Journal of
Medicinal
Chemistry

Volume 47, Number 18 August 26, 2004

Perspective

Discovering Drugs through Biological Transformation: Role of Pharmacologically Active Metabolites in Drug Discovery

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Received March 18, 2004

Introduction

In most cases, the metabolism of drugs leads to pharmacological inactivation through biotransformation to therapeutically inactive molecules. However, drug metabolism can also result in pharmacological activation, where pharmacologically active metabolites are generated.[1−3] Although formation of pharmacologically active metabolites (bioactivation) can be mediated by both phase I (oxidation, reduction, and hydrolysis) and phase II (conjugation reactions) metabolism, bioactivation resulting from phase I metabolism mediated by cytochrome P450 (CYP) enzymes is the most common pathway.

Active metabolites may have superior pharmacological, pharmacokinetic, and safety profiles compared to their respective parent molecules. As a result, a number of active metabolites have been developed and marketed as drugs with improved profiles relative to their parent molecules. Examples of active metabolites of marketed drugs that have been developed as drugs include acetaminophen,[4] oxyphenbutazone,[5] oxazepam,[6] cetirizine (Zyrtec),[7] fexofenadine (Allegra),[8] and desloratadine (Clarinex).[9] Each of these drugs provides a specific benefit over the parent molecule and is superior in one or more of the categories described above.

Full characterization of metabolite profiles and elucidation of metabolite structures for new chemical entities are often not completed until early or sometimes even late in the drug development stage. It is at this stage in development that the degree of contribution of active metabolites to the overall observed therapeutic effect is evaluated. In some cases, full assessment of the pharmacological significance of metabolites has not been made until after drugs have reached the market. Even as technological advances have made early determination of biotransformation profiles feasible, it remains uncommon for drug candidates to be screened for the presence of active metabolites during the lead optimization stage of drug discovery. As a result, with the exception of the classical prodrug approach, biological transformation as a method of drug design has not been widely exploited. One of the reasons for this is the difficulty of detecting and characterizing metabolites within a timeline consistent with the rapid pace of drug discovery. In addition, rapid chemical synthesis of metabolites by traditional methods is often challenging. It is also often difficult or impossible to predict a priori whether a certain compound could form a pharmacologically active metabolite. For example, the same biotransformation, the hydroxylation of an alkyl side chain, produces a pharmacologically inactive metabolite of phenobarbitone, whereas hydroxylation on the alkyl side chain of flutamide[10] leads to a metabolite that is more active and long lasting than the parent compound (Figure 1). Sulfation of minoxidil leads to the formation of a pharmacologically active metabolite,[11] whereas

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10.1021/jm040066v CCC: $27.50
Published on Web 07/29/2004

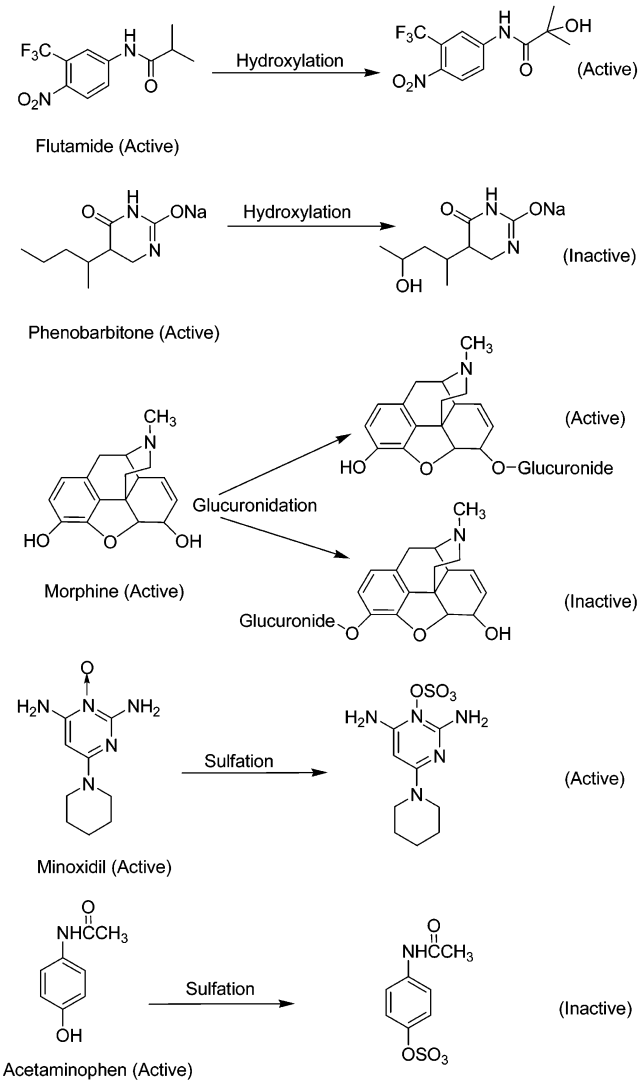

Figure 1. Examples of common biotransformation reactions that led to pharmacologically active or inactive metabolites.

sulfation of acetaminophen leads to bioinactivation (Figure 1). The 6-*O*-glucuronide metabolite of morphine is pharmacologically active, whereas morphine 3-*O*-glucuronide is not (Figure 1).[12] In silico methods have the potential to predict and assess the formation of metabolites from a precursor compound and whether these metabolites are active against known pharmacological targets. However, a robust in silico method that could fulfill these objectives across a wide variety of chemical space has yet to be developed. Therefore, experimental methods are needed to generate, detect, and characterize important active metabolites. In this paper, we will discuss the importance of embracing the concept of active metabolites as part of the drug design process and will review experimental observations that can trigger the search for active metabolites. We will also discuss methodological advances in the detection, generation, and structural characterization of active metabolites.

Definition of an Active Metabolite

Here, we define an active metabolite as a pharmacologically active metabolic product with activity against the same pharmacological target as the parent molecule. This is in clear distinction from classical prodrugs, which involve a biologically inactive compound being converted to a biologically active one by chemical or enzymatic means (usually via hydrolytic biotransformation). The concept of active metabolite as defined here is also distinct from reactive metabolites, which are chemically reactive intermediates formed during metabolism that may elicit toxicological activity through covalent binding to biomolecules. The importance of an active metabolite is measured by the degree of its contribution to the total pharmacological activity of a given dose of the parent compound and by its intrinsic activity and the relative concentration at the site of action.[13,14]

Generation of Chemical Diversity through Biological Transformation

Current drug discovery paradigms involve (1) random or directed screening of natural or synthetic product libraries, (2) combinatorial chemistry and molecular modification of pharmacologically active synthetic or natural products, and (3) a de novo rational drug design. As described above, discovering drugs by methods of biological transformation may also be a viable approach to drug design. It is therefore beneficial to have methods in place to allow screening of compounds for therapeutically useful biotransformation products or active metabolites during the drug discovery phase.

During lead optimization, drug candidates are routinely screened for metabolic stability or in vivo systemic exposure and rank-ordered according to the rate and extent of metabolism or systemic exposure level.[15] In the case of metabolic screening, this is usually performed in vitro after incubations of the drug candidates with subcellular fractions such as liver microsomes or intact cellular systems (e.g., hepatocytes) containing a full complement of drug-metabolizing enzymes. Compounds with low metabolic stability are then excluded from further consideration because most therapeutic targets require compounds with an extended pharmacokinetic half-life. The same is true with in vivo exposure studies, where high-clearance compounds are discarded. In these early screens, the concentration of the parent compound is typically the only measurement made. Consequently, there is no information on the number, identity, and pharmacological significance of metabolites that may have been formed. Even when metabolic profiling is completed and metabolites are identified, the information is typically used to direct synthesis of analogues with improved metabolic stability through the modification of metabolic soft or hot spots.[16,17] Thus, the information is rarely used for the purpose of searching for pharmacologically active products as new analogues. However, rapid metabolism of parent compounds can lead to the formation of pharmacologically active metabolites that may have comparatively superior "developability" characteristics. As a result, metabolic instability, which otherwise may be considered a liability, can be used as an advantage during lead optimization.

Advantages of Tracking Biological Transformation during the Drug Design Process

There are a number of advantages for screening drug candidates for active metabolites during drug discovery. The primary reason is that the process may lead to the discovery of a drug candidate with superior drug developability attributes such as (1) improved pharmacodynamics, (2) improved pharmacokinetics, (3) lower probability for drug−drug interactions, (4) less variable pharmacokinetics and/or pharmacodynamics, (5) improved overall safety profile, and (6) improved physicochemical properties (e.g., solubility).

Other advantages of early screening for active metabolites include the potential for modifications of the entire chemical class (chemotype) to improve overall characteristics.[1,18] Further, early discovery of active metabolites provides a more complete patent protection of the parent molecule. Additionally, tracking active metabolites at the drug discovery stage will allow for the correct interpretation of the pharmacological effect observed in preclinical species in relation to a predicted effect in humans. In other words, if an active metabolite is responsible for significant activity in a species used for preclinical efficacy determination, there is a significant risk that the effect will be dramatically different in humans unless similar levels of metabolite are achieved in humans.

Active metabolites possessing one or more of the above attributes have been found for a number of marketed drugs and have led to the introduction of the metabolites as commercial products. Several examples of active metabolites that have become marketed drugs are discussed below.

Desloratadine (Clarinex) now marketed as a second-generation antihistamine agent is an active metabolite of loratadine (Claritin) (Figure 2). It is approximately 10-fold more potent in vivo in human than the parent drug.[19,20] Desloratidine has lower oral clearance with greater systemic concentrations at equivalent oral doses and has a longer plasma elimination half-life compared to the parent compound in human. As a result of its superior pharmacokinetics and pharmacodynamic profiles, the standard human dose is half as much as that of loratadine. It also shows less pharmacokinetic variability in comparison to the parent drug.[21,22]

Morphine-6-*O*-glucuronide, a widely used analgesic, is an active metabolite of morphine possessing pharmacological activity greater than that of the parent compound (Figure 1).[23,24] Upon chronic treatment, the pharmacokinetic profile of morphine-6-*O*-glucuronide led to higher plasma concentrations than morphine, contributing to its better clinical effect.[25] Morphine-6-*O*-glucuronide also showed an improved side effect profile, with a lower incidence of nausea and vomiting commonly observed with the parent drug.[23]

Cetirizine (Zyrtec), a second-generation antihistamine, is an active metabolite of hydroxyzine (Atarax) (Figure 2). Cetirizine has greater affinity for the H_1 receptor and, in contrast to hydroxyzine, is a nonsedating agent at least partially because of its lack of distribution to brain tissue.[7,26]

Fexofenadine (Allegra), another antihistamine agent, is an active metabolite of terfenadine (Figure 2).[8,27]

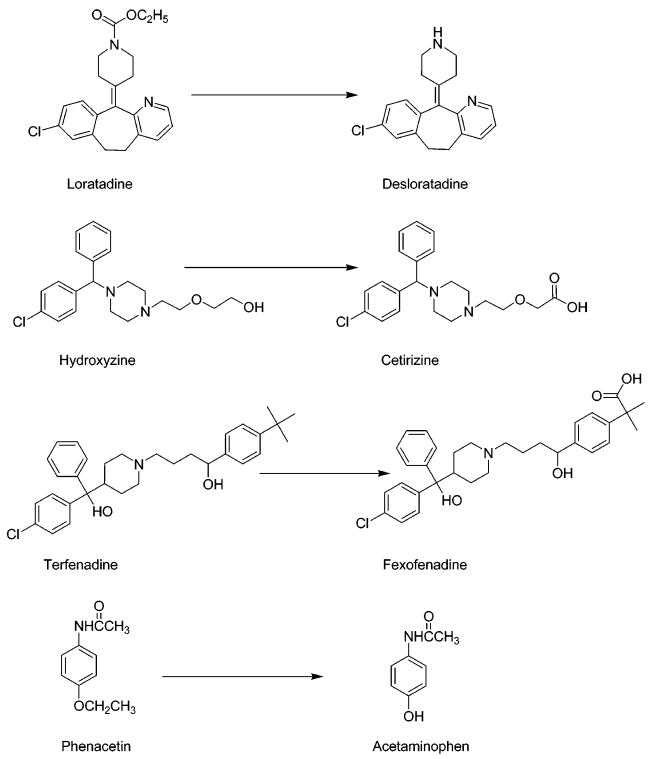

Figure 2. Examples of biotransformation reactions that led to active metabolites that have been developed and marketed as drugs.

Terfenadine undergoes extensive first-pass metabolism that is predominantly mediated by CYP3A4 and forms fexofenadine as the major circulating metabolite. Terfenadine was shown to promote the inhibition of cardiac ion channels (potassium ion currents) leading to prolongation of the QT_c interval and ventricular arrhythmia.[28,29] Coadministration of terfenadine with CYP3A4 inhibitors such as ketoconazole significantly altered its pharmacokinetic profile, resulting in an increase in plasma concentration potentially leading to fatal cardiac arrhythmia.[30] In contrast, no change in the QT_c interval was observed with fexofenadine when administered alone or in combination with ketoconazole. As a result, terfenadine was withdrawn from the market and was replaced with fexofenadine.

In most cases, further metabolism of active metabolites leads to the formation of fewer total metabolites compared to the parent compound. As a result, the use of an active metabolite as drug may lessen the chance for off-target toxicity that may be caused by one or more extra metabolites formed from the parent compound, even when the parent compound itself has no intrinsic toxicity. Moreover, most active metabolites are products of phase I functionalization reactions and as such are more susceptible to phase II conjugation reactions. Phase II conjugation reactions result in the formation of secondary metabolites that, in general, are safely cleared from the body. For example phenacetin, which is no longer in use in humans, is metabolized to a number of metabolites. Of the many phenacetin metabolic pathways, the O-deethylation pathway leads to the formation of acetaminophen (Figure 2) (a more analgesic agent than phenacetin), whereas N-hydroxylation of phenacetin leads to the formation of a toxic metabolite that induces methemoglobinemia and hemolytic ane-

mia.[31] On the other hand, the corresponding active metabolite, acetaminophen, is predominantly cleared via phase II conjugation reactions (sulfation and glucuronidation) and has a greater margin of safety relative to phenacetin.[32]

In general, drug metabolism reactions convert lipophilic compounds to more hydrophilic, more water-soluble products. An improvement in the solubility profile is an added advantage, particularly in the current drug discovery paradigm where many drug candidates generated during lead optimization have poor aqueous solubility.

An active metabolite can serve as a modified lead compound around which new structure−activity relationships can be investigated during the lead optimization stage of drug discovery. For example, this approach was used in the discovery of ezetimibe, a cholesterol absorption inhibitor.[1,18,33] In these studies, a lead candidate (SCH48461) gave rise to a pharmacologically active biliary metabolite upon oral administration to rats that was approximately 30-fold more potent than the parent molecule. Further optimization of the metabolite through structural modification led to the discovery of ezetimibe, a molecule that was approximately 400-fold more potent than the initial lead candidate.

In summary, tracking active metabolites at the drug discovery stage not only is important to correctly interpret the pharmacological effects in preclinical species but may also lead to the discovery of a lead candidate with superior drug developability characteristics.

Detection of Active Metabolites during Drug Discovery

The exploration of the potential for formation of active metabolites can be carried out with varying degrees of direction from information gathered through metabolism, pharmacokinetics, and biological/pharmacological assays. An example of undirected screening of active metabolites would be the modification of chemical libraries by subjecting them to metabolizing systems and subsequently using these modified libraries for high-throughput screens, either against the intended target or more broadly. This example is a way to generate increased molecular diversity from a given chemical library. However, this approach requires significant "deconvolution" efforts when activity is found in mixtures. To increase the success rate and decrease the number of compounds screened to a manageable size, the search for active metabolites could be limited to those compounds or chemotypes showing high clearance rates in in vitro metabolic stability or in vivo exposure screens.

On the other hand, activity assays may serve as a more rationale approach to the exploration of active metabolites. This is most often and most effectively done in the setting of an in vivo efficacy experiment that allows for both pharmacodynamic (PD) and pharmacokinetic (PK) information to be gathered. Analysis of the relationship between the PD endpoint and the PK profile will sometimes demonstrate an apparent disconnect between the two data sets and point to the possibility that an active metabolite is responsible for some of the activity. These disconnects can serve as clear trigger points for the initiation of active metabolite searches.

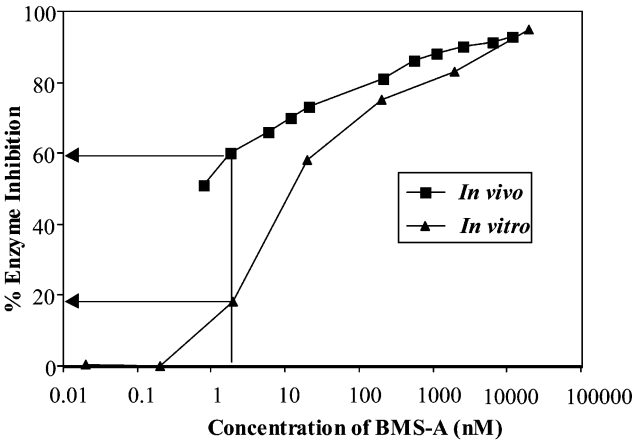

Figure 3. In vitro and in vivo plasma enzyme inhibition vs concentration of BMS-A.

For example, Van Heek and co-workers observed a lead candidate that underwent extensive first-pass metabolism and yet elicited a significant level of pharmacological activity.[33] To evaluate the biological activity of the in vivo biotransformation products, they collected samples of bile from rats dosed with a lead compound and directly administered the bile samples to bile duct cannulated rats via an intraduodenal cannula. In a control study, the parent compound prepared in blank bile was dosed in a similar fashion to the recipient rats. Results from this study indicated that the in vivo activity elicited by the bile samples was higher than the parent control sample, clearly indicating the presence of an active metabolite(s) that was more potent than the parent compound. To identify the active component, the bile sample was then fractionated and each fraction was tested for biological activity. The structure of the metabolite was then established following the detection of the active fraction. As mentioned before, further modification of the active metabolite led to the discovery of ezetimibe.

Another example is the recent pharmacological evaluation of a BMS drug candidate (BMS-A), where higher in vivo potency was observed than was expected from either the PK data or from in vitro activity data.[34] The study involved enzyme activity measurements in rat plasma in vitro and ex vivo. To measure the enzyme activity in vitro, known concentrations of the drug candidate were spiked to fresh plasma collected from rats at concentrations that would give a reasonable dose−response curve. An appropriate concentration of the enzyme substrate was then added to each sample, and the enzyme activity was measured. The resulting dose−response curve is shown by the right-hand side curve in Figure 3. The in vitro data were then compared with the enzyme activity data obtained using ex vivo plasma collected from rats administered intravenously with an appropriate dose of the drug candidate. The actual concentrations of the drug in the plasma samples were determined by LC/MS/MS (liquid chromatography and tandem mass spectrometry). As shown in Figure 3, the inhibitory activity appears to increase when the compound was given in vivo compared to the in vitro measurements at similar nominal concentrations of the

parent compound. It was discovered later that the higher activity of the ex vivo plasma samples, as shown by the leftward shift in the graph (Figure 3), was due to the presence of an active metabolite in the samples from the in vivo study. The metabolite was then isolated, and partial structural identification led to the synthesis of a hydroxylated analogue that became the lead compound for the chemotype.

An additional clue that could point toward the presence of an active metabolite is the observation of a greater pharmacological effect upon extravascular administration of a compound relative to parenteral administration. In most cases, the systemic concentrations are lower when the compound is administered orally because of the first-pass effect, especially during the first few hours after dose. As a result, greater in vivo activity is expected to be observed when the compound is administered intravenously compared to when it is administered orally at a similar nominal dose, provided the pharmacological site of action is not a first-pass organ. If the converse is observed, then an active metabolite(s) may be playing a role. This was exactly what was observed when enzyme activity was measured ex vivo upon administration of the BMS-A drug candidate discussed above.[34] In these studies, the concentration of the parent compound as well as the enzymatic activity was measured in ex vivo plasma samples. It was found that in samples where 80% inhibition of the enzyme activity was measured, the concentration of the parent compound was 46 nM after oral administration and 132 nM after intravenous administration. This difference in apparent potency after oral and intravenous administration was evident at all levels of enzyme inhibition. The data clearly showed that the enzyme activity was inhibited more significantly upon oral administration and was consistent with the presence of an active metabolite.

An additional trigger point for the search for active metabolites based on PK/PD disconnects is the observation of a prolonged PD effect for a drug candidate despite a short pharmacokinetic profile.[3] In particular, if a compound shows a relatively high in vivo activity and a prolonged pharmacodynamic effect compared to other compounds of the same class, while demonstrating similar pharmacokinetic profiles, then there is a strong indication of the presence of an active metabolite(s). Recently, on the basis of this type of observation, we were able to identify a number of active metabolites with superior "developability" characteristics compared to the parent compounds.[35] In one such case, several drug candidates (BMS-B, -C, -D, and -E) from the same chemical class were observed to have essentially similar PK (similar plasma concentrations, similar protein binding, and target tissue concentrations) and similar in vitro activity profiles. However, what was intriguing was the dissimilarity in the apparent in vivo activity data of these compounds, with some showing many-fold more activity than others. For example, BMS-B was about 20- to >40-fold more active in vivo than BMS-C, -D, and -E. Since the result was suggestive of the presence of active metabolites, a rapid bioassay-guided method was designed to generate and detect the active metabolites (vide infra). In this method, compounds were incubated with rat liver microsomes for a specified period of time, after which the incubation was terminated. The biological activity was determined in the initial and final incubation mixture, without isolation, using an in vitro cell-based assay. The IC_{50} (concentration value resulting in 50% inhibition) values were determined to be 12, 11, 41, and 60 nM in the initial incubation mixtures and 19, 51, 585, and 490 nM in the final incubation mixture for BMS-B, -C, -D, and -E, respectively. The amount of the parent compounds remaining in the final incubation mixture was determined to be <1%, <4%, <1%, and 20% of the initial concentration of BMS-B, -C, -D, and -E, respectively. The data showed that despite the almost complete loss of parent compounds in the final incubation mixture, a significant level of activity remained for compounds BMS-B and -C, suggesting the formation of active metabolites. The active metabolites were subsequently isolated, their structure determined, and their in vitro and in vivo activities confirmed. In this case, the metabolite of compound BMS-B became the lead candidate for the discovery program.

In addition, if a compound or a compound class is coadministered in vitro or in vivo with compounds that inhibit metabolism (e.g., aminobenzotriazole, ketoconazole, etc.) and shows a reduced pharmacological effect, then the formation of a biologically more active metabolite or active metabolite with extended PK compared to the parent compound is highly probable.

The appearance of unique metabolites in preclinical animal or human in vitro systems such as liver microsomes and hepatocytes can also serve as a trigger in the evaluation of biological activity. For example, morphine is eliminated largely through glucuronidation of the phenolic 3-OH (major metabolite, inactive) and the 6-OH group (minor metabolite, active) in human. On the other hand, only the 3-*O*-glucuronide is formed in rats and mice.[24] In this example, additional pharmacological activity would be expected in humans compared to rodents. However, this situation could just as easily be reversed and the preclinical species would then overpredict the activity in humans. Thus, the presence of a significant concentration of circulating metabolite in a species used for efficacy studies should also warrant further investigation to see if the metabolite contributes to the overall therapeutic effect in the preclinical species.

The above discussion shows that active metabolites can be found during the evaluation of lead compounds. To that effect, there are a number of experimental observations that can serve as signals that the biotransformation of compounds to pharmacologically active products is playing a role in the overall activity of the parent compound.

Methods for Assessing and Evaluating the Biological Activity of Metabolites

To assess the biological activity, and hence usefulness of metabolic products, several approaches can be used. The more traditional approach is to take samples of interest (i.e., microsomal incubations, plasma samples, etc.) and isolate and purify any metabolites present, after which the structure of the metabolites is determined and each is tested for biological activity. An alternative approach is to use bioassay-guided methods where biological samples containing biotransformation products are first evaluated for their pharmacological

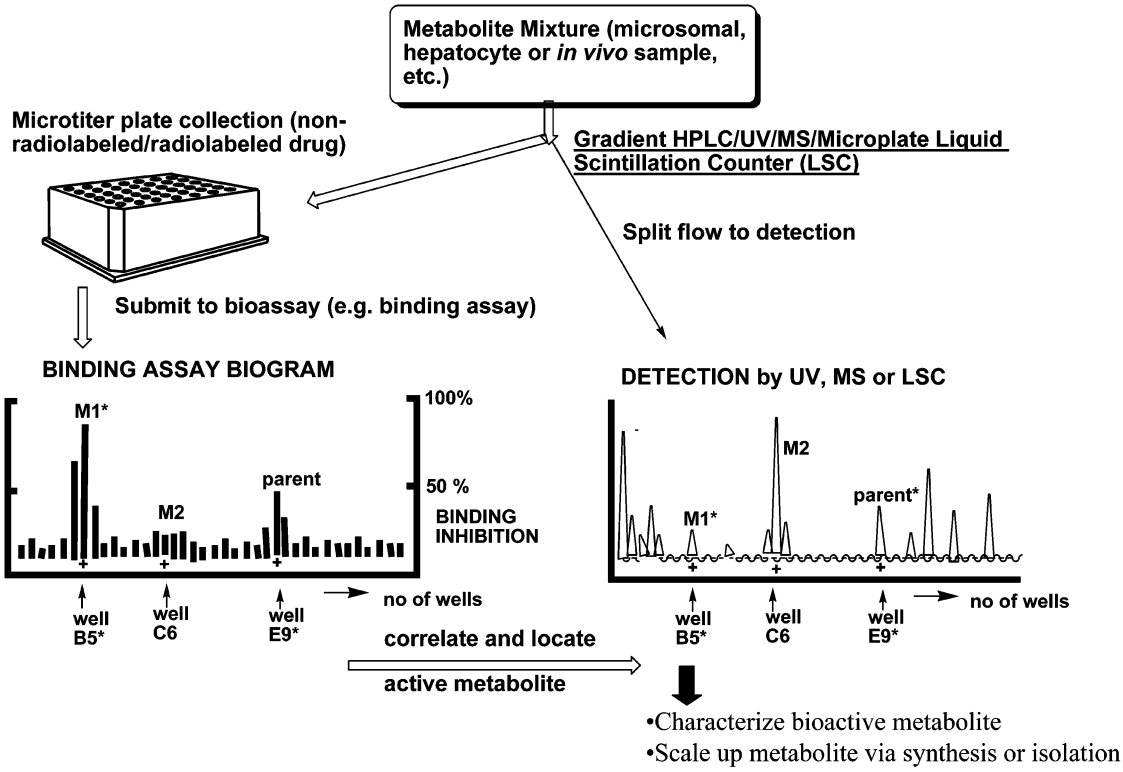

Figure 4. Schematic illustration of the "HPLC biogram" approach.

activity without any effort to isolate or structurally characterize the metabolites. This method potentially has higher throughput capability and utilizes the most recent advances in biotechnology and analytical instrumentation. The bioassay methods may be based on the assessment of the pharmacological activity using in vitro ligand binding,[36,37] cell-based assays,[35] or in vivo pharmacological assays.[33] Metabolites can be generated by any of the in vitro and in vivo methods discussed in the following sections. Biological activity in the sample mixture can then be evaluated as is or after fractionation of the sample mixture by using chromatographic techniques. The structural identity of the active metabolite can then be determined and its in vitro and in vivo activity confirmed after isolation and/or after further biological or chemical synthesis.

A systematic approach to profiling active metabolites using a 96-well plate format was recently described.[38] The approach is based on rapid bioassay-guided metabolite detection and characterization, and a schematic illustration of this bioassay "HPLC biogram" method is shown in Figure 4. Drug metabolite mixtures (generated by various methods described below) are separated and fractions collected into 96-well plates. The fractions are then subjected to one or more relevant activity (e.g., receptor ligand binding) assays. Fraction collection utilizes a time-based protocol, resulting in a direct relationship between a well's position in the plate and a corresponding area on the HPLC chromatogram. This allows assignment of activity to a particular component of the mixture. The active peaks are correlated back to HPLC/UV/MS profiles and are followed up with UV/MS spectral analysis for structure characterization.

The "biogram" methodology has been used in the discovery of bioactive natural products and other mixtures, including but not limited to evaluation of substances of interest (hits) from initial high-throughput screens, biological assessment of impurities and racemic mixtures (chiral HPLC required), and isolation of active component in a mixture.[39] Figure 5 shows an example of the use of the "biogram" method in detecting and characterizing a very minor but bioactive metabolite. When applied in metabolite studies, the "biogram" approach allows early recognition of active metabolites in a rapid and efficient manner while minimizing time and effort spent on inactive metabolites. The "biogram" approach also facilitates decision-making in initiating reisolation or scale-up of active metabolite for full structural and biological evaluation.

There are a number of important determinants for a successful "biogram" experiment. The fundamental requirement is the availability of a robust bioassay. To detect a minor active metabolite, the bioassay employed should be as sensitive as the analytical assay (e.g., LC/UV/MS) and should have a good "signal/noise" ratio. If these conditions are met, the biogram method can provide rapid and efficient detection of minor active metabolites previously undetected by physical methods. False positives, such as interfering endogenous materials, detergents, pigments, metal ions, etc., are an important consideration in any biogram study. Therefore, it is very important to run appropriate blank and control samples. The "biogram" method usually consumes a relatively small amount of metabolite sample (mixture). Nevertheless, a larger quantity of metabolite mixture may be needed for any follow-up experiments and can be achieved by several biological transformation techniques described below.

Methods for Generation of Metabolites

There are a number of in vitro and in vivo biotransformation techniques available to generate metabolites.

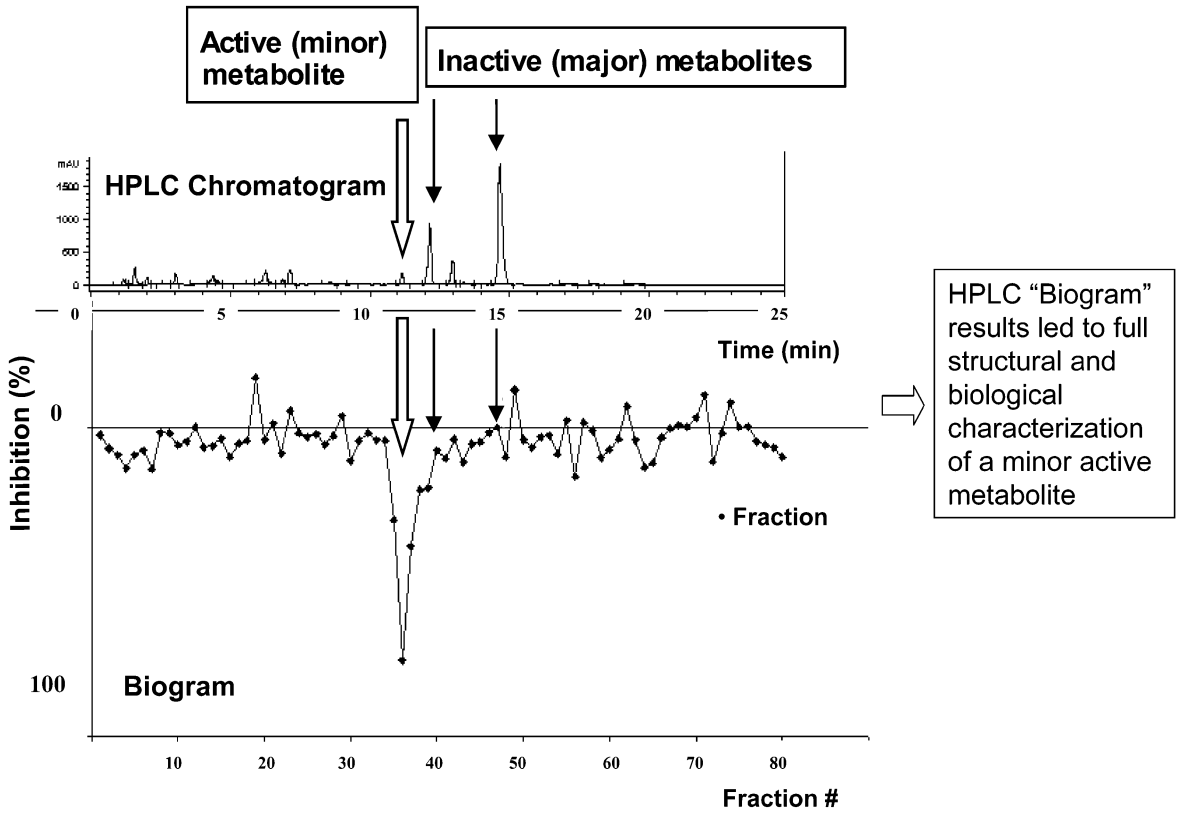

Figure 5. Example of the "biogram" method used in the identification of a minor metabolite with a high degree of pharmacological activity. The two major metabolites, denoted with solid arrows in the chromatogram, were found to show little or no enzyme inhibition, while the minor component was found to possess almost all of the enzymatic inhibitory potency.

The in vitro techniques include the use of subcellular fractions prepared from cells that mediate drug metabolism, intact cell-based systems, intact organs, and isolated enzymes. In vivo methods involve the use of biological fluids (plasma, bile, urine, etc.) obtained from laboratory animals or humans dosed with the parent molecule. Microbial methods can also be used to generate metabolites. Each of these methods is briefly discussed below.

Subcellular Fractions

Subcellular fractions prepared from organs expressing drug metabolizing enzymes include the cytosolic fraction (the cytoplasmic portion of cells), the S9 fraction (a supernatant fraction obtained by centrifugation of organ homogenate, such as liver at 9000*g*−10000*g*), and microsomes (the pellet obtained by centrifugation of an organ homogenate, such as liver at 100000*g*). Organs such as intestine, liver, kidney, lung, and skin are known to mediate xenobiotic metabolism. Liver is the major site of drug metabolism. As a result, liver subcellular fractions are often employed for studying metabolic reactions and generation of metabolites. For example, a rat liver microsomal system was recently used to prepare 25 mg of a glucuronide metabolite.[40]

Subcellular fractions can be used to prepare metabolites formed by a number of enzymes such as CYP, flavin monooxygenase, myeloperoxidase, ketoreductase, alcohol dehydrogenase, prostaglandin H synthase, uridine diphosphoglucuronosyltransferase, sulfotransferase, etc. As described before, CYP enzymes are the most dominant enzymes responsible for the metabolism and bioactivation of various drugs. cDNA expressed enzymes can also be used for metabolite generation. Microsomal fractions (e.g., Supersomes) prepared from baculovirus-insect cell systems are widely used for high-level expression of human CYP enzymes. NADPH-CYP oxidoreductase (CYPOR) is often coexpressed in these systems to maintain efficient CYP activity.[41,42] The use of cDNA expressed enzymes for metabolite generation requires that the enzyme that is responsible for its generation be identified. This type of "reaction phenotyping" investigation is accomplished by a combination of different approaches, including the use of a panel of heterologously expressed recombinant enzymes (e.g., Supersomes), isoform-specific chemical inhibitors, or inhibitory antibodies.[43−45]

Liver microsomes immobilized by hydrophobic binding to phospholipid coated octadecylsilica or covalently immobilized on Sepharose beads, etc. have also been used to prepare metabolites such as glucuronides.[46−49] These methods are particularly useful when the utility of free microsomes is limited because of low compound solubility in in vitro incubation mixtures.

Primary Cell-Based Systems

Intact cells such as primary hepatocytes, contain both soluble and membrane-bound enzymes including the relevant cofactors at or near the appropriate physiological concentrations. As a result, they have greater physiological relevance and can mediate both phase I and phase II metabolism. Cryopreserved hepatocytes are now available for convenient use, and the hepatocytes can be pooled together from different donors.[50] Immortalized human liver cell lines (vide infra) in which enzymes such as CYPs are individually expressed can

also be used for relatively large-scale generation of metabolites. Tissue slices such as liver slices contain both phase I and II drug metabolizing enzymes and can be used to generate sufficient quantities of metabolites.[51]

Cell Lines Containing Heterologously Expressed Drug-Metabolizing Enzymes

Recently, several cellular systems that express high levels of CYP enzyme have been used as "CYP bioreactors" for rapid and large-scale biosynthesis of metabolites. These include baculovirus-infected insect cells,[52] immortalized human liver epithelial cells,[53] and *E. coli*[54,55] that have been engineered to express large quantities of individual CYP enzymes. These CYP bioreactor systems are capable of generating appreciable quantities of CYP-derived metabolites using simple equipment. Although CYP bioreactors are still at an early development stage, it has been demonstrated that they are a promising technology for large-scale and low-cost production of metabolites. Currently tested CYP bioreactors, such as suspension cultures of insect cells and attachment cultures of immortalized human cell lines, have several unique advantages over conventional metabolism systems and chemical synthetic approaches. These include rapid and stereospecific synthesis of human CYP-derived metabolites at very high yield and ease of metabolite isolation and identification, as bioreactor mediums are relatively clean compared to other biological fluids.

As discussed above, the microsomal fractions (Supersomes) prepared from baculovirus-insect cell systems have wide applications in in vitro metabolism studies. In addition, suspension cultures of the baculovirus-insect cell systems were developed as CYP bioreactors for biosynthesis of milligram quantities of metabolites at minimal cost.[52] Metabolite production by the insect cell CYP bioreactor consists of three steps: (1) growth and maintenance of insect cells in suspension culture until a desired cell density is reached, (2) transfection of the insect cells with baculovirus containing cDNA for a CYP enzyme and/or CYPOR, and (3) metabolite production by the addition of the parent drug to the suspension culture. High metabolite yield is achieved using this system. For example, metabolite yields for testosterone, diazepam, and diclofenac in the appropriate insect cell CYP bioreactors were more than 85% at a 100 μM substrate concentration.[52]

Immortalized human liver cell lines transfected with individual human CYP genes were originally developed for identifying potential mutagens, cytotoxins, and chemotherapeutics agents.[56,57] More recently, the system has been applied for the biosynthesis of milligram quantities of CYP-dependent metabolites.[35,53] Studies with several CYP substrates in the immortalized human cell CYP reactors showed 75−100% conversions after 2−3 days of incubation.[53] The immortalized human cell CYP bioreactor system is particularly suitable for metabolite production from compounds that show intermediate to high rates of metabolism. Briefly, typical biosynthetic reactions were initiated by transferring drugs into a flask after the cell lines have formed a monolayer. Incubations were carried out in a serum-free medium at 37 °C for 2−3 days. By use of this procedure, the biotransformation of testosterone using a CYP3A4 bioreactor was recently explored, resulting in the formation of 6β-OH testosterone with greater than 90% yield.[53] These systems are relatively clean, and the metabolites in the incubation media can be directly identified by LC/MS or LC/NMR with no further purification.

Microbial Cell-Based Systems

CYP-dependent monooxygenases capable of carrying out the oxidative biotransformations of xenobiotics have been found in bacteria, yeast, and fungi.[58] Usually, the fungal systems are two-component microsomal systems similar to liver monooxygenases, and the bacterial systems are soluble three-component systems similar to the adrenal mitochondrial monooxygenases.[58] Although phase I metabolism is of more importance for identifying metabolites with pharmacological and toxic effects, some microbial phase II enzymes have been described, including glucuronosyltransferase, arylsulfotransferase, and glutathione *S*-transferase.[59−61] Smith and Rosazza first proposed in 1974 that microbial biotransformations could be used for the preparation of mammalian drug metabolites.[62] Once identities of the microbial and mammalian drug metabolites have been established, usually by LC/MS, the microbial metabolites can be prepared in sufficient quantity to be used in biological activity tests, preparation of analytical standards, and further elaboration by medicinal chemists. The advantages of using microorganisms for production of metabolites are that the cultures are relatively easy to maintain and grow and that scale-up to produce milligram or gram amounts is readily accomplished. In some cases, it is not possible to obtain all of the mammalian metabolites by microbial transformation but because of the simplicity of the methods and the capability of supplying large amounts of metabolites, the technique remains a useful option.

A typical procedure for determining whether a microbial system will be useful would begin by screening a group of bacterial or fungal strains to determine if the desired mammalian metabolite is produced. Fungal strains, especially *Cunninghamella elegans*, Actinomycete bacterial strains, and *Streptomyces griseus*, are among the most useful. In a typical procedure, a collection of strains such as the group listed below is grown at 28 °C in shake flasks on a medium known to be favorable for hydroxylations.[62] Nutrisoy is often added because it can induce monooxygenase activity.[63] Some fungal strains typically screened in our laboratories to obtain hydroxylated metabolites of drug candidate compounds are *Cunninghamella elegans*, *Aspergillus niger*, *Aspergillus foetidus*, *Beauvaria bassiana*, *Cunninghamella echinulata*, and *Aspergillus ochraceus*. Actinomycete strains typically screened are *Streptomyces griseus*, *Saccharopolyspora hirsuta*, *Nocardioides luteus*, *Amycolatopsis orientalis*, *Amycolata autotrophica*, and *Streptomyces antibioticus*. For example, a *Streptomyces griseus* bacterial strain was used recently to produce a total of 49 g of (8*S*)-hydroxymutilin (BMS-303786), 17 g of (7*S*)-hydroxymutilin (BMS-303789), and 13 g of (2*S*)-hydroxymutilin (BMS-303782) from 162 g of mutilin.[64]

Production of metabolites by many other strains of fungi and bacteria has been reported.[65−68] Many ex-

amples of microbial preparation of mammalian drug metabolites are given in recent reviews.[67,68] Examples include pravastatin, an HMG-CoA reductase inhibitor that is a 6α-hydroxylated derivative of compactin (mevastatin) produced by biotransformation using *Streptomyces carbophilus*. In fact, this microbial biotransformation process is used to manufacture pravastatin rather than a chemical synthesis.[69] *Cunninghamella elegans* was reported by Zhang and co-workers to produce eight metabolites, including the two major mammalian metabolites (11-hydroxy and N-demethylated), after incubation with the tricyclic antidepressant amitryptyline.[70] Several of these metabolites are known to have pharmacological activities.

In Vivo Methods

Metabolites can be generated in vivo following compound administration to preclinical animals or human. In vivo samples such as plasma,[35,37] urine,[69] and bile[33] can be used to obtain metabolites. Metabolites can also be obtained from drug-metabolizing organs such as liver obtained from animals pretreated with the desired compound.

In vivo methods of generating active metabolite can be especially useful when active metabolites are formed extrahepatically because extrahepatic systems are not as widely used as hepatic systems for in vitro generation of metabolites.

Recent Advances in the Structural Characterization of Metabolites

Until recently, the majority of the effort to identify metabolites and assign exact structures was undertaken in the late stage of the drug development process. The identification process involved, in most cases, a lengthy and labor-intensive isolation of metabolites and the use of radiolabeled compounds. Fortunately, these processes have now been obviated or greatly reduced in scale because of the recent advances in high-pressure liquid chromatography−mass spectrometry (LC/MS), high-pressure liquid chromatography−mass spectrometry/mass spectrometry (LC/MS/MS), and liquid chromatography−nuclear magnetic resonance spectroscopy (LC/NMR). Below, we will briefly discuss the recent advances in the LC/MS or LC/MS/MS and LC/NMR technologies with respect to metabolite structure identification.

LC/MS or LC/MS/MS

By coupling liquid chromatography with mass spectrometry (LC/MS or LC/MS/MS), it is now possible to quantify and determine on-line the identity of drugs and metabolites of diverse structures in different biological systems with unprecedented speed, sensitivity, and specificity.[71−76] LC/MS, particularly in combination with atmospheric pressure ionization (electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)) techniques, can now be used to analyze polar, nonvolatile, and thermally labile drugs and metabolites.[77−79] Less polar molecules can be analyzed with the recently introduced atmospheric pressure photoionization technique.[79] Advances in computer software technology have also greatly facilitated LC/MS data acquisition and interpretation.[80,81] LC/MS is a robust technique and is amenable to automation.[82] The ability to analyze samples from different sources and chemotypes on-line with such great speed and sensitivity using LC/MS has indeed revolutionized the drug discovery and development process.

For detection and structural elucidation of metabolites, various tandem mass spectrometric (MS/MS) experimental techniques such as product ion, precursor (parent) ion, and neutral-loss scans are used.[73−76,79,82−84] The major types of instruments used for tandem mass spectrometric studies are triple quadrupole, ion trap, time-of-flight mass spectrometers, and to a lesser extent magnetic sector and Fourier transform ion cyclotron resonance (FTICR) mass spectrometers. The recently introduced coupled mass spectrometers, such as quadrupole-time-of-flight (Q-TOF)[85] and quadrupole-linear ion trap[86,87] mass spectrometers, are also becoming popular.

Multiple-stage mass spectrometric (MS/MS^n, $n > 2$) experiments can be performed by both ion trap and FTICR mass spectrometers. Ion traps are more widely used for structural identification of metabolites.[73,88,89] Ion trap mass spectrometers are highly sensitive, and structural information can be obtained with low quantities (picogram) of metabolite.[90]

For exact mass determination, high-resolution mass spectrometers such as double-focusing magnetic sector or FT-ICR have traditionally been used. The recently coupled highly sensitive quadrupole time-of-flight mass spectrometers (Q-TOF) in combination with electrospray ionization can also be used for high-resolution measurements.[85,91] With Q-TOF, a mass accuracy of greater than 5 parts per million can be achieved and the elemental composition of metabolites can be determined. Quite recently, a triple quadrupole mass spectrometer based method demonstrated the capability to provide enhanced resolution and accurate mass measurements.[84] Exact mass measurement capability in combination with neutral loss and precursor ion scan modes by triple quadrupole mass spectrometers can greatly enhance the identification of metabolites.

As described above, significant advances in mass spectrometric technology in terms of ionization techniques, mass analyzer capability, and data reduction have greatly facilitated the identification of metabolites. These, along with significant advances in liquid chromatography technologies, have made LC/MS the method of choice for initial identification of metabolites in complex biological matrices.[36,72]

LC/NMR

Nuclear magnetic resonance (NMR) is the premier analytical method for the determination or confirmation of molecular structures. NMR can unambiguously solve regiochemistry questions that are frequently encountered in metabolite structure characterization through the use of the appropriate repertoire of 1D and 2D homo- and heteronuclear experiments. [92,93]

The past 15 years has seen multiple advances in NMR hardware and software that has broadened the application of NMR to drug discovery and development.[94] The advances that have the largest impact include increases in sensitivity and solvent suppression pulse schemes. The gain in sensitivity can be directly attributed to advances in probe design. For example, the amount of

material required to obtain a 1D carbon spectra in a reasonable acquisition time has dropped from more than a hundred milligrams to submilligram quantities over the past few years. Probe design has gone through a metamorphosis, and a wide selection of probes is now available for various applications. Small-volume probes, such as high-resolution magic angle spinning nanoprobes,[95] flow probes for LC NMR (400−100 μL), and capillary probes (10−25 μL),[96−99] have made a tremendous impact on metabolite and impurity identification because of their high signal to noise performance. The recent introduction of cryoprobes promises a further 3- to 4-fold boost in signal to noise ratios.[97,100−102]

A prelude to the advances in probe design was the development of solvent suppression pulse sequences.[103] Solvent suppression sequences have been automated for effortless incorporation into any NMR experiment. The combination of the gain in sensitivity due to probe design and the ease of solvent suppression is the main driver for the improvements in the identification of small quantities of metabolites and in the development of methods that couple NMR with other analytical techniques such as liquid chromatography (LC/NMR) and mass spectrometry (LC/NMR/MS).[104−109] As a result, LC/NMR in combination with mass spectrometry has become a major contributor of metabolite identification.

Flow or direct injection NMR was introduced in the mid 1990s for 96-well plate analyses. Shortly after that, flow-probe NMR technology was integrated with LC and MS as necessitated by the need for structural characterization of low-level metabolites. This integration allowed the determination of the exact molecular structure of metabolites in one HPLC injection. To increase the sensitivity of LC/NMR further, an automated solid-phase extraction has been incorporated into an LC NMR system.[110−112] Recently, with the introduction of capillary probes, capillary electrophoresis-NMR is also becoming a reality.[98]

One-dimensional proton LC/NMR often provides enough information about the structure of metabolites. Various 2D NMR experiments can be used to confirm the structure or for identification of more complex structure.[92,93]

In summary, advances in NMR instrumentation and techniques have made the characterization of metabolite structures a routine part of the drug discovery and development process.

Conclusions

The numerous examples discussed above demonstrate that biological transformation can be a useful approach for discovering drugs. It is therefore useful to screen drug candidates for possible conversion to active metabolites during the discovery stage. Active metabolites may show superior pharmacology, pharmacokinetics, and safety profiles in comparison to their parent molecules, and the inherent benefits that metabolites often display make their study a worthwhile endeavor. As discussed above, a number of active metabolites of marketed drugs have already been developed as drugs in their own right. Also discussed in this paper are several examples where active metabolites were identified during the discovery phase and either became lead compounds or led to the structural alterations of the chemotype. Consequently, the discovery of drugs through biological transformation can be an integral part of the drug discovery process and it may therefore be beneficial to have a process in place to screen drug candidates in search of active metabolites.

There are a number of experimental observations during the preclinical pharmacokinetic and pharmacodynamic evaluations of drug candidates that can trigger the search for conversion of drug candidates to active metabolites. Approaches such as the rapid bioassay guided method described herein can be used to generate and detect active metabolites. Different biological methods are now available for production of active metabolites. Large-scale synthesis of metabolites by biological methods is now feasible because of the recent advances in biotechnology, particularly in the area of CYP bioreactors and microbial methods. Recent advances in LC/MS and LC/NMR technologies have also expedited the structural characterization of metabolites with high sensitivity, specificity, and speed. These advances have made the routine identification and evaluation of metabolites a reality.

Acknowledgment. We gratefully acknowledge Dr. David Rodrigues for his critical review of this manuscript.

Biographies

Aberra Fura received his Ph.D. in Chemistry in 1992 from Cornell University. After 2 years of postdoctoral fellowship at the Department of Chemistry at University of California, Berkeley, he moved to Cytomed inc., a biopharmaceutical company in Cambridge, MA, where he began his career in drug metabolism, pharmacokinetics, and bioanalytical sciences. He moved to Bristol-Myers Squibb, Pharmaceutical Research Institute in 1997, where he is currently a Principal Scientist in the Metabolism and Pharmacokinetics Department. His interest is in the application of drug metabolism and pharmacokinetics to drug discovery, in vitro and in vivo correlations, and LC/MS and new technology in drug metabolism. He is a co-inventor of several patents in the drug design area based on biological transformation methods, active metabolites, and structure−metabolism relationship.

Yue-Zhong Shu received his Ph.D. degree in Pharmaceutical Science from Toyama Medical and Pharmaceutical University (Japan) in 1988 on the chemistry and biotransformation of bioactive natural products. He went on for postdoctoral appointments at University of Saskatchewan and Virginia Tech on the metabolism and synthesis of central nervous system drugs and naturally occurring heterocyclic mutagens. He started his industrial career at the Drug Metabolism Department of Pfizer Central Research Division in 1991. Later, he joined Bristol-Myers Squibb, Pharmaceutical Research Institute, where he is currently Associate Director of Discovery Biotransformation. His research is centered around the mammalian and microbial metabolism of synthetic and natural product drug candidates through application of bioorganic, biochemical, and bioanalyitcal methods, aiming at addressing potential safety and efficacy issues related to drug metabolism.

Mingshe Zhu received his B.S. degree in Biochemistry and his M.S. degree in Chemistry. He received his Ph.D. degree in Analytical Toxicology from SUNY at Albany in 1994. He completed 2 years of postdoctoral training in drug metabolism with Professor Sidney Nelson at the University of Washington. He is currently a Senior Research Investigator at Bristol-Myers Squibb, Pharmaceutical Research Institute and has been involved in the drug metabolism studies to support drug development and registration. His current main responsibility is the development and implementation of new technologies

and approaches in drug metabolism. His research interests include application of LC/MS and radiodetection techniques, development of cell-based P450 bioreactors, metabolic bioactivation, and reaction phenotyping of metabolizing enzymes.

Ronald L. Hanson received his Ph.D. in Biochemistry from the University of Wisconsin. After 2 years as a Postdoctoral Fellow at Harvard Medical School and 7 years as an Assistant Professor of Biochemistry at Columbia University, he worked for 8 years at Sandoz Pharmaceuticals in the diabetes drug discovery section in East Hanover, NJ. For the past 16 years he has worked in process research and development for Bristol-Myers Squibb in New Brunswick, NJ, where he is currently a Senior Principal Scientist. His research is in the area of biocatalysis by microbial cells and enzymes.

Vikram Roongta received his Ph.D. in Chemistry from Purdue University, IN, in the area of biomolecular structure determination by NMR. He joined University of Minnesota as a Staff Scientist and for the first 4 years managed the Chemistry Department NMR Facility and for the following 4 years managed the Biochemistry Department NMR Facility and performed research on solution structures of proteins by NMR. For the past 6 years he has worked in Discovery Analytical Sciences Department supporting drug discovery at Bristol Myers Squibb and is currently a Group Leader. His research is in the area of applications of NMR in small-molecule structure determination in drug discovery.

W. Griffith Humphreys, Ph.D., is currently Associate Director in the Metabolism and Pharmacokinetics Department at Bristol-Myers Squibb, Hopewell, NJ. He received his graduate training at the University of Virginia in Chemistry and then completed a Postdoctoral Fellowship at Vanderbilt University in molecular toxicology. He oversees a group responsible for drug metabolism and pharmacokinetic studies during the candidate optimization phase of drug discovery. His interests include drug metabolism, predictive metabolism and toxicology models, in vitro and in vivo correlations, and strategies for candidate optimization.

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JM040066V