Metabolism and Toxicity of Drugs. Two Decades of Progress in Industrial Drug Metabolism

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The science of drug metabolism and pharmacokinetics (DMPK) has developed significantly over the past 20 years, and its functional role in today's pharmaceutical industry has matured to the point where DMPK has become an indispensable discipline in support of drug discovery and development. While contributions to the lead optimization phase of discovery efforts have been particularly noteworthy in helping to select only the best drug candidates for entry into development, it should be recognized that the scope of DMPK spans the continuum of discovery through clinical evaluation and even into the post-marketing phase; as such, the breadth of DMPK's involvement is almost unique in contemporary pharmaceutical research. This perspective summarizes notable advances in the field, many of which have been made possible by technological developments in areas such as molecular biology, genetics, and bioanalytical chemistry, and highlights the critical nature of key partnerships between Drug Metabolism, Medicinal Chemistry, and Safety Assessment groups in attempting to advance drug candidates with a low potential for causing adverse events in humans. Finally, some speculative predictions are made of the future role of DMPK in pharmaceutical research, where current advances in our mechanistic understanding of the molecular processes that control the absorption, disposition, metabolism, elimination, and toxicity of drugs and their biotransformation products will combine to further enhance the impact of DMPK in drug discovery and development.

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1. The Past 20 Years

During the past 20 years that *Chemical Research in Toxicology* has been in existence, the field of drug metabolism and pharmacokinetics (DMPK[1]), and its relationship to drug-mediated toxicity, has evolved significantly in a number of respects. Prior to the early 1980s, for example, DMPK studies in the pharmaceutical industry were largely descriptive in nature and were not aimed at providing mechanistic insight into the fate of candidate drugs in biological systems. Rather, these early studies focused primarily on clinical development efforts in support of product registration, with little attention being devoted to the metabolic and pharmacokinetic characteristics of new chemical entities in preclinical species. The fact that the primary reason for failure of drug candidates in clinical development during the 1960s and 1970s was judged to be due to inappropriate human pharmacokinetics (*1*) led to the progressive integration of DMPK as a key component of the overall drug discovery process. As a consequence of this paradigm shift, selection of drug candidates for entry into development became based, in part, upon their DMPK properties in animals, which, in turn, allowed cautious predictions to be made of their corresponding properties in humans. While attrition in the overall drug development process remained high during the subsequent two decades, attrition due to inappropriate human PK properties fell from 39% to 8%, with preclinical toxicology and lack of efficacy in humans now listed as the major reasons for failure (*2*). In parallel with the increased investment of DMPK resources in the drug discovery and early development process (*3*) came a heightened awareness of the potential role of metabolites as mediators of pharmacological response and drug-induced toxicities, and screens for both on- and off-target biological activities now routinely include those metabolites that represent major circulating species in animals or are predicted to be prominent drug-related materials in humans.

The concepts that emerged from the pioneering academic studies in the 1970s and 1980s on the role of chemically reactive metabolites in drug-induced toxicities have been relatively slow to impact contemporary drug development, largely due to a continued lack of understanding of the basic mechanisms involved in acute cell injury. However, there is general agreement that the exposure of cells to reactive, electrophilic metabolites of foreign compounds is an undesirable outcome (*4, 5*), and most major pharmaceutical companies now have

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[1] Abbreviations: DMPK, drug metabolism and pharmacokinetics; OATP, organic ion-transporting polypeptide; PXR, pregnane X receptor; CAR, constitutive androgen receptor; GR, glucocorticoid receptor; TPMP, thiopurine *S*-methyltransferase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ESI, electrospray ionization; UHPLC, ultra high performance liquid chromatography; AMS, accelerator mass spectrometry; MALDI, matrix-assisted laser desorption–ionization; ADME, absorption, distribution, metabolism, and excretion; Hrms, high resolution mass spectrometry; GSH, glutathione; siRNA, small-interfering ribonucleic acid; PK/PD, pharmacokinetic/pharmacodynamic; FAIMS, high-field asymmetric waveform ion mobility spectroscopy; QWBA, quantitative whole body autoradiography.

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established procedures to detect such short-lived intermediates through appropriate *in vitro* and *in vivo* studies and, by way of appropriate structural modifications to lead compounds, divert metabolism to pathways that yield chemically stable products (*6–8*). In the past two decades, genetic aspects of both drug metabolism and susceptibility to drug toxicity have become widely appreciated, and reaction phenotyping (primarily with regard to human drug-metabolizing enzymes that exhibit genetic polymorphism, for example, CYP2D6 and 2C19) now is a standard component of the *in vitro* profiling of all drug candidates entering development. Similarly, drug candidates are evaluated at an early stage for their potential to cause, or to serve as a victim of, drug–drug interactions that may arise through induction or inhibition of cytochrome P450 enzymes (*9*), reflecting the fact that several recent product withdrawals from the marketplace have occurred as a consequence of serious adverse events triggered by polypharmacy, one component of which altered CYP activity and thereby impacted the clearance of the co-administered agent(s). Thus, screens for both competitive and mechanism-based inhibition of key enzymes, for example, CYP3A4 (*10*), have been established and implemented in a high throughput fashion (*11*). In addition, the field of transporters localized in the plasma membrane and their role in drug uptake, efflux, and drug interactions has become a prominent area of investigation in recent years and is highly relevant to the pharmaceutical industry since it has become apparent that the interplay of drug-metabolizing enzymes and drug transporters can represent a critical determinant of drug disposition, drug interactions, and toxicity in animals and man (*12*). While initial interest focused on P-glycoprotein (*13*), current attention has broadened to encompass a wide spectrum of both organic anion and cation transporters that modulate the passage of many drug candidates across membrane barriers. A case in point is the antihypercholesterolemic agent atorvastatin, which, along with active hydroxylated metabolites that are formed by CYP3A4, is actively taken up into liver tissue by organic ion-transporting polypeptides (OATPs) such as OATP1B1. Rifampicin has been shown to inhibit these transporters (*14*) such that when atorvastatin is dosed together with rifampicin, a significant increase in systemic exposure to both atorvastatin and its metabolites results (*15*).

Overall, one of the key roles of preclinical DMPK in today's pharmaceutical industry is to identify potential liabilities in new chemical entities as early as possible, with the aim of eliminating molecules that exhibit metabolic or pharmacokinetic properties that likely would preclude their successful development as therapeutic agents. In this regard, a close partnership between DMPK scientists and their colleagues in Medicinal Chemistry, Biology, and Safety Assessment groups has become critical in advancing candidates with the highest probability of success into clinical development (*3, 5, 16*). It is noteworthy that most, if not all, of the advances in drug metabolism that have occurred over the past two decades have been stimulated, directly or indirectly, by developments in technology. For example, the tools of molecular biology have made available recombinant human drug-metabolizing enzymes for studies of protein structure, mechanisms of catalysis, reaction phenotyping, metabolic stability screening, and evaluation of inhibitors, yet these biological reagents were not available 20 years ago (*17*). Similar advances in the transporter field have provided transfected cell lines with which to examine *in vitro* the kinetics of drug uptake and efflux and to probe mechanisms of drug–drug interactions mediated by transport proteins. The roles of nuclear transcription factors, for example, pregnane X receptor (PXR), constitutive androstane receptor (CAR), and the glucocorticoid receptor (GR), in the regulation of gene expression were poorly understood in the 1980s, but these receptors now are recognized to act as key determinants of enzyme induction and are available for early *in vitro* assessments of the potential of drug candidates to induce key enzymes, for example, CYP3A, in both animals and humans (*18*). Advances in the field of genetics have had a major impact on drug metabolism and have provided the experimental tools with which to probe the genetic basis for inter-subject variability in human drug pharmacokinetics, metabolism, and toxicity (*19–22*). A growing understanding of pharmacogenomics has led to the implementation of clinical genotyping in connection with the use of certain narrow therapeutic index drugs such as the thiopurine derivatives azathioprine and 6-mercaptopurine for acute lymphoblastic lymphoma (*23, 24*) and the colorectal cancer agent irinotecan (*25*). In the case of the former agents, a genetic polymorphism in thiopurine *S*-methyltransferase (TPMT*3) is now known to be responsible for profound inter-subject variability in drug exposure and associated safety margins that necessitates dosage adjustments, while SN-38, an active metabolite of irinotecan, is cleared primarily via glucuronidation catalyzed by UGT1A1, of which the *28 allelic variant results in the reduced expression of the enzyme and influences the ratio of SN-38 to its inactive glucuronide conjugate.

In the area of bioanalysis, LC-MS/MS technology with electrospray ionization (ESI) has all but replaced alternative analytical methods for the routine qualitative and quantitative determination of drugs and their metabolites in biological fluids (*26*). The first generation of robust triple quadrupole LC-MS/MS systems only became commercially available in the early 1990s with the introduction of the Sciex API 3 mass spectrometer, yet instruments with this basic configuration now represent the mainstay of most industrial drug metabolism laboratories. Recent developments in high resolution instrumentation for LC-MS/MS applications, such as the Waters Q-TOF and Thermo LTQ-FT and Orbitrap systems, provide accurate mass data for all ions in the spectrum, resulting in elemental composition information that is immensely valuable in the structure elucidation of drug metabolites in complex mixtures of biological origin (*27–29*). Advances in HPLC technology (e.g., ultra high performance liquid chromatography or UHPLC) also have contributed to the greatly enhanced capabilities of contemporary LC-MS/MS systems, not only in terms of chromatographic resolution and associated speed of separations, but also with regard to the capacity and robustness afforded by the current generation of columns, autosamplers, pumping systems, and so forth. Accelerator mass spectrometry (AMS), a technique employed originally for applications in the geophysical sciences, has been developed as an exquisitely sensitive tool for biomedical applications, including studies of drug disposition, in which trace amounts of a radioisotope (e.g., ^{14}C) can be detected and quantified in blood or excreta following the administration of a microdose of the agent of interest (*30*). In the area of therapeutic proteins and monoclonal antibodies, matrix-assisted laser desorption–ionization (MALDI) mass spectrometry has become an important technique (in addition to ESI) not only for qualitative applications but also as a reference method against which immunological assays (e.g., ELISA) can be validated for determining the PK properties of these biologics in animals and humans (*31*). From the above selected examples, it may be concluded that the past 20 years have witnessed enormous technological advances that have facilitated the study of drug metabolism in animals and humans and have led to a much

deeper appreciation of the multiplicity of factors that govern the biological fate of foreign compounds in living systems.

2. Today's Changing Environment: Metabolites in Safety Testing (MIST)

The pharmaceutical industry of the early 21st century faces a range of formidable challenges, stemming from factors such as the higher complexity of target diseases for which there are unmet medical needs, the greatly increased costs associated with drug discovery and development, the heightened competition for global market share, and the growing regulatory and societal pressures to demonstrate the safety and effectiveness of newly introduced medicines. Attrition in early drug development remains high, largely due to preclinical toxicology, and increasing attention has been paid over the past years to the role of drug metabolites, in addition to their respective parent compounds, as potential mediators of drug-induced toxicities. In this regard, it becomes important to define a set of operating principles upon which the results of drug metabolism studies can be placed in context with respect to the toxicological evaluation of new chemical entities. In 2002, a position paper sponsored by the US Pharmaceutical Research and Manufacturers Association (PhRMA) outlined a series of considerations related to the issue of metabolites in the safety assessment of drug candidates, and proposed a number of practical approaches to address the problem (*32*). A clear distinction was made between stable drug metabolites, which can be isolated, purified, and identified by standard techniques, and chemically reactive metabolites, which typically cannot. While the PhRMA paper dealt with the former class of metabolites, it was acknowledged that chemically reactive intermediates may play an important role in the characteristic toxicities of certain investigational and approved therapeutic agents, yet there was a lack of consensus within the industry as to how best to deal with these short-lived species. In the period following publication of this position paper (the so-called MIST document), there has been lively debate, with contributions to the literature from both from the pharmaceutical industry (*33–35*) and the US Food and Drug Administration (*36*), over some of its recommendations; in particular, discussion has centered on the appropriate level of investment that should be devoted to assessing the safety of stable drug metabolites, notably those that are formed in humans but are detected only at low levels, or not at all, in animals (*5, 37*). Part of the problem here stems from the fact that our technical capabilities to detect, identify, and quantify drug metabolites have outpaced our ability to understand the role (or lack thereof) of metabolites as the causative agents in various types of toxicity.

Regardless of the final outcome of the above debate, it is clear that progressively more detailed information will be sought on the metabolic profiles of drug candidates in both animals and humans in support of preclinical and clinical safety studies and that particular attention will need to be paid to abundant circulating metabolites in humans that are not well represented in the preclinical species used for safety assessment. This, in turn, will create a need for methodology through which drug metabolites formed in humans (notably those circulating in plasma) can be detected, identified, and quantified at a much earlier stage of clinical development than traditionally has been the case. While the gold standard approach to the assessment of drug metabolism and disposition in man has been the human ADME (absorption, distribution, metabolism, and excretion) study, normally conducted with the aid of an appropriately ^{14}C-radiolabeled analogue, this study usually is not performed until proof-of-concept has been achieved, that is, when the drug candidate is in Phase II clinical development. This timing is dictated by the resource requirements associated with the preparation and formulation of the radiotracer and by the desire to conduct the experiment at a dose of the drug that is likely to fall within the range to be employed therapeutically. The drawback of this timing, however, is that evidence for human-specific metabolites (which are very rare) or metabolites that circulate in humans at higher levels than have been evaluated in animals (which are observed occasionally) is not obtained until relatively late in the development program. From a regulatory standpoint, it could be argued that it is inappropriate to advance a drug candidate into broad, pivotal Phase III clinical trials in the absence of adequate exposure margins in animals for both parent compound and major circulating metabolites. Indeed, there have been instances in recent years where a clinical hold has been placed on development programs until such issues have been addressed, resulting in significant delays in the initiation of the phase III trials and, ultimately, in product registration.

At least two solutions to the above problem have been proposed. The first employs a microdose approach, in which a trace dose of a ^{14}C-labeled analogue is administered to volunteers at a very early stage of development (Phase 0), even before traditional Phase I safety and tolerability studies have commenced (*38*). The fate of the ^{14}C then is followed by AMS, although the current lack of commercial online LC-AMS instrumentation requires that specimens of blood and excreta be subjected to off-line HPLC separation and individual fractions then analyzed to reconstruct a metabolite profile. The advantages of this approach are that a truly tracer dose is administered to volunteers such that the amount of radioactivity that is dosed falls below the limits that would be subject to regulations for the use of radiolabeled materials. Since the mass of compound dosed also is very low, an abbreviated preclinical safety package is required, with concomitant savings in toxicology resources and bulk drug synthesis. However, limitations to this method are that a ^{14}C-labeled tracer is required and that no structural information on metabolites is provided since all drug-related materials in the sample are graphitized prior to analysis for ^{14}C. Moreover, there is an underlying assumption that the ADME properties of the drug candidate of interest following administration of a microdose are representative of those following a therapeutic dose (*39*). An alternative approach, which does not require the availability of a radiotracer, involves the use of new high resolution mass spectrometry (Hrms) and associated data processing techniques to selectively detect drug-related materials in biological specimens by virtue of their accurate masses (*40*). In essence, this technique is based upon the fact that the majority of drug substances exhibit a negative mass defect relative to endogenous background constituents of biological samples with a similar molecular weight. In essence, this is because the fractional mass of a molecule (i.e., the figures after the decimal point) is strongly influenced by the number of hydrogen atoms in that molecule, and drug substances tend to be relatively deficient in hydrogens due to the prevalence of heterocycles and other centers of unsaturation. (The presence of one or more halogen atoms in the parent drug further enhances the negative mass defect of metabolites relative to endogenous materials.) Advantage can be taken of this feature by searching LC-MS/MS data sets post-acquisition to selectively detect components whose accurate masses are consistent with drug-derived products. Following detection of these potential metabolites, information on their molecular structures then may be extracted from

MS/MS spectra with the benefit of the elemental composition information encoded in the accurate masses of their respective parent and fragment ions. It may be anticipated that the various steps in this process will be facilitated through advances in artificial intelligence software routines that process MS/MS data and propose metabolite structures based upon comparisons of parent and metabolite spectra and a knowledge of atom connectivities in the parent molecule. It is probable that this potentially very powerful methodology will prove especially valuable in analyzing plasma samples from first-in-human studies to establish a preliminary human metabolic profile and to reveal products of biotransformation that were predicted on the basis of preclinical studies, and, conversely, metabolites that were not expected. Although this approach is strictly qualitative in nature, semiquantitative estimates of exposure could be based upon comparisons of mass spectrometric responses of individual metabolites with corresponding radiometric, UV, or fluorescence responses from the same compounds detected in preclinical studies. Potentially unique human metabolites detected by this approach would have to be identified and authentic standards prepared by synthesis for further evaluation.

3. Drug Metabolism and Lead Optimization: A Critical Partnership in Drug Discovery

As discussed above, drug metabolism studies continue to play a key role in the lead optimization stage of drug discovery, through the identification of undesirable ADME characteristics in candidate molecules that would impede a successful development program. While each pharmaceutical company approaches lead optimization in a somewhat different fashion, and may have different acceptance criteria for progressing compounds, a key issue in today's environment centers on those factors that may impact drug safety. For example, progress has been made in the application of toxicogenomics approaches to identify characteristic gene expression signatures predictive of specific toxicity end points, although much of the current effort in this area is focused on validation of the methodology (*41*). A more established approach is the identification of toxicophores, defined as structural features within a molecule that have been associated with a particular toxicity in a series of molecules (*42*). In most cases, toxicophores correspond to functionalities that undergo metabolism to chemically reactive intermediates that cause cellular damage by one of a number of mechanisms (*43*). Indeed, it is now appreciated that many functional groups can be subject to metabolic activation (*44*), usually via CYP-mediated oxidation reactions (*45*), but our ability to predict, *a priori,* which elements of a given molecular structure will be subject to such activation remains very limited, and information on this topic generally needs to be derived through the conduct of appropriate *in vitro* and/or *in vivo* experiments. Moreover, even if a given drug candidate is demonstrated to form reactive metabolites, as evidenced by the formation of adducts with nucleophiles such as glutathione (GSH) or through covalent binding to tissue macromolecules, it is rarely possible to predict the associated toxicological risk given current limitations in our understanding of basic mechanisms of cellular injury (*46, 47*). At Merck Research Laboratories, strategies for lead optimization take into account the propensity of drug candidates to undergo metabolic activation, based on the fundamental premise that, in the absence of knowledge of the effects of specific reactive metabolites, exposure of tissues to these electrophilic intermediates represents an undesirable feature of any drug candidate (*4, 48*). Thus, detailed information is generated on the metabolic fate of promising candidates in the lead optimization phase, using liver microsomal preparations from several species (including human) as well as plasma and excreta from a limited number of *in vivo* animal studies, in order to identify potential pathways of bioactivation. These studies are complemented by assessments of covalent binding to liver protein, in which radiolabeled analogs of the compounds-of-interest are incubated, under standard conditions, with hepatic microsomal preparations from animals and humans in the absence and presence of cofactors such as NADPH and GSH (*49*). In addition, *in vivo* covalent binding studies also are performed in the rat, where irreversible binding of radioactivity is measured to both liver and plasma proteins. According to our standard protocol, levels of covalent binding ideally should be <50 pmol equiv/mg protein, which reflects an $\sim$20-fold margin relative to typical levels of covalent binding observed with prototypical hepatotoxins (*48, 49*). However, it should be emphasized that this value represents a target and not a rigorous threshold above which compounds are not advanced into development. Rather, a number of factors are taken into consideration in assessing, on a case-by-case basis, whether a certain level of covalent binding is viewed as acceptable (*48*), and greater weight is given to the *in vivo* results, as opposed to the figures obtained in liver microsomal preparations. It also should be noted that in situations where the mechanism of action of the drug candidate is believed to involve covalent modification of the biological target, covalent binding studies would not be interpretable and therefore are not performed; examples would include agents such as omeprazole, clopidogrel, and β-lactam antibiotics.

Collectively, the above suite of studies provides valuable insight, both qualitatively and quantitatively, into pathways of metabolic activation of drug candidates in mammalian systems, which is utilized by medicinal chemists in their efforts to optimize the properties of molecules *prior to their progression into development*. This latter point is important since the opportunity for informed structural modification is essentially lost after a compound is formally accepted as a development candidate. It should also be emphasized that the object of these studies is *not* to predict toxicity; rather, the goal is to help decide where to place one's bets with regard to the selection of one molecule over others for entry into development since this decision triggers the commitment of substantial resources to the successful candidate. On the basis of this discussion, it will be evident that Drug Metabolism, in concert with Medicinal Chemistry and Safety Assessment groups, has a very important role to play in the lead optimization arena. The following example of a recent study conducted by Preclinical Drug Metabolism at Merck serves to illustrate this point and describes a case of species-selective metabolic activation that correlated with species-selective hepatotoxicty[2].

In the course of lead optimization studies on a series of substituted zwitterionic piperidine derivatives, one compound (Compound A, Figure 1) emerged as a prospective development candidate on the basis of a favorable pharmacological profile (high potency and selectivity against the biological target) and pharmacokinetic characteristics in rats, dogs, and rhesus monkeys suggestive of a once-daily dosing regimen in humans. Preliminary *in vitro* metabolism studies with this compound indicated that two major routes were followed in all species examined, namely, UGT-mediated conjugation of the carboxylic acid moiety to yield the corresponding acyl glucuronide, and CYP-mediated oxidation of the aryl and alkyl substituents denoted as R_1 and R_2. The acyl glucuronide appeared to be

[2] Watt, A. P., B. Sohal, B., and O'Connor, D., unpublished results.

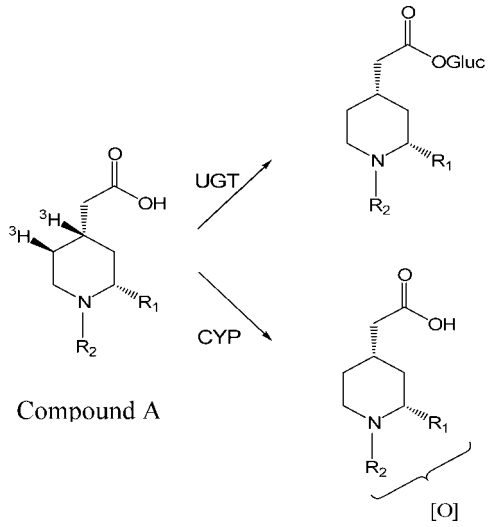

Figure 1. Primary routes of metabolism for piperidine carboxylic acid derivative, Compound A. The locations of the tritium atoms in the radiolabeled tracer are as shown.

Table 1. Covalent Binding of Compound A (10 μM) to Liver Microsomal Preparations from Various Species[a]

species	*in vitro* CB[b] (pmol equiv/mg)	turnover[c] (%)
rat	<5	39
dog	12	41
rhesus	686	84
human	5	<10

[a] [^{3}H]Compound A was incubated with pooled liver microsomal preparations from rat, dog, rhesus, and human for 60 min, and radioactivity irreversibly bound to protein was measured according to a standard protocol (*49*). Parallel incubations that lacked NADPH served as negative controls. [b] CB-NADPH-dependent covalent binding to protein. [c] Turnover, expressed as the fraction of substrate consumed over the 60-min incubation period.

relatively stable from a chemical standpoint, in that little evidence was observed of rearrangement to positional isomers. In order to examine the metabolic fate of Compound A in more detail, a radiolabeled analogue was prepared by tritiation of an unsaturated precursor to afford the [3,4-$^3H_2$] variant depicted in Figure 1 for a series of *in vitro* and *in vivo* studies and for assessment of the propensity of the compound to undergo bioactivation to species that became covalently bound to protein. Interestingly, the results of the latter experiments, conducted according to standard Merck covalent binding protocols (*49*), revealed a striking species difference in the ability of [$^3H_2$]Compound A to alkylate liver microsomal proteins fortified with NADPH. Thus, while covalent binding was low (relative to the <50 pmol equiv/mg protein target value) in preparations from rats, dogs, and humans, high levels of irreversibly bound radioactivity were noted in liver microsomal preparations from the rhesus monkey (Table 1). Turnover of substrate in monkey liver microsomes (84%) was double that in corresponding preparations from rats and dogs and much higher than that in human liver microsomes, suggesting that metabolism in the rhesus differed, qualitatively and/or quantitatively, from that in the other species studied. Parallel incubations in which UDPGA was added as a cofactor to support glucuronidation demonstrated that the covalent binding was dependent upon oxidative metabolism and not upon acyl glucuronide formation (data not shown). The relatively low levels of covalent binding to rat liver proteins *in vitro* were replicated in studies performed in the rat *in vivo*, where a single 20 mg kg^{-1} oral dose led to maximum levels of binding to liver and plasma proteins of only 5 and 12 pmol equiv/mg protein, respectively. On the basis of these observations, an exploratory single dose toxicology study was performed in rhesus monkeys (100 mg kg^{-1}) that demonstrated liver enzyme elevations in plasma and, upon necropsy, histological evidence of hepatic necrosis. In contrast, no evidence of liver injury was obtained from corresponding *in vivo* studies in rats or dogs. Hence, it appeared that Compound A was subject to extensive metabolic activation in the rhesus monkey, leading to species-specific hepatotoxicity.

Table 2. Effect of Trapping Agents on the Covalent Binding of Compound A (10 μM) to Rhesus Liver Microsomal Preparations[a]

trapping agent (200 μM)	*in vitro* CB[b] (pmol equiv/mg)	turnover[c] (%)
none	743	82
GSH	586	76
β-mercaptoethanol	649	81
cyanide	297	77
methoxylamine	735	83

[a] Incubations (60 min) were performed with [^{3}H]Compound A, as described in Table 1, in the presence or absence of a nucleophilic trapping agent, and radioactivity irreversibly bound to protein was measured according to a standard protocol (*49*). [b] CB-NADPH-dependent covalent binding to protein. [c] Turnover, expressed as the fraction of substrate consumed over the 60-min incubation period.

In order to test the above hypothesis, a series of *in vitro* studies were conducted to compare the metabolite profiles and covalent binding properties of Compound A in liver microsomal preparations from the rhesus monkey in the absence and presence of nucleophilic trapping agents such as GSH and mercaptoethanol (thiol nucleophiles), cyanide ion (an effective trap for iminium ions), and methoxylamine (a trapping agent for reactive carbonyl species). These experiments revealed that of the nucleophiles tested, only cyanide ion successfully attenuated the covalent binding of Compound A to protein (Table 2), with no diminution of turnover. When unlabeled Compound A then was incubated in rhesus liver microsomes fortified with NADPH and [^{14}C]cyanide, HPLC analysis indicated the formation of a single ^{14}C-labeled product (Figure 2) which, upon LC-MS analysis (positive ion ESI), afforded an MH^+ ion whose *m*/*z* value was consistent with an elemental composition of (M + O – H + CN). Finally, it was noted that when [$^3H_2$]Compound A served as substrate in incubations with or without added nucleophiles, and the products were analyzed by reverse phase HPLC, significant amounts of radioactivity eluted in the column void volume, indicating that metabolism of Compound A was associated with partial loss of the ^{3}H label in the form of tritiated water.

The above data led to the construction of a proposed bioactivation mechanism for Compound A, which is depicted in Figure 3. According to this scheme, Compound A undergoes CYP-mediated two-electron oxidation, yielding an intermediate iminium species which, upon deprotonation, affords a putative enamine species. A second cycle of CYP catalysis introduces an atom of oxygen to generate an unstable epoxide that undergoes spontaneous ring opening to the dipolar oxy-anion that ultimately is trapped by cyanide to give the stable end-product detected in our experiments. According to this scheme, one or more of the electrophilic intermediates resulting from oxidation of the piperidine ring system would represent likely culprits in the observed covalent binding of Compound A to protein. The mechanistic insight gained from this investigation resulted in the synthesis and evaluation of a series of analogues of Compound A in which the above pathway of metabolism was blocked, thereby lowering the potential liability of reactive

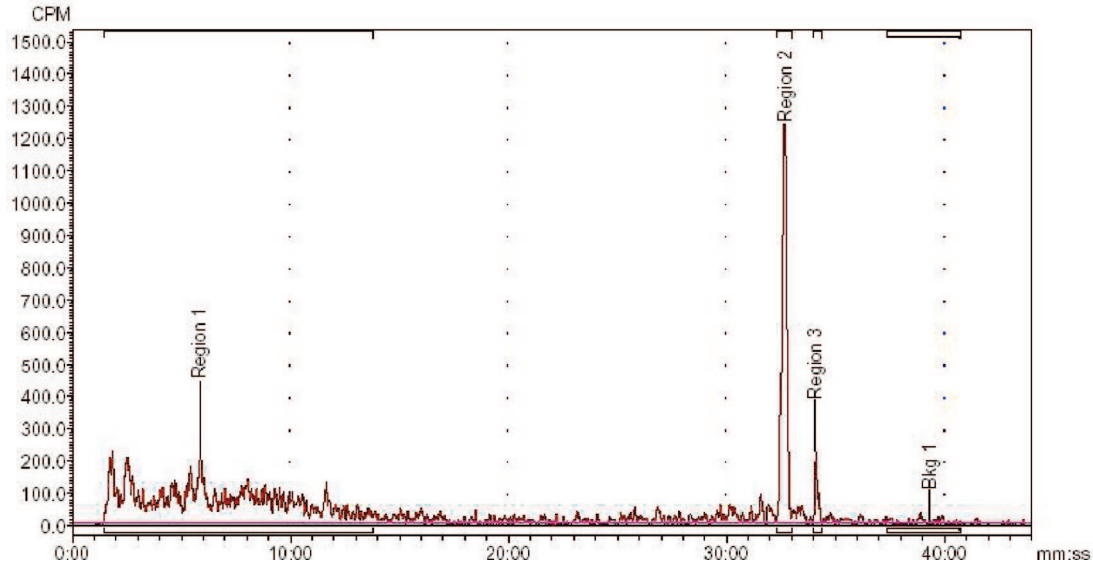

Figure 2. Radiochromatographic analysis of the products of incubation (60 min) of unlabeled Compound A (10 μM) with rhesus liver microsomal preparations fortified with NADPH and K^{14}CN.

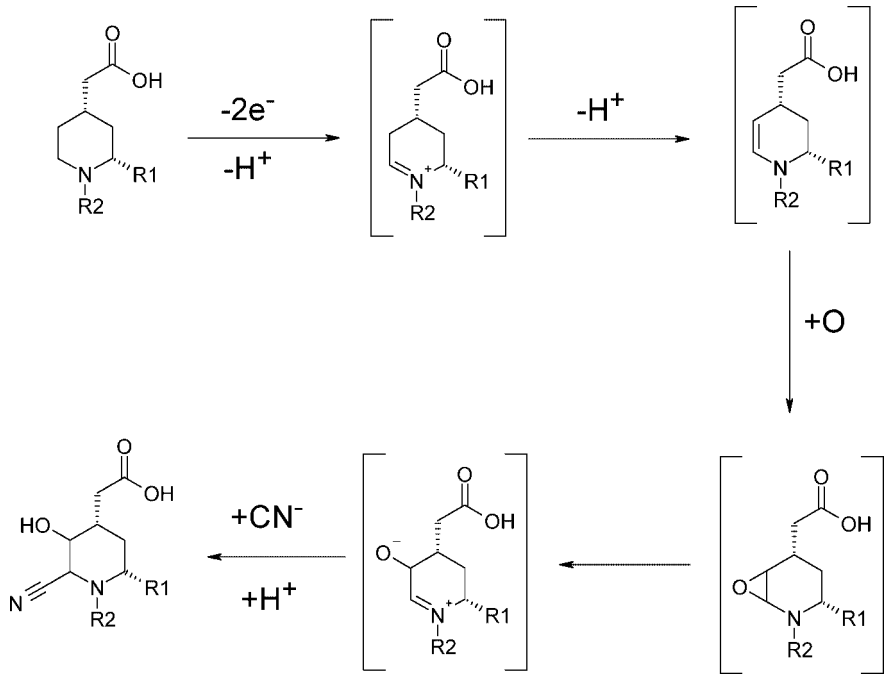

Figure 3. Proposed bioactivation mechanism for Compound A. The intermediates depicted in brackets were not isolated but are inferred.

metabolite-mediated liver toxicity. It should also be noted that in the specific case of Compound A, the rhesus monkey was not representative of humans with respect to its metabolic properties, and therefore, this animal species would have been a poor choice in the preclinical toxicological evaluation of Compound A, had this agent been selected for development.

4. Drug Metabolism in the Future: What Lies Ahead?

On the basis of the advances in drug metabolism made over the past two decades and in particular upon the trends noted in more recent years, one can speculate on areas where the field likely will develop in the future. With regard to the constituent elements of ADME, an increased understanding of the role of transporters in drug disposition (notably, as they pertain to both hepatic and renal uptake and elimination, and CNS penetration) will lead not only to a fuller appreciation of factors that influence the absorption and disposition of pharmaceuticals but also to the interplay between transporters and drug-metabolizing enzymes. In this regard, the AD and ME components of ADME are inextricably linked, and a knowledge of both the enzymology and transporter biology that determine the fate of foreign compounds in living systems, together with the factors that regulate their expression, will be required in order to build predictive models of drug metabolism and disposition in humans (*18*). Such models, which will become available in the near future, will permit the evaluation of drug–drug interaction potential, not only at the level of enzyme induction and inhibition but also through competition for uptake or for efflux transporters (*50, 51*). Indeed, a fuller appreciation of the structure–activity relationships for transporters will impact drug discovery efforts through the targeted use of transporters for selective tissue uptake (or exclusion) and enhanced drug absorption. Progress will continue to be made on the development of novel formulations and drug delivery systems, and these

efforts will be dependent upon appropriate ADME support. A case in point is the exciting new area of siRNA-based therapeutics, where effective delivery of the siRNA to target tissues currently represents the greatest challenge in the development of these agents as a broad therapeutic platform (*52*). To date, siRNA has been formulated as conjugates (e.g., with cholesterol derivatives), liposomes/lipoplexes or as complexes with peptides, polymers, or antibodies, yet very little is known about the *in vivo* stability and metabolic fate of these components that may influence their siRNA-delivering properties.

While the field of *in silico* modeling for ADME applications has not yet realized its full potential in support of pharmaceutical research, it seems likely that this approach also will mature in the coming years, enabling predictions of the most relevant pharmacokinetic, metabolic, and, potentially, toxicity end points (*53, 54*). Similarly, methods to predict the susceptibility of a new chemical entity toward metabolic activation will provide guidance to medicinal chemists engaged in drug discovery programs, although it seems unlikely that *in silico* techniques will replace well-established *in vitro* or *in vivo* experimental approaches in the foreseeable future. In contrast, modeling and simulation of pharmacokinetic/pharmacodynamic (PK/PD) relationships already has demonstrated its value in the design of more efficient, streamlined, clinical trials (*55*), and the tools of pharmacometrics are being adopted widely by both the pharmaceutical industry and regulatory agencies both for decision making in drug development and in the assessment of disease progression (*56*).

The next decade also will bring an appreciation of the functional consequences of genetic polymorphisms in drug transporters (*57–59*), in much the same way as has been achieved in the past two decades in the drug-metabolizing enzyme field (*22*). Developments in the concept of personalized medicine, in which both the selection of a specific therapeutic agent and its associated dosage regimen are tailored to the individual patient on the basis of genetic considerations (*60*), clearly will have ramifications for drug metabolism in that genetic aspects of each of the processes involved in ADME will need to be better defined than is the case today (*61, 62*). Also related to the field of genetics is the increasing availability of genetically engineered animal models that express human enzymes, transporters, and/or nuclear receptors, which offer intriguing possibilities for studies in both drug metabolism and toxicology. For instance, the safety implications of a unique human metabolite formed through CYP3A4 catalysis may be addressed by an appropriate toxicology study conducted in chimeric mice with humanized livers (*63*).

In the area of new technologies, developments in mass spectrometry and allied techniques continue at a rapid pace and undoubtedly will impact drug metabolism in the future, as they have done in a profound way over the past 20 years. This is likely to be especially true in the area of metabolite detection and structure elucidation, on the basis of a new generation of high resolution mass analyzers that may be expected to provide unrivaled LC-MS/MS performance in terms of high sensitivity and dynamic range, low parts-per-million (ppm) accuracy in mass assignments across the entire spectrum, compatibility with high resolution chromatographic interfaces, and automated data processing capabilities designed specifically for drug metabolism applications (*64, 65*). Indeed, it is probable that Hrms-based approaches will have all but replaced current low resolution MS methods for qualitative applications in industrial drug metabolism laboratories over the next 5 years. Orthogonal capabilities for MS, such as those based upon high-field asymmetric waveform ion mobility spectrometry (FAIMS), will provide additional dimensions in terms of the selectivity of detection (*66*), while advances in imaging applications of mass spectrometry (*67, 68*) will complement (and perhaps even replace) today's quantitative whole body autoradiography (QWBA) approaches to the study of tissue levels of drugs and their metabolites. The utility of AMS will be augmented greatly by online coupling to liquid sample introduction devices such that the resulting LC-AMS combinations will find increasing application for microdose studies and for investigations on the metabolic fate of lightly labeled tracers (i.e., pharmacological doses containing only trace amounts of radioactivity). Routine quantitative analyses of drugs and their metabolites probably will continue to employ some variation of today's triple quadrupole-based LC-MS/MS design, although it seems inevitable that significant improvements in performance and versatility of instrumentation will occur and that miniaturization of equipment will lead to reductions in purchase cost. These advances will be accompanied by a higher degree of automation in all aspects of sample handling, analysis, and data reporting such that the productivity of the drug metabolism mass spectrometry laboratory will increase by at least an order of magnitude by the year 2012. Moreover, the information extracted from each biological sample will increase since future generations of LC-MS/MS systems will possess the ability to generate qualitative and quantitative data simultaneously, without sacrificing accuracy, precision, and overall data quality.

In the area of drug safety, drug metabolism will continue to be a prominent contributor to the field, particularly as more is learned about structure–toxicity relationships at the molecular level (*69*). Research on mechanistic aspects of cellular damage caused by chemically reactive drug metabolites holds the potential of dramatically altering the way that the pharmaceutical industry currently deals with metabolic activation issues, although studies in this area face formidable challenges in deciphering the critical molecular targets for injury and their role in signaling pathways for cellular defense or cell death. Recent publications on the application of MS-based proteomics techniques to the problem of reactive metabolite–protein covalent adducts underscore the complexity of the issue but also suggest that the tools are now available to identify key cellular targets of these electrophiles, a knowledge of that could lead to future predictive approaches to toxicities mediated by reactive intermediates (*46, 70–73*). Of particular importance in this area is the recognition that different reactive electrophiles can exhibit a relatively high selectivity for alkylation of different proteins, and for different nucleophilic sites within these proteins, the toxicological significance of which remains to be established. Idiosyncratic toxicities that are believed to involve the human immune system represent some of the most challenging safety problems of all (*74–76*), yet progress in this field likely also will benefit from a deeper understanding of the interaction between reactive drug metabolites and proteins. The scope of drug metabolism activities in support of the safety assessment of new chemical entities also seems likely to include the field of metabonomics since this evolving science provides one avenue to a true systems biology approach to toxicology. Advanced analytical methodologies of the types described above, coupled with gene expression profiling technologies (*77–79*), could have a major impact on early drug development through reduction of attrition due to toxicity problems. Given all of the above prospects, the next 20 years of research on drug metabolism and toxicity promise to be at least as exciting as the previous two decades!

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