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Small molecule drug metabolite synthesis and identification: why, when and how?

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The drug discovery and development process encompasses the interrogation of metabolites arising from the biotransformation of drugs. Here we look at why, when and how metabolites of small-molecule drugs are synthesised from the perspective of a specialist contract research organisation, with particular attention paid to projects for which regulatory oversight is relevant during this journey. To illustrate important aspects, we look at recent case studies, trends and learnings from our experience of making and identifying metabolites over the past ten years, along with with selected examples from the literature.

Keywords: metabolites in drug development; regulatory framework; disproportionate metabolites; metabolite synthesis case studies; active metabolites

Introduction

The significance of the metabolites of small-molecule drugs surfaces at various points in the drug discovery and development process. Earlier on in lead identification, metabolic stability assays and soft spot analysis support the medicinal chemistry design cycle to generate new iterations of scaffolds with improved properties. Analytical workflow initiatives coupled with *in silico* predictions can be used in a semi-automated way to identify major routes driving *in vitro* metabolic clearance at this early stage.[p1] Later in the discovery process and during preclinical and clinical development, more definitive identification of metabolites becomes more pertinent as part of understanding

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clearance and risk mitigation, and to satisfy regulatory requirements.

Whilst safety is the number one reason why metabolites of drugs are accessed and interrogated in the discovery and development process, there are other valuable benefits to be gained. Metabolites might have improved potency, selectivity, solubility or improved drug metabolism and pharmacokinetics (DMPK) properties, and they can also provide valuable data for intellectual property (IP) coverage.[p2],[p3],[p4],[p5]

In this article we focus on accessing metabolites of small-molecule drugs as part of the typical drug discovery and development life cycle, and discuss commonly used methods to synthesise and characterise them. Insights are derived from experiences acquired by a contract research organisation (CRO) specialising in metabolite synthesis for more than 300 client companies, ranging from large pharmaceutical firms to small companies focusing on the development of single products.

The need

Through our interactions with scientists across all types of pharmaceutical companies, we see a wide variety of triggers for when a metabolite becomes of sufficient interest to warrant synthesis and definitive structure elucidation. Scientists are not only keen to meet regulatory guidelines, but are driven to ensure that they are being thorough, yet practical, in ensuring their drug is safe for a patient.

The use of modern analytical methods such as high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy generates a wealth of data from which biotransformation pathways populated with a multitude of metabolites can be assembled.[p6] Amongst these, what is classified as a significant metabolite or xenobiotic pathway of concern, and when should further action be taken to interrogate it?

The answer is not black-and-white, because the decision is based on various factors including systemic exposure, the significance of the chemical structure of the metabolite and whether the metabolite is disproportionate. In order to build a framework that provides guidance and promotes consistency of strategy, regulatory direction has been issued by the U.S. Food and Drug Administration (FDA), European Medicines Agency (EMA) and other regulatory bodies.

Why? The impact of regulatory framework

MIST (Metabolites in safety testing) guidance for small-molecule drugs published in 2020 recommends that if a metabolite constitutes >10% of the total drug-related material in human plasma, and at significantly greater levels than the maximum exposure seen in the preclinical toxicity studies, non-clinical characterization is warranted before large-scale clinical trials.[p7]

Although human-specific metabolites are not common, the formation of a metabolite at higher levels in humans or with lower clearance than in the animal species used in safety testing of the parent drug is more frequently encountered.[p8] If a relevant animal species that forms the metabolite cannot be identified, such disproportionate metabolites are synthesised to ensure clinical safety. This necessitates the generation of material for dosing in an appropriate animal model, along with the development of standards for analytical methods to identify and measure the levels of the metabolite in the non-clinical toxicity studies. Early identification of disproportionate drug metabolites can prevent delays in clinical development, and the FDA strongly recommends that metabolic evaluation in humans is conducted as early as is feasible, rather than late in clinical trials.[p7]

By definition, metabolite studies conducted before human studies might not always accurately highlight what could become significant or disproportionate metabolites, and these are consequently found later during clinical development. Such surprises usually require prompt action to definitively identify metabolite structures and provide sufficient material for further testing. Such was the case during the development of ganaxolone, in which a human disproportionate metabolite became apparent during the ^{14}C-human mass balance study.

Ganaxolone (Ztalmy oral suspension, CV), a neuroactive steroid approved by the FDA for the treatment of seizures associated with cyclin-dependent kinase-like 5 (CDKL5) deficiency disorder (CDD) in patients 2 years of age and older, is subject to complex metabolism, with 59 metabolites identified.[p9] It is a synthetic C-3 methyl analogue of allopregnanolone that binds to allosteric sites of the $GABA_A$ receptor. The major routes of ganaxolone metabolism involve multiple steps, comprising hydroxylation at the 16α-hydroxy position, stereoselective reduction of the 20-ketone to give the corresponding 20α-hydroxysterol, and sulfation of the 3α-hydroxy group. The sulfation reaction yields an unstable tertiary sulphate, which undergoes elimination to form a double bond in the A ring. A combination of these pathways, together with oxidation of the 3β-methyl substituent to a carboxylic acid and sulfation at the 20α position, give rise to two major circulating metabolites in plasma, M2 and its C20-sulfate, M17.

In vitro and animal *in vivo* studies led to the initial belief that alternative biotransformations were responsible for clearance of the drug. The major human liver microsome (HLM) metabolite was identified as 16α-hydroxyganaxolone (M1), mainly through the action of CYP3A4, whereas in human hepatocytes the corresponding glucuronide, M11, was also observed as a major metabolite along with a minor metabolite, M5. A later key finding was the high conversion of this dehydrated metabolite to M2 in hepatocytes, but not in HLMs.

Key intermediates of M2 and M17, M5, M6 and M53, were observed at low levels in human hepatocyte and human S9 incubations of ganaxolone, but their levels were overlooked in the presence of the much more dominant M1 and M11.

None of the preclinical species yielded detectable amounts of M2 or M17, except trace levels in the female rat. However, findings in the ^{14}C radiolabel human mass balance study pointed to the presence of an alternative situation, with a very slow rate of excretion and with only 72.7% ± 9.6% of the administered radioactivity recovered by the end of the study at 720 h.

Subsequently, M2 and M17 were shown to be disproportionate human metabolites that persisted in circulation, highlighting the limitations of traditional animal studies and human *in vitro* systems in predicting major circulating metabolites in humans. Unfortunately, there was an added complication in that minimal

structural information could be gleaned from tandem mass spectrometry (MS/MS), and NMR was needed to elucidate the structure of M2 and other metabolites.

Multiple enzymes are involved in ganaxolone's biotransformation, including cytochrome P450 3A4 (CYP3A4), aldo–keto reductase family 1 (AKR1), sulfotransferases (SULTs) and uridine 5′-diphospho-glucuronosyltransferases (UGTs). Interestingly, it is proposed that the species-specific nature of one sulfation step is the reason that preclinical species fail to produce appreciable amounts of the M5 or M5-derived intermediate metabolites.[p9] The involvement of extrahepatic SULT or SULTs also points to the disconnect between hepatic *in vitro* systems and the *in vivo* situation.

Although M2 demonstrated no functional activity at the $GABA_A$ receptor, either as a modulator or as a direct activator, safety studies including toxicology and carcinogenicity studies were needed. This necessitated the development of a scalable synthesis of M2 that could readily be progressed to a multiple-kilogram scale (unpublished results). The additional evaluation of M17 as a metabolite under the MIST guidelines was not seen as necessary, and no separate studies were conducted for M17.

The initial eight-step synthesis of M2 was unfortunately difficult to scale up beyond low gram quantities. As an alternative, a much shorter regio- and stereoselective synthesis was developed, as shown in Figure 1, which required only three synthetic steps from a commercially available diketone. These steps comprise a regioselective nitroalkane condensation, an oxidation of the resulting nitroalkane to the corresponding carboxylic acid and a diastereoselective enzymatic ketone reduction.

The MIST guidance is somewhat less concerned about phase II metabolites such as glucuronides, since they 'generally render a compound more water soluble and are pharmacologically inactive'.[p7] However, more caution is recommended with potentially toxic conjugates, such as acyl glucuronides of carboxylic-acid-containing drugs. In addition, intermediate reactive metabolites can form stable products such as glutathione conjugates, which can be more readily detected and characterised. This does not mean that glucuronides should be disregarded, owing to the potential for drug–drug interactions (DDIs).

In addition to potential toxicity or other off-target concerns, there is the need to interrogate the potential of circulating metabolites for DDIs. This is not confined just to phase I metabolites, because common metabolites such as glucuronides have also been shown to interact with cytochrome P450 enzymes (CYPs), in particular CYP2C8 as a result of its distinctive active site, which allows binding to anionic and bulky ligands such as glucuronides.[p10],[p11] Several classes of glucuronide conjugates, including acyl glucuronides, *O*-glucuronides, *N*-glucuronides and carbamoyl glucuronides, have been shown to be substrates or time-dependent inhibitors of CYP2C8.[p10],[p12] As well as inhibiting key drug-metabolising enzymes, metabolites might also inhibit transporters in the intestine, liver and kidney. In a recent study investigating 25 drug metabolites, seven were found to be potent inhibitors of either or both of the organic anion transporters OAT1 and OAT3 in the kidney, four of which were inhibitors at clinically relevant concentrations.[p13]

All major metabolites of parent drugs (or those that contribute significantly to pharmacological activity or contain structural alerts for known DDI mechanisms) are now required by major regulatory bodies [the FDA, EMA and Japanese Pharmaceuticals and Medical Devices Agency (PMDA)] to be assessed for DDI potential. They all recommend a similar approach to DDI testing,[p14] with the *In Vitro* Drug Interaction Studies Guidance advising that *in vitro* DDI assessments be conducted before clinical testing starts.[p15],[p16]

In Vitro Drug Interaction Studies Guidance states that metabolites should be evaluated for *in vitro* CYP enzyme and transporter inhibition studies in cases in which circulating metabolites occur at 25% of parent exposure for metabolites that are less polar than the parent drug, and at 100% of parent exposure for metabolites that are more polar than the parent drug. In addition, any metabolite with a structural alert should be evaluated for potential mechanism-based inhibition. A study analysing 120 new molecular entities approved between 2013 and 2018 revealed that 63% of metabolites quantified *in vivo* had >25% of parent exposure and that 75% of these metabolites were tested for CYP inhibition *in vitro*, resulting in the identification of 15 metabolites with potential DDI risk. Interestingly, analysis of the data did not support the use of metabolite polarity as a predictor of the risk of DDIs caused by metabolites, and also revealed several metabolites that inhibited CYPs *in vitro* where the parent drug did not. Overall, results supported the use of a 25% of parent exposure cutoff for *in vitro* CYP inhibition testing to adequately identify metabolites with the potential to cause *in vivo* DDIs.[p17] The EMA also recommends the assessment of metabolites that represent >10% of drug-related exposure observed dur-

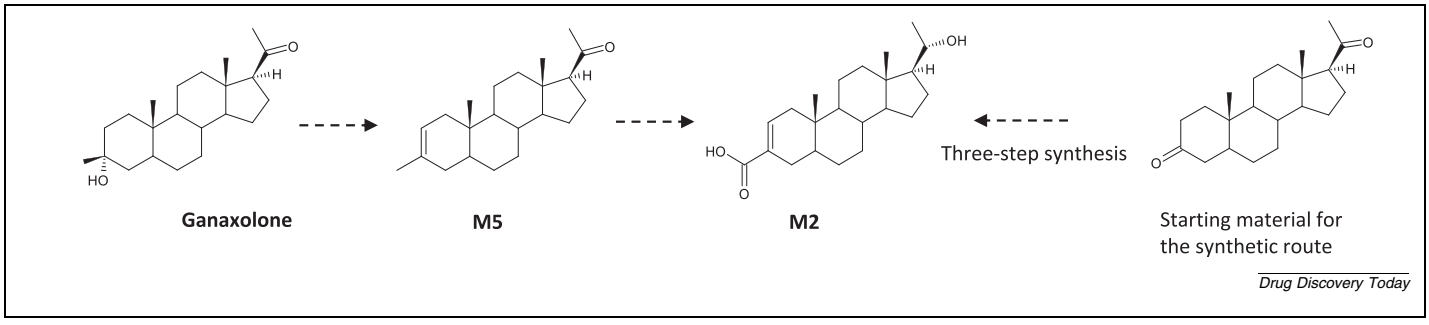


FIGURE 1
Major human disproportionate metabolite M2 of ganaxolone, formed via M5 in humans, and the starting diketone used for the three-step synthetic route.

ing the ^{14}C human *in vivo* AME (absorbed, metabolised, excreted) mass balance study.[p18] This includes structural characterisation, and for phase I metabolites that meet the criteria, establishing any potential inhibitory effects on common drug-metabolising enzymes.

The assignment of structural alerts has been debated widely as a way of flagging the potential for reactive metabolite formation that could cause idiosyncratic drug toxicity,[p19] especially considering that numerous drugs containing structural alerts are devoid of any adverse drug reactions. Analysis of the top 200 drugs (sold and prescribed) in the United States in 2009 revealed a significant proportion containing structural alerts, with the majority involving reactive metabolite formation. Significantly, the majority of these drugs were administered at low daily dose; this seems to be a key factor. There were also competing detoxication pathways and/or alternative non-metabolic clearance routes that mitigated the risk.[p20] However, interrogation of the metabolites of new drugs with structural alerts needs to be performed, and risk-mitigation strategies considered.

When?

We see the synthesis of drug metabolites performed at various stages in the drug discovery and development process, ranging from late discovery or preclinical evaluation to almost all phases of clinical development (Figure 2).

Prior to development, *in vitro* studies provide a key map of the biotransformations of a drug. Such studies can be run preemptively to identify any potential human disproportionate metabolites through a cross-species comparison of metabolites observed in hepatocytes of human and preclinical animal species. The testing of drugs in traditional ^{14}C ADME (absorption, distribution, metabolism and excretion) rodent species and, more rarely non-rodent animal species, has been a key milestone as a drug progresses through preclinical development into phase 1. Often at this stage a full *ex vivo* metabolite study of various samples (blood, plasma, bile, etc.) can take place to enable further understanding of the biological fate of molecules. The definitive human ^{14}C ADME study usually takes place in phase 2, after which metabolite synthesis might be needed for definitive structure identification and pharmacological and safety evaluations. Indeed, it has been strongly recommended that the study results and those of linked studies to characterise metabolites in non-clinical species, as well as an understanding of any possible interactions, should be available before phase 3.[p18]

The FDA's MIST guidance encourages the identification of any differences in drug metabolism between animals used in nonclinical safety assessments and humans as early as possible, to avoid unwelcome surprises late in drug development which can cause costly delays: such as that experienced during the approval of ozanimod (Zeposia®). The two-year delay before the drug could be approved and marketed stemmed from the FDA issuing a 'Refuse to File' letter owing to insufficient preclinical characterization of an active metabolite of ozanimod.[p21]

More recently, there has been an emphasis on the identification and quantification of human metabolites from early clinical studies,[p18],[p22] which may then lead to an earlier need for synthesis of a metabolite. This shift in timing of the mass balance study enables more efficient handling of any metabolites that might need safety testing.[p18]

It is advised that metabolites present at >10% of dose in excreta or drug-related material in plasma should always have structures definitively identified,[p23] which can be challenging if the metabolite profile is complex. Proof of the chemical structure

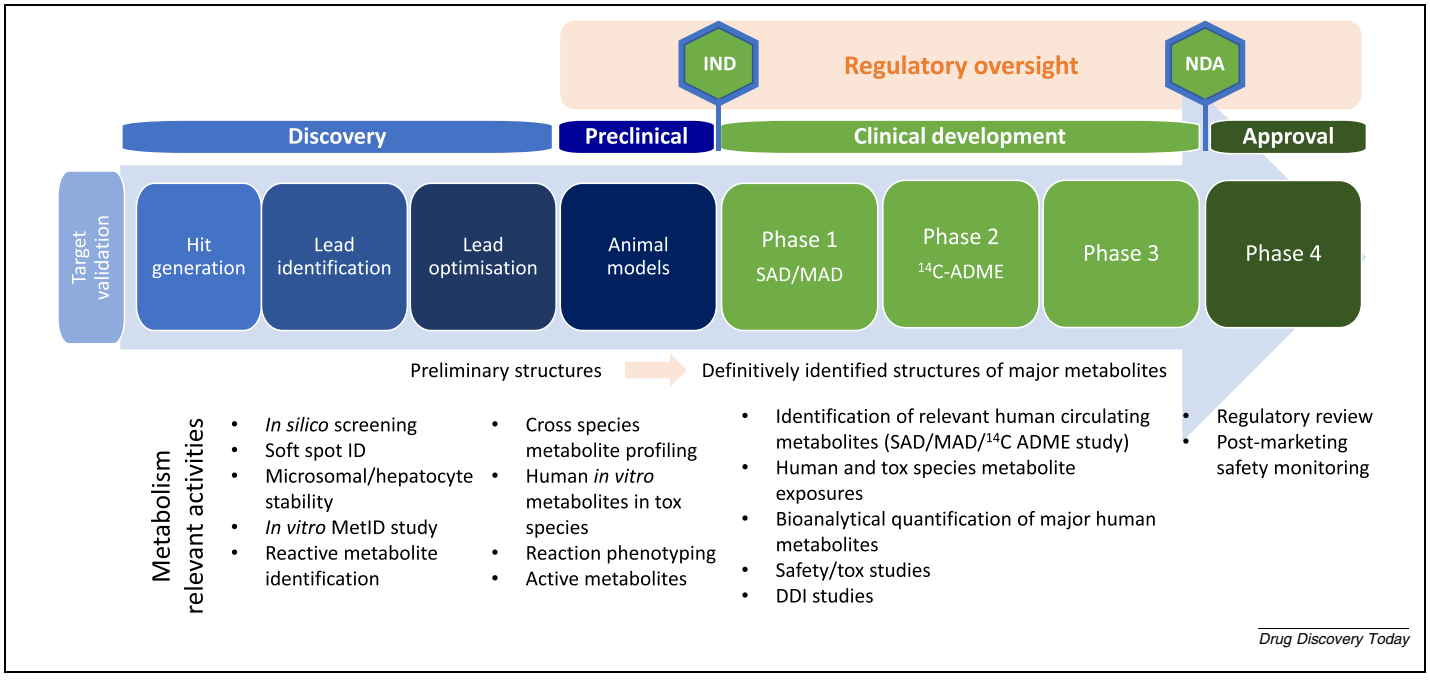


FIGURE 2
Consideration of metabolites in the drug discovery and development process, selected information taken from ref.[p83]

might require additional investigations obtained from comparisons with a synthesised authentic standard of the metabolite. Metabolite reference standards can also be isolated directly from a biological matrix (e.g., human urine from a clinical study), although this is limited by the material availability relative to how much metabolite is needed for further studies. In terms of activity testing of metabolites, although structural similarity to the parent drug is not an indicator that a metabolite will be pharmacologically active, there is a greater likelihood that this might be the case. It is thus recommended that circulating metabolites formed from relatively simple biotransformations, such as hydroxylation, heteroatom demethylation and dehydrogenation, should be evaluated for pharmacological activity.[p23]

A recent paper highlights that a good time to interrogate any major human circulating metabolites missing from *in vitro* studies is through the analysis of plasma samples acquired during the phase 1 multiple ascending dose (MAD) study,[p24] since it is the metabolite profile and exposure of any circulating metabolites at steady-state that are key.

A potential move towards a human first/human only approach is gaining momentum.[p25] Technological advances from the use of ^{14}C-microtracer dosing with accelerator mass spectrometry (AMS) permit earlier human-relevant knowledge to be gained about the metabolism of a drug in phase 1. As such, the assessment of metabolites of human relevance from first time in human (FTIH) studies is now considered standard practice in many pharmaceutical companies. Consequently, metabolites identified in early work can then be synthesised so they are available, for example, as chromatographic standards. At the same time, this enables a reduction in the use of animals during drug development, as resolved by the European Parliament in 2021.[p26] In addition to the information gained on human metabolite profiles and routes and rates of excretion, the use of ^{14}C-microtracing in early clinical development quickly proved useful to Lundbeck in uncovering unknown issues related to the metabolism of a drug. In this instance, the use of a microdose of ^{14}C-labelled drug and analysis by AMS revealed that the unexpectedly low exposure of a drug was almost exclusively attributable to extensive metabolism by aldehyde oxidase (AO).[p27]

Additional guidelines also affect the timelines for accessing metabolites. Various regulatory frameworks have previously addressed DDI studies. The latest International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) draft guidance for drug interaction studies (M12) consolidates recommendations internationally for designing, conducting and interpreting enzyme- or transporter-mediated *in vitro* and clinical DDI studies during drug development. The overall recommendation is that information on DDI potential should be generated as early in the drug development as possible, including the DDI potential of metabolites with significant plasma exposure or pharmacological activity similar to that of the parent drug. Within projects, any major metabolites need to be available for DDI studies to be performed in parallel with the parent drug.

Much work is focussed on understanding the metabolism of drugs in the liver; however, although less common, extrahepatic metabolism can also drive the formation of major human metabolites. It can be more difficult to pinpoint such metabolites at an earlier stage where the phase I or phase II enzymes involved are specific to non-liver tissues, illustrated by the identification of a human disproportionate and rather unusual diglucuronide in the FTIH study of GDC-0810. This was further complicated by species-specific reactions of enzymes involved in *N*-glucuronidation.

Although the glucuronidation of drugs is a common clearance pathway, it is unusual to encounter linked or di-glucuronides/glycosides, not least those that are also major human circulating metabolites. GDC-0180 (Brilanestrant) is a non-steroidal, orally bioavailable selective oestrogen receptor degrader (SERD) developed for the treatment of breast cancer. Metabolite profiling during the FTIH study led to the identification of a novel discrete diglucuronide metabolite (M2) as a primary circulating metabolite that had not been previously detected. M2 is an example of a discrete diglucuronide formed by glucuronidation on two different functional groups to a major circulating metabolite in humans, formed from sequential acyl glucuronidation in the intestines followed by *N*-glucuronidation in the liver.[p28]

There are a few interesting facets to this biotransformation. First, that *N*-glucuronidation is more common in humans owing to the activity of UGT1A4 and UGT2B10, resulting in the absence of M4 in animal species. Second, that acyl glucuronidation to M6 was generally compartmentalised in the intestine rather than the liver owing to the involvement of the UGTs 1A8/1A7 and 1A1. These elements help to explain the unpredicted appearance of the discrete diglucuronide M2 as a major circulating metabolite in humans (Figure 3).

How? Typical routes to metabolite synthesis used at Hypha

Both synthetic and biological approaches are used to generate metabolites of interest, with special attention paid to procedures that are capable of yielding specific stereo- and regioisomers and complex conjugates. Other reviews discuss the comprehensive techniques that can be applied[p29],[p30],[p31],[p32]; however, here we focus on techniques used internally to make the most frequently requested metabolites needed to support drug development programmes. Our experiences show that there is not one magic technique that can be relied upon to access the wide range of metabolite structures needed. Where possible, it is more animal-friendly and cost effective to identify an alternative to biotransformation methods that use mammalian tissues for scaling-up metabolites where surrogate methods can instead readily satisfy requirements.

Late-stage chemical synthesis

Classical organic synthesis is often considered first for accessing metabolites. However, depending on the nature and complexity of the drug candidate and the structure of the target metabolite, the generation of the metabolites is not always straightforward and might necessitate complex syntheses. This could demand valuable medicinal or process chemistry resources to deliver even minor structural changes to the original molecule. Where timely confirmation of metabolite structure or access to reference standards is important, late-stage functionalisation methods com-

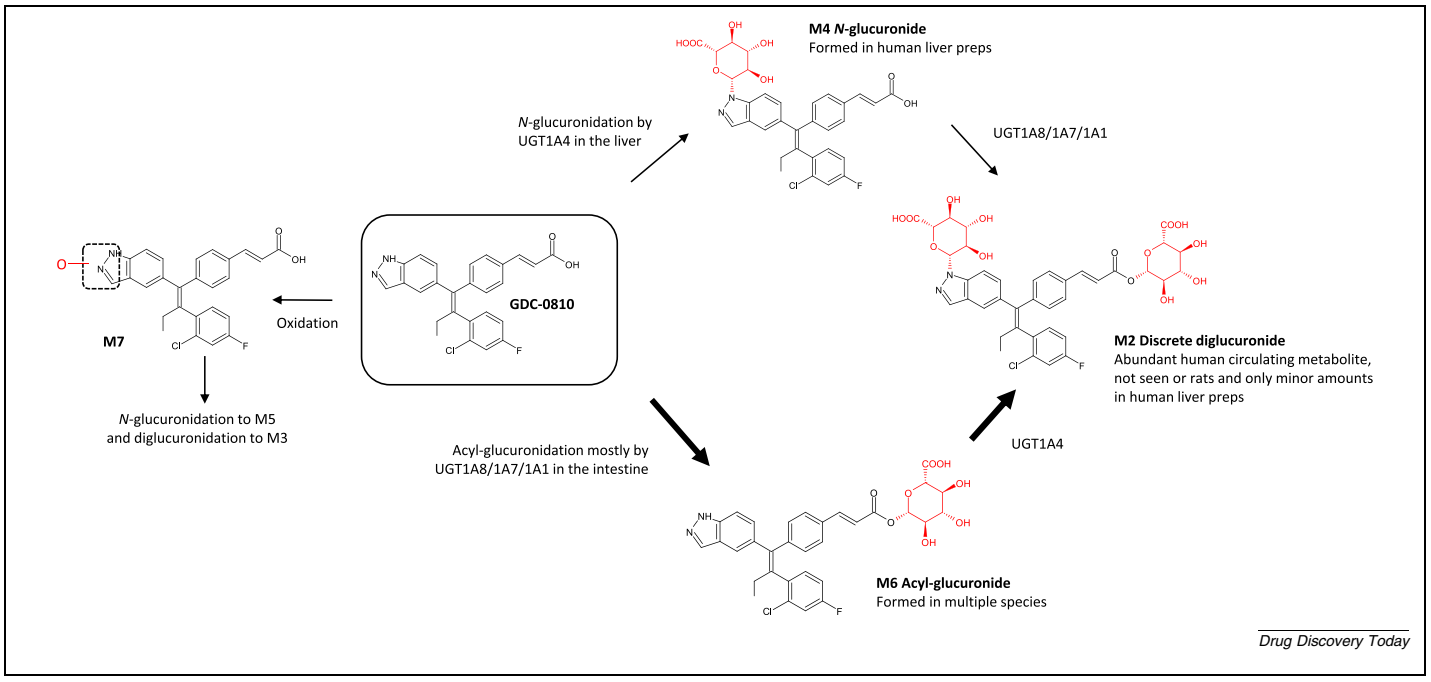


FIGURE 3

Metabolism of GDC-0810, adapted, with permission, from ref.[p28] Metabolites were identified in samples from the FTIH study, various in vitro studies and in vivo rat mass balance studies by comparison with synthesised standards made at Genentech.

monly used for improving drug-like properties are often successful and quick.[p33],[p34],[p35]

Chemical synthesis of phase I metabolites is often challenging in cases where the regiospecific oxidation of non-activated carbons in complex molecules is required, or where metabolites have specific stereochemistry. However, the use of late-stage chemical oxidation, electrochemistry and photochemistry methods for some oxidations is possible.

Late-stage chemical synthesis has been regularly used for making phase II metabolites of drugs, with glucuronides being the most common conjugate required. Although synthetic methods for all classes of glucuronides have been published,[p36] attention still needs to be given to minimising any stability issues, including isomerisation and hydrolysis, during their synthesis and purification.

Late-stage methods are generally conducted as a two-step process, starting with a screen to expose the parent compound to a wide variety of chemical conditions appropriate to the glucuronide type (if known). Once a suitable method has been identified, the reaction will be scaled-up according to the target amount of glucuronide needed, and the material can then be purified. Generally, most synthesised glucuronides are stable; however, some require careful handling and purification following synthesis, as encountered with purification of the *O*-glucuronide of AZD9574.

The main route of metabolism of the poly(ADP-ribose) polymerase 1 (PARP1) inhibitor AZD9574 in human hepatocytes is through glucuronidation (Figure 4).[p37] Screening of AZD9574 in eight different late-stage chemical glucuronidation conditions resulted in the conversion to three different glucuronides across three conditions. Two of these were shown to form the desired glucuronide by liquid chromatography with LC-MS/MS matching with the hepatocyte sample. It was noted at this initial stage that the glucuronide was hydrolytically unstable in acidic conditions. Scale-up of the most promising reaction with 5 g of AZD9574 resulted in the synthesis of 605 mg of the glucuronide. However, due to high lability resulting in release of some of the aglycone under mild conditions, a further purification step was necessary. The solid material was also found to be temperature sensitive and started hydrolysing as soon as the final lyophilisation stage was complete, thus necessitating careful handling. The additional purification step resulted in a total of 481 mg of what was later determined to be an *O*-glucuronide, with minimised levels of the parent drug.

Microbial biotransformation

Where the chemical synthesis of a human metabolite is challenging or even impossible, carefully selected microbes can provide a route to obtain these metabolites due to their expression of phase I and II metabolising enzymes that are homologous to mammalian systems.[p38],[p39] These enzyme types include CYP monooxygenases as well as enzymes that form the products of non-CYP phase I mammalian metabolising enzymes, such as aldehyde oxidase and flavin-containing monooxygenases. The expression of conjugative enzymes, including UGTs, provides the key advantage over chemical synthesis in that multistep reactions in a 'single pot' are possible: for example, sequential hydroxylation and glucuronidation. The scalability of microbial incubations is mostly sufficient to cater for milligram- to gram-scale material requirements, and in contrast to liver fraction incubations, the cost of materials is lower due to the amenability of microbes for biotransformations in multi-litre-volume fermentations, and also because microbes generate the necessary cofactors for metabolic reactions.

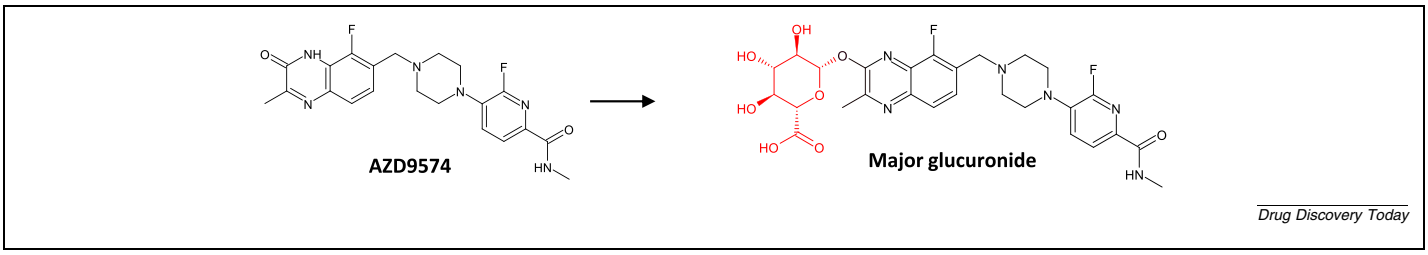


FIGURE 4

Glucuronide of AZD9574 *O*-linked through the lactim tautomer of the quinoxalinone, made by late-stage chemical synthesis.

Microbial biotransformation can also be applied to make stable labelled and radiolabelled metabolites of drugs in cases where chemical synthesis is more challenging. Here, a process for producing the metabolite is defined and optimised using unlabelled material, and then repeated using labelled parent material. In this way, a ^{14}C-radiolabelled major CYP metabolite of deflazacort (DFZ) was generated for transporter studies.

DFZ is a glucocorticoid that exists as an inactive pro-drug that is rapidly converted by esterases to the active metabolite, 21-desacetyl DFZ, after oral administration. The active metabolite is further metabolised by CYP3A4, primarily to 6β-hydroxy-21-desacetyl DFZ (6β-OH-21-desDFZ) (Figure S1). The pharmacokinetic drug interaction potential of 6β-OH-21-desDFZ was not fully characterised and was subsequently required by the FDA after approval of the drug.[(p40)] Chemical synthesis of 6β-OH-21-desDFZ is challenging because multiple steps as well as stereospecific reactions are required, which are time-consuming and costly. Microbial biotransformation was therefore explored as a route to obtain ^{14}C-radiolabelled 6β-OH-21-desDFZ.[(p40)] Unlabelled DFZ was first screened against 37 microbial strains to identify a suitable route to the metabolite. The highest yielding strain was scaled up using 200 mg of DFZ to provide 7.9 mg of a hydroxylated metabolite, the NMR spectra of which matched an authentic commercial standard of the desired β-isomer. The process was then repeated using [2′-^{14}C]-DFZ, and after iterative purification steps generated 62.1 μCi (equivalent to 448 μg) of the metabolite from 5 mCi of parent drug, with a radiochemical purity of 99.2% and chemical purity of 98.7%, at an overall yield of 1.2%.

The metabolite was used for CYP induction and inhibition studies, and the radiolabelled metabolite for further transporter-mediated substrate accumulation studies using bidirectional permeability assays. The metabolite modestly inhibited CYP2C19, CYP3A4, Multidrug resistance protein 1(MDR1), OATP1B1 and OATP1B3 activities ($IC_{50} \geq 20$ μM).[(p40)] However, the metabolite was not expected to drive any clinically relevant DDIs owing to the low plasma exposure at the therapeutic dose of DFZ (0.34 μM).

Gut metabolism

Metabolism by gut microbes has recently gained more attention,[(p41),(p42),(p43)] although, in our experience to date, it is relatively rare that these metabolites are prominent enough to warrant investigation. They can, however, be significant, such as the unexpected involvement of gut microbiota in the reductive metabolism of ozanimod, where oxadiazole ring scission and reabsorption results in the formation of RP101124, a major circulating metabolite.[(p44)]

Ozanimod has a complex disposition, aspects that were discovered late in phase 3 clinical development and which delayed approval of the drug by two years. Its biotransformation results in more than seven active metabolites that are as potent and as selective as the parent drug.

In addition to the generation of active metabolites, the involvement of gut bacteria in ozanimod's metabolism led to the formation of a major inactive circulating metabolite, RP101124, formed as a result of a scission of the oxadiazole ring system (Figure S2). RP101124 was discovered during an *in vivo* ^{14}C-ADME study in rats, in which a lag time was observed before its appearance in circulation. Gut bacteria are also involved in the breakdown of RP101124, via RP112533, through anaerobic decarboxylation, a step which might have contributed to the low recovery in the human mass balance study, where radiolabel was lost as $^{14}CO_2$, in addition to the long half-life of metabolites.

For drugs that act locally in the gut, prominent metabolites in the gastrointestinal tract are of more significance than circulating metabolites. The pan-Janus kinase (JAK) inhibitor izencitinib is a gut-selective drug that is extensively metabolised, but where no one metabolite exceeded 10% of total drug-related material in plasma. However, given that izencitinib is locally acting, it was proposed that two major faecal metabolites (M9 and M18), which together represented more than 50% of the dose, were relevant for evaluating the pharmacological and toxicological properties of the drug. Their structures are unusual, resulting from oxidation and rearrangement of izencitinib to M18 with a proposed one-carbon addition with formaldehyde forming a tetracyclic product M9 (Figure S3). Both metabolites were tested for pharmacological activity and were inactive. The authors point out that toxicological testing of these 'local metabolites' should be the focus rather than systemic exposure, the latter being irrelevant to this locally acting drug.[(p45)]

Where gut metabolites are suspected to be implicated in the fate of an experimental drug, human faecal incubations can be conducted with parent drugs under anaerobic conditions to shed light on whether a metabolite is formed by gut microbiota or in the faecal matrix itself. Sufficient material can usually be generated to purify enough for structure elucidation by NMR spectroscopy and biological testing.

Recombinant enzymes

Recombinant enzymes are used as a tool to make metabolites. Various sources can be used, such as engineered human CYPs,

ancestral CYPs and bacterial derived BM3 (CYP102A1) mutants.[p30],[p46] All but a handful made by us have been CYP-derived metabolites created using PolyCYPs® enzymes, a panel of bacterial CYPs mined from the genomes of actinomycete strains and expressed in *Escherichia coli* together with appropriate redox partners.[p47],[p48],[p49] Scale-up of reactions of each isoform of interest is achieved using cell homogenates or whole cell biotransformation of PolyCYPs expressed in *E. coli* or *Streptomyces lividans*. Where desired metabolites have not been produced by PolyCYPs, human recombinant CYPs have been used to access metabolites. Of 22 CYP metabolites made by recombinant CYPs and structurally identified, four have been made using human recombinant CYPs: one from CYP1A1, one from CYP1A2 and two from CYP2J2. Other human recombinant enzymes have also been useful in making non-CYP-derived phase I metabolites, specifically human recombinant AO and flavin-containing monooxygenases (FMOs) expressed in *E. coli,* but these products are typically scaled up using microbial biotransformation due to the better scalability and lower cost of this method.

Similar to other commercially available CYP enzyme systems for making metabolites,[p30] PolyCYPs enzymes are used to make metabolites via screening and scale-up kits, as illustrated in the synthesis of a prominent CYP3A4 metabolite of BI 894416, where access to a monohydroxylated metabolite was required as part of early development studies. During early development of BI 894416, the appearance of M398(2) was observed, but its structure could not be deduced by LC-MS/MS. Due to multiple sites in the lactam ring system where the biotransformation could occur and creation of a new chiral centre through hydroxylation, use of biotransformation methods to access the metabolite was preferred.[p50]

BI 894416 is a spleen tyrosine kinase inhibitor that was under development for the treatment of asthma. Its metabolism results in several oxidised metabolites, with M398(2) comprising 11.4% of total drug-related exposure in the single ascending dose (SAD) study $AUC_{0–24h}$ pool. Both HLM incubations and the use of PolyCYPs recombinant enzymes were evaluated as a route to make M398(2).

Incubation of BI 894416 in HLMs gave a 5% conversion to M398(2). Screening of BI 894416 against a panel of 18 recombinant microbial PolyCYPs, human recombinant AO and FMO3 resulted in the highest conversion of 40% by PolyCYP 152 to yield both M398(1) and M398(2). The fragmentation pattern of putative M398(2) produced by PolyCYP 152 matched the human plasma sample from the SAD study. A small scale-up (1 mg) yielded 3.6% conversion in HLMs and 65% using PolyCYP 152. M398(2) was purified from both the HLM and PolyCYP 152 incubations, and the position of the hydroxylation of the structure was confirmed by NMR spectroscopy (Figure 5). Scientists at Boehringer used an in-house-developed diastereomeric *in silico* chiral elucidation (DiCE2) prediction tool to identify the metabolite from both sources as the *RSR* isomer.[p51] Further scale-up of the PolyCYP 152 reaction was performed to generate milligrams of M398(2) for additional investigations; however, the programme was terminated before DDI and pharmacological activity studies were conducted.

Purification

Direct isolation of metabolites from biological matrices such as plasma or urine samples can be particularly useful for metabolites with unanticipated structures resulting from multi-step biotransformations, and where the complexity of the metabolite structure makes provision of material by other routes more challenging. This is especially relevant where many possible structures of a metabolite exist, thereby making synthetic approaches unattractive until the structure can be elucidated by techniques such as NMR spectroscopy. Only small amounts of materials (typically tens of micrograms) are needed for structure elucidation using cryoprobe NMR technology. Knowledge of the structure can then enable chemical synthesis of the correct metabolite. These purifications can prove to be challenging, especially if the concentration of the metabolite or metabolites of interest is low or detection by high-performance liquid chromatography (HPLC) with ultraviolet/mass spectrometry (UV/MS) detection is difficult. Selective extraction methods are needed to minimise downstream problems caused by matrix components. Since hydroxylated or conjugated metabolites are usually polar molecules, purification by reversed phase HPLC methods guided by LC-MS assays is usually appropriate. Two or three purification stages using different column chemistries and/or mobile phase modifiers are usually needed to achieve sufficient purity.

Liver S9 fractions and microsome incubations

Rarely, when other biotransformation or chemical synthesis methods fail to make a target metabolite, biotransformations using microsomes or liver S9 fractions can be used, along with the addition of the appropriate cofactors. In our experience, these are reserved as 'back-up' techniques owing to the cost of the materials and the lack of scalability arising from the typically low concentrations of drug that can be dosed in such incubations. Only 7% of metabolites identified in-house were made using these methods. It is also desirable to minimise the use of systems that necessitate large quantities of animal-derived tissues for scaling up in order to reduce the use of animals in drug development.[p52] These biotransformations, however, can be an important technique for producing metabolites that are difficult to make by other means.

Learnings from the past 10 years of making metabolites

Analysis of the 100 most commonly prescribed drugs in 2022 showed that, of the 89 subject to drug metabolism, 75% were cleared via phase I mechanisms, with CYP3A4 being the major enzyme involved in metabolism of 48% of drugs.[p53] Similar statistics were observed for small molecule drugs that were approved by the FDA in 2022,[p54] perhaps not surprising given that we are analysing molecules that emerged from laboratory benches many years ago. In 2008, 90% of all drugs currently approved for clinical use were metabolised by one of only seven CYP isoforms: CYP1A2, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1 and/or CYP3A4.[p55] In 2013, an analysis of 248 clini-

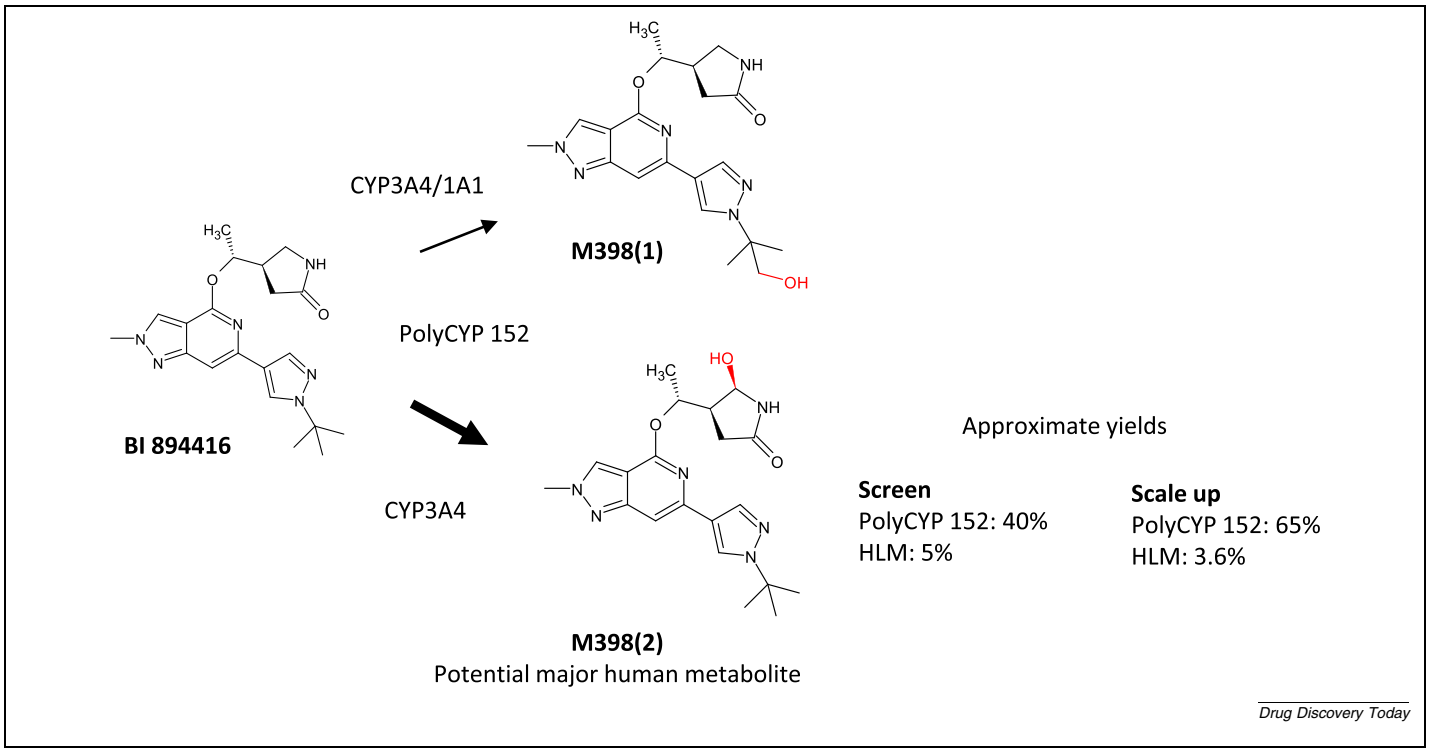


FIGURE 5

Hydroxylated CYP3A4 human metabolites of BI 894416 formed by PolyCYP 152.

cally used drugs with known CYP involvement pointed to the involvement of ten CYPs: CYP3A4 (30.2%), CYP2D6 (20%), CYP2C9 (12.8%), CYP1A2 (8.9%), CYP2B6 (7.2%), CYP2C19 (6.8%), CYP2C8 (4.7%), CYP2A6 (3.4%), CYP2J2 (3%) and CYP2E1 (3%).[p56] A wider comparison of the main biotransformation routes of the top 200 most prescribed drugs with drugs approved by the FDA between 2005 and 2016 revealed that the role of CYP1A2, CYP2C19 and CYP2D6 had decreased as major metabolizing enzymes among the more recently approved drugs.[p57] The contribution of alcohol dehydrogenases (ADHs), aldehyde dehydrogenases (ALDHs) and SULTS had also decreased. Interestingly, the contribution of CYP3A4 as the major biotransformation pathway had increased from 40% in the established drug list to 64% in the 2005–16 FDA approved drug list. An even more recent analysis of the main metabolic route of 245 small and macromolecule drugs approved by the FDA from 2015 to June 2020 points to the continued importance of CYP3A4/5, with 52% of drugs being metabolised by this enzyme. Overall, 80% of drugs in this list were cleared by CYP enzymes, with 15% by UGTs and only 5% by other mechanisms [2% FMO, 2% monoamine oxidase (MAO) and 1% AO].[p58]

We conducted an analysis of 241 metabolites (referred to as the 'Hypha set') where Hypha Discovery was involved in the structure elucidation of the metabolites (Figure 6). The analysis revealed that 60% of the requests derived from phase I oxidative mechanisms and 37% from phase II mechanisms, particularly via UGTs, with a smaller percentage arising from mixed phase I and II pathways and gut metabolism. Metabolites made were prominent in *in vitro* and/or *in vivo* studies, and of those attempted to be made by the client before approaching us, generally could not be made by facile chemical synthesis. Where the metabolism pathway was known, metabolism via CYP3A4 was the dominant route, mirroring the published data on approved drugs. However, significantly more glucuronide conjugates were observed as major metabolites in the Hypha set.

The majority of biotransformations observed result from phase I mechanisms, including hydroxylations, multiple oxidations, *N*-oxidation and ring opening/hydrolysis reactions. Not surprisingly, hydroxylation is the most common biotransformation observed, resulting mainly (where known) from CYP-mediated oxidations of alkyl and aryl moieties in drugs. Where drugs featured nitrogen-containing heterocycles, selected AO-mediated hydroxylation was also observed, along with *N*-oxidations catalysed by CYPs or FMOs. Examples of known CYP-mediated hydroxylated metabolites made in this set where structures are in the public domain include eight hydroxylated (and corresponding keto) metabolites of ruxolitinib,[p2] a radiolabelled hydroxylated metabolite of DFZ[p40] and, more recently, three hydroxylated metabolites of rezafungin.[p59],[p60]

Rezafungin is an echinocandin that was approved by the FDA to treat candidaemia and invasive candidiasis in adults with limited or no other treatment options. It has a quaternary ammonium side that confers high stability and a long half-life, thereby preventing the spontaneous degradative metabolism to a ring-opened peptide observed in previously approved echinocandins. Instead, metabolism is diverted to the alkyl portion of the terphenyl, pentyl ether side chain. In the single-dose human mass balance study, although rezafungin was the most abundant circulating component in plasma, calculated at 69% of the total plasma radioactivity exposure (by AUC0-t), three hydroxylated metabolites were also in circulation, comprising 9.8%, 5.5% and 6.9% of the total plasma radioactivity exposure.

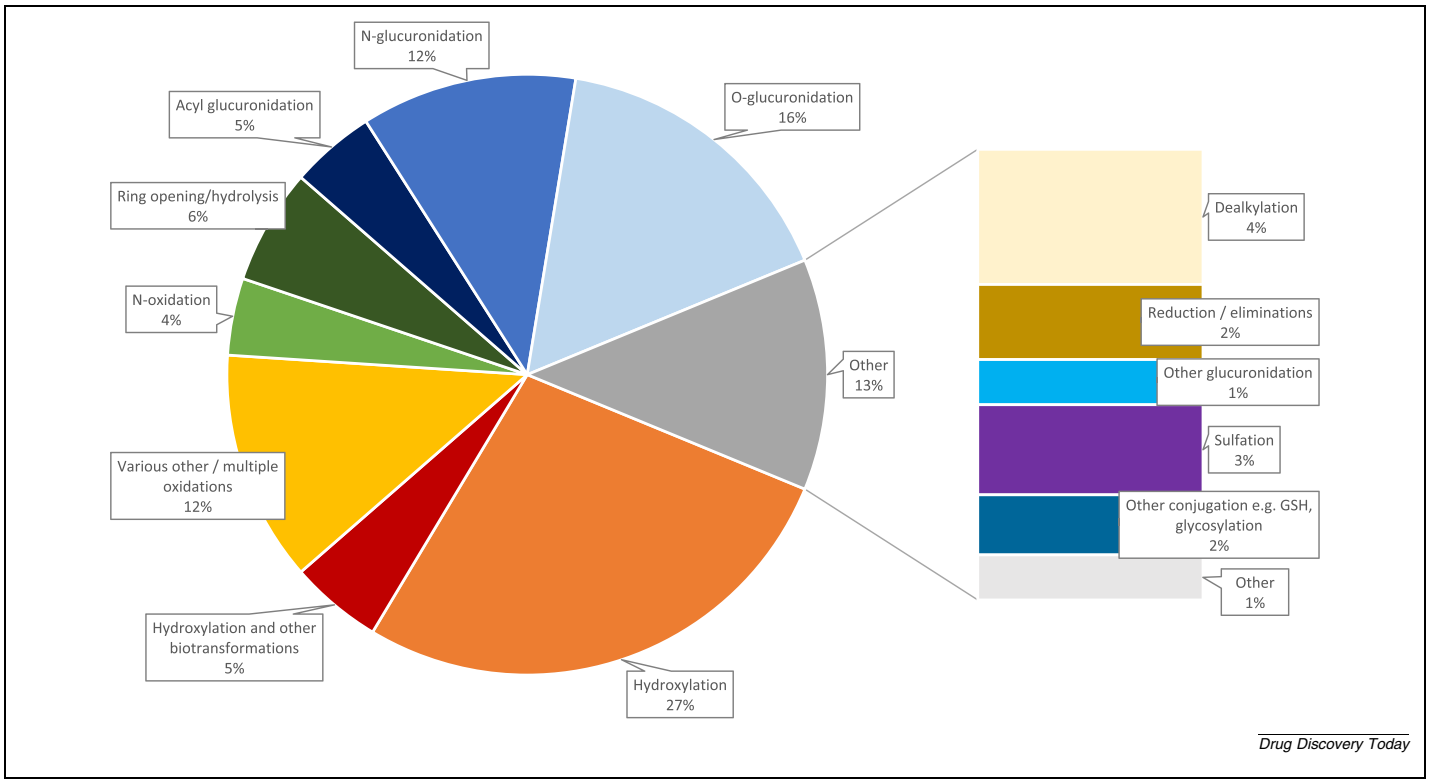


FIGURE 6

An analysis illustrating the broad routes of formation of 241 metabolites made for clients where Hypha Discovery was involved in the structure elucidation step.

The rate of formation of these metabolites is slow and is consistent with the slower elimination of rezafungin. Owing to the structural complexity of this cyclic lipopeptide, microbial biotransformation was used to make the metabolites for identification, biological testing and to act as reference standards.[p60] Following screening of rezafungin against a panel of microbes, a fungal strain was identified that produced all three hydroxylated metabolites, and a minor des-pentyl metabolite formed via *O*-dealkylation. Leaving the biotransformation longer resulted in generation of the minor despentyl metabolite, but at the expense of the hydroxylated target metabolites. The structure of each metabolite was elucidated by NMR spectroscopy to reveal three isomers resulting from hydroxylation of the terphenyl, pentyl ether side chain (Figure S4). Biological testing revealed that the metabolites were inactive.

There is perhaps a surprising number of metabolites that are made from or characterised by glucuronidation (34% of total metabolites) among the Hypha set, possibly reflecting the challenge in accessing these metabolites versus those that are more straightforward to make by facile chemical synthesis. The need for glucuronides might also represent a divergence from the CYP-based mechanisms that have previously dominated, as the latest drugs move through the various stages of clinical development. *O*- and *N*-glucuronides constitute the majority of these, as well as acyl glucuronides derived from some carboxylic-acid-containing drugs.

Nitrogen-containing aromatic heterocycles are commonly used in medicinal chemistry for multiple reasons, including reduced lipophilicity when compared with phenyl groups, increased metabolic stability and the ease of substitution. Looking at the metabolism of commonly used five-membered aromatic nitrogen-containing heterocycles for example, indicates that the more nitrogen atoms that are present in the ring system, the less likely it is that it will be subject to phase I type oxidations of the ring itself. For example, pyrroles, azoles, imidazoles and thiazoles are extensively metabolised by CYPs, often resulting in oxidative ring opening.[p61] In contrast, pyrazoles, triazoles and tetrazoles are more resistant to oxidative cleavage, but can be subject to *N*-glucuronidation.[p62],[p63],[p64],[p65] *N*-containing heterocycles can also act as substrates for AO metabolism.[p66]

As highlighted previously, interspecies variability in *N*-glucuronidation can be a barrier for accurate prediction of *in vivo* glucuronidation of drugs in humans, as was discovered during the development of LEO compound 1. LEO compound 1 is an oral interleukin-17A (IL-17A) protein–protein interaction modulator under development for the treatment of psoriasis and other inflammatory disorders. It is metabolised through multiple phase I and phase II routes, including various CYP3A4-mediated hydroxylations and *N*-dealkylation, as well as *N*-sulfation and *N*-glucuronidation (Figure 7). Conjugation reactions occur in the pyrazole moiety, with an *N*-glucuronide being a major metabolite in humans; only small amounts of the *N*-glucuronide were observed in other species.

The interspecies variability in *N*-glucuronidation is particularly high, especially for aliphatic tertiary amines and, relevant to LEO compound 1, aromatic *N*-heterocycles. In addition, *N*-glucuronidation rates in humans are reported to be much higher than in animals, largely due to UGT1A4 and UGT2B10.[p67] This

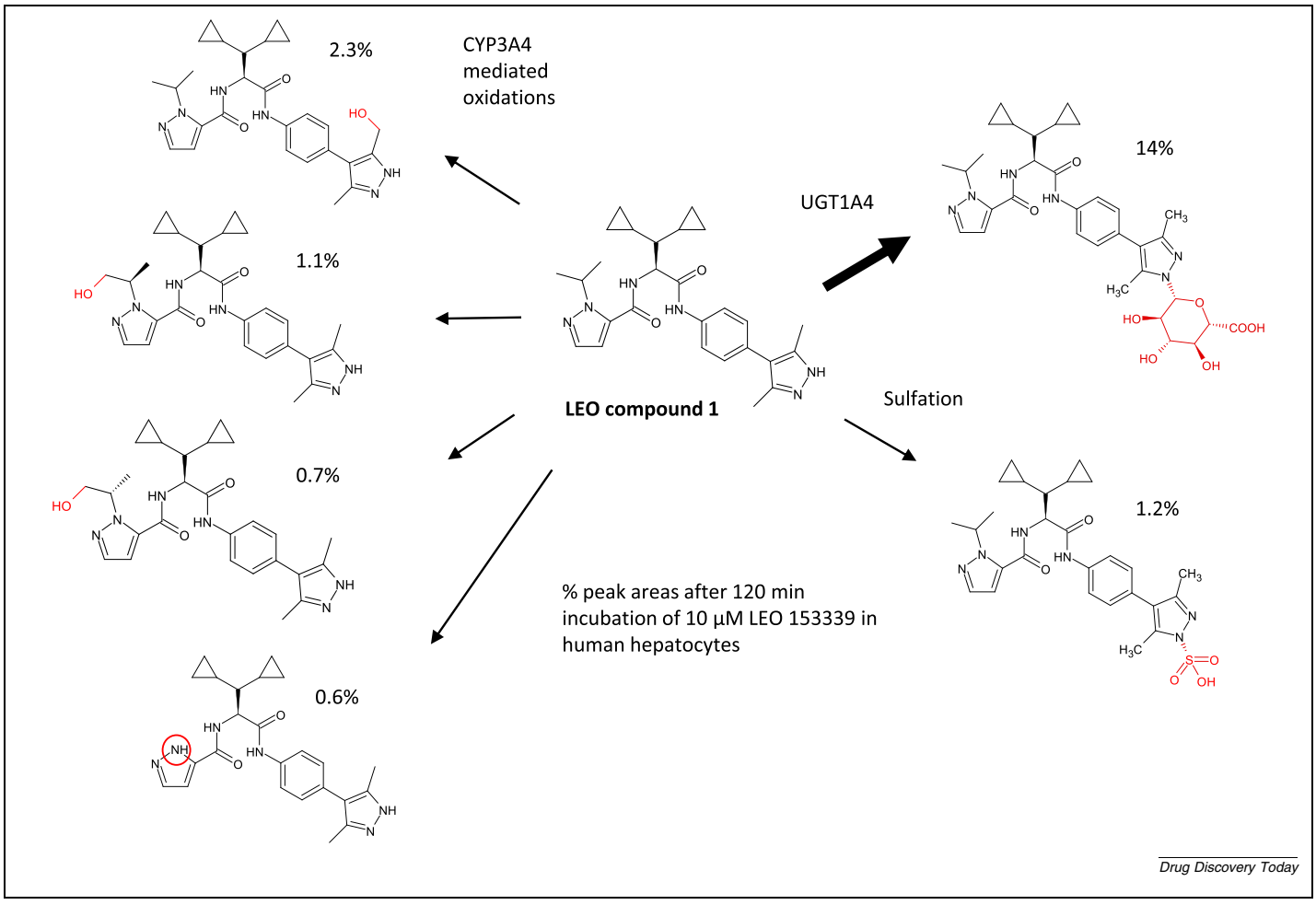


FIGURE 7

Metabolism of LEO compound 1 in human hepatocytes to a major *N*-glucuronide.

species difference in UGT activities can therefore be a barrier for accurate prediction of *in vivo* glucuronidation of drugs in humans. In fact, rodents lack a human UGT1A4 homologue gene.[p68] UGT1A4 was shown to be responsible for the major human metabolite of LEO compound 1.

Originally, the *N*-glucuronide was produced in human female liver S9 incubations to generate tens of milligrams as a reference standard. High conversions (79%) were obtained using this route. However, owing to the amount of material subsequently needed, a late-stage chemical synthesis method was developed to make both unlabelled and deuterated metabolite. Initially, chemical glucuronidation resulted in only very low conversion; however, various modifications in reaction temperature, solvent and reagent stoichiometry allowed the identification of conditions that provided a higher and cleaner conversion, increasing isolated yield from 1.1% to approaching 10%. Microbial biotransformation was also explored as a route to make the *N*-glucuronide, but this was only possible at a maximum of 10% in extract yield prior to purification. The late-stage chemical route was therefore used to scale up to multiple grams. An additional purification step, not used in the previous production route via S9, was introduced to purify low gram amounts of the metabolite. Profiling of the *N*-glucuronide in dogs suggested excretion in the bile, hydrolysis and reabsorption of the parent, but this was not predicted to lead to significant enterohepatic recirculation.[p69]

Acyl glucuronides are a relatively common metabolite type requested for synthesis since this phase II metabolite is specifically mentioned in the FDA's MIST guidance as having the potential to cause idiosyncratic drug toxicity through the direct acylation of proteins or, if subject to intramolecular rearrangement, through the formation of aldehydes that lead to protein glycation.[p7] However, it is important to note that many acyl glucuronides of carboxylic-acid-containing drugs are not a cause for concern,[p70] exemplified here by the acyl glucuronide of AZD5991.

AZD5991 is a potent and selective inhibitor of Mcl-1 that entered clinical development for the treatment of haematological malignancies.[p71] In human hepatocytes, AZD5991 is biotransformed to several metabolites, with an *S*-oxide and an acyl glucuronide being major human metabolites. The abundant glucuronide metabolite required further characterisation during preclinical development, and chemical synthesis of it was not possible at the time. Separately, an *in vivo* rat bile duct cannulated study revealed abundant excretion of the glucuronide in bile, enabling milligram quantities to be isolated. The material

obtained from bile excreta was of sufficient quantity and purity to obtain an NMR spectrum that revealed an acyl glucuronide structure. This material was also sufficient to test in an acyl migration assay, which showed that it did not undergo acyl migration under physiological conditions. To support future bioanalytical-method development and validation needs for clinical studies, significantly larger quantities were required than those that could be obtained from direct purification of preclinical materials. AZD5991 was therefore screened against a panel of microbes, which revealed extensive metabolism via phase I and phase II pathways.[p72] As observed in preclinical studies, a prominent glucuronide was formed by several microbes. The glucuronide produced was demonstrated by LC-MS/MS and NMR to match that observed from human hepatocyte incubations and excreta in rat bile. Experiments pointed to earlier dosing resulting in greater stability, with over 90% conversion of AZD5991 to the acyl glucuronide (Figure S5).

There is also evidence to suggest that on-target pharmacological studies of acyl glucuronides of drugs could be warranted.[p73] This is particularly relevant where acyl glucuronidation constitutes the primary clearance mechanism, or where the pharmacological target is in the extracellular matrix and does not require penetration by the acyl glucuronide conjugate.

Glucuronides can also be responsible for clinically relevant DDIs, such as those attributed to the acyl glucuronides of clopidogrel and gemfibrozil, which selectively inhibit CYP2C8.[p74],[p75] As humans readily oxidise acidic drugs, there is also a potential complication arising from the presence of acyl glucuronides of oxidised metabolites of the drug, which might later alter conjugate reactivity if oxidation occurs on a moiety nearby. Issues can also arise as a result of β-glucuronidase-mediated hydrolysis of *O*-glucuronides to the parent drug, the propensity for which differs owing to marked species differences in expression of β-glucuronidases.[p76]

In addition to 'simple' hydroxylations and glucuronidations, metabolites arising from multiple or more complex biotransformations are often seen. These have generally already proven more difficult to make, and the substrate set analysed will thus be biased towards such metabolites. Phase I metabolites involving multiple enzymes provide the most challenge, such as the M2 metabolite of ganaxolone previously discussed. Other notable phase I biotransformations observed involve oxidation of *N*-linked pyrrolidine ring moieties followed by ring openings or rearrangements. The example shown in Figure S6 was an outcome of a collaboration between Hypha Discovery and the University of Dundee Drug Discovery Unit (DDU) on a series of lead compounds discovered for the treatment of neglected tropical diseases; the drug molecule was originally identified during a previous collaboration between DDU and GlaxoSmithKline.[p77] Some oxidised metabolites prove to be susceptible to degradation on handling and pose significant purification challenges, such as hemiaminal metabolites that present purification issues to varying degrees, depending on reactivity.

Requirements for metabolites

The quantities of metabolites requested vary widely (Figure 8), with 10–50 mg or sometimes higher amounts typically requested for use, for example, as certified reference standards for quantitative bioanalysis. Frequently, metabolites are needed for on– and off-target pharmacological testing and DDI studies. Larger amounts are less commonly needed for *in vivo* toxicity studies for drugs further along the developmental pathway.

Where multiple metabolites of a drug are needed, and where these can be produced in a 'one pot' approach such as through microbial biotransformation or use of a recombinant enzyme system, the reaction is tailored to the metabolite produced at lowest yield. Hence, significantly greater quantities of the major metabolite (or metabolites) may be produced and purified at the same time.

Adding value from metabolite synthesis

Meeting regulatory requirements is the prime goal for many of the projects undertaken. However, additional benefits can be reaped by evaluating metabolites, both major and minor, in target and off-target assays. In rare but notable cases, a metabolite might prove superior to the parent drug, or provide important structure–activity relationship (SAR) insights and support intellectual property protection around a chemical series.[p78]

Added SAR: an in vivo active aldehyde oxidase metabolite

Although metabolite surprises during clinical trials are never welcome, an interesting example of key learnings arose from a metabolite of PF-5190457, a spiro-azetidino-piperidine drug, during late phase 1 clinical trials (Figure S7).[p79] The metabolite circulates at ~25% of the parent drug in humans, and both the parent and metabolite significantly inhibit food uptake in rats at similar doses. The largely AO-mediated biotransformation revealed some intriguing differences about the metabolite compared with the parent drug, and unearthed some previously unknown structure-activity relationships. Although the metabolite has lower binding affinity and potency for inhibiting GHSR1a-induced inositol phosphate accumulation versus the parent, it has increased GHSR1a-induced β-arrestin recruitment potency. The authors demonstrated that although the metabolite had 25-fold lower affinity binding to GHSR1a than the parent, it was four- to sevenfold more potent in recruitment of β-arrestin. Follow-up work characterising the pharmacology of the parent compound and the metabolite PF-6870961 revealed that PF-6870961 had no off-target interactions in a full screen of binding and enzymatic targets. The studies revealed some new insights into SARs that might affect the future design of such GHSR1a inverse agonists. The metabolite is postulated to form hydrogen bonds in an amino acid region that confers biased inverse agonism of β-arrestin recruitment, possibly through disrupted flexibility in these regions.

The widening of IP coverage and patenting of active metabolites is a desirable outcome from studies of metabolites, such as undertaken for CD-14547, a novel mammalian target of rapamycin (mTOR) inhibitor. The metabolites, which were originally observed in plasma samples from an *in vivo* rat pharmacokinetics study, were included in the CD-14547 patent as potentially useful treatments for skin conditions such as acne, atopic dermatitis, actinic keratosis and psoriasis.[p80] To identify the metabolites, CD-14547 was screened against a panel of 20 PolyCYPs enzymes. Seven isoforms biotransformed the parent drug to a maximum

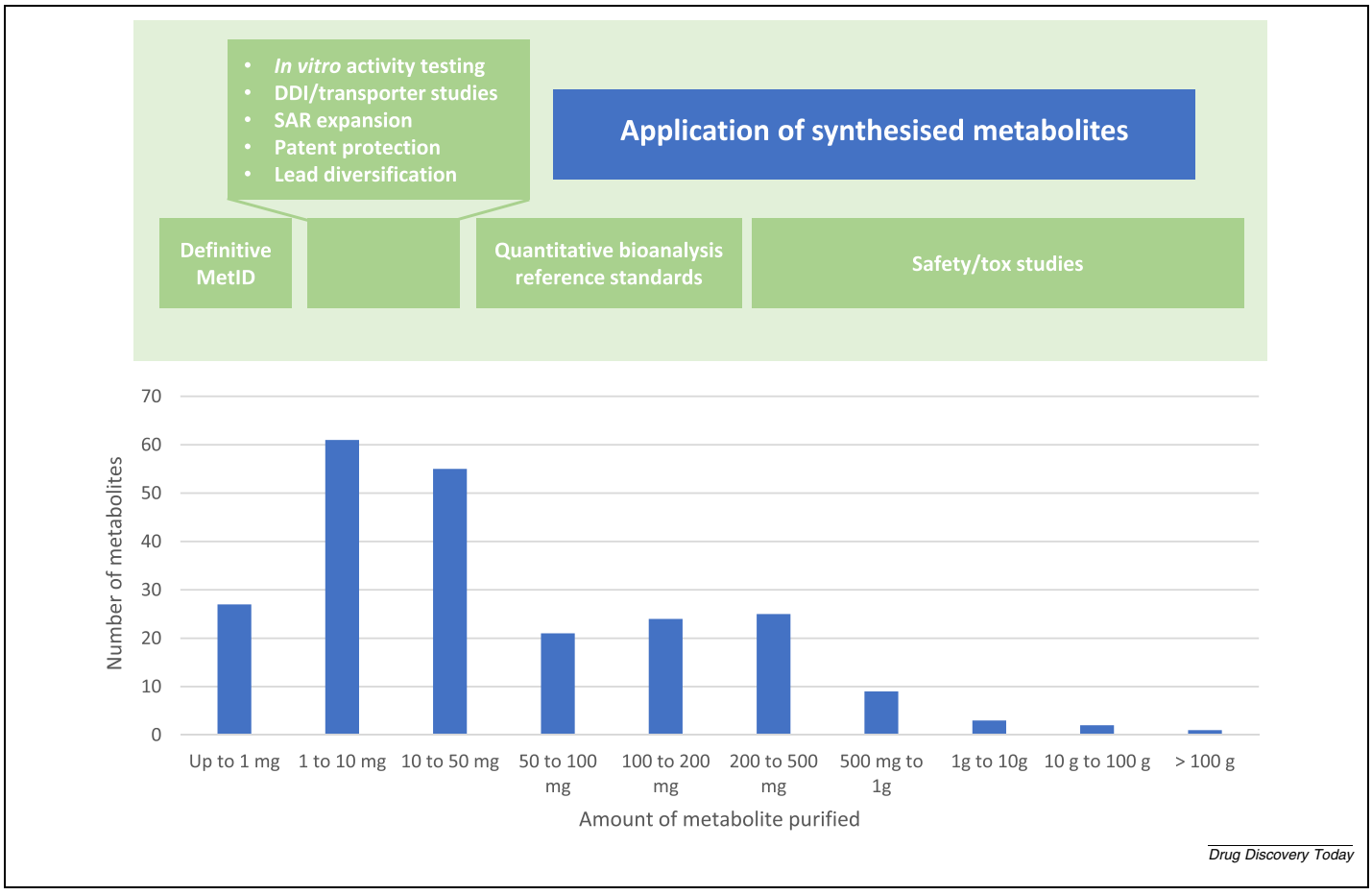


FIGURE 8
The range of quantities made for 228 metabolites and their use.

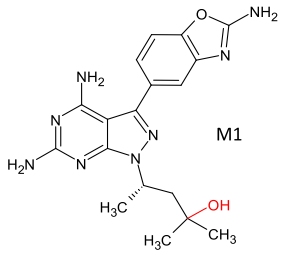
conversion of 91.5%, with PolyCYP 359 providing the best conversion to three hydroxylated metabolites in similar proportions to those seen in the rat plasma samples. The PolyCYP 359 enzyme reaction was scaled up to generate material for structure elucidation and *in vitro* pharmacology assays. NMR spectroscopy of M1 revealed a hydroxylation at the penultimate carbon in the alkyl side chain that generated a tertiary alcohol group, with M2 and M3 being diastereoisomers formed by hydroxylation of the non-equivalent terminal methyl groups (Figure 9). In the pharmacological assay, M1, M2 and M3 all inhibited AKT phosphorylation in human skin A-431 cancer cells with IC_{50} values of 13 nM, 7 nM and 3.7 nM, respectively, compared with an IC_{50} of 2 nM for the parent drug. A further structurally uncharacterised metabolite (CD-14547-H2) was also found to be active, with an estimated IC_{50} value of 3.6 nM.

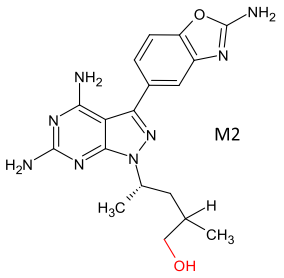
Metabolite synthesis to support lead optimisation

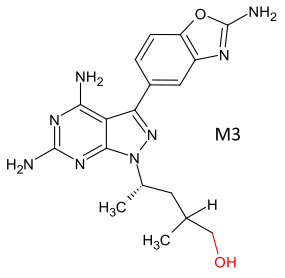
Although the majority of metabolite synthesis projects completed by Hypha Discovery have been to support small-molecule drug development programmes, when performed in late lead-optimisation, beneficial properties associated with polar modifications akin to oxidised metabolites can be captured and incorporated into the eventual development candidate. For instance, improving aqueous solubility by hydroxylation can boost the oral bioavailability of poorly soluble compounds, and there are numerous precedents of hydroxylated metabolites possessing improved metabolic stability, target selectivity and sometimes potency profiles when compared with the parent drug.[(p81),(p82)] A common medicinal chemistry challenge in the optimisation of drug leads is to maintain control of lipophilicity while increasing potency, with ligand lipophilicity efficiency (LLE, or lipE, a function of target IC_{50} and LogP) used as the metric to measure 'druglikeness'. Although a hydroxylated metabolite with equivalent potency to the parent drug will in itself improve LLE, more significant gains in LLE will result from the detection of a more potent hydroxylated metabolite. This means that, in contrast to the synthesis of metabolites to support drug development programmes, producing and testing metabolites at the lead optimisation stage can provide information that is often complementary and orthogonal to conventional medicinal chemistry drug design strategies, by empirically probing for hitherto unknown polar interactions with a target protein.

Concluding thoughts

Although the majority of the metabolite projects we have worked on were related to drug compounds in phase 2 and 3 clinical trials, it is clear from our interactions with many of our clients that metabolites pertinent to regulatory guidelines are being targeted and identified earlier in development. We anticipate that this trend will increase with the greater focus on







Compound	IC_{50} (nM)
CD14547 (parent)	2.0
CD14547 M1	13
CD14547 M2	7.0
CD14547 M3	3.7

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FIGURE 9

Novel mTOR inhibitor metabolites of CD14547. An enzyme reaction catalysed by PolyCYP 359 was scaled up using 82.5 mg of parent compound in 272 ml of enzyme incubation to generate material for structure elucidation and in vitro pharmacology assays, yielding 25.8 mg of M1, 3.5 mg of M2 and 2.1 mg of M3, at a total isolatable yield of 38%.[p80]

human studies earlier in clinical development, particularly if the human first/human only approach is embraced across the industry. As expected, the vast majority of metabolites we are commissioned to make are deemed to be major according to the regulatory guidance, and among these, many are disproportionate human metabolites. However, there is also interest in the interrogation of metabolites for pharmacological activity and beneficial DMPK properties. This does not just apply to those that would be classified as major metabolites from a regulatory standpoint. Companies are interested in ensuring comprehensive IP coverage as well as not discounting structures that could deliver an improvement on the parent drug. Timing is key!

Even though some metabolites formed by simple single-step biotransformations, such as hydroxylated or glucuronidated metabolites, can be challenging to produce by facile chemical synthetic approaches, these can generally be made in a late-stage manner using either biocatalytic methods or late-stage chemical functionalisation. Although initial MetID (metabolite identification) studies are generally useful in pinpointing structural changes to specific moieties, they are not always accurate at predicting structures. After a metabolite structure has been definitively identified by NMR, some quite different and unexpected biotransformations become evident. Although not an everyday occurrence, be open to expecting the unexpected when it comes to the metabolism of drugs! As a result of unanticipated structural changes, more complex multi-step biotransformations can provide a challenge, and often these require more effort to make and scale up once metabolite structures have been accurately determined.

There is a growing prevalence of more structurally complex drug candidates: for example, beyond-rule-of-five (bRo5)-molecules and those that act through non-traditional mechanisms, such as proteolysis targeting chimeras (PROTACs), molecular glues and covalent binders. It will be interesting to see the net result of the interplay between this growing structural and mechanistic complexity and the drive to reduce metabolic clearance in the design phase on the biotransformation mechanisms of these molecules. This will affect the type and number of metabolites that might need to be definitively identified and assessed during clinical development and the challenges around accessing metabolites for these studies.

Competing interest statement

All listed authors were employed by Hypha Discovery at the time any client project work described in the paper was conducted.

CRediT authorship contribution statement

Julia Shanu-Wilson: Writing – original draft, Formal analysis, Data curation. **Samuel Coe:** Data curation. **Liam Evans:** Writing – original draft. **Jonathan Steele:** Writing – original draft. **Stephen Wrigley:** Writing – original draft, Formal analysis, Data curation.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.drudis.2024.103943.

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