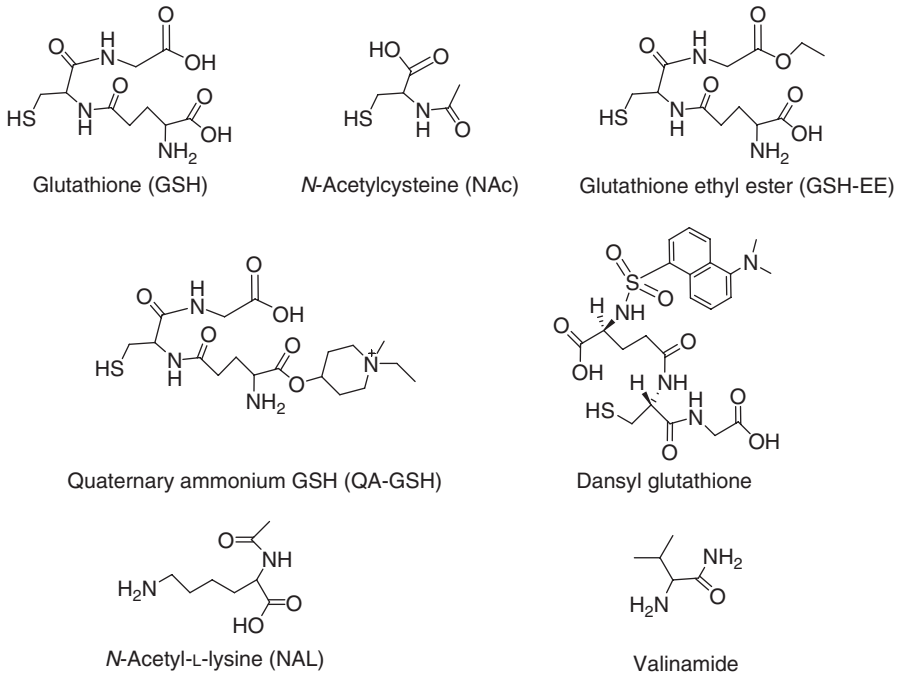

Figure 22.2 Structures of GSH and amino acid derivatives-related trapping agents.

This conjugation reaction may or may not require catalysis by glutathione-*S*-transferases (GSTs), depending on the chemical reactivity of the metabolites. The resulting GSH adducts are subject to hydrolysis of the tripeptide moiety, catalyzed by γ-glutamyltranspeptidase and dipeptidase. These hydrolytic reactions lead to the formation of cysteinylglycine and cysteine adducts, with the latter undergoing *N*-acetylation to afford the corresponding mercapturic acids [*N*-acetylcysteine (NAc) adducts]. Mercapturic acids usually are eliminated via urinary excretion. Although conjugation reactions with GSH generally represent detoxification, the thiol adducts sometimes serve as carriers for reactive metabolites, causing cellular damages in tissue compartments distal from the site where drug metabolism takes place. Possible scenarios include (i) retro-Michael reactions regenerating reactive species from which the GSH adducts are originated, (ii) further bioactivation of cysteine adducts via the β-lyase pathway, and (iii) exacerbation of tissue oxidative stress due to participation of thiol adducts in cellular redox cycling [8,9]. From a metabolite profiling perspective, identification of stable thiol adducts formed via the mercapturic acid pathway represents a bioanalytical option for the detection of reactive metabolites with short degradation half-lives in biological systems (see below).

22.2 DETECTION OF PEPTIDE AND PROTEIN ADDUCTS

Reactive species formed during drug metabolism processes usually are short lived, with their fate partitioning between reaction with biological molecules and further degradation to stable products. This has presented a technical challenge for direct detection

of reactive metabolites even with state-of-the-art instrumentation. An alternative is to identify derivatives resulting from reactions of the metabolites with the so-called trapping agents (Fig. 22.2). Such an approach has proved valuable during investigation of bioactivation mechanisms and identification of potential biological targets susceptible to covalent structural modification. In drug discovery and development settings, the data are utilized to construct an SAR aiding medicinal chemistry's effort to design and synthesize molecules with a low propensity to bioactivation.

22.2.1 Detection of GSH-Related Thiol Derivatives

Many reactive metabolites are electrophiles prone to react with nucleophilic amino acid residues of proteins, leading to the formation of covalently bound drug–protein adducts. One such amino acid is cysteine in its reduced form. Although the outcome can be very informative, analysis of protein adducts is relatively complex and resource consuming. In order to simplify bioanalytical procedures while not losing essential structural information, studies of bioactivation are often carried out via identification of the stable products resulting from nucleophilic "trapping" of electrophilic metabolites. For example, experiments are routinely performed to search for GSH adducts. The peptide GSH is an important endogenous substance, and one of its cellular functions is to scavenge reactive species (see above). GSH conjugation reactions also occur in incubations with liver microsomal or hepatocyte preparations when the thiol peptide is present. Detection of GSH adducts by MS is relatively straightforward due in part to the fact that MH^+ ions of those adducts produce characteristic fragments resulting from the neutral loss of 129 Da upon collision-induced dissociation (CID) [10]. A conventional approach is to analyze biological samples obtained from either *in vivo* or *in vitro* using the full scan functionality of an ion trap or triple quadrupole MS [11]. Initial identification of potential adducts is based on searching MH^+ ions corresponding to the combined molecular weights of GSH and the putative metabolites. Any molecular ion meeting the criteria is subject to CID, and further analysis focuses on the characteristic fragment ions such as those derived from the neutral losses of pyroglutamate (129 Da) and cysteine (75 Da). The probability of success for a full MS scan-based probe of GSH adducts is highly dependent on the experience of investigators when measured by the number of "false negative results." An alternative is to use a neutral loss scan of 129 Da for detecting GSH adducts with triple quadrupole MS operated at positive ionization mode. Subsequently, the MH^+ ions identified are subject to CID, generating product ion spectra for structural analysis. One of the major limitations related to the neutral loss scan of 129 Da is its low sensitivity and selectivity; the former results from the inherited deficiency of full scan mode, while the latter is mainly due to interference by the biological matrix. This may be improved by scanning for those precursor ions $(M-H)^-$ that produce a specific fragment at m/z 272 with a triple quadrupole MS [12]. The ion of m/z 272 represents a diagnostic fragment of GSH adducts under the negative ionization mode, corresponding to the cleavage of the carbon–sulfur bond at the peptide moiety. Both the aforementioned approaches are relatively low throughput with application of "low" resolution MS (see below).

In order to improve sensitivity and selectivity, multiple reaction monitoring (MRM) has been used as an alternative to full scan for triggering the acquisition of enhanced product ion (EPI) spectra on a triple quadrupole linear ion trap MS, such as API4000 Q-Trap [13]. Thus, it was demonstrated that up to 114 MRM scans can be constructed

to monitor mass transitions resulting from the neutral losses of 129 and/or 307 Da, with the MH^+ ions proposed to target-specific GSH adducts whose structures are derived from a database of known bioactivation reactions including those observed for compounds with similar substructures. Detection of the desired signals by the MRM survey scan triggers a data-dependent acquisition of EPI, from which the structures of GSH adducts are deduced. As such, investigators should be able to obtain product ion spectra of targeted GSH adducts, and the sensitivity of detection could be as high as ~180-fold better than the full-scan-based neutral loss survey scan. However, this approach would only enable identifying the GSH adducts "predicted" by a database based on which MRM survey scans are constructed. In other words, GSH adducts formed via "novel" bioactivation mechanisms are likely to escape from the detection.

Several derivatives of GSH have been synthesized for trapping reactive metabolites. One such agent is GSH ethyl ester (GSH-EE, Fig. 22.2) used *in vitro* to improve the sensitivity of detection [14]. The experiment was performed on a microbore liquid-chromatography-micro-electrospray-ionization tandem MS instrument. Detection of GSH-EE adducts was based on selected reaction monitoring (SRM) for simultaneously monitoring multiple MH^+ to $[MH\text{-}129]^+$ transitions, where $[MH\text{-}129]^+$ ions correspond to the neutral loss of pyroglutamate from the GSH-EE moiety. It appears that the sensitivity of detection was ~10-fold better for GSH-EE adducts relative to GSH adducts. In a separate study, GSH and GSH-EE were added together into liver microsomal incubations in an attempt to increase the probability and certainty of identifying reactive metabolites by LC-MS analysis [15]. In this case, paired MH^+ ions with their mass difference of 28 Da were clearly indicative of GSH and GSH-EE adducts. Another GSH derivative synthesized for trapping purpose incorporated a quaternary 4-hydroxy-*N*-methylethyl piperidinium (QA-GSH, Fig. 22.2) [16]. Similar to GSH, QA-GSH "traps" electrophilic reactive metabolites, but detection of the resulting adducts by LC-MS was based on M^+ and $(M + H)^{2+}$ ions, as the agent already contains a fixed positive charge. Formation of QA-GSH adducts *in vitro* was semiquantifiable when the so-called QA-GSH standards were added during the sample processing and employed as internal standards for calibration purposes. Thus, the M^+ and $(M + H)^{2+}$ ions were monitored using either a multiple single reaction monitoring (mSRM) battery or a precursor ion scan of m/z 144, with the latter corresponding to the 4-hydroxy-*N*-methylethyl piperidinium ion liberated from QA-GSH adducts. Both GSH-EE and QA-GSH belong to a class of "artificial" trapping agents, and their trapping capability is dictated by chemical reactivity. For comparison, formation of GSH adducts in liver microsomal incubations sometimes can be enhanced via adding GST or hepatic cytosolic fraction [17]. A larger quantity of the metabolite formed should obviously improve the probability of detection. The experimental setup also better mimics the *in vivo* biological environment since GST is present in abundance in hepatocytes. Semiquantification is achievable in principle by employing a synthetic GSH adduct as an internal standard during LC-MS analysis.

The fragmentation of GSH adducts often is dominated by cleavages of the peptide bonds, and, as a result, the product ion spectra sometimes do not offer sufficient clues for structural analysis of drug portion of the adducts. Certain types of GSH adducts are chemically unstable, further complicating bioanalysis. An alternative trapping agent is NAc (Fig. 22.2) with a molecular weight of 163 Da. Unlike the corresponding GSH adducts, NAc adducts lack a peptide moiety and consequently their fragmentations are likely associated with drug portion of the molecules, hence more structural information

on the trapped reactive species and better understanding of the mechanism underlying bioactivation. In addition, NAc adducts can be detected *in vivo*, formation of which takes place via the mercapturic acid pathway following GSH conjugation reactions with reactive metabolites (see above). The conventional approach described above for detection of GSH adducts has also been employed for analysis of NAc adducts, such that identification of potential adducts focuses initially on searching MH^+ ions corresponding to the sum of 161 Da plus the molecular weights of putative reactive metabolites, followed by MS/MS analysis of the MH^+ ions in question. Similar to GSH adducts, NAc adducts produce fragments corresponding to the neutral loss of 129 Da, although the fragmentation is due to the cleavage of the thioether bond on the cysteine side of the molecule. On that basis, a neutral loss scan of 129 Da can be used for screening NAc adducts. In a recent report, NAc adducts formed either *in vitro* or *in vivo* were detected using a quadrupole linear ion trap MS with the polarity switching technique [18]. In the study, neutral loss scans of 129 Da, based on either full scan or MRM, triggered the acquisition of parent ion spectra and fragment-rich EPI spectra in a single LC-MS run for structural assignment. This method employs a generic data-acquisition protocol for detection of various NAc adducts and may therefore be suitable for fast screening of samples derived from *in vitro* and *in vivo* experiments. However, one of the limitations in such an approach is that it would only be able to identify those NAc adducts "predicted" by investigators. In addition, the neutral loss scan of 129 Da appears to exhibit lower sensitivity for detection of NAc adducts than for GSH adducts due likely to the fact that the two fragmentations are associated with cleavage of the chemical bonds with different strength. The thioether bond of an NAc adduct is much stronger than the peptide bond of a GSH adduct.

A recent development in instrumentation is related to the data acquisition with alternating low/high collision energy on high resolution MS (HRMS) such as a hybrid quadrupole time-of-flight (Q-TOF) MS. This improvement of methodology, in general, has increased not only bioanalytical throughput but also the sensitivity of detection for drug metabolites, including trapped reactive species [19,20]. The experimental setup starts with scans of ions at a low level of collision energy, followed by ramping collision energy to different levels in order to trigger CID. The first scan function at low collision energy detects the intact molecular ions, whereas the second scan at higher levels of collision energy generates MS^E spectra, where "E" represents collision energy. The MS^E spectra are equivalent to the ones obtained from nonselective tandem MS/MS scans, containing fragmentation information for the ions registered in the low collision energy scan [20]. However, MS^E spectra acquired by Q-TOF MS are not specific, as they lump together all fragments of the molecular ions that reach the detector at a given time. Such deficiency complicates the data interpretation but has been somewhat compensated by coupling HRMS with UPLC, which separates efficiently the analytes from each other as well as from the biological matrix [21]. Accurate mass-based postacquisition data processing software also aids interpretation of the MS^E spectra [22,23]. Consequently, a single sample injection onto UPLC-HRMS usually is sufficient to obtain both MS and MS^E spectra of the metabolites of interest. In this regard, detection of a diclofenac–GSH adduct may serve to demonstrate the utility of HRMS. Initial scans at low collision energy under the positive ionization mode of a sample from human liver microsomal incubations registered an MH^+ ion at m/z 583.1267, which corresponds to the exact mass of [diclofenac + GSH + O − Cl − H] (Fig. 22.3a). Subsequent automated switch to a scan at high collision energy

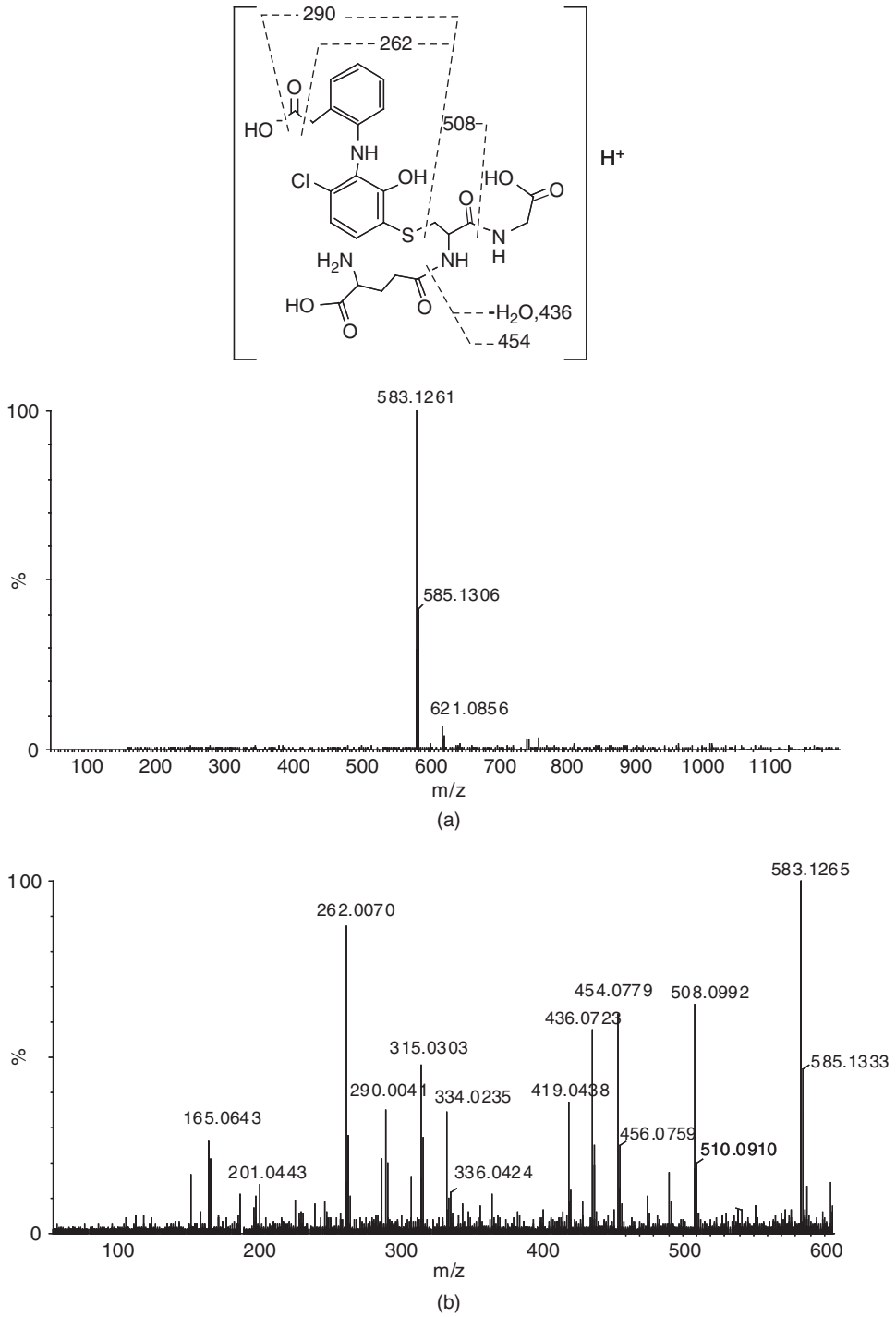

Figure 22.3 Detection by HRMS of a GSH adduct derived from diclofenac bioactivation.

produced an MS^E spectrum (Fig. 22.3b), followed by data processing of the spectrum by Metabolynx (Waters) for assignment of the fragment ions. The result supports the idea that diclofenac undergoes bioactivation to form reactive quinone imine or epoxide species, which are trapped by GSH conjugation reactions [24,25]. Similar to their counterpart with low mass resolution, HRMS also is equipped for searching GSH adducts via neutral loss scans [26]. In this case, consecutive pairs of low and high ionization energy spectra are generated for MH^+ ions satisfying the neutral loss of 129.0426 Da within a window of ±20 mDa. Once the instrument registers an MH^+ ion with the specified neutral loss, it switches to MS/MS mode to collect an MS^E spectrum, providing sufficient information for subsequent structural analysis using data-processing software. The entire exercise often requires only a single sample injection, significantly enhancing the bioanalytical throughput. In principle, such an approach should be applicable for detection of not only GSH adducts but also drug metabolites with known characteristic neutral losses.

22.2.2 Detection of Amino acid- or Peptide-Related Adducts

Although GSH and NAc are the commonly used trapping agents, their utility to some extent is limited by their ability to form conjugates with reactive metabolites primarily via the sulfhydryl functional group. Some reactive species, such as quinones or quinone imines, may prefer to react with amines depending on their chemical reactivity, whereas certain thiol adducts could escape from detection by LC-MS analysis because they remain highly unstable and undergo degradation or rearrangement either in biological systems or during sample preparation [27,28]. In this context, individual amino acids or small synthetic peptides containing representative amino acid residues may serve as alternatives to GSH and NAc for trapping reactive metabolites formed *in vitro*.

It was therefore demonstrated that hydroxymethylvinyl ketone (HMVK) reacts readily with three model amino acids, namely, L-valinamide, *N*-acetyl-L-lysine (NAL), and NAc (Fig. 22.4) [29]. HMVK is a putative metabolite of 1,3-butadiene, which has been classified as a human carcinogen. Exposure to butadiene usually is associated with occupation and/or cigarette smoke. In the study, formation of adducts between HMVK and the model amino acids was monitored by HPLC with UV detection, while structural assignment was based on NMR analysis of purified products. Several HMVK-amino-acid adducts were identified, including those resulting from mono- and di-Michael addition(s) of the electrophile to the nucleophilic acceptors (Fig. 22.4). Among the amino acids tested, the reactive potential was in the order of NAc > valinamide > NAL, implying a free cysteine residue is a preferred site of modification. The detection of multiple different types of adducts has reinforced the idea that a variety of proteins are potential biological targets for HMVK *in vivo*. Similarly, cysteine, lysine, histidine, tyrosine, and serine were shown in a separate study to be able to trap quinone methides of different electrophilicity [30]. With HPLC-UV detection, three quinone methides, which were 4-methylene-2,5-cyclohexadienone derivatives, were observed to react rapidly with cysteine to form thioether derivatives with little or no competition from hydration in aqueous solutions. The α-amino groups of lysine, histidine, tyrosine, and serine were the primary sites of modification by the quinone methides, although alkylation also occurred with the side chain amino groups of lysine and histidine. The order of reactivity was cysteine thiol > N-terminal amine > *N*ε-lysine ∼ *N* Im-histidine, but this pattern of reactivity did not hold for peptides or proteins. For the

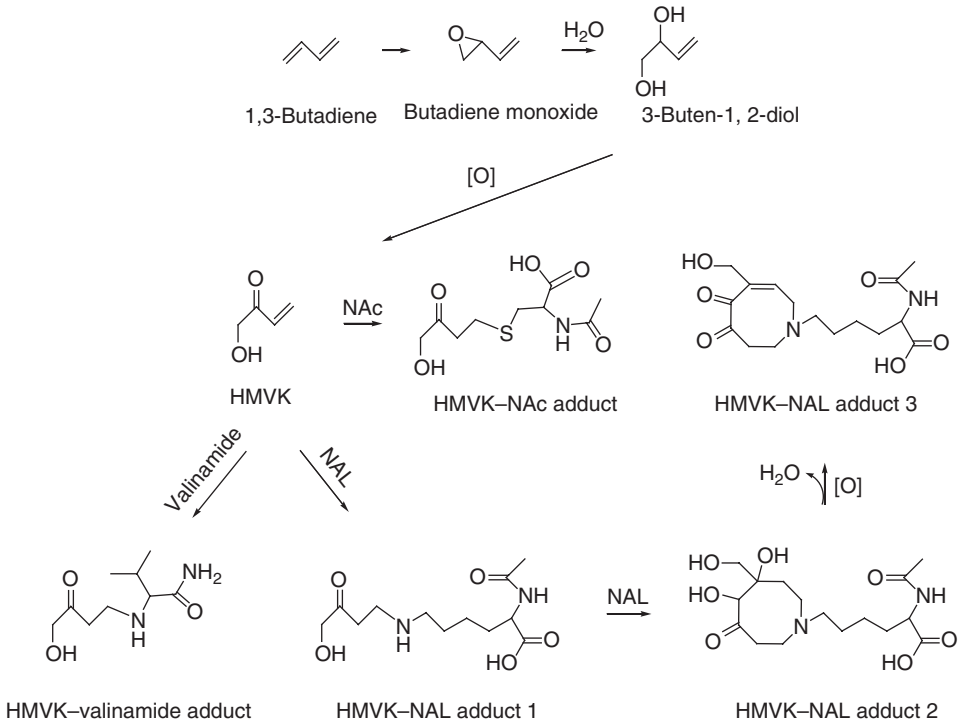

Figure 22.4 Structures of *in vitro* trapped adducts of HMVK with valinamide, NAL, and NAc.

synthetic tripeptide Gly-His-Lys and human hemoglobin, their primary sites of modification by the quinone methides were *N*α-glycine and *N*ε-lysine, respectively, based on LC-MS/MS analysis of the samples prepared with and without treatment by a protease.

Peptides may therefore represent better trapping agents when the focus of investigation is on the identity of modified amino acid residues. A generic experimental setup may include incubations of test compounds with human liver microsomes or recombinant P450s in the presence of a trapping peptide, followed by sample workup and LC-MS/MS analysis. For example, reduced oxytocin was employed as a model biological target of alkylation by the 2,4-diene derivative of valproic acid, an anticonvulsant agent that causes severe but rare liver injury [17]. Initial LC-MS scans registered an MH^+ ion of m/z 1491, consistent with a nucleophilic addition of two diene molecules to a single reduced oxytocin molecule. Subsequent CID of the MH^+ ion led to the assignment that the alkylation occurred at Cys-1 and Cys-6, suggesting that the sulfhydryl residues of proteins are vulnerable to attack following valproic acid bioactivation. Recent efforts include design and synthesis of trapping peptides that incorporate various nucleophilic amino acid residues in an attempt to capture different types of reactive species in a single experiment [31]. One such trapping agent was the 11-amino-acid peptide ECGHDRKAHYK containing not only cysteine but also other potential targets for nucleophilic addition/substitution, such as lysine and histidine. In incubation of a drug in question with the peptide in a bioactivation system, samples were partially purified and drug–peptide adducts retained on a cation-exchange chip

surface were analyzed by a surface-enhanced laser desorption ionization-time of flight (SELDI-TOF) MS. Identification of those adducts was based on parent ion scans for MH^+ corresponding to a combined mass of the trapping agent plus putative metabolites. Although application of an SELDI-TOF MS eliminated the requirement for separation by LC of the analytes, the instrument, in general, exhibits a relatively poor precision in comparison with conventional LC-MS and is incapable of generating fragmentation data to aid structural elucidation of the observed adducts as it lacks a CID function. Such an experimental setup was driven primarily by industrial research, characteristics of which include a rapid turnaround time to answer a much simplified question about bioactivation, that is, whether reactive metabolites are produced from a given test compound. Peptide-based trapping agents have the advantages of mimicking biological protein targets better than individual amino acids and providing information on the sites of modification. However, similar to GSH adducts, drug–peptide adducts usually offer insufficient or no information at all for the drug side of trapped products even if they are subjected to fragmentation. This can be very unsatisfactory for those whose research focus is on an SAR with respect to bioactivation. In this context, data from trapping experiments with GSH, NAc, and various peptides are likely complementary to each other.

22.2.3 Detection of Protein Adducts

Formation of protein adducts is at the center of a working hypothesis relating bioactivation to drug-induced AEs (see above). Identification of modified proteins should therefore represent a step forward to better understanding of the pathogenesis of tissue injury. When the modified protein is a drug-metabolizing enzyme, studies of the corresponding protein adduct not only bear toxicological significance but also shed light on interactions between the enzyme and its substrate, hence the relevant topography of enzyme active site. Similar to any analyte in question, detection of protein adducts usually begins with separation or partial purification of those adducts from biological matrix, and the techniques may fall into two major categories, one of which is one- and two-dimensional gel electrophoresis. An alternative is chromatographic separation, represented by HPLC and size exclusion, ion exchange, and affinity chromatographies. Popular means of detection includes immunochemical and MS analyses.

Immunochemical detection coupled with electrophoretic separation essentially constitutes the so-called Western blotting (see ***Bioanalytics for Human Microdosing***), capable of identifying multiple modified proteins including the antigen(s) that may be responsible for causing pharmacotherapy-related hypersensitivity. Polyclonal antibodies are usually produced by immunization of laboratory animals, such as mice or rabbits, with a carrier protein conjugated to a drug or metabolite in question. Antibodies raised are present in serum, termed *antisera*, and can be used as is or subject to purification before application. Detection of protein adducts resulting from drug treatment is therefore based on recognition by the antisera or purified antibodies of the adducts separated by one- and two-dimensional gel electrophoresis. A classic example involves identification of acetaminophen (APAP)-related protein adducts, in which polyclonal antisera was obtained from rabbits immunized with APAP linked to keyhole limper hemocyanin (KLH) via NAc [32]. After characterized for its specificity toward APAP–protein adducts by a competitive enzyme-linked immunosorbent assay (ELISA), the antisera was utilized for analysis of the serum samples collected from

patients administered with APAP. The results revealed that the subjects with increased serum transaminase levels had APAP–protein adducts in their circulations, whereas those without signs of liver injury did not exhibit immunochemically detectable signals for protein adducts. A reversed sequence of the aforementioned detection procedure is viable to determine if patients are "immunized" by drug treatment. For example, sera from patients who experienced tienilic acid-induced hepatitis recognized CYP2C9 protein but not other members in the CYP2C family on immunoblotting, suggesting the presence of anti-CYP2C9 antibodies in these patients [33]. Additional experiments with mutants of CYP2C9 identified three regions on the enzyme protein that form a binding pocket interacting with the antibodies. Similarly, antisera against proteins modified by halothane was present in patients who developed drug-treatment-related hepatotoxicity [34]. Detection of antibodies that recognize either native or modified proteins is of mechanistic importance, as it implicates an activated immune system in the pathogenesis of idiosyncratic drug toxicity (see above). In this regard, a patient treated by halothane several years earlier experienced desflurane-induced hepatotoxicity [35]. Subsequent analysis of serum samples from the patient by ELISA revealed that the sera exhibited reactivity toward liver microsomal proteins from rats treated with halothane. This led to the hypothesis that the patient was sensitized by the early halothane therapy and consequently became susceptible to desflurane treatment on the basis that metabolism of both halothane and desflurane generates putative trifluoroacetic chloride, a reactive species capable of binding covalently to proteins and, presumably, activating the immune system [35].

More recently, chromatographic separation coupled with MS detection has become a popular method for analysis of protein adducts. Advantages of an LC-MS/MS-based approach reside with not only sensitivity and accuracy but also a capacity of generating data for structural elucidation. In this context, LC-MS/MS analysis, together with the use of various types of proteases, often provides information on molecular weight of protein adducts and the amino acid residues modified. In one instance, such a method was used for detection of APAP–albumin adducts in patients administered with the analgesic [36]. An earlier experiment established that *N*-acetyl-*p*-benzoquinone imine (NAPQI) formed during APAP bioactivation reacts with the only Cys residue of serum albumin, and digestion of the protein adduct by pronase E gives rise to an NAPQI-Cys-Pro-Phe (NAPQI-CPF) adduct. Thus, serum specimens from three patients overdosed with APAP were analyzed by LC-MS/MS, following treatment of the samples with protease E. Serum from healthy volunteers served as controls. The tripeptide adduct was observed only in those who were ingested APAP, and its level was ~10-fold higher in a patient with severe hepatotoxicity. From the same samples, the LC-MS/MS assay also was able to pick up signals for the NAc and Cys adducts of NAPQI, providing additional data in a relatively straightforward analytical process that enables better understanding of the fate of NAPQI [36]. With respect to covalently modified drug-metabolizing enzyme, studies of the resulting protein adduct aid structural elucidation of the enzyme active site, complementary to the data collected by other techniques. For example, grapefruit juice intake can cause significant increases in systemic exposures of CYP3A substrate drugs such as cyclosporine and midazolam. Two major components in grapefruit juice, bergamottin (BG) and 6′, 7′-dihydroxybergamottin (DHBG), were identified as irreversible inhibitors (inactivators) of CYP3A, possibly via modification of the enzyme apoprotein. Subsequent effort was focused on studies of the protein adducts formed during enzyme inactivation [37,38]. Recombinant or purified

enzymes were used in order to simplify bioanalysis. In one study, DHBG was incubated with purified CYP3A4, and the samples were injected sequentially onto two different HPLC columns for chromatographic separation of analytes from the matrix before MS analysis. Two protein adducts were detected, one of which exhibited the molecular ion corresponding to the intact CYP3A4 plus 386.8 Da, suggesting covalent binding of an oxygenated DHBG derivative to the enzyme apoprotein. The second adduct appeared to be a "bis-adduct," with its molecular weight equal to the apoprotein modified by two oxygenated DHBG derivatives [37]. Similarly, a protein adduct detected in a separate study by LC-MS showed an increase of mass by 387 Da from the native enzyme when BG was incubated with purified CYP3A5, implying BG was converted to DHBG followed by protein alkylation [38]. A proposed mechanism has therefore attributed the loss of CYP3A activity in grapefruit juice drinkers to oxidation of the furan moiety of DHBG to an epoxide species that alkylates the enzyme apoprotein and consequently inactivates the enzyme. Detection of the bis-adduct resulting from interactions of DHBG with CYP3A4 implicates a large enzyme active site capable of accommodating more than one substrate molecule. Additional information concerning amino acid residues critical to enzyme activity may rely on studies of the peptides derived from digestion by proteases of the drug-protein adduct. One example involves raloxifene, which causes irreversible inhibition of CYP3A4. The loss of enzyme activity was attributed to alkylation by raloxifene of CYP3A4 apoprotein, as the inactivated enzyme maintains an intact heme-Fe moiety that produces spectrally detectable P450 similar to controls [39]. Further analysis by LC-MS/MS of inactivated CYP3A4 following digestion by protease K and trypsin, respectively, revealed that the Cys-239 and Tyr-75 residues were targets of alkylation by a reactive species, designated as extended quinone or diquinone methide, formed during raloxifene metabolism [40,41]. These data collectively suggest that Cys-239 and Tyr-75 are important to CYP3A4 activity.

22.2.4 Detection of DNA Adducts

DNA consists of deoxyguanylate, deoxyadenylate, deoxythymidylate, and deoxycytidylate, while purines and pyrimidines, including adenine, guanine, cytosine, uracil, and thymine, are the base units for constructing DNA molecules. Electrophilic reactive species formed during drug metabolism may react with the nucleophilic sites of purine and/or pyrimidine base units, causing covalent modification of, and lesions to, the nucleic acids. In response to the insult, cells activate repair enzymes or polymerases to identify and correct the damage in order to maintain normal cellular function. The affected cells undergo programmed cell death or mutation when damages overwhelm the repair mechanism, resulting in tissue injury or triggering cancer development. From a chemistry perspective, the nucleobase units of DNA molecules are "hard" nucleophiles that are susceptible to the attack by "hard" electrophiles (see below) such as epoxides, aldehydes, ketones, and quinones.

In this context, acetaldehyde (Fig. 22.5a), classified as a possible human carcinogen, is present in various foodstuffs and is an oxidative metabolite formed following alcohol consumption or cigarette smoking [42]. In an attempt to analyze DNA adducts resulting from exposures to acetaldehyde, calf thymus DNA was incubated with the suspected carcinogen, in the presence or absence of $NaBH_3CN$, and the resulting samples were hydrolyzed with aqueous HCl to release adenine- and guanine-related products for

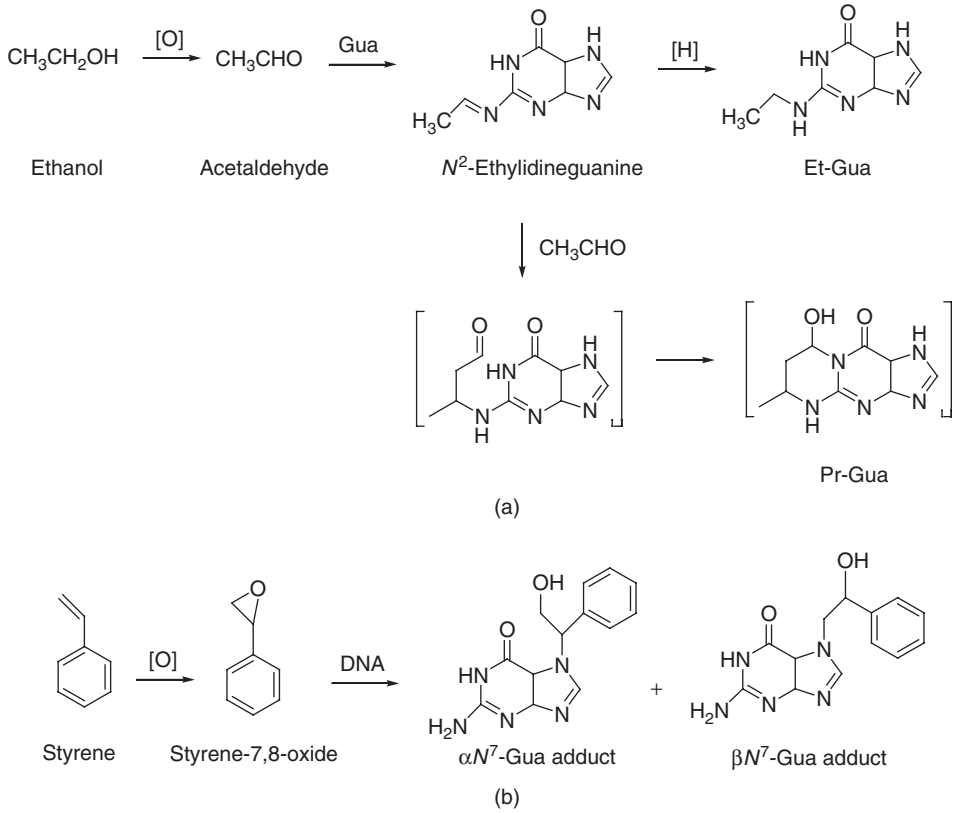

Figure 22.5 *In vitro* reactions of acetaldehyde with calf thymus DNA (a) and styrene-7,8-oxide with salmon testis DNA (b).

analysis by LC-MS/MS with selective ion monitoring (SIM). N^2-Ethylguanine (Et-Gua) or 1,N^2-propanoguanine (Pr-Gua) was the major adduct, depending on whether incubations contained or lacked $NaBH_3CN$. Thus, the underlying mechanism likely involves the reaction of acetaldehyde with the DNA molecule, resulting in the formation of an unstable Schiff's base N^2-ethylidineguinine adduct, chemical reduction of which by $NaBH_3CN$ (followed by hydrolysis of the DNA adduct) produces Et-Gua. In the absence of the reducing agent, N^2-ethylidineguinine undergoes further reaction with an additional molecule of acetaldehyde to give Pr-Gua (Fig. 22.5a). When acetaldehyde was incubated with cultured HL-60 cells, both Pr-Gua and Et-Gua were detected by LC-MS/MS, suggesting that alkylation of DNA by the aldehyde occurs in intact cells [42]. Covalent DNA modification also took place following incubations of calf thymus DNA with 5′-acetoxy-N'-nitrosonornicotine (5′-acetoxy-NNN), a precursor to the reactive species 5′-hydroxy-NNN resulting from tobacco smoking [43]. The reaction mixtures were subject to enzymatic hydrolysis by phosphodiesterase and alkaline phosphatase, and the adducts identified by LC-MS/MS with selected reaction monitoring (SRM) of ($MH^+ \rightarrow [MH\text{-}116]^+$) mass transitions were pyridyloxobutyl-dGuo (POB-dGuo), POB-dCyt, and POB-Thd. The $[MH\text{-}116]^+$ fragment formed upon CID corresponds to the loss of the deoxyribose moiety from the nucleobase adduct MH^+ ion. Similar to aldehydes, epoxides, such as styrene-7,8-oxide, also are potential

mutagens and/or carcinogens. In incubations of styrene-7,8-oxide with salmon testis DNA, several adenine and guanine adducts were characterized by LC-MS/MS and UV detection following hydrolysis of modified DNA in aqueous HCl [44]. It appeared that alkylation at the N^7 of guanine accounted for 93% of total DNA modification (Fig. 22.5b), followed by 4% at the N^3 of adenine and the remaining 3% distributed among N^2 of guanine, C^1 of adenine, and N^6 of adenine. The N^7 of guanine has been perceived as a high reactivity center because of its nucleophilicity and accessibility, which is followed by the N^3 of adenine with similar nucleophilicity but slightly more steric hindrance [45]. Other positions on the nucleobase units can also become a main target of alkylation, depending on a specific electrophilic reactive species, as in the case of the reaction between acetaldehyde and DNA, where modification occurred primarily at the N^2 of guanine.

Detection of DNA adducts formed *in vivo* is likely most relevant to biological and pathological processes, and numerous studies have been conducted in laboratory animals. For example, acrylamide (AA, Fig. 22.6a) is a chemical with various applications

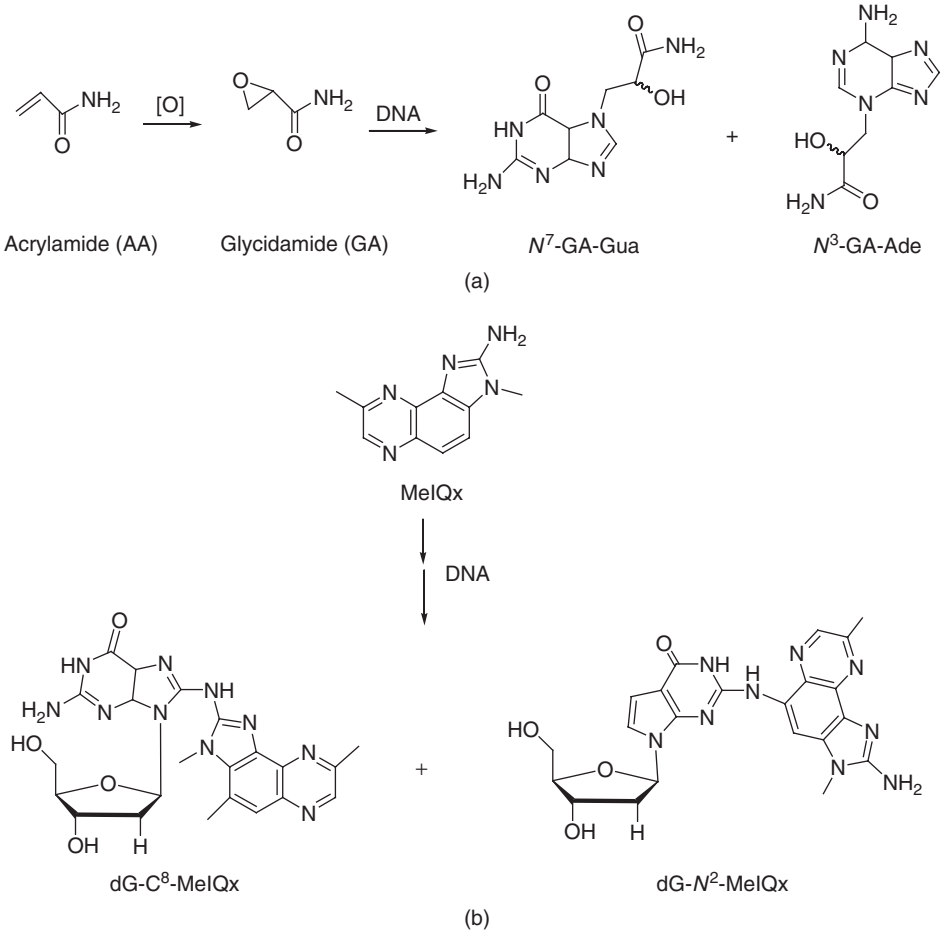

Figure 22.6 Formation of DNA adducts in mice dosed with acrylamide (a) and in rats dosed with MeIQx (b).

in industry, and its human exposures are associated with occupation as well as cigarette smoking and ingestion of fried starchy foods. AA is suspected to undergo bioactivation, getting converted to glycidamide (GA), which, being an epoxide, alkylates DNA [46]. Following a single dose from 1 to 50 mg/kg of AA to mice by intraperitoneal injection, liver, lung, and kidney were collected at 6 h for DNA extraction [47]. The resulting samples were subject to neutral thermal hydrolysis and partial purification by a size exclusion column. Two adducts, namely, N^7-GA-Gua and N^3-GA-Ade (Fig. 22.6a), were quantified by LC-MS/MS based on calibration curves generated with synthetic standards. The results revealed that formation of both adducts exhibited a dose-dependent response, with the level of N^7-GA-Gua being ~100-fold higher than that of N^3-GA-Ade in rat tissues examined. Another example involves 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, Fig. 22.6b), a structural representative of more than 20 heterocyclic aromatic amines formed during meat cooking. MeIQx is a rodent carcinogen and is considered as an agent of potential cancer risk to humans. Thus, MeIQx-C^8-dG and MeIQx-N^2-dG (Fig. 22.6b) were detected and quantified in liver samples from rats treated with MeIQx, based on LC-MS/MS SRM of ($MH^+ \rightarrow [MH\text{-}116]^+$) mass transitions [48]. The observed higher level of MeIQx-N^2-dG relative to that of MeIQx-C^8-dG implies that the former adduct is likely an important contributor to MeIQx-induced genotoxicity in rodents. In a separate study, the neutral loss of deoxyribose was employed to trigger data-dependent acquisitions of the MS^3 product ion spectra [constant neutral loss (CNL)-MS^3 scan] for putative adducts [49]. In addition to MeIQx-N^2-dG and MeIQx-C^8-dG, an adenine adduct was identified via the CNL-MS^3 scan of liver samples from rats dosed with MeIQx. The structure of the adenine adduct was assigned as MeIQx-N^6-dA according to the product ion spectrum. Most significantly, DNA adducts were also detected in humans, one of which is N^2-ethylidene-dG derived from reactions of acetaldehyde with DNA (see above). In this case, DNA isolated from human lung and liver samples was subjected to hydrolysis in the presence of $NaBH_3CN$, converting chemically unstable N^2-ethylidene-dG to the corresponding quantifiable N^2-ethyl-dG [50]. Quantification of N^2-ethyl-dG was based on LC-MS/MS SRM of ($MH^+ \rightarrow [MH\text{-}116]^+$), using synthetic [^{15}N]N-ethyl-dG as a stable isotope labeled internal standard. The results revealed an average of 0.1 adducts per million nucleotides in human livers and a similar level (0.13 per million nucleotides) in human lungs. There was no apparent difference between cigarette smokers and nonsmokers with respect to the level of N^2-ethyl-dG in their lungs, although the comparison was confounded by a small number of subjects investigated ($n = 4$) and a significant period of hospitalization without cigarette smoking before the samples were collected [50]. Nevertheless, the ability to detect and quantify DNA adducts in patients is clearly very important toxicologically.

22.2.5 Detection of Adducts Trapped by Nonbiological Agents

Although GSH and NAc often serve as trapping agents, some compounds known to form reactive species causing covalent protein binding *in vivo* do not produce trapped adducts, which may be attributed to the instability of the adducts or the nature of reactive metabolites. In the latter instance, electrophiles and nucleophiles are generally grouped as "soft" or "hard" according to their chemical properties. Soft nucleophiles are large in size with diffuse charge, such as the thiol group of GSH, and react readily with soft electrophiles, for example, α,β-unsaturated keto group. Hard electrophiles

are small with intense charge and tend to react with hard nucleophiles. As a result, soft nucleophilic trapping agents may not be able to effectively trap hard electrophilic metabolites and vice versa, leading to a false assurance that bioactivation is absent. Therefore, there is a need to explore nonbiological chemicals as alternatives for trapping different types of reactive species.

Cyanide ion is one of the most commonly used supplemental agents for trapping reactive metabolites. It is a hard nucleophile and traps certain hard electrophiles (e.g., iminium ion) with which GSH and NAc may not react. Another advantage is that the resulting cyanide adducts are generally stable and readily identifiable by MS because of the facile loss of 27 Da (HCN) upon CID. For example, metabolism of 1-benzylpyrrolidine in rabbit liver microsomes leads to the formation of a pyrrolidinium ion, identification of which was based on detection by GC-MS of mono- and bis-cyano adducts (Fig. 22.7) when incubations were performed in the presence of sodium cyanide [51]. Formation of the pyrrolidinium was attributed to the observed covalent protein binding by 1-benzylpyrrolidine. Metabolism of [^{3}H]phencyclidine in rabbit liver microsomes, while generating an iminium species, also was associated with covalent protein binding [52]. The degree of covalent protein binding was reduced by 75% in incubations containing 0.1 mM sodium cyanide, suggesting that the reactive iminium ion is responsible for phencyclidine-related protein modification. It should be noted that sodium cyanide at high concentrations may inhibit drug-metabolizing enzymes, leading potentially to decreases in the formation of corresponding cyanide adduct [51]. Several studies revealed that an optimal concentration of sodium cyanide was 1 mM or less when used with rabbit or human liver microsomes [52,53].

In order to facilitate LC-MS/MS detection, a mixture of $Na^{13}C^{15}N$ with NaCN (1:1) has been exploited for trapping reactive metabolites [54]. The isotopic patterns of the corresponding cyanide adducts are characteristic and easily recognizable during LC-MS analysis. This approach not only is useful with nominal MS application but also enhances HRMS detection of adducts trapped by cyanide. In a recent report, cyanide adducts were detected by UPLC coupled with TOF MS in 8 of 12 tested compounds following incubations with human liver microsomes in the presence of 1 mM of a mixture of KCN and $K^{13}C^{15}N$ (1:1) [55]. The exercise further explored the use of the so-called mass defect filter for identification of the trapped adducts during data mining processes, and its success highlighted that a combination of HRMS and a stable-isotope-mixed CN trapping agent could significantly increase the throughput for screening reactive metabolites formed in an *in vitro* system.

Methoxyamine is a hard nucleophile prone to react with hard electrophilic aldehyde to form the corresponding oxime adducts [56]. DBOPN ([1*S*, 5*R*]-3-cyano-1-(3-furyl)-6-{6-[3-(3 R-hydroxy-6,8-dioxabicyclo[3.2.1]octanyl)]pyridine-2-yl-

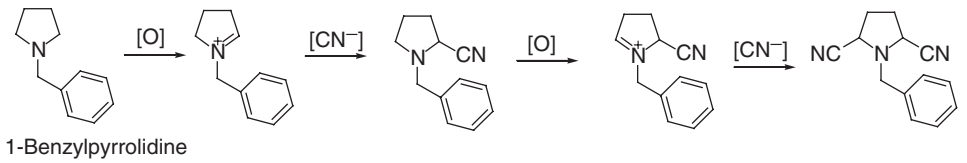

Figure 22.7 Formation of mono- and bis-cyano adducts of 1-benzylpyrrolidine.

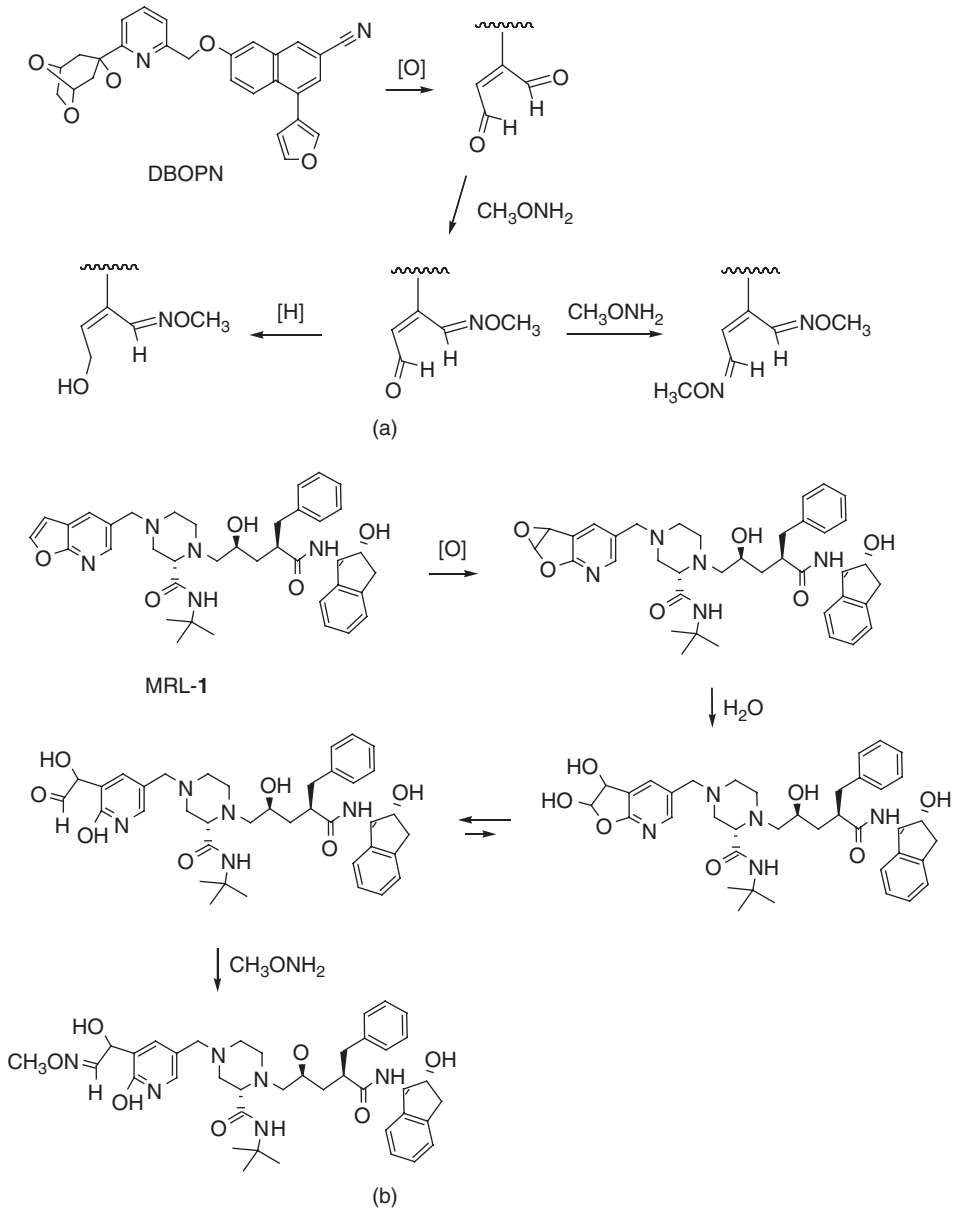

Figure 22.8 Formation of methyloxime adducts of DBOPN (a) and MRL-**1** (b) with methoxyamine.

methoxyl}-naphthalene; Fig. 22.8a) is a 5-lipoxygenase inhibitor designed for the treatment of asthma and inflammatory bowel disease. The compound undergoes metabolism to form reactive metabolite(s) that covalently bind to plasma proteins in the rat and monkey as well as bind to liver microsomal proteins *in vitro* [57,58]. The observed covalent protein binding decreased significantly in the presence of methoxyamine, suggesting that an aldehyde derivative was likely the culprit reactive species. Subsequent studies with rat liver microsomes containing methoxyamine

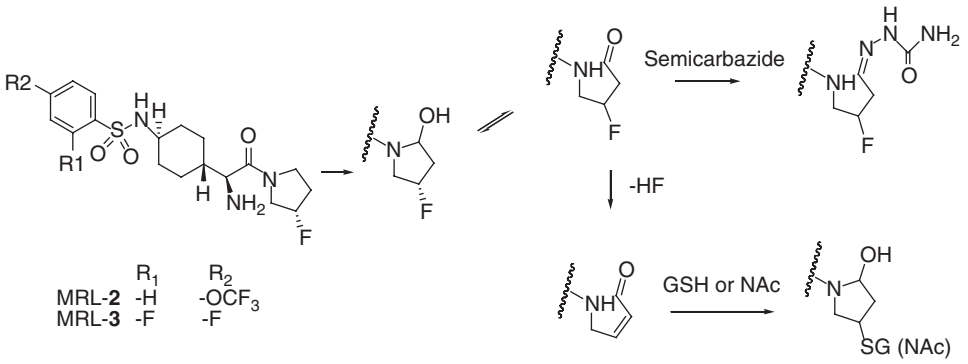

Figure 22.9 Formation of adducts of MRL-**2** and MRL-**3** with semicarbazide or GSH/NAc.

led to identification of five adducts by LC-MS/MS and NMR, four of which were derived from the mono- and bis-addition of methoxyamine to the oxidized furan moiety of DBOPN (Fig. 22.8a). Similar oxime adducts were also detected after incubating DBOPN with liver microsomes from dog, monkey, and human in the presence of hydroxylamine. Another example involves MRL-**1**, a furanopyridine derivative with a potential for anti-HIV treatment (Fig. 22.8b) [59]. MRL-**1** caused covalent protein binding to rat, dog, monkey, and human liver microsomes in an NADPH-dependent manner, implying bioactivation and formation of reactive species. In liver microsomal incubations containing methoxyamine, metabolism of MRL-**1** produced a ring-opened dihydrofurandiol tautomer that was trapped in the form of an *O*-methyloxime derivative (Fig. 22.8b). These two case studies clearly indicate that the furan ring represents a structural alert for bioactivation with the potential to form reactive epoxide and aldehyde derivatives in biological systems.

Semicarbazide is another small nucleophilic molecule capable of reacting with an aldehyde or ketone functional group to afford the corresponding stable semicarbazones. For example, two dipeptidyl peptidase-IV (DPP-IV) inhibitor analogs, MRL-**2** and MRL-**3** (Fig. 22.9), were subject to bioactivation in liver microsomes [60]. Covalent protein binding of the ^{3}H-labeled MRL-**2** and MRL-**3** in rat liver microsomes was NADPH-dependent, suggesting P450-mediated bioactivation. Subsequent trapping experiments with GSH, NAc, and semicarbazide led to the identification of several adducts by LC-MS/MS analysis. A possible mechanism underlying the observed bioactivation involves hydroxylation at the C^5 of the pyrrolidine moiety, with the resulting β-fluoroaldehyde partitioning between reaction with semicarbazide and elimination of HF. The latter process affords an α,β-unsaturated carbonyl that is trapped by conjugation reactions with GSH or NAc (Fig. 22.9). This case study highlights the value of different trapping agents in mechanistic studies of bioactivation.

22.3 SEMIQUANTITATIVE DETERMINATION OF BIOACTIVATION

Bioactivation of test compounds may involve phase I (e.g., epoxidation) and/or phase II reactions (e.g., acyl glucuronidation). If metabolism of the compounds in question is mainly through phase I reaction, incubations with liver microsomes are likely appropriate for evaluation of bioactivation. In cases where compounds undergo both

phase I and II metabolism, hepatocytes should be a better choice of *in vitro* systems for assessing bioactivation potentials. In general, radiolabeled tracers are required for quantifying covalent protein binding. ^{14}C-Labeled compounds are preferred from a chemical and metabolic stability perspective. On the other hand, ^{3}H tracers are often a suitable alternative to the corresponding ^{14}C tracers because the former are relatively easy to synthesize and possess higher specific radioactivity. However, the ^{3}H label may be lost if oxygenation of the test compound occurs directly at the carbon center where the ^{3}H label is attached. This can usually be tracked with appearance of $^{3}H_2O$ during sample analysis, and the data should be incorporated into the assessment of covalent protein binding. To quantify covalent protein binding is a resource-consuming exercise and has been a subject of other review articles [11,54].

Semiquantitative approaches employing radiolabeled trapping agents may become an attractive alternative to covalent protein binding analysis from a resource-saving perspective. For example, bioactivation of test compounds may be determined following trapping experiment using [^{35}S]cysteine and [^{14}C]sodium cyanide [61]. Four drugs, namely, clozapine, diclofenac, R-(+)-pulegone, and troglitazone, known to cause hepatotoxicity, were incubated in human liver microsomes in the presence of [^{35}S]cysteine or [^{14}C]sodium cyanide. The radioactive peak areas of the trapped [^{35}S]cysteine adducts exhibited a reasonable correlation with the extent of covalent protein binding determined via a conventional exhaustive wash method using radiolabeled test compounds. To include both [^{35}S]cysteine and [^{14}C]sodium cyanide in the trapping experiments enabled simultaneously trapping both soft and hard electrophilic reactive metabolites and therefore improved the accuracy of "predicting" the extent of covalent protein binding. In a separate study, a high throughput trapping assay incorporated the use of potassium [^{14}C]cyanide to assess the degree of bioactivation of unlabeled compounds in question [62]. These compounds were incubated with liver microsomes in the presence of [^{14}C]cyanide, and the resulting cyanide adducts were concentrated, while excess unreacted tracer was removed via solid-phase extraction. The cyanide adducts then were eluted from the solid-phase extraction columns and subject to liquid scintillation counting. The extent of bioactivation potential was estimated based on the amount of radiolabeled cyanide adducts derived from the test compounds. Such an approach can be useful for ranking compounds within the same structural class during drug discovery.

Dansyl glutathione (dGSH, Fig. 22.2) is a fluorescent-labeled GSH derivative, with a fluorescence tag attached to the GSH moiety [63]. Its advantage resides with the fact that while reactive species trapped by dGSH are characterized by LC-MS/MS analysis, the amount of dGSH adducts formed is quantifiable via the fluorescent detection. A different fluorescence-based assay was demonstrated to employ a GSH-embedded 96-well plate for high throughput screen of reactive species formed *in vitro* [64]. The 96-well plates are initially coated with poly(2-hydroxyethylmethacrylate) (pHEMA) polymeric membrane, followed by attachment of glutathione disulfate (GSSG) to the surface of pHEMA. The immobilized GSSG is reduced to GSH by treatment with DL-dithiothreitol (DTT) before use. Incubations of test compounds with liver microsomes are performed in the DTT-treated 96-well plate, and the resulting reactive metabolites are trapped via their reactions with GSH coated on the plate. Remaining unreacted GSH are "destroyed" by adding *N*-(iodoacetylamino-ethyl)-1-naphthylamine-5-sulfonic acid (1,5-I-AEDANS) at the end of each incubation, and the resulting GSH-1,5-AEDANS is quantified by a fluorescence measurement. The extent of GSH adducts formed in the

incubation is derived from a reciprocal relationship relative to the amount of GSH-1,5-AEDANS. This method provides a rapid semiquantitative assessment of bioactivation *in vitro* via P450-catalyzed pathway, while its limitations are associated with the preparation of GSSG-immobilized 96-well plates and low sensitivity of detection for the reactive species tested (e.g., 500 nM for NAPQI).

22.4 A GENERIC PROTOCOL FOR *IN VITRO* TRAPPING STUDY

Liver microsomal preparations are suspended in phosphate buffer (100 mM, pH 7.4) containing EDTA (1 mM), $MgCl_2$ (0.1 mM) with the final protein concentration of 1 mg protein/mL. A trapping agent dissolved in the phosphate buffer is added into the suspension with a final concentration of 5 mM for GSH or NAc, 1 mM for KCN/$K^{13}C^{15}N$, or 1 mM for a DNA base. A test compound dissolved in methanol also is added to the suspension to give a final concentration of 10–50 μM. Incubations then are performed in the presence of 1.2 mM of NADPH at 37°C for 30–60 min. Reactions are terminated by adding 3× of acetonitrile, and the mixture is sonicated for 5 min and centrifuged at 20,800*g* at 4°C for 10 min. The resulting supernatant is collected, while the protein pellet is extracted twice with 1 mL of aqueous methanol (75%, v/v). The extracts and the supernatant are combined and evaporated to dryness under nitrogen at room temperature. The residues are suspended in 300 μL of aqueous acetonitrile (66%, v/v), an aliquot (75 μL) of which is subjected to LC-MS/MS analysis [65]. Further details can be found in experimental protocols published previously [10,11].

22.5 DATA INTERPRETATION AND UTILIZATION

Identification of reactive metabolites and their protein/nucleic acid adducts has been a focus of academic research in the area of toxicology and will likely be a part of future investigations exploring the pathogenic process leading to clinical onset of toxicity. A clear mechanistic understanding should significantly improve the detection and subsequent treatment of various tissue injuries resulting from pharmacotherapy. Although a rare example, APAP–protein adducts quantified in the circulation have been shown to serve as a possible biomarker for hepatotoxicity in patients suspected of drug overdose [66]. An accurate and timely diagnosis in this case is critical, as treatment with NAc is most effective if the antidote is given within ~16 h of APAP administration. On the other hand, identification of GSH adducts and related metabolites formed via the mercapturic acid pathway may be of toxicological importance in addition to simply "trapping" reactive metabolites for bioanalysis. A number of thiol adducts are implicated as culprits in drug-induced AEs. For example, terbinafine metabolism generates 7,7-dimethylhept-2-ene-4ynal, an allylic aldehyde that reacts readily with GSH via 1,6-Michael addition [67]. The resulting GSH adduct remains an electrophilic α,β-unsaturated carbonyl and also undergoes a retro-Michael reaction to convert back to the allylic aldehyde. These reactive species are suspected of covalently modifying MRP2 protein, leading to a compromised transporter activity and consequently drug-induced cholestasis. Alternatively, GSH adducts can undergo metabolism to form the corresponding cysteine adducts, which may be subject to bioactivation mediated by

renal β-lyase. The resulting reactive species are capable of modifying kidney proteins, causing nephrotoxicity such as that illustrated in patients exposed to haloalkenes [68].

From a pharmaceutical research perspective, one of the primary purposes of measuring bioactivation is to guide medicinal chemistry effort in drug discovery. There are numerous successful examples in terms of minimizing bioactivation during lead optimization processes [11]. Many of the scenarios reported usually involve the identification by LC-MS/MS of GSH adducts, followed by chemical intervention via incorporation of an electronegative functionality, such as F, CN, or heterocyclic moiety, into the structural series in order to reduce bioactivation potential. A few case studies also suggest that selection of a "meaningful" trapping agent should rely on the knowledge of specific toxicity. For example, MRL-**4** (Fig. 22.10a) is a potent cGMP-dependent protein kinase inhibitor for prevention and/or treatment of coccidiosis, but the compound promoted mutation in Ames tests when assays were performed in the presence of rat liver S9 fraction. This observation indicates that mutagenic effect shown by MRL-**4** was dependent on its bioactivation. Further studies of MRL-**4** metabolism in incubations with rat liver S9 containing guanine or cyanide led to the identification by LC-MS/MS of two possible reactive species (Fig. 22.10a). The first was likely an iminum ion capable of reacting with both the trapping agents to afford *N*-cyanomethyl

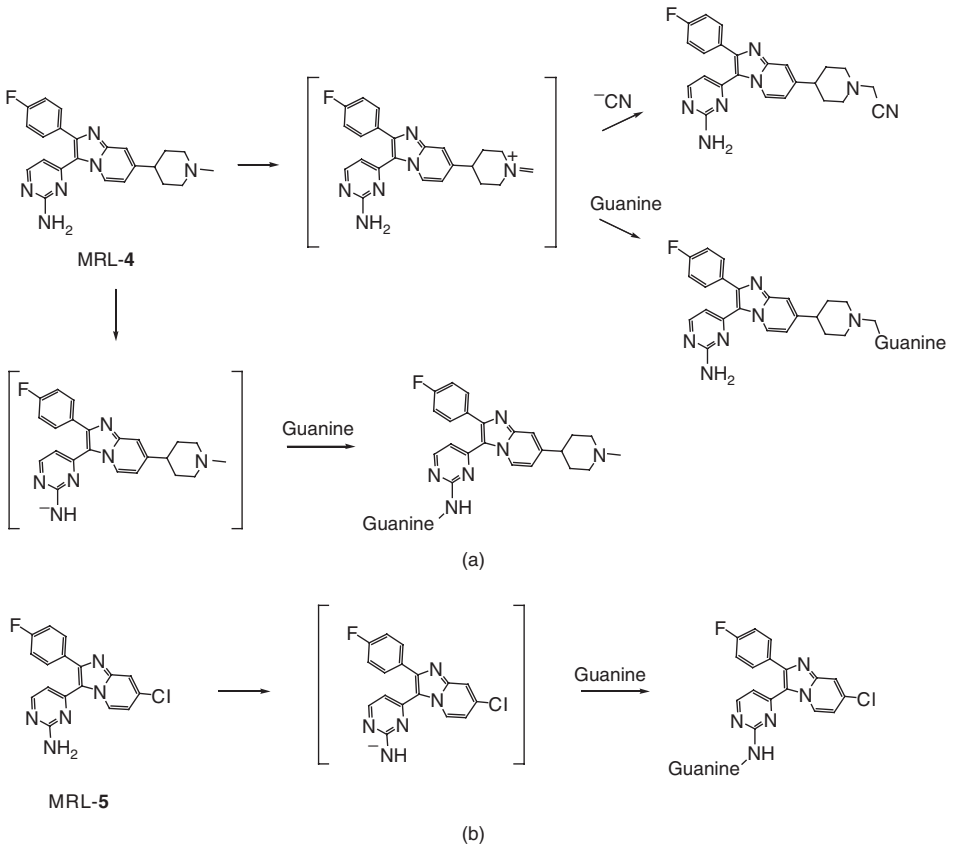

Figure 22.10 Bioactivation of MRL-**4** and MRL-**5** and formation of CN and guanine adducts.

and *N*-guaninemethyl piperidine derivatives, respectively. The second reactive metabolite presumably was a nitrenium ion, formation of which could begin with oxidation of the aminopyrimidine moiety to yield an *N*-hydroxyarylamine, followed by dehydration or a loss of sulfate/glucuronic acid if sulfation/glucuronidation of the hydroxyamine took place. The putative nitrenium ion reacted only with guanine, producing a guanine adduct. Subsequently, MRL-**5** (Fig. 22.10b) was synthesized, which retained the arylamine functionality but replaced the piperidine moiety with chlorine. The compound, when evaluated for mutagenic and bioactivation potential, was Ames positive and formed a guanine adduct in incubations with rat liver S9. Collectively, these data support the idea that bioactivation is responsible for the mutagenic effect of MRL-**4** and related arylamine analogs, whereas guanine adducts in this case serve as a better indicator for the formation of "toxicity-relevant" reactive species. Further lead optimization efforts eliminated arylamine from the structural class and consequently produced compounds that were Ames negative.

This example highlights the importance of minimizing bioactivation, while at the same time emphasizes the significance of data interpretation in relation to the knowledge of toxicity in question. Metabolites with different chemical reactivity are likely to target different biological molecules, some of which are biologically/toxicologically important, but others may simply serve as "scavengers" of reactive species. Such a scenario represents a dilemma for decision making in drug discovery, where data interpretation in many cases is prospective rather than retrospective. A core issue is the level of confidence with which one could whether claim a drug candidate would or would not cause harm to the patient according to a metabolism/bioactivation data set. Although this is a complex question, it appears that daily dose load is an important factor in determining the risk level of a compound, presumably due to saturable detoxification pathways. An empirical analysis suggests that drugs with a daily dose at 10 mg or less are associated with low incidence of drug-induced AEs [69]. Several recent studies attempted to test the hypothesis on whether reactive metabolites are the culprits responsible for clinical drug toxicity. In one study, 37 compounds were divided into 3 classes according to their safety status, including safe drugs (no warnings), warnings for toxicities, and black box warning/withdrawn from the market [70]. Bioactivation was measured by covalent protein binding quantified in incubations with human liver microsomes. The result suggests that majority of the compounds (30 out of 37) evaluated could be placed into their designated safety categories when daily dose was factored into the data analysis. A separate investigation of 50 drug products also was able to discriminate "toxic" from "safe" agents, using a criterion that normalized the extent of GSH adducts formed in human liver microsomal incubations to the daily doses prescribed [71]. Additional aspects deserving careful consideration during the selection of drug candidates include therapeutic areas and patient populations. A decision then can be made to balance the benefit and risk in order to satisfy unmet medical needs.

Last but not least, it should be noted that bioactivation represents only one of the several possible mechanisms underlying drug-induced AEs. Compounds can be intrinsically cytotoxic or can form metabolites that are cytotoxins but not chemically reactive. One such example is ximelagatran, a direct thrombin inhibitor prescribed for anticoagulation. The drug was removed from the market following clinical observations that ximelagatran treatment was associated with a significant elevation of liver enzymes and thus a high risk of severe liver injury [72]. Subsequent attempts to connect the

clinical AE and ximelagatran metabolism failed to identify GSH adducts or detect covalent protein binding in incubations with human liver microsomes or hepatocytes [73]. A probable cause of ximelagatran-induced hepatotoxicity remains obscure, although some evidence implies that the observed liver enzyme increases on drug treatment is immune mediated and dependent on specific genotypes of the major histocompatibility complex [74]. The parent drug also directly affected the plasma membrane fluidity and lipid composition in human hepatocyte incubations [75]. Similarly, troglitazone could serve to demonstrate that sometimes there are multiple mechanistic explanations for a pharmacotherapy-related AE in question. The drug was an agonist of the peroxisome-proliferator-activated receptor in the thiazolidinedione structural class for treatment of diabetes. Troglitazone was withdrawn from the market following numerous clinical reports of severe liver injury characterized by hepatocyte necrosis and bile duct proliferation. Metabolism of the drug leads to the formation of several reactive species, most of which are derived from P450-catalyzed oxidation of the chromane and thiazolidinedione moieties [76,77]. While these metabolites in principle could initiate pathogenesis of liver injury, the parent drug itself exhibited cytotoxicity to HepG2 cells expressing limited P450 activity [78]. Troglitazone also was subject to extensive sulfation in patients; the resulting sulfate derivative was the most abundant circulating metabolite with its plasma concentrations several-fold higher than that of parent drug [79,80]. Both troglitazone and the sulfate metabolite exhibited strong inhibition of BSEP *in vitro*, and their IC_{50} values and clinical exposures were within a similar concentration range. Because BSEP is a hepatic canalicular transporter regulating bile flow and removal of bile acids from liver, a compromised transporter function on drug treatment could lead to an accumulation of toxic bile acids in hepatocytes and subsequent cholestatic liver injury in susceptible patients. On that basis, inhibition of BSEP by troglitazone and its chemically inactive metabolite represents an alternative to bioactivation as a hypothesis to explain the drug-induced hepatotoxicity. Collectively, the above case studies suggest that clinical AEs, including liver injury, do not necessarily follow the path of forming reactive metabolites from the offending drug. With respect to pharmaceutical research, a balanced approach appears preferable such that the effort of minimizing bioactivation potential does not result in a delay of pivotal preclinical safety studies. It is conceivable that further clarification of the relationship between bioactivation and pharmacotherapy-related AEs should affect greatly the strategy of drug discovery and development, leading eventually to improved drug safety.

ACKNOWLEDGMENT

The authors thank Huifang Yao for providing the HRMS spectra of a GSH adduct resulting from diclofenac bioactivation.

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Abstract

A number of small-molecule drugs that cause severe adverse effects (AEs) undergo metabolism to form reactive species capable of covalently modifying proteins and/or nucleic acids. This has led to a working hypothesis that eliminating compounds susceptible to bioactivation in drug discovery should eventually produce safer medicines. A technical challenge associated with this strategy is to identify reactive metabolites that are often short lived either in biological systems or during sample preparation. Current bioanalytical options include capture of reactive metabolites with various trapping agents, such as glutathione (GSH) derivatives, synthetic peptides, cyanide, and nucleobase derivatives. These agents "trap" different types of reactive species, presenting the first hint on the mechanism of bioactivation. *In vitro* incubations usually are carried out in the presence of an appropriate trapping agent, with liver microsomes or hepatocytes serving as the source of enzymes responsible for bioactivation. The resulting samples are subjected to chromatographic separations and mass spectrometry (MS)-based detection of the trapped stable products. Similar procedures are applicable to specimens from preclinical species and humans for identification of GSH-related adducts formed *in vivo* via the mercapturic acid pathway. Sample analyses by triple quadrupole or ion trap mass spectrometers follow primarily a stepwise manner from neutral loss and/or parent ion scans to multiple-stage mass fragmentations in order to acquire sufficient data for metabolite structural assignment. Recent developments in instrumentation have centered on coupling ultraperformance liquid chromatography (UPLC) and high resolution MS; this combination is able to achieve a superior separation of analytes and acquisition of accurate mass spectra, hence a significant increase in sample throughput and improvement of metabolite detection. Use of accurate mass-based data-processing software further simplifies data interpretation, enabling quick structural assignment of the trapped products from which reactive species and bioactivation mechanisms are deduced. From a bioanalytical perspective, these experiments often require minimal sample preparation and method development, with a throughput usually satisfying the need of medicinal chemistry to construct a structure–activity relationship (SAR) with respect to the formation of reactive metabolites. The database accumulated, together with preclinical and clinical safety information, should also lead to better understanding of drug treatment-related adverse effects (AEs).

Keywords

bioactivation; bioanalysis; drug metabolism; DNA adduct; glutathione adduct; mass spectrometry; protein adduct; reactive metabolite; toxicity