Drug-Protein Adducts: An Industry Perspective on Minimizing the Potential for Drug Bioactivation in Drug Discovery and Development

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Running title: Drug bioactivation and covalent binding to proteins

It is generally accepted that there is neither a well defined nor consistent link between the formation of drug-protein adducts and organ toxicity. Since the potential does exist, however, for these processes to be causally related, the general strategy at Merck Research Laboratories has been to minimize reactive metabolite formation to the extent possible by appropriate structural modification during the lead optimization stage. This requires a flexible approach to defining bioactivation issues in a variety of metabolism vectors, and typically involves the initial use of small molecule trapping agents to define the potential for bioactivation. At some point, however, there is a requirement to synthesize a radiolabeled tracer and to undertake covalent binding studies in vitro, usually in liver microsomal (and sometimes hepatocyte) preparations from preclinical species and human, and also in vivo, typically in the rat. This Perspective article serves to provide one pragmatic approach to addressing the issue of bioactivation from an industry viewpoint based on protocols adopted by Merck Research Laboratories. The availability of a dedicated Labeled Compound Synthesis group, coupled to a close working relationship between Drug Metabolism and Medicinal Chemistry, represents a framework within which this perspective becomes viable; the overall aim being to bring safer drugs to patients.

I. Introduction

The concept that toxicities can stem from drug bioactivation in vivo continues to be problematic for pharmaceutical researchers, inasmuch as while the detection of a bioactivation process is relatively straightforward, the downstream consequences of this process remain indeterminable. This is due to the relatively little progress which has been made in understanding the molecular events which occur following drug haptenization of a protein. Since experimental tools are available to investigate drug bioactivation processes, and medicinal chemists are well versed in the art of optimizing structure-activity relationships, it is natural to assume that this latter skill set also can be exploited to prepare drugs which do not undergo metabolic activation. This goal has been the credo at Merck & Co., as one element of attempting to bring safe drugs to market.

The concept that small organic molecules can undergo bioactivation in vivo, and that the resulting electrophiles can adduct to biological macromolecules and subsequently elicit organ toxicity, has its origins in studies performed during the early 1930's to mid 1950's. Such investigations included the work of Fieser (*1*) who investigated the hepatotoxicity of polycyclic hydrocarbons, and that of Miller and Miller (*2, 3*), who studied the hepatotoxic effects of *p*-dimethylaminoazobenzene in the rat and reported that aminoazo dyes become tightly bound to the protein constituents of liver tissues.

These early studies laid the foundation for the "Covalent Binding Theory" of xenobiotic induced liver and lung toxicity, which emerged during the 1970's and 1980's

through a series of investigations performed at the National Institutes of Health by Brodie, Mitchell, Gillette and Boyd (*4-8*) who examined the bioactivation of a wide variety of small organic molecules and correlated this process with organ toxicity. The molecules investigated in these early studies, and in more recent work, include 4-ipomeanol (*6-8*), acetaminophen (*9*), halothane (*10*), vesnarinone (*11*), isoniazid (*12, 13*), furosemide (*14*), clozapine (*15, 16*), and bromobenzene (*17-19*). Many of these investigations shared the common experimental approach of using enzyme inducers and inhibitors to modulate the extent of bioactivation which, in turn, influenced the degree of covalent binding and tissue damage observed in vivo; the dogma postulated being that the processes of bioactivation, covalent binding and tissue damage were intrinsically linked.

The above dogma was challenged by several investigators during the 1980's. For example, Tirmenstein and Nelson (*20*) reported that despite normalizing the dose level of acetaminophen (APAP) and its regioisomer, 3′-hydroxyacetanilide, to provide comparable levels of covalent binding in vivo in the mouse (~ 1.3 - 1.6 nmol drug equivalents/mg liver homogenate protein), APAP was hepatotoxic, but 3′-hydroxyacetanilide was not. From findings such as these, it was proposed that it was the adduction of "critical proteins", namely proteins required for specific cell function and viability, which was important for organ toxicity. While there are increasing numbers of reports of the identities of proteins which are modified by reactive drug metabolites (*18, 21-23*), few have attempted to distinguish "critical" from "non critical" proteins, or have investigated whether there are species differences in the types of proteins adducted. The absence of such information has essentially limited our ability to predict *a priori* whether

bioactivation, and the accompanying covalent binding of drug-related material to proteins will, or will not, result in organ toxicity in preclinical species or human. Moreover, even if a drug candidate that is subject to metabolic activation were to fail to cause organ damage in a conventional preclinical safety assessment program, the concern remains that the drug-protein adducts formed in humans may have the potential to act as haptens and elicit an immune-mediated adverse event (*24*). This represents a significant concern for the pharmaceutical industry since it is generally acknowledged that no animal model exists for human immune-mediated toxicities. Thus, conventional preclinical safety studies may fail to identify those drug candidates which have the potential to elicit a hypersensitivity reaction in humans.

II. Covalent Binding Risk Assessment in Drug Discovery

In light of our inability to predict which reactive metabolites will be toxic, and which will not, the default position for Merck has been to minimize reactive intermediate formation to the extent possible by appropriate structure modification during the lead optimization stage. Initially, efforts are made to identify reactive intermediates by the use of chemical trapping agents, such as reduced glutathione (*25*) or cyanide (*26, 27*), to form stable adducts that are amenable to characterization by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and/or NMR spectroscopy. These experiments, normally conducted in liver microsomal preparations, provide valuable indirect information on the identity of the original electrophile. Parallel efforts may involve the

conduct of covalent binding studies, although these require the early availability of radiolabeled lead candidate compounds.

Regardless of the specific approach, there needs to be a readiness to identify the mechanism by which bioactivation occurs and a willingness to abrogate the potential liability through iterative structural modifications. Indeed, understanding the mechanism of metabolic activation is critically important if medicinal chemists are to minimize this process through rational changes in structure. It is these two phases, namely that of understanding the mechanism of bioactivation and applying chemical intervention to minimize bioactivation, which highlights the importance of a close working relationship between Drug Metabolism and Medicinal Chemistry from the point of project inception. This proactive approach to addressing bioactivation is justified when considering the issue of patient safety and the economic risks in pursuing drugs which do covalently modify proteins. These risks are somewhat compounded by the typically low incidence of idiosyncratic reactions to drugs in the patient population (Table 1) which can result in safety issues being identified only after the patient population has become relatively large (Phase III clinical trials and beyond); a time when the economic commitment to drug development already has been considerable.

For those compounds which possess the requisite characteristics of a development candidate (high degree of efficacy and potency in animal models, acceptable pharmacokinetics in preclinical species, appropriate physico-chemical properties, etc), a radiolabeled analog of the compound (preferably ^{14}C-labeled) is prepared and in vitro

covalent binding experiments are performed in liver preparations from animals and humans. Hepatic microsomes are employed to assess metabolic activation catalyzed primarily by oxidative enzymes, while the use of freshly isolated hepatocytes may serve to reveal metabolic activation processes that depend upon the presence of a full complement of cellular enzyme systems. Covalent binding studies also are carried out in intact rats, where the level of adducts to both liver and plasma proteins is determined at appropriate intervals after administration of a standard oral dose of the test compound (Section III).

Having determined the level of covalent binding of a drug candidate, the question then becomes, "How much apparent covalent binding is acceptable in deciding whether to advance a drug candidate into development?" Merck's approach to this question has been to take the levels of covalent adducts typically found in the livers of animals given a dose of a prototypic hepatotoxin (e.g. APAP, bromobenzene, furosemide or 4-ipomeanol) (Table 2) associated with the expression of hepatic necrosis (approximately 1 nmol drug equivalents/mg protein), and to reduce this figure by 20-fold to give a conservative target 'threshold' value for in vivo covalent binding of 50 pmol drug equivalents/mg total liver protein. This value also corresponds to a level of radioactivity that is approximately 10-times background under normal conditions, and thus provides a suitable dynamic range for measurement of covalently bound drug-protein adducts. It should be emphasized that the figure of 50 pmol drug equivalents/mg protein is viewed as a *target* upper limit, and not as an absolute threshold above which a compound would not be advanced into development, since it is recognized that other factors must be taken into consideration in

arriving at this decision. In this regard, it is important to point out that several drugs which are known to undergo bioactivation and have associated clinical adverse events (AEs) are still marketed. This fact provides some initial insight into how we should view covalent binding data in the context of risk assessment. The AEs for such drugs include agranulocytosis (amodiaquine & clozapine), hepatotoxicity (diclofenac), hepatic failure (bicalutamide), and Stevens-Johnson syndrome (lamotrigine). Clearly, in using these agents, the risks have to be weighed against the benefits, and a number of factors taken into consideration. In the context of new drug discovery and development, these factors, which can be regarded as "qualifying considerations" for the purposes of this text (Figure 1), include the intended clinical use of the drug candidate (is it an unmet medical need or are current therapies inadequate?), the severity of the indication (is the prognosis disabling or life-threatening?), the dosing regimen (will the drug be used acutely, or chronically/prophylactically?), and the intended clinical population (will the drug be used for a pediatric indication where the tolerance for toxicity will be less?).

When assessing covalent binding data in liver microsomes in vitro, the degree to which drug clearance is dependent on Phase 2 enzymes also should be borne in mind. For instance, a compound providing levels of covalent binding in liver microsomes of > 50 pmol drug equivalents/mg, but which is metabolized almost exclusively in hepatocyte preparations or in vivo by Phase 2 enzymes, where covalent binding is < 50 pmol drug equivalents/mg protein, would still be considered viable. Also worthy of consideration is the projected therapeutic dose. Uetrecht has reported (*28*) that drugs given at a daily dose of 10 mg or less are rarely, if ever, associated with a significant degree of idiosyncratic

drug reactions. From the foregoing considerations, it is apparent that the decision process regarding the weight to place on covalent binding data is not digital but multifactorial. Thus, the propensity for metabolic activation should be viewed as one of several potential liabilities which need to be taken into account when making programatic decisions on a lead compound.

As an example of the application of the above criteria, it is instructive to consider ethinylestradiol (EE), the usual estrogenic component of the oral contraceptive pill. While the daily oral dose is very low (35 μg), hence satisfying at least one of the risk-benefit considerations, EE produces high levels of covalent binding in vitro (1.2 nmol drug equivalents/mg protein following incubation of 250 μM EE for 20 min in rat liver microsomes) (Table 2). Understanding routes of metabolism (the compound is eliminated predominantly by sulfation and glucuronidation, with the gut making a significant contribution to this process) (*29, 30*), and the mechanism of bioactivation (in the absence of cytosolic catechol O-methyl transferase, a reactive *o*-quinone will be formed), an informed decision can be made not to view Phase 1-mediated metabolic activation as a serious liability.

Another practical issue that needs to be taken into account, namely that of appropriate location of the radiolabel, is perhaps obvious but is critical if false negative results are to be avoided. This is best exemplified in the case of isoniazid where, had the primary metabolite acetylisoniazid been labeled in the pyridine moiety (as opposed to the acetyl group) for covalent binding studies, no radioactivity would have become

associated irreversibly with liver proteins (Table 2). This example highlights the potential need to undertake covalent binding studies with more than one radiolabeled form of the compound-of-interest, often prompted by a better understanding of the metabolic disposition of the drug candidate.

The process of covalent binding risk assessment in drug discovery can be summarized as attempting to minimize reactive metabolite formation to the extent possible by appropriate structure modification during the lead optimization stage. Studies performed in vitro (*e.g*. liver microsomes) allow species differences in covalent binding to be explored and an insight gained into the mechanism of metabolic activation. Studies performed in vivo (typically in the rat) provide information on the levels of covalent binding which will occur during evaluation in safety studies, and also provide an opportunity to assess the degree of metabolic activation in tissues other than the liver. The decision tree employed at Merck (Figure 1), along with the risk-benefit (qualifying) considerations, provide a rationale for compound evaluation; target covalent binding values of $\leq$ 50 pmol drug equivalents/mg protein both in vitro and in vivo are desirable, although values in excess of this figure in vitro and/or in vivo may be acceptable under qualifying conditions.

III. Standardization of Covalent Binding Methods

Since covalent binding data have the potential to influence the fate of a program lead compound, it is essential that these data become available at an early stage when

there is time to optimize the lead structure or to explore alternative chemical templates. If covalent binding data from different research sites are to be viewed consistently, it is imperative that data be derived under standardized conditions and that assay conditions be validated accordingly. At Merck, several research sites participate in drug discovery efforts, and therefore an inter-site validation of a covalent binding method, based on the use of rat and human liver microsomes, was undertaken.

In brief, the method employed involves the incubation of 10 μM drug substrate with rat or human liver microsomes (RLM, HLM; 1mg protein/ml) (± NADPH) for a 1 hr incubation period (total final volume of 200 μl), at which point the reaction is stopped and protein precipitated by the addition of acetone (800 μl). Precipitated protein is collected using a Brandel cell/membrane harvester and the protein washed with 80% aqueous methanol (1.5 liter per 96 well plate; 30 ml per filter). Individual filter discs are punched from the Brandel harvester mat, the protein solubilized, and aliquots taken for protein assay and determination of radioactive content by liquid scintillation counting. In addition to evaluating test compounds, two control compounds also are routinely investigated. The first of these is used by all sites and allows the performance of the assay in different laboratories, and on different sites, to be monitored. The second control is program specific, and allows the Project Team to monitor the impact of Medicinal Chemistry intervention on improving the stability of subsequent lead compounds towards metabolic activation.

In the context of standardizing methodology for assessing the covalent binding of drugs to liver microsomal protein in vitro, compounds were selected on the basis of a well characterized bioactivation process. Knowledge of the enzyme system(s) responsible for metabolic activation also was desirable. The compounds chosen covered a wide range of covalent binding values (~50 to >2500 pmol drug equivalents/mg) and included diclofenac, imipramine, L-746530 and MRL-A (Figure 2). Four Merck sites were involved in this validation process wherein four compounds were radiolabeled and rat and human liver microsomes prepared on one site were shipped to the remaining three sites for evaluation in a standard covalent binding protocol. The percentage coefficient of variations (CVs) obtained for this assay generally were < 20%, although there were some exceptions where CVs were as high as 40% (Tables 3 & 4).

Covalent binding studies in pooled hepatocyte preparations using the Brandel tissue harvester method also have been undertaken (10 μM drug, 2 hr incubation period with 1 x 10^6 cells/ml, N = 3 preparations, Brandel extraction). Due to the inherent variability of covalent binding values originating from the use of individual preparations of freshly isolated hepatocytes, or even from different batches of pooled cryopreserved hepatocytes, data from these studies are not necessarily considered to be on the critical path for decision making purposes. Rather, these data are viewed in the broader context of better defining the metabolic disposition of the compound, the mechanism of bioactivation, and the level of covalent binding observed in all experimental systems, both in vitro and in vivo. For information, however, the mean values (with CVs in parenthesis) of covalent binding obtained for the compounds under investigation in

pooled (N=5) cryopreserved human hepatocytes were: 6 (17%) pmol drug equivalents/mg for imipramine, 81 (8%) pmol drug equivalents/mg for MRL-A, 31 (24%) pmol drug equivalents/mg for diclofenac, and ~359 (15%) pmol drug equivalents/mg for L-746530.

Studies performed in vivo also have been standardized. Rats were dosed orally at 20 mg/kg and animals exsanguinated at 2, 6 and 24 hr (N=3 animals per time point), their livers removed, and the samples stored frozen until analysis. This in vivo assay may, in addition, be performed at a lower dose of 2 mg/kg to evaluate the impact of dose on the extent of metabolic activation. For guidance, approximately 20 μCi of ^{14}C labeled compound or 40 μCi of ^{3}H labeled compound typically is dosed to a 300 g rat. The dose levels of 2 and 20 mg/kg were chosen arbitrarily to reflect a typical "low" and "high" dose. It should be noted that the use of a tritium tracer carries with it the potential to underestimate levels of covalent binding, either through loss by exchange (generation of tritiated water) or through metabolism (a concern which also applies, albeit to a lesser extent, to a ^{14}C tracer). Metabolism studies usually provide insights into the potential for such tracer loss, and investigators need to be prepared to undertake covalent binding and drug disposition studies with an alternative tracer, should the need arise.

It should be clear also from the foregoing discussion that our approach to assessing metabolic activation makes no effort to scale values of covalent binding determined in vitro (liver microsomes or hepatocytes) to those determined in vivo. Rather, our experiments are designed to highlight the potential for metabolic activation in several metabolic systems so that a structure based resolution of this finding can be

instigated. In this regard, our choice of relatively high drug concentrations, 10 μM for in vitro and up to 20 mg/kg for in vivo studies, reflect our desire to balance maximizing analytical sensitivity with standardizing protocols.

IV Characteristics of Compounds Investigated

Diclofenac typically is prescribed to patients at 50 mg tid and can undergo both CYP3A4- and CYP2C9-mediated bioactivation processes (*31*-*33*). Following incubation of 1 mM diclofenac with human liver microsomes, protein adduct formation was reported to be mediated by CYP3A4 (*33*) since the initial product of CYP3A4 catalysis, 5-hydroxydiclofenac, is subject to further oxidation to a reactive *p*-benzoquinoneimine intermediate. In support of this contention, an N-acetylcysteine adduct of 5-hydroxydiclofenac was identified in urine from human subjects dosed with diclofenac (*34*). On the other hand, Tang et al. (*32*) have reported that an isomeric *p*-benzoquinoneimine, derived from 4′-hydroxydiclofenac, is formed by CYP2C9 in vitro at more clinically relevant concentrations (< 50 μM), whereas the 5-hydroxylation of diclofenac, in agreement with Shen et al. (*33*), becomes a significant pathway in vitro at higher concentrations (> 100 μM). Indeed, CYP2C9 has been shown to mediate the formation of the putative quinoneimine which can be trapped with reduced glutathione to form 4′-hydroxy-3′-(glutathion-S-yl)diclofenac (*32*). Bougie *et al*. (*35*) have also shown that the product of CYP2C9-mediated oxidation and acyl glucuronidation, namely the 4′-hydroxydiclofenac acyl glucuronide metabolite, had an apparent role in diclofenac-induced hemolytic anemia.

Imipramine is used in the dosage range of 75 to 225 mg per day and has been associated with abnormalities in liver function which tend to occur during the second month of treatment (*31, 36, 37*). Imipramine 2-hydroxylation is a major route of metabolic clearance in humans (*31*), and is catalyzed almost exclusively by CYP2D6 (*38*). By analogy with rat (*39*), it is proposed that imipramine undergoes metabolic activation in humans to an arene oxide which covalently modifies proteins.

L-746530 undergoes CYP3A-mediated bioactivation (Section V). Following oxidation on the dioxobicyclo portion of the molecule at the methylene position alpha to the ring oxygen, an aldehyde is formed which can be trapped in vitro using semicarbazide. Alternatively, the aldehyde can form a Schiff base adduct to proteins in vitro resulting in levels of covalent binding of ~ 2 nmol drug equivalents/mg (Tables 3 & 4). The furan moiety of L-746530 also can undergo metabolic activation, as described in Section V for its close structural analog, L-739010.

The levels of covalent binding following incubation of 10 μM diclofenac with rat or human liver microsomes (~290 and ~57 pmol drug equivalents/mg) highlight a quantitative species differences, binding being higher in rat liver microsomes. Had diclofenac been a development candidate under the current Merck paradigm, efforts would have been made to explain this species difference in bioactivation, with the goal of more effectively assessing the potential risk to humans. The covalent binding values obtained for imipramine (~ 460 and ~130 pmol drug equivalents/mg in rat and human

liver microsomes) would have warranted a similar discussion. The values for covalent binding of L-746530 and MRL-A (> 1500 pmol drug equivalents/mg) are sufficiently high that it is unlikely that these compounds would be advanced today as drug candidates.

V. Biomarkers of Metabolic Activation

In some instances, reactive intermediates can form adducts with small molecule trapping agents. Characterization of these adducts using LC-MS/MS and NMR techniques can provide indirect information on the structure of the reactive species from which they are derived, thereby defining a potential bioactivation mechanism and hence a rationale on which to base a chemical intervention strategy. The use of trapping agents also provides a means by which a relatively large number of compounds can be evaluated rapidly, thereby allowing prioritization of compounds for radiolabeling prior to classical determination of covalent binding. A caveat to using a trapping agent is that there probably is no single small molecule which can serve as a universal surrogate for a complex protein macromolecule; nevertheless, several traps have found widespread utility and are discussed below. Also, certain reactive metabolites are so reactive that they are presumed to be unable to escape from their site of formation, or react rapidly with water of the medium, and therefore are not efficiently trapped. Products of hydrolysis can, however, yield information about their precursor reactive intermediates. A good example is the trifluoroacetic acid metabolite of halothane which is excreted in

the urine of patients receiving this agent and is formed, in part, by the hydrolysis of the reactive trifluoroacyl halide intermediate (*31*).

A. Glutathione

Reduced glutathione (GSH), an abundant physiological nucleophile by virtue of its cysteine sulfhydryl group, captures reactive xenobiotic metabolites to form *S*-substituted adducts. Once GSH stores are depleted, however, cellular proteins may be adducted, and it is believed that in certain cases cellular function is compromised and organ toxicity can ensue (*40*). Hence, GSH serves as a natural trapping agent for chemically reactive metabolites and has been used extensively in vitro to study a broad range of reactive intermediates including quinoneimines (acetaminophen), nitrenium ions (clozapine), arene oxides (carbamazepine), quinones (estrogens), imine methides (3-methylindole), and Michael acceptors (valproic acid metabolites), for which references are provided in Table 5.

Typically, in vitro experiments are conducted at 0.2 - 5 mM GSH concentration. Glutathione adducts can be analyzed by LC-MS/MS using either the full scan mode to search for anticipated conjugates, or by constant neutral loss scanning for 129 Da (γ-glutamyl moiety) to detect all GSH-related species (Figure 3). However, it should be recognized that some GSH adducts are not stable and are subject to chemical degradation/rearrangement or enzymatic degradation (e.g., catalyzed by γ-glutamyltranspeptidase; SectionVI A), thereby escaping detection. In certain cases, *N*-

acetyl cysteine (NAC) can be used in place of GSH as a nucleophilic trap, although this agent may be less efficient than GSH if the conjugation reaction in question is catalyzed (even in part) by glutathione-S-transferase enzymes.

B. Potassium Cyanide

The cyanide anion (CN^-) is a "hard" nucleophile that can be used to trap certain electrophilic drug metabolites. An example is presented in Figure 4, in which 1 mM KCN (a mixture of CN and $^{13}C^{15}N$ at 1:1 ratio) was used as the trapping agent. The detection of cyano adducts by LC-MS was greatly facilitated by the presence of prominent isotopic "doublets" that differed in mass by 2 Da (mono-adducts) or 4 Da (bis-adducts); further, the MS/MS spectra of these adducts were characterized by a neutral loss of 27/29 Da ($HCN/H^{13}C^{15}N$). This technique has its origins in the work of Gorrod and co-workers (*26, 27*), and has been highlighted recently in a comprehensive review of bioactivation reactions of nitrogen-containing xenobiotics (*41*).

C. Methoxylamine and Semicarbazide

Both methoxylamine and semicarbazide can form a Schiff base with aldehydes, a process mimicking reactions between aldehyde metabolites with lysine residues on proteins. Typical conditions require the addition of 5 mM of either trapping agent to the incubation mixture followed by LC-MS/MS analysis. This approach was taken for both L-739010 and L-746530 (*42, 43*) and is presented in Figure 5.

VI. Bioactivation - 'Structure Alerts' Past and Present

Over the last few decades, considerable literature has accumulated on the subject of chemical induced toxicity, a representative selection of which is summarized in Table 5. The chemicals listed share the common feature of being able to form a reactive intermediate capable of alkylating proteins. Table 5 also contains two recent examples from Merck Research Laboratories (L-754394 and L-739010) where metabolic activation issues were identified and simple modifications made to the parent structures to produce drug candidates with markedly reduced bioactivation potential.

A. Piperazine Bioactivation

MRL-A, a 3-acyl-N^1-methylpiperazine derivative (Figure 6), was found to undergo CYP3A4-mediated piperazine bioactivation (*44*). A mechanism for this process was proposed whereby formation of the end-product metabolites M1 and M2 involved a six electron oxidation of the piperazine ring, attack by GSH, and hydrolysis of the glutamic acid residue to afford a cysteinylglycine conjugate of the piperazinone. Attack (aminolysis) by the cysteinyl amine moiety resulted in opening of the piperazinone ring leading to a thiazolidine thioaminal intermediate which, in turn, underwent ring closure to the imidazoline products observed. The structures of adducts M1 and M2, elucidated by ^{1}H-NMR and high resolution mass spectrometry, were consistent with this sequence of events. In keeping with the proposed mechanism, it was found that while MRL-A alkylated proteins extensively, simple alkyl substitutions (methyl, isopropyl) alpha to the

N^1-methyl functionality afforded derivatives which did not undergo metabolic activation and therefore were more attractive as drug candidates.

B. Pyrazinone Bioactivation

When a substituted pyrazinone derivative (MRL-B, Figure 7) was incubated with liver microsomal preparations from rats and humans fortified with GSH, two isomeric GSH conjugates (GSH-1 and GSH-2) were detected using LC-MS/MS (*45*). The benzylic alcohol, resulting from hydroxylation of MRL-B at the 6-methyl position, also was observed. The proposed origins of GSH-1 and GSH-2 pointed to two distinct, cytochrome P-450-mediated metabolic activation processes. Thus, it was proposed that MRL-B had undergone a two-electron oxidation, either directly or *via* dehydration of the hydroxymethyl metabolite, to generate an electrophilic imine-methide. Capture of this intermediate by GSH afforded adduct GSH-1. In the competing activation process, it was proposed that oxidative attack of the pyrazinone ring generated an unstable epoxide intermediate, which was attacked regiospecifically by GSH to afford a cyclic carbinolamide. Tautomeric ring-opening of the latter species to the acyclic methyl ketone, followed by *syn/anti* isomerization of this substituted amidine, would lead to the formation of a new ring system which, following elimination of the elements of water, would generate the stable imidazole derivative, GSH-2. Subsequent studies performed in the rat in vivo using radiolabeled MRL-B indicated that this compound gave rise to appreciable levels of covalent adducts to plasma and liver proteins. However, replacement of the 6-methyl group by chlorine, which prevented formation of the imine

methide intermediate and rendered this heterocycle less susceptible to epoxidation, led to an analog whose potential for reactive metabolite formation and protein alkylation was greatly diminished.

C. Bioactivation of a Series of Aryloxy Derivatives

In one of our drug discovery programs at Merck, sites of metabolic activation within a new structural series were identified rapidly using online LC-MS^n on an ion trap mass spectrometer (*46*). Following incubation of MRL-C (Table 6) with rat or human liver microsomes, reactive intermediates were trapped as their corresponding glutathione conjugates. Mass spectral characterization of these conjugates allowed potential sites and mechanisms of bioactivation for MRL-C to be proposed. These studies, coupled to informed structural modification of the series to block sites susceptible to metabolic activation, resulted in a significant reduction in the propensity of this compound class to label liver microsomal proteins in vitro (Table 6).

One strategy to reduce metabolic activation of MRL-C was to explore aromatic fluorine substitution as a means of blocking sites of oxidative metabolism, the aim being to take advantage of the electron-withdrawing effects of fluorine to reduce the π-electron density of the aromatic ring and hence render this moiety less susceptible to cytochrome P-450 catalyzed metabolism (*47, 48*). In addition, the carbon-fluorine bond is stronger than the corresponding carbon-hydrogen bond, and thus is less prone to oxidative cleavage. Unfortunately, fluorinated derivatives such as MRL-D (Table 6) all underwent

extensive metabolic activation that was accompanied by oxidative dehalogenation and formation of GSH adducts. Therefore, an alternative strategy was pursued whereby a pyridine ring was incorporated as an isosteric replacement for the phenyl ring; this modification resulted in reduced levels of metabolic activation of MRL-E. Despite this improvement, MRL-E remained sub-optimal and provided levels of covalent binding well in excess of the target threshold value of 50 pmol drug equivalents/mg. Further improvements were made via chlorination (MRL-F) or trifluoromethylation (MRL-G) of the pyridine, the latter substitution resulting in a compound which failed to produce GSH adducts in either rat or human liver microsomal incubations. Furthermore, the levels of covalent binding to liver microsomal protein determined for MRL-G were significantly lower than those obtained in any other members of the series (Table 6). Further structural refinements in other parts of the molecule (data not shown) provided levels of covalent binding which were <50 pmol drug equivalents/mg in both rat and human liver microsomal preparations, while retaining desirable pharmacokinetic and pharmacodynamic characteristics.

VII. Concluding remarks

Despite 70 years of research, it is still not possible to accurately predict the potential for toxicity of a compound which has been shown to undergo metabolic activation. However, considerable advances have been made in the areas of analytical methodology (mass spectrometry and NMR spectroscopy) and labeled compound synthesis. As a result, it has become easier to identify reactive intermediates, by using

either small molecule trapping agents or by undertaking covalent binding studies using radiolabeled compounds, and these approaches have now become a routine part of candidate drug evaluation at Merck Research Laboratories. Importantly, these investigations are undertaken at an early stage, during the discovery phase, when a mechanistic understanding of a bioactivation problem enables Medicinal Chemistry to provide a structure-based solution. Clearly there is a need to standardize methods for evaluating the potential for a drug candidate to under-go bioactivation in order to ensure consistency of data and uniformity in the decision making process. Future advances in the areas of immunology, genetics and proteomics may well provide a more coherent relationship between the characteristics of a drug-protein adduct and a toxicity outcome. Indeed, an important problem to address in the field of toxicology has been the characterization of protein targets of reactive electrophiles; in this regard, the work of Badghisi and Liebler offers new LC-MS/MS / bioinformatic based approaches to mapping protein modifications at the level of amino acid sequence and should prove of value in such studies (*49*). Our current practice of stabilizing drug candidates against metabolic activation hopefully will contribute to bringing equally effective, but safer drugs to market.

Acknowledgments

The authors would like to thank Dr. Maria Beconi, Mr. Stephen Day, Ms. Ann Mao, Ms. Rebecca White, and Dr. Timothy Schulz-Utermohl for their contributions to the global covalent binding assay development and validation project at Merck Research

Laboratories. Our thanks go also to Drs. Ronald Franklin, Ralph Stearns, Wei Tang, Sanjeev Kumar, and Mr. Randy Miller, for valuable discussions during the preparation of this manuscript. For the preparation of radiolabeled compounds our thanks go to Drs. David Melillo, Dennis Dean, Matthew Braun, Frank Tang, Chad Elmore, Eric Soli, Allen Jones, Daniel Dubé and Mr. Wensheng Liu.

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Table 1 Incidence of clinical toxicity for drugs withdrawn from use or are still marketed

Compound	Marketed / Withdrawn	Toxicity	Incidence	Ref
Amiodarone	Wyeth-Ayerst, -Cordarone	Non-specific hepatic disorders Rash	1 - 3% ~1%	50
Amodiaquine	Withdrawn from prophylactic use	Hepatotoxicity and agranulocytosis	1:2000	51
Amoxicillin	Generic	Cutaneous ADR	5.1%	52
Bicalutamide	AstraZeneca, - Casodex	Hepatotoxicity	~1%	50
Carbamazepine	Generic	Rash Blood dyscresia Hepatotoxicity	1% 0.2% 0.2%	53
Cephalosporins e.g.Cefotaxime Cefradine Cefaclor Ceftriaxone Cefuroxime	Generic	Cutaneous ADR	2.1%	52
Cimetidine	Generic	Cutaneous ADR	1.3%	52
Clozapine	Novartis	Agranulocytosis	0.8%	53
Cotrimoxazole (trimethoprim-sulfamethoxazole combination)	Generic	Cutaneous ADR	3.4%	52
Diazepam	Generic	Cutaneous ADR	0.1%	52
Diclofenac	Generic	Hepatotoxicity	15%	31
Doxycycline	Generic	Cutaneous ADR	0.5%	52
Erythromycin	Generic	Cutaneous ADR	2.0%	52
Hydralazine	Generic	Systemic lupus erythmatosis	1-21%	52, 55
Indomethacin	Generic	Cutaneous ADR	0.2%	52
Isoniazid	Generic	Cutaneous ADR	0.6%	52
Lamotrigine	GlaxoSmithKline, - Lamictal	Rash	1%	50
Methyl dopa	Generic	IgG antibodies Severe hemolysis	10-36% 1%	56
Penicillin	Generic	Allergic reaction Anaphylaxis Anaphylactic fatality	8% 1:10,000 1:100,000	57
Phenobarbital	Generic	Epidermal necrolysis	1:5000	58
Phenytoin	Generic	Epidermal necrolysis	1:5000	58
Procainamide	Squibb	Systemic lupus erythmatosis	5-10%	59
Sulphamethoxazole	Generic	All idiosyncratic reactions. Hepatotoxicity	2-3% 1:10,000 – 50,000	60
Ticlopidine	Sanofi	Agranulocytosis	1-2%	61
Tienilic acid	Ticrynafen (Withdrawn)	Increased transaminases. Hepatotoxicity	7% 0.7%	56
Vesnarinone	Not marketed	Agranulocytosis	1-2%	11

Table 2 Summary of covalent binding data for prototypical hepatotoxins in vivo and in vitro

Compound	Species	Dose	Covalent binding to liver protein homogenate in vivo	Evaluation of covalent binding in vitro (1)	Reference
Acetaminophen	Mouse	250 mg/kg ip Phenobarbital induced animals	1.3 - 1.6 nmol equiv./mg @ 3 hr	Not determined	23
Amodiaquine	Rat	18 mg/kg portal vein	Only negligible levels of binding determined in vivo	Following incubation of 10 μM amodiaquine (AQ) with rat or human liver microsomes, levels of covalent binding were ~ 600 pmol equiv./mg (rat) and ~180 pmol equiv./mg (human). Incubation of AQ-quinoneimine with rat or human liver microsomes produced levels of binding >3 nmol equiv./mg protein	51, 62
Bromobenzene	Rat	~ 300 mg/kg ip	5.6 nmol equiv./mg @ 4 hr	Following incubation of 1 mM bromobenzene with rat liver microsomes (45 min, 2 mg protein), the level of covalent binding was ~ 20 nmol equiv. (~ 13 nmol equiv./mg)	17 - 19
Ethinylestradiol	Rat	5 mg/kg iv 50 mg/kg iv 100 mg/kg iv	< 5 pmol equiv./mg @ 3 hr	250μM ethinylestradiol (EE) incubated with rat liver microsomes (20min) = 1.2 ± 0.06 nmol equiv./mg (< 10% EE metabolized)	63 - 65
Furosemide	Mouse	400 mg/kg i.p.	1.2 nmol equiv./mg @ 3 hr	50-250 μM Furosemide incubated with mouse liver microsomes: 4 – 6 nmol equiv./mg	14, 31
Iproniazid	Rat	200 mg/kg ip	0.26 nmol equiv./mg @ 6 hr	Isopropyl – [2-^{3}H] hydrazine (1mM) covalently labeled rat and human liver microsomal protein; 0.58 and 0.37 nmole equiv./mg (15 min incubation time).	66, 67
4-Ipomeanol	Rat	45 mg/kg ip	Liver - 1.9 nmol equiv./mg @ 24 hr Lung - 3.8 nmol equiv./mg @ 24 hr	In liver and lung microsomes in vitro, the level of covalent binding observed following a 6 min incubation with 1 μmol [3,5-^{14}C]-ipomeanol approximated to 3-6 nmol equiv./mg protein	6 - 8
Isoniazid	Rat	200 mg/kg acetylisoniazid	0.5 nmol equiv./mg @ 24 hr	Acetyl – [^{14}C] – hydrazine (1mM) covalently labeled rat and human liver microsomal protein; 0.55 and 0.16 nmol equiv./mg (15 min incubation time).	67
Phenacetin	Mouse	500 mg/kg ip	Liver - 0.60 nmol equiv./mg @ 4 hr Kidney - 0.53 nmol equiv./mg @ 4 hr Lung - 0.64 nmol equiv./mg @ 4 hr	Incubation of *p*-[^{3}H]-nitrosophenetole with rat liver microsomes (4.5 mg) resulted in levels of covalent binding which approximated to 40 nmol equiv. following a 1 hr incubation.	68

(1) Covalent binding data have been normalized to an incubation period of 1 hr to allow for comparison of data, unless otherwise stated

Table 3 Covalent binding of 10 µM imipramine, MRL-A, diclofenac, and L-746530 following incubation with rat liver microsomes

	Covalent binding, pmol drug equiv/mg protein[a]				
	Site 1	**Site 2**	**Site 3**	**Site 4**	**Intersite**
Compound & Study Day	**Mean (%CV)**	**Mean (%CV)**	**Mean (%CV)**	**Mean (%CV)**	**Mean (%CV)**
[^{3}H]Imipramine					
1	471 (6)	348 (5)	555 (10)	549 (11)	
2	402 (9)	300 (6)	461 (6)	525 (21)	
3	496 (4)	349 (4)	461 (15)	638 (12)	
Interday	456 (11)	332 (8)	492 (11)	571 (10)	463 (22)
[^{3}H]MRL-A					
1	2622 (6)	2670 (4)	2872 (31)	2354 (3)	
2	2277 (4)	1842 (6)	2905 (4)	1896 (6)	
3	2457 (0)	2128 (1)	4216 (11)	3571 (1)	
Interday	2457 (7)	2214 (19)	3331 (23)	2607 (33)	2652 (18)
[^{14}C]Diclofenac					
1	259 (6)	402 (11)	484 (13)	211 (5)	
2	291 (2)	219 (7)	275 (8)	203 (7)	
3	359 (13)	196 (21)	299 (5)	279 (9)	
Interday	303 (17)	272 (40)	352 (33)	231 (18)	290 (18)
[^{14}C]L-746530					
1	1875 (7)	1876 (4)	2231 (8)	1520 (29)	
2	1757 (4)	1563 (6)	1728 (11)	1357 (3)	
3	1982 (4)	1720 (1)	2011 (8)	2608 (4)	
Interday	1871 (6)	1720 (9)	1990 (13)	1828 (37)	1852 (6)

[a] Values are means (N=3) with %CV in parenthesis.

Table 4 Covalent binding of 10 µM imipramine, MRL-A, diclofenac, and L-746530 following incubation with human liver microsomes

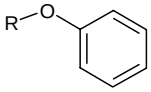
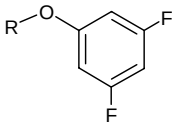
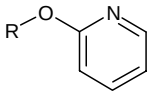
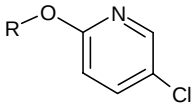
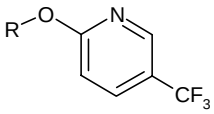
	Covalent binding, pmol drug equiv/mg protein[a]				
	Site 1	**Site 2**	**Site 3**	**Site 4**	**Intersite**
Compound & Study Day	**Mean (%CV)**	**Mean (%CV)**	**Mean (%CV)**	**Mean (%CV)**	**Mean (%CV)**
[^{3}H]Imipramine					
1	132 (2)	86 (7)	142 (10)	184 (8)	
2	106 (3)	129 (35)	113 (7)	113 (12)	
3	104 (8)	138 (10)	118 (22)	162 (27)	
Interday	114 (14)	117 (24)	125 (12)	153 (24)	127 (14)
[^{3}H]MRL-A					
1	1739 (5)	1343 (17)	1667 (18)	2077 (9)	
2	1495 (9)	2143 (21)	2360 (12)	1735 (18)	
3	1636 (5)	1469 (4)	2075 (3)	1731 (21)	
Interday	1623 (8)	1652 (26)	2034 (17)	1848 (11)	1789 (11)
[^{14}C]Diclofenac					
1	51 (2)	42 (17)	77 (7)	61 (14)	
2	58 (2)	85 (24)	56 (16)	49 (8)	
3	53 (4)	42 (10)	60 (14)	51 (27)	
Interday	54 (6)	57 (44)	65 (18)	54 (12)	57 (9)
[^{14}C]L-746530					
1	2183 (16)	1856 (18)	1779 (4)	2626 (7)	
2	2055 (3)	2827 (11)	2245 (12)	2201 (6)	
3	1901 (1)	2015 (23)	1579 (13)	2534 (8)	
Interday	2046 (7)	2232 (26)	1868 (18)	2453 (9)	2150 (12)

[a] Values are means (N=3) with %CV in parenthesis.

Table 5 Examples of compounds with toxicities which are known to undergo metabolic activation

Compound	Toxicity	Reference
Aryl oxidation to either an epoxide or a quinone		
Benzo[a]pyrene	Lung toxicity	69
Bromobenzene	Hepatotoxicity	70
Carbamazepine	Teratogenicity	71
Phenytoin	Drug induced hypersensitivity, teratogenic.	72, 73
Naphthalene	Lung, but covalent binding higher in liver and kidney.	74, 75
Estrogens	Carcinogenicity (Breast, liver, endometrial, kidney)	63-65, 76, 77
Tamoxifen	Endometrial cancer	78-80
Raloxifene	Jaundice accompanied by elevated liver enzymes	81
Practolol	Skin and eye lesions	82
Furan epoxidation, ring opening to yield an aldehyde		
4-Ipomeanol	Lung	6-8
Furosemide	Teratogenicity	14, 31
L-739010	Hepatotoxicity in dogs	42, 43
Pulegone	Hepatotoxicity	83
L-754394	Bone marrow toxicity in dogs	84
Formation of quinoneimines		
Acetaminophen	Hepatotoxicity	9, 23, 85, 86
Amodiaquine	Hepatotoxicity	62
Diclofenac	Hepatotoxicity	32-34, 87-90
Phenacetin	Kidney toxicity	68
Formation of isocyanate		
Troglitazone	Hepatotoxicity	91, 92
Formation of imine methide		
3-Methylindole	Pneumotoxicity (in ruminant)	93, 94, 95
Formation of a Michael acceptor		
Valproic acid	Hepatotoxicity	96-99
Formation of an acyl glucuronide conjugate		
Bromofenac	Hepatotoxicity. Withdrawn in 1998 – 6 deaths	100
Benoxaprofen	Hepatotoxicity. Withdrawn from market	100, 101
Ibufenac	Hepatotoxicity. Withdrawn from market	100, 102
Zomepirac	Hepatotoxicity. Withdrawn from market	100, 103-105
Miscellaneous		
Halothane	Idiosyncratic hepatoxicity	10
Isoniazid	Hepatotoxic in humans following N-acetylation and liberation of acetylhydrazine	12, 13
Clozapine	Agranulocytosis - nitrenium ion implicated	15, 16
Tienillic acid	Immunogenic, hepatitis	106, 107

Table 6 Irreversible binding of radioactivity to human and rat liver microsomal protein upon incubation with a series of tritiated analogs (MRL-C though MRL-G) at a 10 μM concentration for 1 hr in the presence of an NADPH-regenerating system. The irreversible binding was assessed both in the absence and presence of 5 mM GSH.

		Covalent binding to liver microsomal protein (pmol drug equiv/mg protein) [a]			
		Human		Rat	
	Structure [b]	No GSH	5-mM GSH	No GSH	5-mM GSH
MRL-C		3870 ± 303	647 ± 64	1490 ± 133	325 ± 16
MRL-D		1690 ± 315	472 ± 76	841 ± 94	120 ± 47
MRL-E		911 ± 109	294 ± 18	535 ± 21	139 ± 50
MRL-F		303 ± 81	100 ± 19	190 ± 43	54 ± 17
MRL-G		88 ± 4	27 ± 11	111 ± 21	24 ± 5

[a] Parallel experiments demonstrated that covalent binding of radioactivity to microsomal protein at time zero and in the absence of an NADPH-regenerating system was <5 pmol drug equivalents/mg protein incubation in all cases.

[b] For all compounds, the tritium label was incorporated at the same position within the functional group labeled 'R'.

Figure Legend

Figure 1. Decision tree for assessing the suitability of lead compounds for development based on metabolic activation considerations. (As indicated in the text, a liability with regard to metabolic activation is viewed as only one of a number of criteria in the selection process).

Figure 2. Compounds used for the inter-site evaluation of covalent binding in vitro

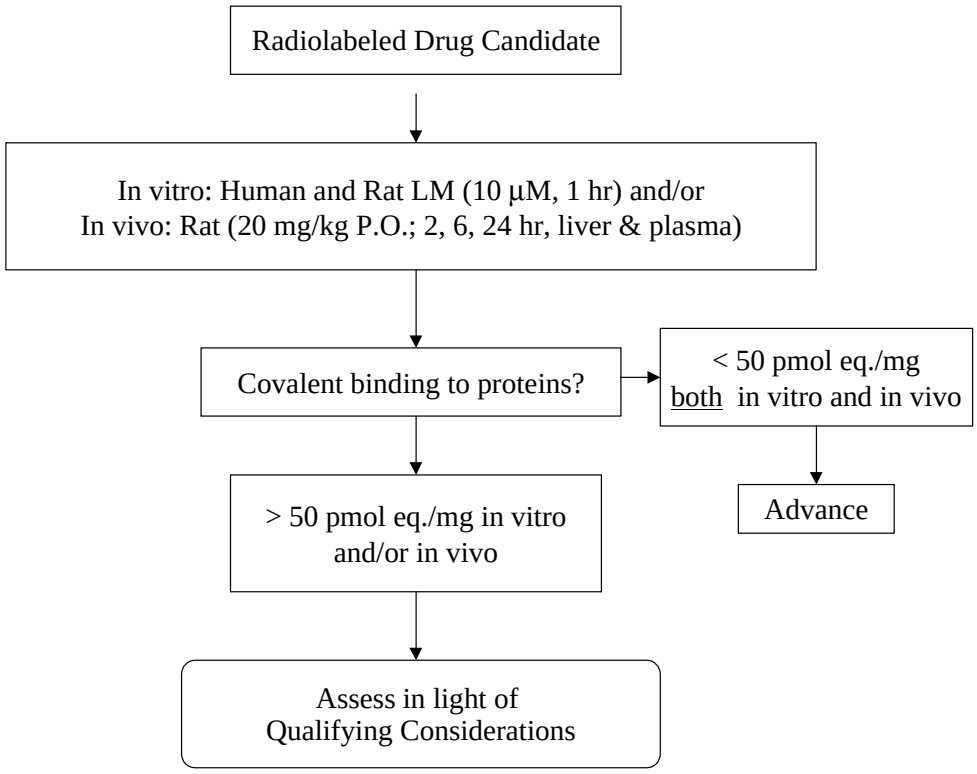
Figure 3. Proposed mechanism of bioactivation of acetaminophen and tapping of the electrophilic intermediate by glutathione. The origin of the characteristic neutral loss of 129 Da upon collisional activation of the MH^+ species of the glutathione conjugate in a tandem mass spectrometer is as indicated.

Figure 4. Use of cyanide to trap an iminium ion intermediate.

Figure 5. Bioactivation of L-739010 or L-746530 (Fig. 2) and trapping of an aldehyde intermediate using (A) semicarbazide, and (B) methoxylamine.

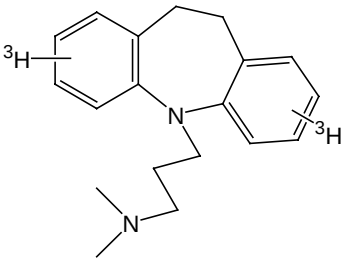
Figure 6. Novel bioactivation of a 3-acyl-N^1-methyl piperazine derivative. The structures shown in brackets represent proposed intermediates which were not isolated.

Figure 7. Novel bioactivation of a substituted pyrazinone derivative (*45*). Structures shown in brackets represent proposed intermediates which were not identified.

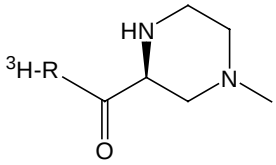


Qualifying considerations

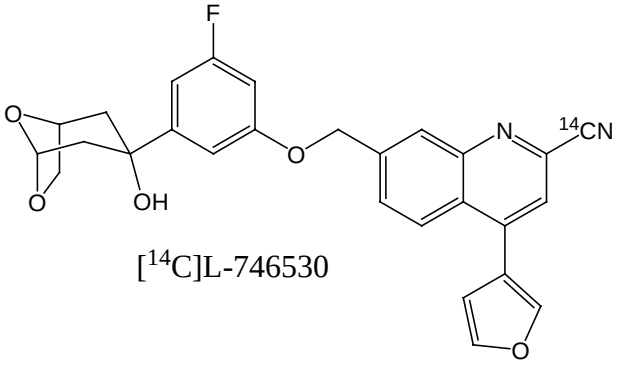
- Chemical tractability of structural series?
  - What potential exists to modify structure?
  - Evidence of informed chemical intervention?
  - Has metabolic activation been minimized relative to the preceding compound(s)?
- Availability of existing treatments?
- Is the prognosis disabling or life-threatening?
- Is the anticipated clinical daily dose < 10 mg?
- Are the metabolic clearance routes predominantly non-Phase 1?
  - Consider studies in hepatocytes.
- Expected duration of therapy?
  - Will the drug will be used chronically/prophylactically?
- What is the intended target population?
  - Is the drug intended for a pediatric indication?



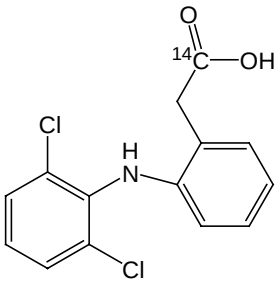
[^{3}H]Imipramine



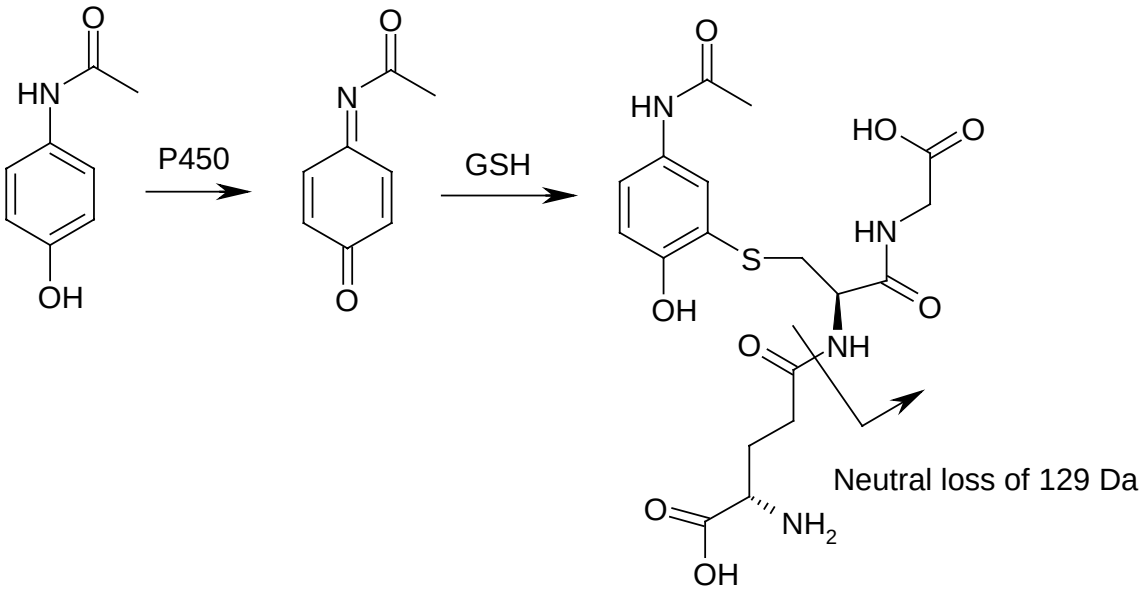
[^{3}H]MRL-A

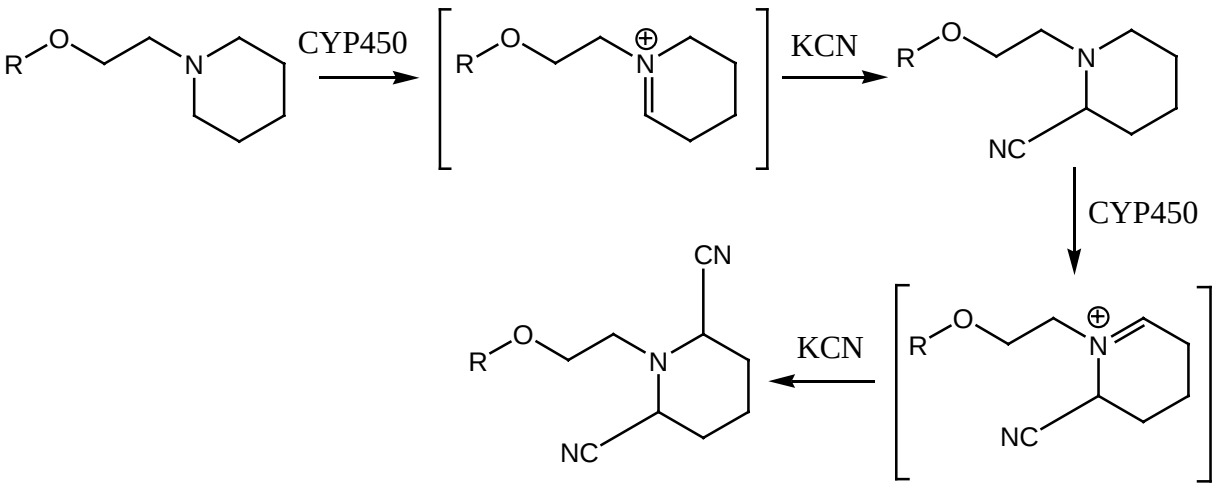


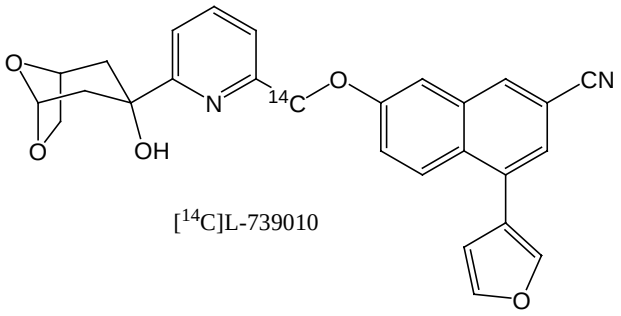
[^{14}C]L-746530



[^{14}C]Diclofenac

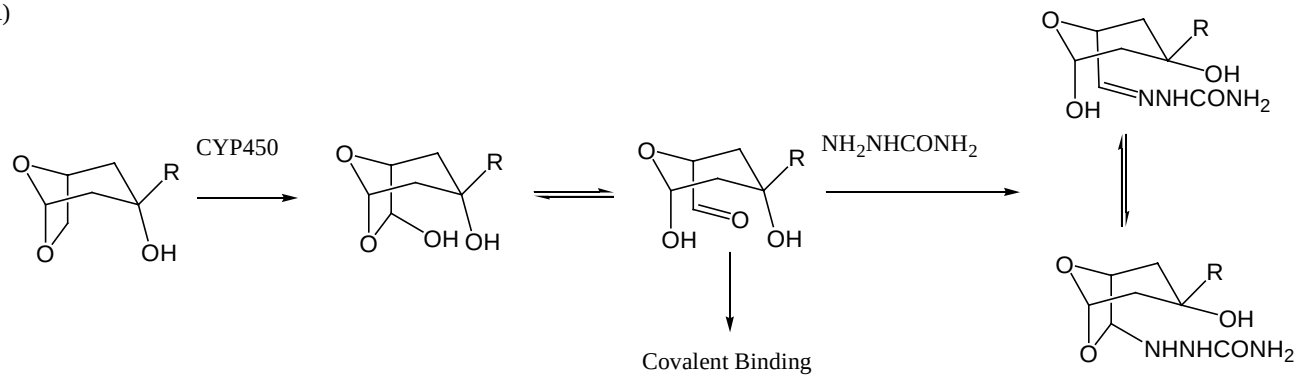

P450
GSH
Neutral loss of 129 Da


R
O
N
CYP450
KCN
NC
CYP450
KCN
CN

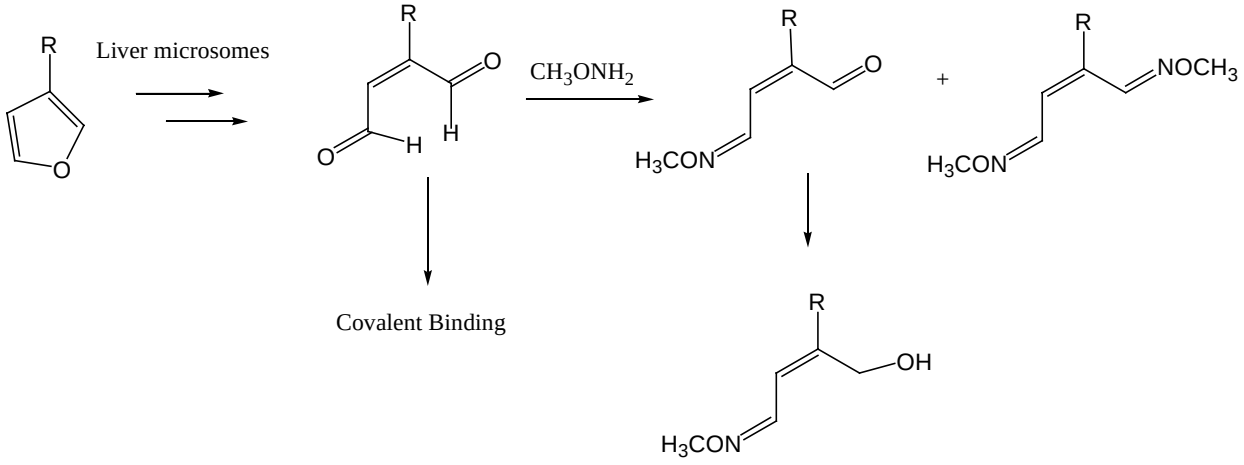


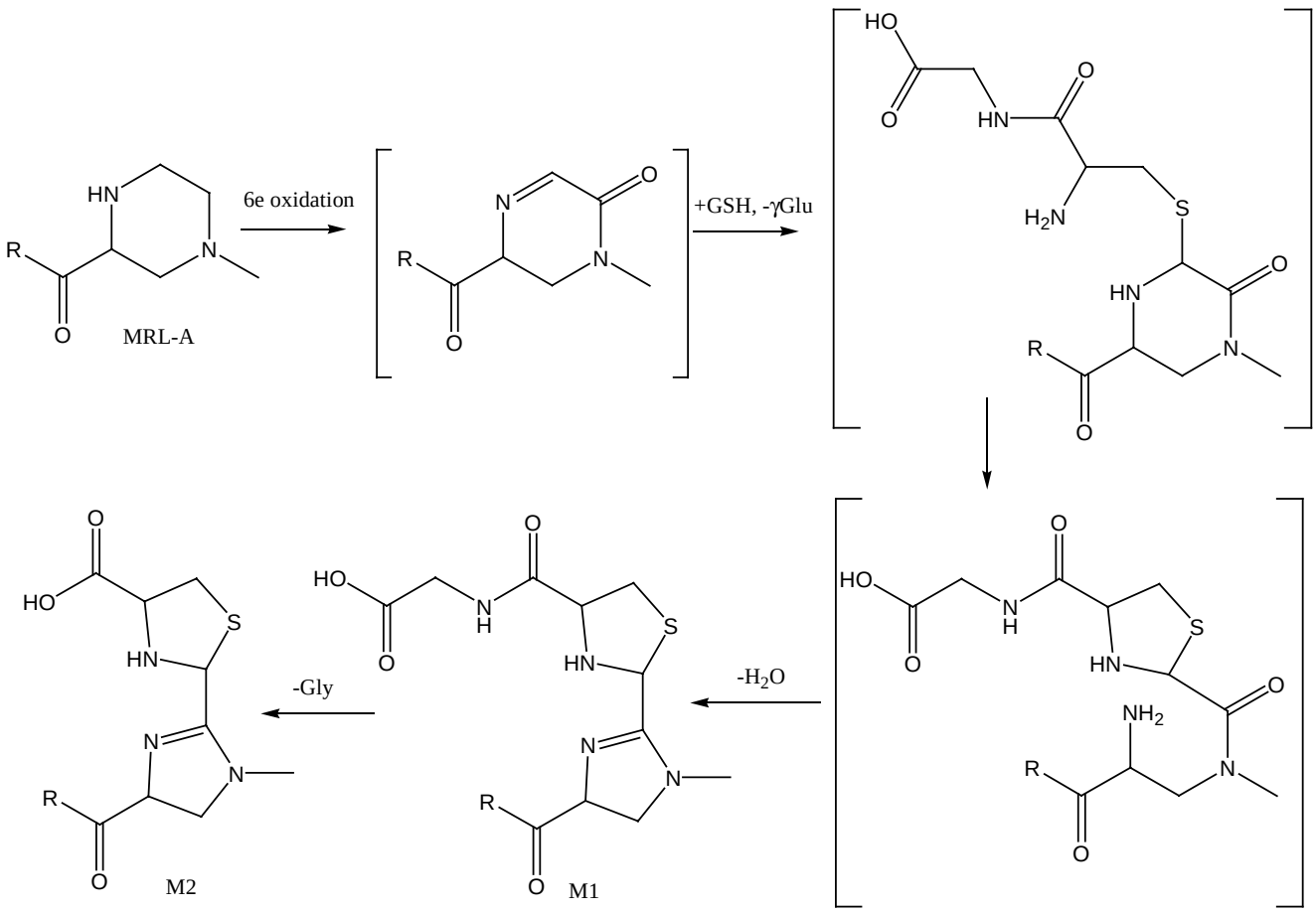
[^{14}C]L-739010

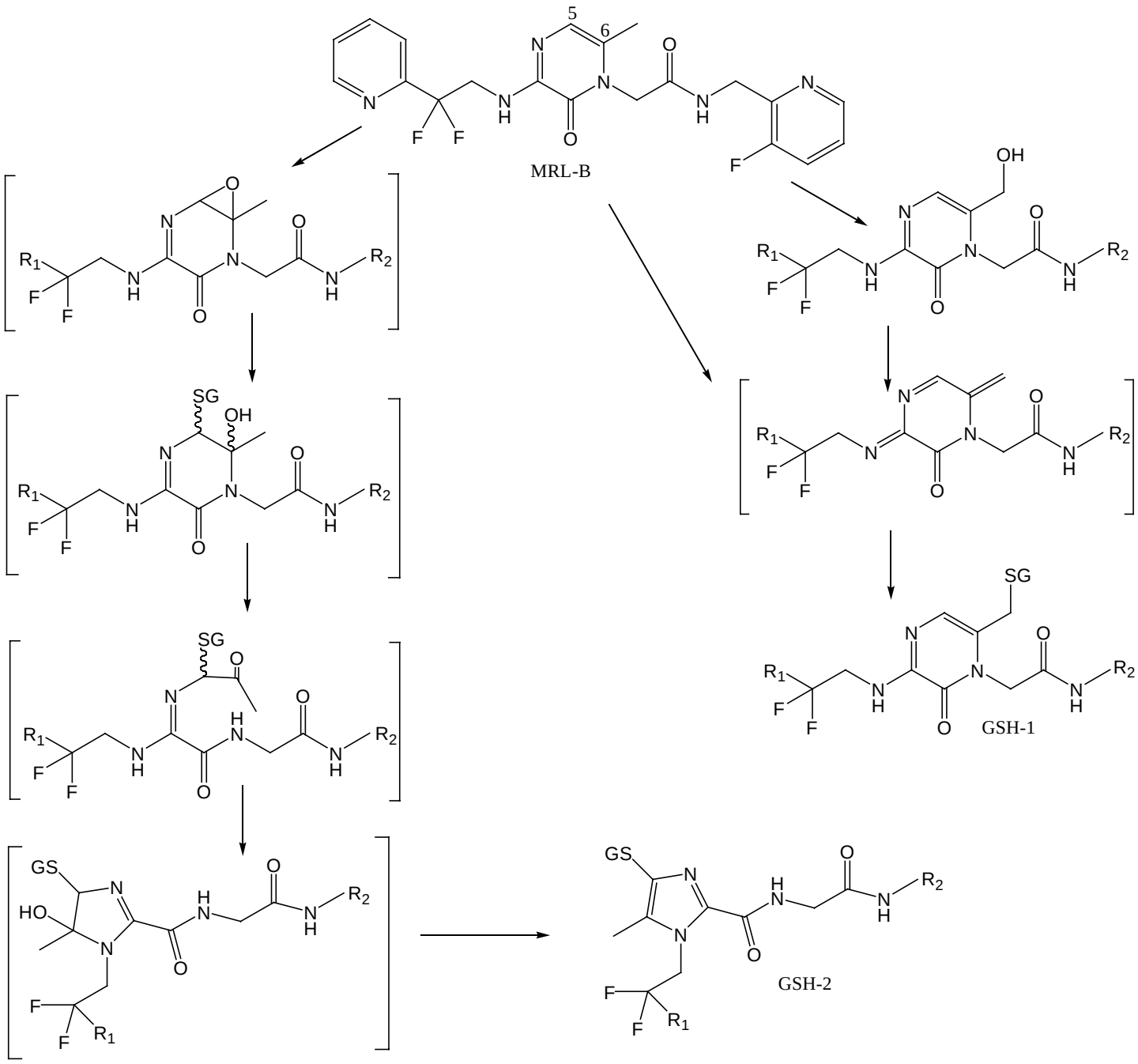
(A)



(B)




6e oxidation
+GSH, -γGlu
MRL-A
-Gly
-H2O
M2
M1


MRL-B
GSH-1
GSH-2