A Holistic Strategy for Characterizing the Safety of Metabolites through Drug Discovery and Development

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The subject of metabolites in safety testing has had much debate in the recent past and has shown itself to be a complex issue with no simple solutions to providing absolute assurance of drug safety. Much of the attention has focused on the ability to identify metabolites and then demonstrate that their risk has been adequately characterized, either through their exposure in toxicology species or, failing this, by direct safety testing. In this review, we summarize our forward operational strategy that combines the principles summarized in the FDA Guidance, together with discussions at scientific meetings and literature opinions. It is a balance between the primary goal of assuring patient safety with one of reasonable investment. A key principle in striking this balance is to build stepwise information on metabolites through the drug discovery and development continuum. This allows assessments to be made from early nonclinical studies onward as to whether or not metabolite safety is underwritten by exposure in toxicology species. This strategy does not require absolute quantitation of the metabolites in early clinical trials but relies upon comparison of relative exposures between animals and humans using the capabilities of modern analytical techniques. Through this strategy, human disproportionate metabolites can be identified to allow a decision regarding the need for absolute quantitation and direct safety testing of the metabolite. Definitive radiolabeled studies would be initiated following proof of pharmacology or efficacy in humans, and nonclinical safety coverage would be adequately assessed prior to large-scale clinical trials. In cases where metabolite safety is not supported through the parent compound toxicology program, approaches for the direct safety testing of metabolites with regard to general and reproductive toxicology, safety pharmacology, and genetic safety have been defined.

Contents

1. Introduction: History and Perspective

The debate around the requirements for identification and safety characterization of drug metabolites has been going on in scientific meetings and the literature for much of the past decade. Following the deliberations of a multidisciplinary committee sponsored by the Pharmaceutical Research and Manufacturers of America in 2000, a paper detailing the proposed practical and scientific strategy for this topic was published in 2002 (*1*). This publication served to fuel even greater debate on the subject, resulting in further publications

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reflecting, among other areas, the limitations of assessments based primarily on percentage abundance (*2*), considerations of pharmacological activity (*3*), and considerations of chemical reactivity (*4*). A continuing underlying theme throughout this debate has been the practical question of what should we actually be doing and when in order to adequately assess the safety risk associated with drug metabolites during drug development. There is clearly no absolute answer to this question, and all participants in the debate, including the regulatory agencies, have continued to state the need for scientific consideration on a case-by-case basis (*5*). Within our own organization, we have reviewed and refined our practices in this area in order to ensure that these provide an appropriate level of quality and scientific rigor to the characterization of drug metabolites. This was reviewed again upon the issue of the guidance on metabolites in safety testing (the MIST[1] guidance) by the Food and Drug Administration (FDA) in 2008 (*6*).

The objective of this review is to describe the holistic approach that is taken to metabolite characterization as molecules progress through the drug discovery and development continuum and the building of the overall metabolite safety assessment that occurs through this process. The application of modern analytical technology (*7*) and in vitro metabolism techniques (*8*) allows access to metabolite information from studies with unlabeled material that was not possible 10 to 20 years ago. Thus, by the time of phase 2 development, when the definitive radiolabeled ADME (absorption, distribution, metabolism, and excretion) studies are performed, there already exists a relatively detailed understanding of the metabolic fate of the new chemical entity (NCE). This information may have already prompted specific safety assessment of metabolite molecules, should human exposure not be adequately supported within the toxicology program with the parent molecule.

2. Metabolite Characterization Strategy through Drug Discovery and Development

Definitive data on the total metabolite profile of a NCE in humans can only be gained from an in vivo study in which a radiolabeled compound is administered, and blood/plasma and excreta samples are collected to profile and quantitate radioactive components. Prior to such a study, it is generally not possible to conclusively confirm that all metabolic products have been identified, and all drug related material has been eliminated from the body. Exceptions may occur for compounds which are not metabolized and eliminated entirely by the renal route, but such cases are relatively rare among small drug molecules. However, a wealth of information on metabolites can be obtained prior to radiolabeled studies starting with in vitro studies in the drug discovery phase and extending to metabolite scouting in plasma samples from animals and humans obtained from routine studies in the drug discovery and development program. The term metabolite scouting refers to a series of experiments to identify metabolites using a combination of modern analytical technology and the accumulated knowledge base of xenobiotic transformations (including in silico tools, scientific literature and expert knowledge) that permits a fairly comprehensive metabolite profile to be established without the use of a radiolabeled compound. The MIST guidelines identify metabolites for safety concern as those representing greater than 10% relative AUC (area under the plasma concentration time curve) to the parent molecule. This can generally be regarded as a first point of reference as information is generated on the metabolites of an NCE. As information becomes available, it is therefore possible to consider the question, is the metabolite likely to be present at a significant level in man, and if so, is it likely to be present at sufficient levels within the toxicology species to support the human exposure? From in vitro studies, this question is answered by cross-species comparison of metabolic profiles that may indicate metabolic transformations unique or disproportionate in man. A unique human metabolite is one that is only found in man, whereas a disproportionate human metabolite is present in higher concentrations in humans than in the animals used in nonclinical studies. Metabolite scouting from early clinical studies provides the opportunity to make a semiquantitative assessment of metabolites present in human plasma and by reference to plasma samples from animal toxicology studies it can be determined if significant human metabolites are sufficiently represented in the toxicology species to underwrite the safety of human exposure. Apart from assessing the abundance of metabolites to determine if human safety is adequately supported, there are other important pieces of information that can be gained from structural considerations. The structure of a metabolite will indicate if it is likely to contribute to the desired or primary pharmacological response and is therefore important for PKPD (pharmacokinetic−pharmacodynamic) considerations. If a structural alert is apparent, this may flag a specific safety concern for the compound.

Thus, there are essentially three stages at which critical information on the metabolite profile of an NCE are obtained: (i) in vitro human and animal studies, (ii) circulating human metabolites from phase 1 multiple dose studies, and (iii) the definitive human and animal radiolabel ADME studies. Brief details of the experimental approach and information that can be obtained at each of these stages are provided in the following section; further details of the typical analytical strategies for metabolite identification have been reviewed recently (*9*). Broadly speaking, these are the major sources of metabolite information that will be obtained; however, they may be supplemented by additional studies and specific investigations as judged to be scientifically appropriate to individual programs. While the human radiolabeled ADME study provides the definitive data with regard to identifying metabolites of an NCE, information that arises earlier in the drug discovery or development program may trigger more detailed evaluation of specific metabolites if they are deemed to pose a potential safety risk. For example, if the nonquantitative analysis from metabolite scouting in early clinical studies provided evidence for a unique (or disproportionate) human metabolite, this may prompt synthesis of an authentic standard to permit quantitative bioanalysis and enable safety studies with direct administration of the metabolite to be conducted if deemed necessary. Committing resources to chemical synthesis and quantitative analysis will only be undertaken if there is sufficient evidence to indicate that this is required to complete the safety assessment, as will be discussed within this review.

[1] Abbreviations: ADME, absorption, distribution, metabolism and excretion; AMS, accelerator mass spectrometry; AUC, area under the plasma concentration time curve; C_{avg}, average concentration; FDA, Food and Drug Administration; GLP, Good Laboratory Practice; GMP, Good Manufacturing Practice; HPLC, high performance liquid chromatography; LC-ARC, liquid chromatography−accurate radioisotope counting; LC-MS, liquid chromatography coupled to mass spectrometry; LTQ, linear trap quadropole; MIST, metabolites in safety testing; MS/MS, tandem mass spectrometry; MS^n, mass spectrometry to the n^{th} power; NCE, new chemical entity; NMR, nuclear magnetic resonance; NNRTI, non-nucleoside reverse transcriptase inhibitor; Q-TOF, quadropole-time-of-flight; P450, cytochrome P450 enzyme; PKPD, pharmacokinetic−pharmacodynamic; S9, the supernatant fraction obtained from liver homogenate by centrifuging at 9000*g* for 20 min; this fraction contains cytosol and microsomes; PPAR, peroxisome proliferator activator receptor; SS, steady state; UV, ultraviolet.

2.1. In Vitro Metabolite Profiling in Animals and Humans. These studies will generally be performed as part of the early assessment of potential drug candidates. A typical starting point for this phase of metabolite profiling is the use of liver preparations from human and the major toxicology species. Whereas in vitro samples are the major focus during compound selection, the analysis of in vivo samples from relevant animal species is also a common and important activity, in order to assess the identity and abundance of circulating metabolites and thus provide some context around in vitro−in vivo correlations for the compound in question. In this context, the term abundance refers to the amount of metabolites relative to the parent compound and also the relative amounts across species. Mass spectrometry remains the technique of choice for metabolite identification during drug discovery because of its speed, sensitivity, and selectivity. The ability of mass spectrometry to provide specific structural information, however, remains limited, and thus partial Markush structures are commonly the output of metabolite identification studies at this stage. Further structural information is possible via a number of techniques, including nuclear magnetic resonance (NMR) spectroscopy (*10*), derivatization (*11*), and deuterium exchange experiments (*12*). All, however, require additional resources, and it is often necessary to make a judgment as to the potential benefits of the extra structural information versus the investment in generating the data. Specific approaches to mass spectrometric metabolite profiling in drug discovery are many and varied. Historically, the functionality of triple quadrupole mass spectrometers has been used to detect and identify metabolites, using precursor ion and/or constant neutral loss experiments and tandem mass spectrometry (MS/MS) analysis, respectively (*13, 14*). In addition, ion trap instruments have been used to provide MS^n (mass spectrometry to the power of n) data in the hope of generating additional structural information (*15*). The recent development of hybrid linear ion trap instruments, such as the QTrap (Applied Biosystems, Bedford, MA) (*16*), combines many of the advantages of these two approaches and has become a common tool for metabolite identification (*17, 18*). In recent years, the use of high resolution mass spectrometry (both to provide molecular formulas and thus clarify ambiguous structural assignments, and to facilitate metabolite detection (the mass defect approach (*19*))) has become commonplace (*20, 21*), facilitated by the introduction of new high-resolution instruments such as the quadropole-time-of-flight (Q-TOF, Waters Corporation, Milford, MA) and the LTQ-Orbitrap (Thermo Scientific, Waltham, MA). Together with the increased utilization of high-resolution mass spectrometry, high-resolution chromatography systems are becoming the preferred method throughout the industry. The improved resolution not only allows better separation of metabolites but also separates drug-related from endogenous components, thereby facilitating metabolite identification in complex matrices (*22, 23*). Another area of significant growth in recent years is the development of software packages in support of metabolite identification studies. Systems such as Metworks (Thermo Scientific), Metabolynx (Waters Corporation), and Lightsite (Applied Biosystems) all represent user-friendly and effective tools to facilitate metabolite detection and identification. In addition to these supporting packages, the use of in silico prediction tools to augment metabolite identification experimentation is an area of potential growth. Packages such as METEOR (*24*), which uses chemical rules and a database of metabolic reactions, or Metasite (*25*), which utilizes conformational positioning within P450 enzyme active sites, can be used to aid the detection and subsequent identification of metabolites during drug discovery. In combination, these tools give a relatively comprehensive picture of the in vitro metabolic pathways of compounds in the discovery phase, providing appropriate confidence around safety and clearance understanding to underwrite progression into drug development.

2.2. Metabolite Scouting in Human and Animal Plasma. In keeping with the principles of the MIST guidance, the key focus of metabolite scouting in early development is to provide confidence in safety via an assessment of apparent disproportionate metabolites in human circulation. Thus, it is essential to ensure that the process involves an appropriately robust comparison between the profiles of circulating metabolites in humans and those in the relevant toxicology species at steady state following multiple dosing. Typically, human plasma samples from the first multiple escalating dose study in healthy volunteers are used for the analysis and compared to animal plasma samples from multiple dose toxicology studies (*7*). The ability to analyze human and animal plasma samples simultaneously (or as close to it as is feasible) greatly facilitates the comparison, and this may dictate the choice of toxicology study from which samples are used depending on the relative timing of the toxicology program and associated plasma sample availability. In order to maximize the amount of drug-related material and thereby facilitate metabolite identification, the analysis will typically involve samples generated following the highest dose of compound, although samples derived from other dosing cohorts may provide value depending on the specific issues and/or strategy being deployed for a given program. Following a simple sample preparation step in order to maximize the recovery of metabolites, the typical approaches to metabolite detection and identification are as described for the discovery phase. In many cases, extra diligence regarding structural characterization may be appropriate in order to properly underwrite compound safety such that use of additional techniques such as NMR analysis may be a more common activity. Although potentially limited by the low concentrations of drug related material in the circulation for some compounds, NMR can still be a valuable tool during the metabolite scouting phase where plasma exposures are relatively high or where urine samples can be utilized to generate more material (*26*). One key difference between the discovery phase and metabolite scouting in early development is the need to provide appropriate quantitative data for metabolites in the absence of radiolabeled material and (in the majority of cases) an authentic standard. In the discovery phase, the use of ultraviolet (UV) and/or mass spectrometry to provide approximate relative abundance assessments is usually considered sufficient, in spite of the limitations of both technologies. The key to abundance assessments in early development is that relative, rather than absolute, abundance is the desired target in order to assess the disproportionality of human circulating metabolites. Many technologies have been considered and utilized for metabolite quantitation (*9, 26−28*); however, their use will not always be appropriate for metabolite scouting. The simplest approach to the scouting process is to analyze human and animal plasma samples simultaneously using appropriate liquid chromatography−mass spectrometry (LC-MS) approaches such that online comparison of the metabolite profiles in terms of retention time and LC-MS data can be made. An outline of such an approach is described in Figure 1. Appropriate matrix matching methodologies can be employed where necessary to ensure that the relative quantitation is not confounded by endogenous material, even though in most cases, it is likely that the relatively low interference from matrix components in plasma samples will

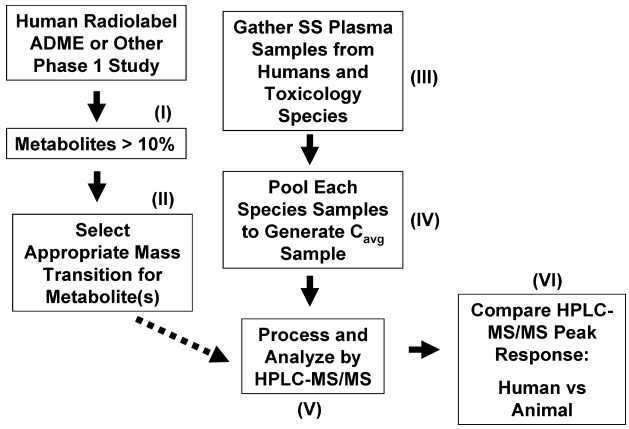

Figure 1. Approach to determine whether toxicology species are adequately exposed to human metabolites. In this approach, it is not necessary to obtain authentic standards of metabolites or develop and validate bioanalytical assays. (I) The approach begins with an inspection of the circulating metabolite relative abundances from the single dose human radiolabeled ADME study. Metabolites present at 10% of the parent drug exposure (or at 10% of total radioactivity for those compounds that are extensively metabolized) are considered as requiring a demonstration of the presence in animals. This process can also be triggered by observation of metabolites in earlier clinical studies that do not use the radiolabeled drug. (II) From the ADME study or metabolite scouting, the retention time on HPLC and appropriate mass transition(s) can be identified and used in subsequent comparisons. (III) After deciding which metabolites require a closer look, one can gather pharmacokinetic samples from clinical trials in which the drug is dosed to steady-state. If animal plasma samples remaining from toxicokinetic analysis are available, these can be gathered as well. Alternately, if such samples are too old or unavailable, new ones can be generated by dosing relevant animal species to steady-state at the NOAEL dose level. (IV) Pooling of samples from each species is done in such a manner as to provide a pool for each species in which parent drug and metabolites will be present at C_{avg} (*29*). (V) The pooled samples are analyzed using HPLC-MS/MS (by the method applied to plasma samples from the human radiolabel ADME study if this is available), applying the specific mass transitions already identified for each metabolite of interest. To avoid possible matrix differences between human and animal plasma (i.e., endogenous interferences and ion suppression), an equal volume of animal control matrix can be mixed with the human sample and vice versa. Thus, the sample type becomes identical, and any potential matrix interferences will be the same. The samples are processed using a technique that will not selectively remove the metabolite(s) of interest, such as simple protein precipitation using miscible organic solvent. An internal standard can be used to ensure corrections for variability in analyte extraction or HPLC injection volumes. Injection volumes should be such that the peak intensities do not approach the maximum response on the mass spectrometer. (VI) Finally, the peak responses for the total ion current chromatograms for the mass transitions of interest are compared. If the metabolite peak in steady-state animal pooled plasma exceeds that observed for the human sample, then the metabolite exposure coverage has been achieved. If not (i.e., human disproportionate or unique metabolite), then further work is required to qualify the safety of the human metabolite.

result in a proportionate mass spectrometric response regardless of the species. The observation of disproportionate metabolites in human plasma should give rise to subsequent investigations (e.g., formal metabolite monitoring or activity assessment) as described elsewhere in this review. While absolute amounts of metabolites are not obtained by this method, a degree of semiquantitation can be obtained by the combination of MS and UV response, knowledge of the chemical structure, and any metabolite standards that may be available. In this way, an excessive number of metabolites that are of only minor abundance in human plasma are unlikely to be flagged for qualification within the animal species. If the appropriate methodologies are applied to ensure that metabolite characterization is optimized, this pragmatic approach to metabolite scouting should provide ample confidence that adequate safety considerations have been made in advance of the definitive metabolite identification studies in later development.

2.3. Definitive Human and Animal Radiolabel ADME Studies. These studies are generally conducted during phase 2 clinical development once a compound has been shown to provide the desired pharmacological response in a patient population. In common with metabolite scouting in early development, the primary objectives of the definitive metabolism package are the comparison of circulating metabolites between humans and animal species and obtaining a picture of the total disposition of drug-related material. Typically, plasma and excreta samples are derived from ADME studies using the clinical route of administration in humans, the major toxicology species (one rodent and one nonrodent), mouse, and rabbit (as additional carcinogencity and teratogencity species respectively), the exception being for inhaled compounds, where the logistics of dosing radiolabeled material via this route typically mean that ADME studies are conducted using oral or intravenous administration. While plasma is the main focal point for analysis, excreta samples can also provide valuable data, both as a source of additional material to facilitate structural elucidation (e.g., by NMR) and on occasions to provide additional information to help understand clearance pathways. The key difference between the definitive studies and metabolite profiling conducted earlier in discovery or development is the presence of radiolabeled material, which enables detection of all drug-related components (provided that the position of radiolabel incorporation is appropriate) and the reliable quantitation of the metabolites using conventional radiochemical detection methods. Depending on the levels of drug-related material in the samples (and hence the levels of radioactivity), metabolite profiling can be achieved either using conventional radiochemical detection in line with high performance liquid chromatography (HPLC) analysis or more commonly during the analysis of plasma samples, using techniques designed to enable low-level counting, such as the LC-ARC (liquid chromatography−accurate radioisotope counting) system (*30*) or fraction collection into plates followed by off-line counting (*31, 32*). The ultimate in sensitivity for analysis is accelerator mass spectrometry (AMS), a technology that detects individual isotope ions (as opposed to scintillation events resulting from radioactive decay). AMS has been increasingly applied to ADME studies in recent years and can provide metabolite profiles at extremely low levels of drug-related material (*33, 34*). However, the approach is cumbersome (since it cannot be directly interfaced with HPLC) and expensive, provides no direct structural information, and thus should be considered only when other technologies are unable to deliver the data necessary to support safety. Once the approach to radiochemical profiling has been established, structural elucidation is achieved using the techniques and approaches described previously. The timing and definitive nature of these studies dictates that a significantly higher level of diligence in structural characterization is usually appropriate. A possible strategy for the definitive metabolism package is to conduct one animal ADME study (usually in rodents) in advance of the human study in order to validate the position and choice of radiolabel to ensure that the human ADME analysis will not be confounded. The other animal studies can then be performed in parallel with the human ADME study to facilitate the comparison of metabolite profiles. This approach fits well with the principles of the MIST guidelines and is particularly appropriate if GMP (Good Manufacturing Practice) material is required for the human study, as any requirements to synthesize an alternative radiolabeled analogue are best determined prior to initiation of the GMP process.

3. Dealing with Potential Pharmacologically Active and Disproportionate Metabolites Prior to Definitive Studies

Information pointing to the potential for humans to be exposed to metabolites that may not be adequately represented in the safety species may arise from in vitro systems prior to human experience or from qualitative or semiquantitative analysis of human and animal plasma samples. Early knowledge on potential circulating metabolites is primarily considered with respect to possible pharmacological contribution. Metabolites that bear close-in structural resemblance to the parent compound may possess similar pharmacology and should therefore be synthesized to allow assessment of activity and potential contribution to the pharmacodynamic response. Unless there is strong evidence that a specific metabolite is likely to make a significant contribution to the pharmacodynamic response, it is not considered appropriate to set up validated bioanalytical methods for metabolites during toxicology and first in human studies given the additional complexity and cost that multiple analyte assays impart. Should semiquantitative metabolite analysis during early clinical studies add further evidence for a pharmacological contribution from a metabolite, then a specific assay can be established for future studies. As a rule of thumb, unless a metabolite is expected to contribute more than 25% relative potency to parent (based on free exposure and pharmacological activity *1, 2*), then routine quantitation will not be employed. Where in vitro data in combination with circulating metabolite data from animals indicate the potential for unique human or pharmacologically active metabolites, particular emphasis is placed on the qualitative assessment of these entities during early clinical studies (single and multiple escalating doses). Different analytes will provide different mass spectral responses; however, it is still possible to assess if a specific metabolite poses an exposure risk based upon the relative mass spectrometric (or UV) response in humans and animals. This can be accomplished by comparing the relative signal for a metabolite to that of the parent compound in each species and comparison of the relative concentrations of the parent compound in the different species that has been obtained by a quantitative assay. For example, if a particular metabolite provides a signal 50% relative to the parent compound in humans compared to only 10% in a toxicology species, but the absolute concentrations (AUC) are 20-fold higher in the toxicology species, then it can be estimated that the metabolite is present at 4-fold greater concentration in the animal species (10% $\times$ 20 versus 50% $\times$ 1) irrespective of the absolute concentration. While it may not be known if the metabolite AUC in humans is greater than 10% of the parent compound AUC, it can clearly be shown that adequate exposures are obtained within the toxicology program to underwrite clinical safety. Unless other factors (e.g., pharmacological activity, structural alerts, or lipid solubility) require specific quantitation of the metabolite, no absolute quantitation is therefore required at this time. If, however, these analyses do suggest an exposure risk in humans, then the particular metabolite will be synthesized to allow accurate quantitation and if necessary direct safety testing of the compound.

4. Identifying Metabolites for Further Qualification from Definitive ADME Studies

As previously stated, the human radiolabeled study provides the definitive data with regard to metabolite exposure and elimination from the body. From these studies, information is obtained from two different sources, namely, the plasma and the excreta. The considerations on these two sources serve different purposes and are thus treated separately. The first consideration is the circulating metabolites with regard to their pharmacological activity for the primary target and any potential off-target activities. This is consistent with the MIST guidelines and the focus on metabolites judged to be present at significant levels. Metabolites in the circulation that exceed 10% exposure relative to the parent will be considered for qualification. A pragmatic approach is required for extensively metabolized compounds where many metabolites may exceed this 10% level. In such situations, reference to total drug-related material is generally regarded as most appropriate.[2] If exposure in the toxicology species exceeds the human exposure for those metabolites judged to require qualification, then their safety assessment is supported by the toxicology program. For metabolites where this is not the case, then specific testing may be required. A different focus is applied to excreted metabolites. Metabolites in excreta are themselves not of concern per se, but depending on their structures, they could be indicative of exposure to chemically reactive intermediate metabolites. For example, observation of a mercapturic acid conjugate in excreta can indicate the earlier presence of a reactive electrophile metabolite. For this consideration, if the sum of human excretory metabolites that derive from a common chemically reactive precursor intermediate metabolite exceeds 10 mg/day, then assurance is needed that comparable or greater quantities of this reactive intermediate would have been generated in nonclinical safety assessment species (corrected for body weight). This threshold of 10 mg/day is based upon a conservatively estimated minimal amount representing a potential hazard should a reactive intermediate be involved (*2, 35*). If human exposure to these reactive metabolites exceeds animal exposure, then safety testing of the metabolite will be undertaken (it should be noted that safety testing of the downstream metabolite(s) of the reactive metabolite offers no value). Technical limitations prohibit direct in vivo testing of a chemically reactive metabolite; therefore, innovative approaches are needed to provide assurance that an animal species is exposed to a human reactive metabolite so that a risk assessment can be made. An alternate species can be identified that produces the reactive intermediate, or a direct precursor of the reactive intermediate could be administered to animals. Alternately, it is possible that alterations in the metabolite profile in an animal species to yield a greater flux of drug-related material through the reactive metabolite pathway could be effected by coadministration of an inhibitor or inducer of drug metabolizing enzymes.

5. Steady State versus Single Dose Considerations

The MIST guidelines indicate that metabolite exposure should be considered at steady state. This is clearly appropriate from the perspective that most drugs are administered chronically, and therefore, a steady state will be established for parent compound and metabolites within the circulation. The relative abundance of parent drug and metabolites may well be different from the single dose situation because of differences in elimination rate and therefore degree of accumulation. However, for the traditional assessment of metabolite abundance it has generally been the practice to administer a single dose of radiolabeled compound. The main reason for this is the safety

[2] On the basis of recommendations for updated guidelines on nonclinical safety studies (June 2009), the classification of metabolites requiring qualification may be refined to those exceeding 10% of total drug-related material for all molecules. The strategy described here can be readily amended to incorporate this change in definition.

considerations around the administration of radioactivity to humans and the limits this places on the administered dose. It is possible to divide the radiolabeled doses over several days to achieve steady state, but this would reduce the level of radioactivity available for isolating and identifying metabolites. It would also impose greater restrictions on the volunteers, making such a practice generally unacceptable. It is possible to dose with unlabeled compound to steady state and then administer a single radiolabeled dose. This has advantages for compounds that may show autoinduction, such as voriconazole (*36*), as metabolite profiles are then more representative of the steady state situation. However, any specific metabolite accumulation will not be observed as only cold metabolite will have accumulated prior to the labeled dose, and quantitation based on the specific activity of the radiolabel dose will not be possible (however, the use of UV detection may provide information to allow quantitation). It is therefore generally not practical to obtain metabolite profiles at steady state from radiolabeled ADME studies; it is, however, straightforward to conduct metabolite scouting on plasma samples from human and animal multiple dose studies and thus immediately obtain representative steady state profiles on which to make cross-species comparisons. In addition, it should always be possible to identify potential metabolite accumulation in human radiolabeled studies from the concentration time profiles of drug and radioactivity. If radioactivity is eliminated more slowly than the parent and shows potential for accumulation, then further investigation can be carried out to identify the slowly eliminated metabolites and conduct further specific analysis or pharmacokinetic modeling if required.

6. Plasma Protein Binding Considerations

Consideration should always be given to the plasma protein binding of drug and metabolites and actual measurements performed if necessary. While first assessments of metabolite abundance in plasma (or serum) will always be based on total concentrations, it should be borne in mind that pharmacological activity is more likely dependent on free plasma concentrations (*37*). In cases where there is strong evidence for pharmacological activity of metabolites, it is particularly important to consider free exposures. Metabolites are generally less lipophilic than the parent drug and hence may be expected to show reduced binding to plasma proteins. This may result in relatively higher free concentrations of the metabolite and thus a higher than expected pharmacological contribution based simply on relative potency and total plasma concentrations. The overall effect of such changes in binding between parent and metabolites can have the most impact when the protein binding of the parent drug is particularly high and hence relatively small changes in binding can dramatically impact free exposure. It is therefore appropriate to assess the potential importance of plasma protein binding to the understanding of the PKPD of specific molecules on a case-by-case basis. An interesting example in this regard is the angiotensin II receptor antagonist losartan and its pharmacologically active metabolite EXP3174. In this case, the metabolite has higher plasma protein binding than the parent compound (*38*) because of oxidation of the alcohol function to an acid and the inherent high plasma protein binding of carboxylic acids to albumin. The contribution of the metabolite to the pharmacodynamics of losartan, therefore, required consideration of the plasma protein binding (*39*). For metabolites where there is no evidence for pharmacological activity, it is generally not necessary to establish the extent of plasma protein binding.

7. Case study to Highlight the Building of Metabolite Knowledge through the Drug Discovery and Development Process

The development of the metabolite profile for an NCE is illustrated by the non-nucleoside reverse transcriptase inhibitor (NNRTI), lersivirine (UK-453061). At the time at which this compound was selected for development, in vitro metabolism studies were conducted alongside the nonclinical pharmacokinetic studies which provided the basis for the predicted human pharmacokinetic profile (*40*). The in vitro studies identified N-dealkylation and hydroxylation pathways of metabolism by standard mass spectrometric techniques. These metabolites were used in the nonclinical assessment to examine the impact of enzyme autoinduction that was observed with this compound in toxicology species (*41*). When the compound entered human testing, plasma samples from first-in-human studies were used for metabolite scouting purposes. Using standard chromatographic and mass spectrometric techniques outlined previously, the researchers confirmed the hydroxylated metabolites (with information on the relative abundance of the two positional isomers), but only trace levels of the N-dealkylated metabolite were detected in human plasma. In addition, a glucuronide conjugate of the parent was observed in significant amounts together with a carboxylic acid metabolite formed by oxidation of the alcohol function (Figure 2). These mass spectrometric experiments also indicated the presence of additional glucuronide conjugates of the hydroxylated and N-dealkylated metabolites. The information from these studies, prompted action to confirm that the carboxylic acid metabolite was also present in toxicology species at sufficient exposure in order to demonstrate that its safety had been assessed. In addition, quantitation of the extent of glucuronide formation, by hydrolysis and quantitation of the parent in urine, was performed in order to aid understanding of the clearance pathways and to inform the drug−drug interaction strategy. A human radiolabeled ADME study with lersivirine was subsequently conducted (approximately 3 years later) and confirmed the major routes of metabolism identified through the metabolite scouting. Additional information from this study concerned minor metabolic products as well as definitive quantitation of the individual routes both in the systemic circulation and the excreta. This example serves to illustrate the relatively complete metabolite profile that can be obtained from nonradiolabeled experiments during early clinical development. Other examples where nonradiolabeled metabolism data have been shown to provide an accurate representation of the overall metabolism profile, as determined in radiolabeled ADME studies, include the peroxisome proliferator activated receptor (PPAR) agonist, muraglitazar (*42*).

8. Considerations for Safety Qualification of Metabolites

The FDA guidance on Safety Testing of Drug Metabolites (*6*) states that, in general, equal circulating concentrations of major metabolites (10% or greater of parent) is sufficient to qualify a metabolite for safety if found in one of the standard species administered the parent drug during the course of standard toxicology studies to support clinical trials as outlined in the M3 guidance (*43*). Thus, having the human metabolite present at approximately equal concentrations to humans in either rodents or nonrodents for general toxicity, rats or rabbits for reproductive toxicity, and rat, or mice for carcinogenicity would suffice to qualify the safety of the metabolite, barring some unusual safety consideration. When a disproportionate human metabolite is not covered by these initial standard

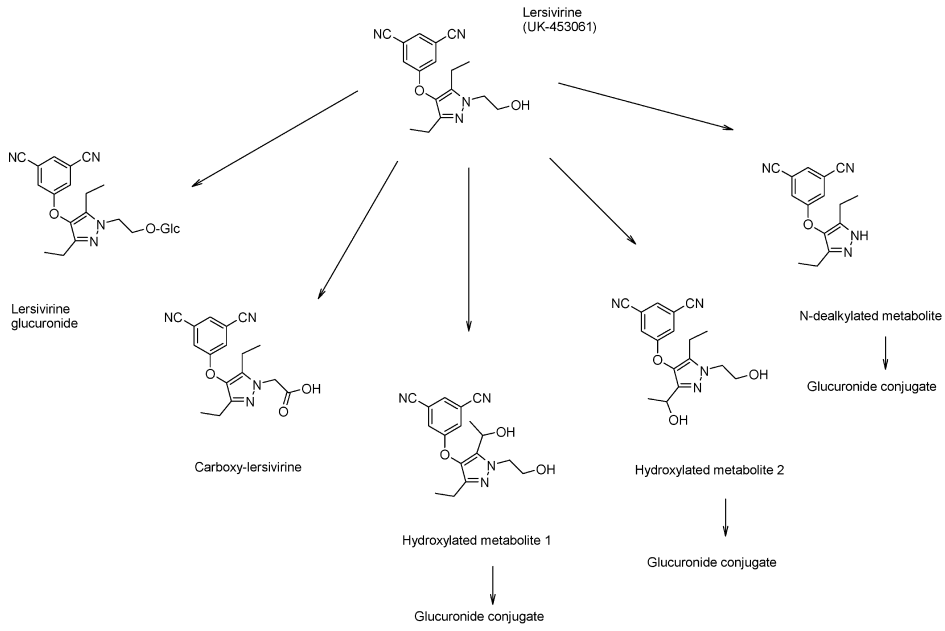

Figure 2. Major metabolic pathways for lersivirine (UK-453,061). Nonclinical in vitro studies demonstrated the formation of the hydroxylated and N-dealkylated metabolites. Metabolite scouting in human plasma identified the glucuronide conjugate of the parent and the carboxylic acid metabolite together with additional conjugates.

species, then two alternatives remain: (1) look for another nonclinical species that generates the human metabolite following administration of the parent that would serve to run a toxicity study to assess the safety of the metabolite, or (2) synthesize and administer the metabolite to one nonclinical species to assess the toxicity of the metabolite of interest. Administration of a metabolite should not be undertaken lightly as complicating factors are recognized both in terms of the ability to dose a metabolite for safety assessment and also the kinetic behavior of a preformed metabolite (*44*). In the case of genotoxicity, it is necessary to consider the adequacy of the assessment for mutagenic and clastogenic potential. A first step would be to determine if there was adequate literature to confidently risk assess the metabolite of interest. If not, then the following options would be examined: (1) determine if the metabolite was present in sufficient quantities when the parent drug was tested in vitro; (2) use an alternative in vitro preparation that generates the metabolite in question (e.g., human S9, recombinant P450 enzymes, etc.); (3) synthesize and directly test the metabolite in vitro; (4) assuming that the metabolite was not found in the rat micronucleus assay, consider conducting a mouse micronucleus assay if the metabolite was generated in that species or the direct administration of the metabolite via a suitable route of administration. Other standard factors that would be considered for the level of testing needed to qualify a metabolite would be the risk/benefit of the indication, presence of structural alerts or class effects, structural similarity to the parent, and pharmacological activity and/or potency. It is notable that the FDA guidance on Metabolite Safety (*6*) does not generally require qualification of metabolites for the standard battery of GLP (Good Laboratory Practice) safety pharmacology studies. This is sensible for the following reasons: (1) in vitro tests can provide the pharmacologic profiles and potencies of metabolites of concern before entry to phase 1, and provisions can be made for appropriate clinical monitoring at that time; (2) human pharmacokinetic information is needed to determine if there are disproportionate or unique human metabolites that were not predicted by the animal or in vitro human metabolism preparations, and measures of safety pharmacology will have been collected and characterized in humans in parallel. The practice of starting at low doses in phase 1 and escalating to higher dose ranges in a stepwise manner into the predicted efficacious range greatly reduces the risk of an adverse event from a pharmacologically potent, unpredicted disproportionate human metabolite. One additional consideration for safety pharmacology pertains to abuse potential. It is possible (although unlikely) for a metabolite to have pharmacologic potency that could impart abuse potential at circulating concentrations less than the 10% threshold put forth in the FDA Metabolite guidance. Therefore, the practice of assessing the pharmacologic contribution of metabolites at circulating concentrations will establish relevance to human safety on this basis.

9. Discussion

The approach outlined to the characterization and safety assessment of drug metabolites provides a holistic strategy to the building of knowledge and understanding as a compound progresses through the drug discovery and development continuum. The main considerations that are taken within this approach as they occur in nonclinical studies and phase 1 and 2 clinical development are presented as a decision tree in Figure 3. This process is intended to deliver a full and thorough understanding by the time the compound reaches phase 3 clinical development, at the same time matching the level of resources invested to the risk of compound attrition. Within any drug development process, it is vital that the costs of programs are carefully managed, especially given the high attrition that is a recognized feature of drug discovery. This economic consideration means that it is not viable practice to conduct human and associated animal radiolabeled ADME studies during the earliest phases of the clinical investigation of an NCE. For the majority of drug metabolites, actual quantitative data will only be available in the latter stages, once a human radiolabeled study has been performed. However, through the application of qualitative and semiquantitative assessments during nonclinical and early clinical phases, it is possible to establish if human metabolites are adequately represented within the toxicology species and to mitigate the need for actual

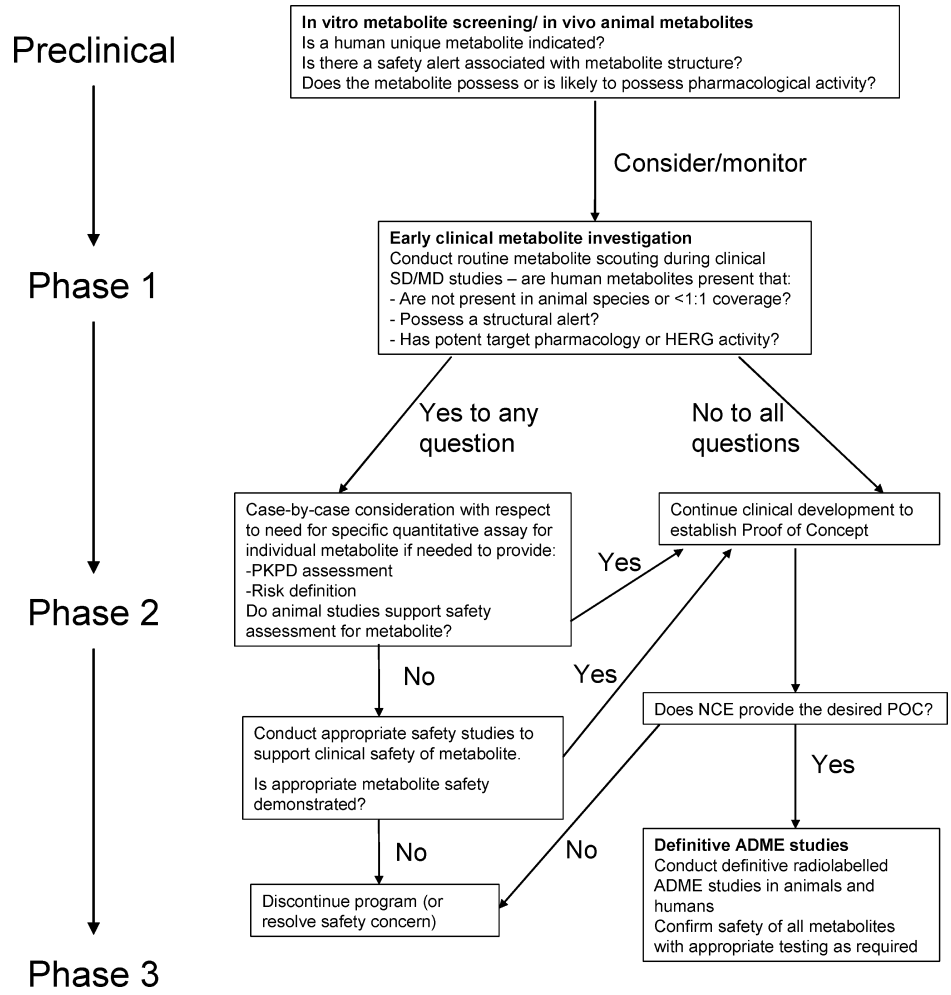

Figure 3. Decision tree for assessing metabolite safety risk through drug discovery and development.

quantitation or further safety testing. This strategy also means that a fairly comprehensive understanding of the metabolites in the systemic circulation is available during the earliest phases of clinical development. This permits not only consideration of the metabolites with regard to their safety risk but also their potential to contribute to drug efficacy and may also provide useful information regarding the drug−drug interaction strategy for the drug candidate. The driving principles of this strategy to operationalize the final FDA guidance Metabolite Safety are as follows: (1) when data arises to indicate that there may be safety alerts, either through disproportionately higher amounts of metabolite in humans versus animal species or through potent pharmacology, then steps will be taken to assess risks, up to and including additional nonclinical studies to ensure clinical trial subject safety, regardless of phase of development; (2) focus expensive research investments on candidates that provide potential benefit to patients where possible (i.e., trigger radiolabeled ADME studies on candidates that have shown proof of pharmacology/concept as a rule without compromising on safety). Execution of these two competing principles is enabled by the combination of in vitro metabolite profiling across species and early phase 1 metabolite scouting methods in animals and humans that do not require a radiolabeled drug. This data set provides sufficient identification of possible safety risks associated with metabolites in humans that may not have been adequately assessed in the nonclinical safety program to that point in time because of unexpected species differences in metabolism. Thus, in this review, Pfizer has described their strategy to take a resource effective approach to operationalizing the FDA guidance on Metabolite Safety in a way that assures human safety coupled to minimizing unnecessary increases in investments on candidates that may fail to show a benefit to humans. This strategy has similarity to other approaches (*7*); however, it places greater reliance on relative abundance between animal species and humans and thus requires less resources in terms of metabolite isolation, chemical synthesis, or bioanalytical methodology. As with any approach that does not employ radiolabeled compounds, there is a risk that metabolic products may be missed because of an unexpected and undetected biotransformation pathway or extremely low analytical response. The risk of such a situation impacting on human safety is considered to be extremely low given the low frequency of unique human metabolites (*45*), and definitive studies with a radiolabeled compound will be completed prior to large scale clinical studies in any case. The utilization of a relative abundance approach compared to absolute quantitation may be seen to raise a further risk, in that the relative importance of an individual metabolite may be over- or under-represented depending on the analytical response obtained. Recognizing the different analytical response that may be obtained between chemical structures, one will not dismiss metabolites simply on the basis of low analytical signal. Where metabolite standards are available because of ease of synthesis or pharmacological testing, then

these will be utilized to improve the quantitative assessment. In the case of a metabolite which is identified in human plasma samples and ultimately found to be of only low abundance, then this potentially means that additional analysis will be performed in animal samples to demonstrate exposure, which may ultimately be deemed unnecessary. This is considered only a minor pitfall, as this can be achieved by simply monitoring the relevant mass spectral transition already established rather than any actual quantitative bioanalysis. In cases where it is not possible to readily establish that a human metabolite is adequately represented within the toxicology species, then this will require more detailed consideration. This will involve further work to establish quantitation and may result in specific chemical synthesis in order to achieve this. We believe that this strategy is consistent with the principles of the FDA guidance to identify "differences in drug metabolism between animals used in nonclinical safety assessments and humans as early as possible during the drug development process" and provide study reports that qualify metabolites "to the FDA before beginning large-scale clinical trials".

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