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Opportunities for Accelerating Drug Discovery and Development by Using Engineered Drug-Metabolizing Enzymes

Elizabeth M.J. Gillam and Valerie M. Kramlinger

School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, Brisbane, Australia (E.M.J.G.) and Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee (V.M.K.)

ABSTRACT

The study of drug metabolism is fundamental to drug discovery and development (DDD) since by mediating the clearance of most drugs, metabolic enzymes influence their bioavailability and duration of action. Biotransformation can also produce pharmacologically active or toxic products, which complicates the evaluation of the therapeutic benefit versus liability of potential drugs but also provides opportunities to explore the chemical space around a lead. The structures and relative abundance of metabolites are determined by the substrate and reaction specificity of biotransformation enzymes and their catalytic efficiency. Preclinical drug biotransformation studies are done to quantify in vitro intrinsic clearance to estimate likely in vivo pharmacokinetic parameters, to predict an appropriate dose, and to anticipate interindividual variability in response, including from drug-drug interactions. Such studies need to be done rapidly and cheaply, but native enzymes, especially in microsomes or hepatocytes, do not always produce the full complement of metabolites seen in extrahepatic tissues or preclinical test species. Furthermore, yields of metabolites are usually limiting. Engineered recombinant enzymes can make DDD more comprehensive and systematic. Additionally, as renewable, sustainable, and scalable resources, they can also be used for elegant chemoenzymatic, synthetic approaches to optimize or synthesize candidates as well as metabolites. Here, we will explore how these new tools can be used to enhance the speed and efficiency of DDD pipelines and provide a perspective on what will be possible in the future. The focus will be on cytochrome P450 enzymes to illustrate paradigms that can be extended in due course to other drug-metabolizing enzymes.

SIGNIFICANCE STATEMENT

Protein engineering can generate enhanced versions of drug-metabolizing enzymes that are more stable, better suited to industrial conditions, and have altered catalytic activities, including catalyzing non-natural reactions on structurally complex lead candidates. When applied to drugs in development, libraries of engineered cytochrome P450 enzymes can accelerate the identification of active or toxic metabolites, help elucidate structure activity relationships, and, when combined with other synthetic approaches, provide access to novel structures by regio- and stereoselective functionalization of lead compounds.

Introduction

The point of preclinical drug discovery and development (DDD) studies is to provide an effective drug candidate that can be tested safely in vivo. Most new chemical entities are destined to fail before they reach the market, due to suboptimal therapeutic effects, problems with their pharmacokinetic profiles, off-target activities or toxicities, or for commercial reasons unrelated to their structure or activity (Kola and Landis, 2004). Therefore, it is important that wherever possible, drugs fail early and cheaply to minimize the costs associated with development, especially in the clinical stages.

Studying the metabolism of a new drug candidate is essential to DDD since the biotransformation of a chemical: influences the concentration reached in the circulation and tissues, and its duration of action within the body; determines the products to which it is converted, whether inert, pharmacologically active, or toxic; as well as influencing their respective concentrations and duration of action. Only a minor proportion of drugs (∼5% (Saravanakumar et al., 2019) are eliminated as the parent compound without some degree of biotransformation (e.g., entirely by renal filtration).

Many different enzymes contribute to the metabolic clearance or bioactivation of novel drug candidates, both to functionalize and conjugate chemicals, processes formerly called phase I and phase II (Josephy et al., 2005). However, the most dominant quantitatively and qualitatively are the cytochromes P450 (P450s), monooxygenases that catalyze a diverse array of biotransformation reactions, including aliphatic and aromatic hydroxylation and epoxidation, heteroatom dealkylation and oxidation, and various other chemistries reviewed separately elsewhere in this special issue (Isin, 2022).

ABBREVIATIONS: CPR, NADPH-P450 reductase; DDD, drug discovery and development; DDI, drug-drug interaction; LC-MS, liquid chromatography–mass spectrometry; LSF, late-stage functionalization; Met ID, metabolite identification; P450, cytochrome P450; SAR, structure-activity relationship.

The aims of drug metabolism studies in DDD include: determining which parts of the molecule are metabolic soft spots, i.e., subject to biotransformation, and, therefore, positions that could be changed to affect metabolic stability; identifying the main metabolites in humans and other species, including any active, toxic, or reactive products; ensuring species chosen for preclinical toxicity studies generate metabolites found in humans; characterizing which enzymes produce these metabolites in humans to predict drug-drug interactions (DDIs); predicting the extent of interindividual variability in pharmacokinetics, including due to pharmacogenetic factors and tissue-specific metabolism; and determining the intrinsic clearance in vitro to predict the likely dose needed for in vivo and clinical studies (Davies et al., 2020).

Applications of Drug-Metabolizing Enzymes in Preclinical Drug Development

Drug metabolism studies in DDD fall into several general categories and can sometimes be done concurrently (Fig. 1). First, the assessment of metabolic stability is undertaken to identify how rapidly a lead candidate is degraded (metabolic stability studies). This involves incubating the lead candidate with a suitable metabolic system such as liver microsomes, postnuclear supernatants (i.e., S9 fractions, supernatants from $9000g$ centrifugation), or hepatocytes, along with appropriate cofactors, then tracking the depletion of the parent compound from the incubation, typically using liquid chromatography–mass spectrometry (LC-MS). Information from such studies reveals whether the structure needs to be altered to increase or decrease the likely bioavailability and half-life and thereby modulate the concentration of drug likely to be achieved in the circulation and its duration of action (Balani et al., 2005).

Secondly, metabolites are identified structurally [metabolite identification, Met ID]. Again, this involves incubating the lead candidate with a suitable metabolic system and appropriate cofactors and analyzing the products generated [e.g., by LC-MS or liquid chromatography-nuclear magnetic resonance (LC-NMR)] to infer metabolite structures. Such studies allow qualitative identification of metabolic soft spots that can be targeted by medicinal chemistry to modulate pharmacokinetics [e.g., by fluorination (Obach et al., 2016; Fig. 1)]. If warranted, the metabolites can then be characterized for therapeutic and off-target or toxic effects. However, this type of additional study, as well as unambiguous metabolite identification, usually requires the synthesis of the relevant metabolites or their recovery from incubations with appropriate enzyme preparations in significant amounts (i.e., milligram quantities or more). Synthesis of pure metabolic standards can be challenging due to the need to make precise stereo-, regio-, and chemoselective modifications to often complex parent molecules at unactivated positions, leading to lengthy delays in accessing metabolites in any quantity (Humphreys, 2022). Therefore, pending access to pure metabolite standards, metabolic soft spots are frequently deduced from incomplete structural identification (e.g., by LC-MS rather than NMR), where the type of modification (e.g., aromatic hydroxylation) is known, but not its exact position, complemented by "expert intuition" to identify sites that are
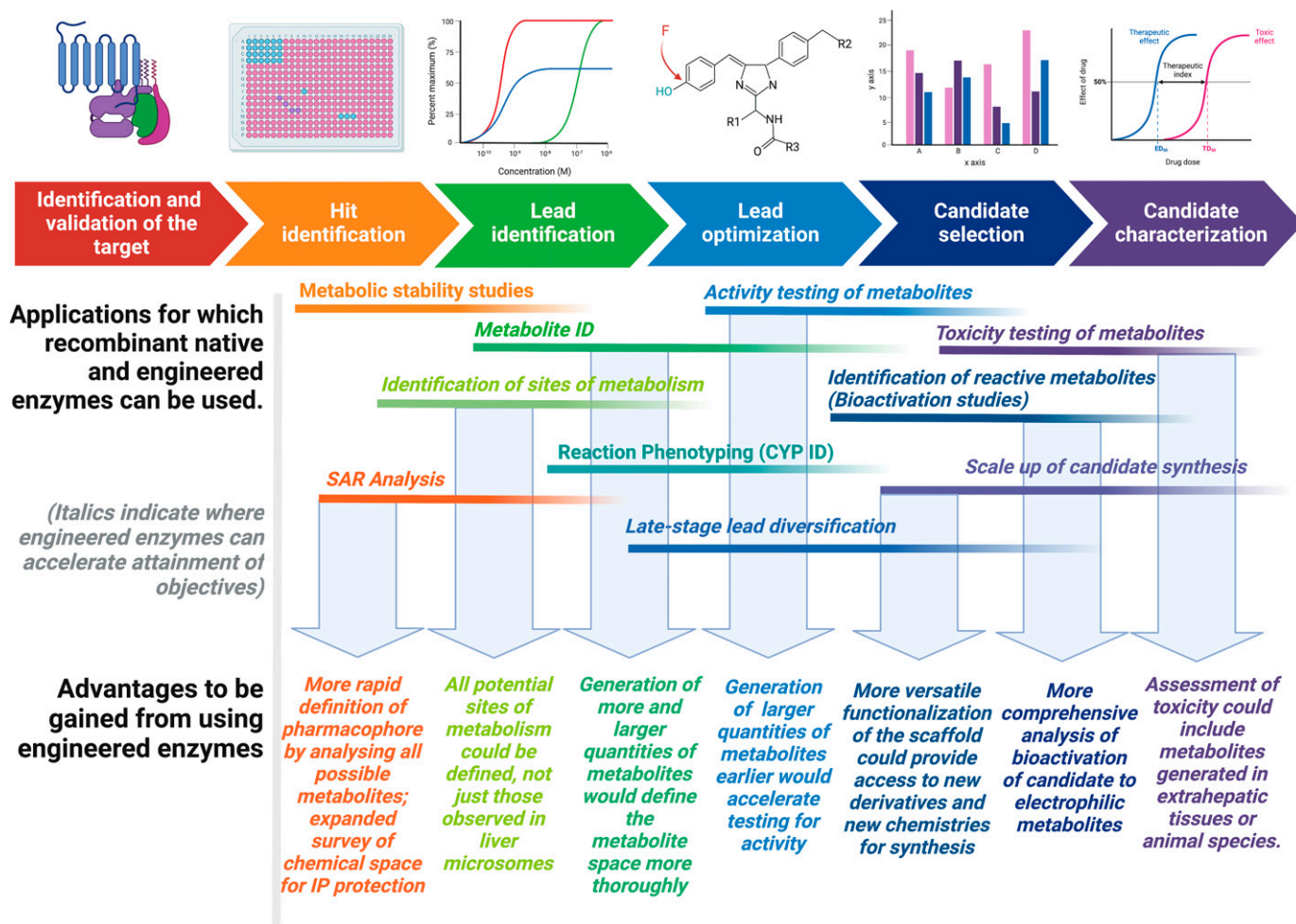

Fig. 1. Current and possible future opportunities for improving the efficiency of DDD by using recombinant and engineered enzymes.

more chemically prone to oxidation. Such "best guesses" may be more or less accurate depending on the specific three-dimensional constraints of the enzyme active site. Unfortunately, it is not yet possible to predict with certainty how a given structure will interact with the conformationally dynamic active sites of multiple enzymes. Artificial intelligence methods such as AlphaFold may facilitate such in silico predictions in the future (Ivanov et al., 2022).

Met ID allows potential metabolic pathways to be proposed, at least tentatively, pending full structural characterization of metabolites. Identification of metabolites that are pharmacologically active adds to information on structure-activity relationships (SARs) that is typically acquired in the hit-to-lead and later stages of DDD and allows better navigation and protection of the intellectual property space(Fig. 1). On the other hand, identification of toxic metabolites provides information on which to base go/no-go decisions concerning likely safety or selectivity of a lead candidate (Humphreys and Unger, 2006).

Thirdly, reaction phenotyping (cytochrome P450 identification, (CYP ID)] is performed to identify which enzymes contribute to metabolic clearance and to quantify their contribution. CYP ID can be done by incubating the drug with a metabolic system (e.g., microsomes) in the presence of selective inhibitors of particular enzymes to see which diminish the metabolism of the drug; correlating the rate of metabolism of the lead candidate with that of other typical probe substrates of specific forms across a set of liver microsomal samples; immuno-inhibition studies using antibodies raised to particular enzymes; or in high-throughput, by testing whether the lead compound of interest inhibits the metabolism of any of a set of easily measured marker substrates that are each selective for a particular enzyme. Alternatively, the drug can be incubated directly with individual recombinant enzymes. Since different enzymes have different degrees of polymorphism in human populations and tissue-specific distribution, as well as discrete substrate ranges and affinities, information from such studies allows prediction of DDIs and pharmacogenetic and other interindividual variability and an understanding of the implications of tissue-specific metabolism.

A final type of study involves incubating the lead candidate with a suitable enzyme preparation (microsomes, S9 fractions, or recombinant enzymes), cofactor/s and a sacrificial nucleophile, such as glutathione or an abundant protein, to detect the production of reactive metabolites (bioactivation studies). Any electrophilic metabolites produced will be scavenged by the excess nucleophile and can then be detected and characterized as the relevant adducts by LC-MS.

Although most of the above aims have remained constant over the last few decades, the ways in which they are addressed have shifted with the advent of recombinant DNA technologies (Cusack et al., 2013). Whereas previously it was necessary to rely on tissue fractions such as liver microsomes or postnuclear supernatants, the development of recombinant expression methods for the major human P450s from the early to mid-1990s (Crespi et al., 1990; Barnes et al., 1991; Crespi, 1991; Crespi et al., 1991; Fisher et al., 1992a; Crespi et al., 1993; Gillam et al., 1993; Penman et al., 1993; Sandhu et al., 1993; Penman et al., 1994; Gillam et al., 1995; Richardson et al., 1995; Crespi and Penman, 1997) allowed a more reductionist approach. In particular, the contribution of individual enzymes to the metabolism of novel compounds could be quantified directly using incubations with recombinant P450s. Methods were developed to assess inhibitory potential for DDIs in high-throughput fashion and on a miniaturized scale using form-selective, fluorogenic, and luminogenic marker substrates (Crespi and Stresser, 2000; Stresser et al., 2002; Trubetskoy et al., 2005; Cali et al., 2006; Chougnet et al., 2007; Cali et al., 2012). Once the nuclear receptors that control the expression of P450s were identified and coupled to simple reporter systems in the late 1990s and early 2000s, it became possible to assess whether new drugs could affect the expression of specific P450s and thereby predict a wider range of DDIs (Sueyoshi and Negishi, 2001; Corcos et al., 2002; Moore et al., 2002; Goodwin et al., 2003; Kliewer, 2003; Raucy, 2003; Persson et al., 2006).

Attention focused initially on the "big five" P450s responsible for the majority of hepatic drug metabolism, i.e., CYP3A4, CYP2D6, CYP2C9, CYP2C19, and CYP1A2; however, almost all human drug-metabolizing P450s are now available as recombinants in at least some form, often coexpressed with NADPH-P450 reductase (CPR), with or without cytochrome b_5. These recombinant systems have been commercialized and widely adopted, although products from many of the early mammalian cell expression systems are no longer available. Several less well studied extrahepatic enzymes that contribute to the metabolism of specific chemicals remain unavailable commercially, however, and the focus of recombinant studies has been entirely on human enzymes rather than any P450s from animal species relevant to safety or efficacy testing.

Limitations of Using Tissue Preparations or the "Big-Five" Human Recombinants in Drug Development

Caveats apply to the use of recombinant human P450s since the membrane lipid profile in a recombinant host, plus the relative expression of CPR and cytochrome b_5, is typically different to that in liver. The presence or absence of b5 may have both quantitative and qualitative effects on metabolite production (e.g., to shift the ratio between two metabolites) that are not possible to predict and that differ according to the enzyme and substrate in question. Likewise, the effect of CPR:P450 ratio is not well defined. However, typically, higher rates are observed with liver microsomes, an effect attributed to better coupling of the P450 with the reductase and b5 in the original source tissue. Another caveat is that it is necessary to modify the N-termini of P450s to achieve recombinant expression in some hosts (e.g., bacteria). Therefore, tissue fractions remain a reference against which studies with recombinant enzymes, such as for reaction phenotyping and Met ID, are benchmarked (Fig. 2). Moreover, tissue fractions are still a mainstay for assessment of metabolic stability, initial metabolite identification, and estimation of intrinsic clearance, where a more holistic view is needed. Parallel improvements in methodologies for culturing hepatocytes have provided an opportunity to analyze drug metabolism at the whole-cell level that captures all biotransformation pathways, at least for liver.

However, liver tissue fractions from a given pool of individuals do not account for possible differences in metabolism that might be encountered across all individuals in a population, across different species used for safety testing, or across extrahepatic tissues. Furthermore, human tissue fractions are a scarce and nonrenewable resource and animal ethics concerns apply to the use of animal tissues (Fig. 2). Although recombinant enzymes are a renewable resource, they still cannot anticipate all the variation in metabolism that could be encountered due to pharmacogenetic variation, species differences, and extrahepatic metabolism. For example, the antiepileptic drug phenytoin is associated with a cutaneous adverse reaction in ~5% of patients and a rare hypersensitivity reaction. Phenytoin is metabolized in the liver to the phenol metabolite by CYP2C9 and CYP2C19. However, the extrahepatic form, CYP2C18, which is present in skin but not liver, has been shown to produce a reactive quinone metabolite that can form adducts with proteins, a process that might underpin the common cutaneous adverse reaction and contribute to the more serious idiosyncratic hypersensitivity reaction. Had the potential for significant bioactivation of phenytoin in the skin been obvious from studying extrahepatic enzymes, such reactions may have been better anticipated.
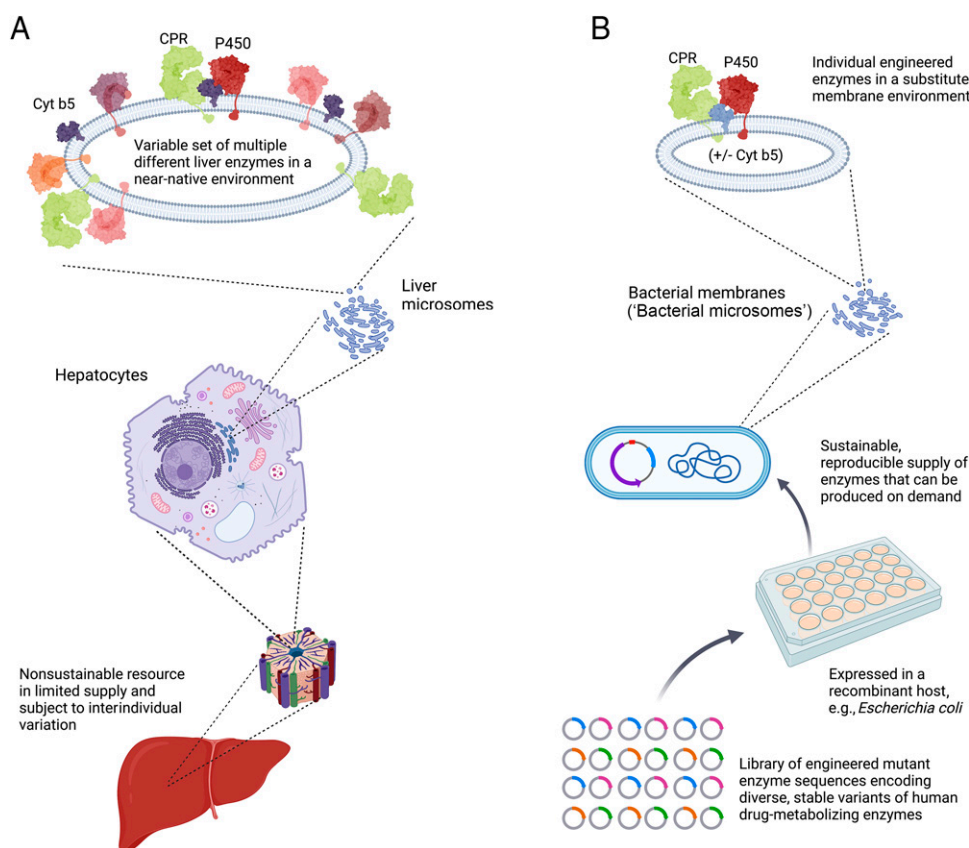
Fig. 2. Comparison of native and engineered metabolic systems. Hepatocytes and subcellular fractions, such as microsomes prepared from liver tissue (A), represent a metabolic system that better reflects enzyme activity in human or animal liver but which is a non-sustainable resource and subject to interindividual variability. Libraries of engineered enzymes expressed in a heterologous host such as *E. coli* (B) provide a renewable resource with activities that overlap but are different to, and possibly expanded from, those found in human or animal liver.

Recombinant enzymes can, however, be used in ways not possible with tissue fractions, e.g., to generate authentic metabolites for structural identification (Rushmore et al., 2000; Vail et al., 2005; Schroer et al., 2010; Fessner et al., 2020). Unfortunately, low activities and yields mean it is difficult to obtain significant amounts of any but the most dominant metabolites, and large amounts of enzymes are often needed since the enzymes are usually not stable for more than 1 to 2 hours of incubation.

On a commercial level, human liver preparations and recombinant enzymes are costly when obtained from commercial suppliers and typically too resource-intensive to set up in-house for pharmaceutical companies (Humphreys, 2022). Table 1 compares some of the alternative enzyme preparations available for different DDD purposes against several specific criteria.

Applications of Drug-Metabolizing Enzymes in Lead Optimization and Improving Drug Synthesis

Late-stage functionalization (LSF) of lead candidates (Guillemard et al., 2021) involves making targeted changes to structures to improve pharmacological or pharmacokinetic properties (e.g., late-stage oxygenation to improve pharmacokinetic properties(Stepan et al., 2018; Fig. 1). The example has already been discussed above of fluorinating metabolic soft spots to enhance stability (Obach et al., 2016). Other types of functionalization, such as oxygenation, can also be useful, as reviewed recently (Charlton and Hayes, 2022). However, targeted regio- and stereoselective modification can be challenging on complex structures with chemically similar sites. Using enzymes offers advantages of

greater selectivity in the site of modification since the topography of the enzyme's active site directs the modification to specific sites and faces of the molecule (e.g., Le-Huu et al., 2016). Moreover, biocatalysis shortens synthetic routes (Simić et al., 2022), can be done under mild conditions, and reduces the reliance on solvents and other toxic chemicals (Kinner et al., 2022).

Whereas LSF involves making targeted changes to a lead candidate, lead diversification (Obach et al., 2018) involves changing the structure in a greater variety of ways to explore the surrounding chemical space so as to better define SARs, explore functional group tolerance, and find compounds with better pharmacological or pharmacokinetic properties (Fessner, 2019). Screening for active metabolites can provide indications about where to direct further optimization efforts (Fredenhagen et al., 2019).

For the same reasons that they are useful in LSF and lead diversification, enzymes can be useful in the large-scale production of drugs, and increasingly, biosynthetic enzymes are being incorporated into synthetic pathways (Fig. 1). Applications of P450s in drug synthesis have been reviewed previously (Sakaki, 2012; Di Nardo and Gilardi, 2020). Notable examples were the use of microbial P450s in corticosteroid biosynthesis (Hogg, 1992), CYP105A3 in the production of statins (Watanabe et al., 1995), CYP725A4 in taxol biosynthesis (Biggs et al., 2016), and the plant CYP71AV1 in the semisynthetic production of artemisinin (Paddon et al., 2013). A notable advance was the use of mammalian P450s in the total synthesis of hydrocortisone in yeast (Szczebara et al., 2003). However, to date, no drug-metabolizing enzymes per se have been incorporated into drug syntheses.

TABLE 1

Comparison of available enzyme preparations for studying drug metabolism with respect to characteristics desired for DDD purposes

Feature or requirement	Liver subcellular fractions or hepatocytes prepared from humans or laboratory animals	Recombinant human enzymes	Microbial enzymes (engineered or native)	Engineered enzymes drawn from recombination or ancestral sequence resurrection of homologous animal sequences
Similarity of activity profiles compared with those seen in vivo that are attributable to human P450s	Identical for human preparations; different but usually overlapping for animal preparations.	Comparable	Different but overlapping or can be engineered to be overlapping	Different but usually similar and overlapping
Ability to cover variability in metabolite profiles in mammalian species used for safety or efficacy testing	Incomplete	Incomplete	Possible depending on degree of diversity engineered into enzymes	High in the case of recombined or ancestral enzymes, since engineering method draws on functional diversity of homologous sequences from mammals
Ability to anticipate novel metabolite profiles in extrahepatic tissues	Poor unless tissue fractions are produced from the tissue of interest	Good if enzymes found extrahepatically are studied	Possible if microbial enzymes serendipitously match the activity of extrahepatic forms.	Good for recombined or ancestral enzymes, since the engineering method draws on functional diversity of homologous sequences including extrahepatic forms.
Ability to anticipate consequences of pharmacogenetic variability in patients	Limited by the availability of tissue representing different genotypes	Genetic variants are not usually expressed so limited without further engineering	Poor	Possible if genetic variants are prepared and expressed.
Metabolite yield	Limited by cost and availability of tissue fractions; only major metabolites can be accessed	Only major metabolites can be accessed; limited by poor catalytic efficiency	Catalytic efficiency can be relatively high for specific pathways; yield can be improved by engineering	Catalytic efficiency can be relatively high for specific pathways; yield can be improved by engineering
Ability to access minor metabolites in quantity	Poor	Generally poor but possible if specific P450s show good activity toward the desired metabolite	Depends on specific P450s showing good activity toward the desired metabolite	Generally good since diverse but overlapping metabolite profiles are generated by recombination so screening can reveal catalysts with the desired metabolite profile.
Sustainability and reproducibility	Poor since dependent on adventitious collection of human tissue. Reproducibility of animal tissue fractions is usually good, but animal ethics concerns impact sustainability	Excellent	Excellent	Excellent
Stability under industrial conditions (long incubations, above-ambient temperatures)	Limited/poor	Limited/poor	Can be stabilized via engineering	Can be stabilized via engineering
Ability to generate novel chemical diversity	Poor	Poor (limited to poorly studied forms)	Good—dependent on choice of microbial enzyme and engineering performed	Good—novel metabolites are found frequently within diverse metabolic profiles
Potential for scale up as biocatalysts for medicinal chemistry	Poor	Generally poor without further engineering	Good and improved by engineering	Good and improved by engineering

Microbial Systems as an Alternative for DDD Applications

Microbial enzymes have been advocated for metabolite generation and lead diversification for several decades (Griffiths et al., 1991), in addition to their roles in drug syntheses noted above (Table 1). Although they may not serve a physiologic role in xenobiotic metabolism, many bacterial P450s have shown useful activities toward drugs and drug-like molecules, especially those from the CYP102 (Cusack et al., 2013), CYP105 (McLean et al., 2015), CYP106 (Virus et al., 2006; Schmitz et al., 2012; Lee et al., 2015; Bakkes et al., 2017; Schmitz et al., 2018), CYP107 (Schmitz et al., 2018), CYP109 (Bakkes et al., 2017), CYP116 (Klenk et al., 2017), CYP154 (Bracco et al., 2013; Bakkes et al., 2017), and CYP264 (Ringle et al., 2013) families. Recent studies made possible by genome mining have illustrated the catalytic potential present in microbial CYPomes (Agematu et al., 2006; Palmer-Brown et al., 2019; Schmitz et al., 2019; Hilberath et al., 2020; Schmitz et al., 2021). Other activities have been revealed by studying the biosynthetic pathways of secondary metabolites, especially antibiotics (Xue and Sherman, 2001; Rudolf et al., 2017; Fredenhagen et al., 2019; Schmitz et al., 2019).

Nonetheless, routine screening using microbial cultures is not widely implemented in Pharma for practical reasons: culturing diverse microbes (e.g., multiple strains of fungi and bacteria) requires different media, conditions, and expertise that are beyond the scope of most drug metabolism and pharmacokinetics (DMPK) groups(Humphreys, 2022). However, where feasible [i.e., with well characterized, easily cultured organisms (e.g., *Cunninghamella elegans*)], microbial cultures offer renewable, stable metabolic systems that often produce much higher yields of product than can be obtained with tissue fractions or recombinant human enzymes (Li et al., 2008; Quinn et al., 2015). Alternatively, microbial enzymes from diverse sources can be expressed and screened in a standard heterologous host (Weis et al., 2009). For example, a set of 213 mostly Actinomycete P450s in 12 different P450 families were expressed in *Escherichia coli* and shown to generate testosterone metabolites in yields sufficient for structure elucidation by NMR (Agematu et al., 2006). Two P450s, CYP105D and CYP107Z from *Streptomyces platensis,* were found to metabolize a broad range of drugs to metabolites seen in humans (Hilberath et al., 2020).

With microbial enzymes, more analytical effort is needed to ensure that the metabolites obtained exactly match those produced by human enzymes or in species used for safety testing (Humphreys, 2022). However, where a microbial catalyst can be identified, scale-up is usually more straightforward and cost effective than with recombinant enzymes or tissue fractions and is cost-competitive with medicinal chemistry (Salter et al., 2019). Outsourcing of microbial screening to contract research organizations that specialize in microbial diversity or sourcing cultures in a plate-based format may circumvent issues with internal expertise and resources in DMPK groups; however, there are implications for cost.

Engineering P450 Enzymes for DDD

Increasingly, *engineered* recombinant enzymes are providing ways to address challenges in drug development and are being incorporated into synthetic strategies for generating chemical diversity or making targeted modifications to drug scaffolds (reviewed in Fasan, 2012). Engineered enzymes differ from the corresponding native ones in that a change has been made to the coding sequence of the gene that alters the amino acid sequence and, therefore, the structural and/or functional properties of the protein, potentially overcoming many of the limitations of recombinant human enzymes (Fig. 2; Table 1). The ideal commercial biocatalyst is highly thermostable, cheap to produce, tolerant of a wide range of reaction conditions (e.g., the presence of organic solvents), uses no or cheap cofactors, shows high yield toward the (single) product of interest, tolerates high substrate concentrations, and is not inhibited by products. All these characteristics affect the balance between the yield of accessible product and the costs associated with the biocatalytic process. Thermostable enzymes allow bioreactors to be run at higher temperatures to maximize yields and reduce microbial contamination but also provide greatly prolonged half-lives at mild temperatures. In our experience, they are also easier to express at high levels and more cheaply produced (Gumulya et al., 2018). Solvent tolerance facilitates loading of substrates that are marginally soluble in aqueous mixtures and raises the prospect of one-pot chemoenzymatic syntheses (Dennig et al., 2015). Factors such as ease of product workup are also relevant and motivate strategies such as immobilization of the biocatalyst. To date, protein engineering has been successful to modify substrate specificity (Kumar et al., 2005), improve yields of a particular metabolite (e.g., a minor metabolite) (Hunter et al., 2011), increase enzyme stability (Salazar et al., 2003; Kumar et al., 2006b; Li et al., 2007b; Romero et al., 2013; Gumulya et al., 2018; Gumulya et al., 2019), enhance activity supported by both redox partners and alternatives (e.g., peroxides as

oxygen surrogates) (Joo et al., 1999; Kumar et al., 2006a; Gumulya et al., 2018; Strohmaier et al., 2020), and improve solvent tolerance (Wong et al., 2004; Kumar et al., 2006b; Gumulya et al., 2018).

Bacterial P450s, especially variants of the fatty acid hydroxylase P450$_{BM3}$ (CYP102A1), have received the most attention in efforts to engineer catalysts of drug biotransformation. P450$_{BM3}$ has been chosen due to its high catalytic rate and coupling efficiency with its natural substrate and because it is self-sufficient as a fusion of a P450 domain with a diflavin P450 reductase (Narhi and Fulco, 1986). Early studies showed that the substrate specificity of P450$_{BM3}$ could be expanded by targeted mutations in the active site or substrate access channel (reviewed recently in Thistlethwaite et al., 2021). Both rational and random mutagenesis have been used to good effect, although turnover and coupling efficiency with non-natural substrates are typically much lower than with the natural fatty acid substrates.

Importantly, P450$_{BM3}$ has been the model system for the development of many methods for enzyme engineering, especially via directed evolution (Jung et al., 2011). One notable success was the structure-guided recombination of CYP102A1 with CYP102A2 and CYP102A3 by SCHEMA by the Arnold group to generate mutant libraries enriched in functional P450s, which have been shown to be useful in producing authentic drug metabolites and diversifying lead compounds (Otey et al., 2006; Sawayama et al., 2009; Rentmeister et al., 2011; Lall et al., 2020) and have led to the commercialization of a set of CYP102 mutants for use in DDD. However, other groups have also demonstrated the usefulness of engineering P450$_{BM3}$ in metabolite synthesis (Cha et al., 2014; Kang et al., 2014; Ryu et al., 2014; Venkataraman et al., 2014; Di Nardo et al., 2016; Le et al., 2019; Nguyen et al., 2021) or in the functionalization of pharmaceutical intermediates (Chu et al., 2016; Munday et al., 2017; O'Hanlon et al., 2017; Li and Wong, 2019; Cao et al., 2021). The reader is directed to an excellent recent review on the application of P450$_{BM3}$ and its mutants to drug metabolism (Thistlethwaite et al., 2021). Other bacterial P450s have been studied recently, especially from the CYP105 (McLean et al., 2015) and CYP106 (Virus et al., 2006; Lee et al., 2015; Schmitz et al., 2018) families, suggesting that useful catalytic diversity can be sourced outside the CYP102 family (Weis et al., 2009).

Eukaryotic enzymes have received less attention, possibly since they are harder to work with (less stable intrinsically, harder to express as recombinants). However, early work by the Halpert and Guengerich laboratories demonstrated that substrate preference and other properties could be modulated by random and rational engineering, as reviewed previously (Kumar, 2010; Gillam and Hayes, 2013). Studies in the author's laboratory showed that DNA shuffling could be used to develop libraries of biocatalysts with varied regioselectivity that could be mined for forms with desired properties, e.g., enhanced production of minor metabolites or novel activities (Huang et al., 2007; Johnston et al., 2007; Hunter et al., 2011; Behrendorff et al., 2013). More recently, ancestral sequence reconstruction has been applied to enhance the thermostability and solvent tolerance of drug-metabolizing P450s and to access forms with altered catalytic properties (Gumulya et al., 2018; Gumulya et al., 2019; Harris et al., 2022; unpublished data).

Engineering Coupling to Electron Transfer Partners

Much effort has been directed toward addressing the often inefficient coupling of the P450 and its obligatory redox partners by substituting alternative redox partners (Park et al., 2012; Lee et al., 2015; Sagadin et al., 2018). In many cases, the native redox partner of microbial P450s is not known, so a substitute is necessary (Ortega Ugalde et al., 2018). With at least one notable exception (CYP101A1), a functional P450 system can usually be achieved (Sagadin et al., 2018).

P450s can be combined with non-natural redox partners in artificial fusions that capture some of the same advantages inherent in CYP102. This idea is not new: the first such fusions were attempted between mammalian P450s and a yeast reductase in the late 1980s and early 1990s (Murakami et al., 1987; Sakaki et al., 1994; Shiota et al., 1994), then developed further using mammalian reductases (Fisher et al., 1992b; Shet et al., 1994; Chun et al., 1996; Chun et al., 1997). More recently, the CPR domain from P450$_{BM3}$ has been used to good effect (Fairhead et al., 2005; Dodhia et al., 2006; Degregorio et al., 2011a; Degregorio et al., 2011b), but electron transfer rates do not yet compare with those seen in P450$_{BM3}$. Further optimization of the linker connecting the two domains and their interface and relative orientation may enhance electron transfer rates and, therefore, the product yields that can be obtained in this system.

The same approach has been used for microbial enzymes, especially to reduce the number of separate components in the electron transfer pathway (Bakkes et al., 2017), including fusing with the reductase domain of P450$_{BM3}$ (Ortega Ugalde et al., 2018), mitochondrial adrenodoxin plus an *E. coli* ferredoxin reductase (Ringle et al., 2013), *E. coli* flavodoxin and flavodoxin reductase (Bakkes et al., 2015), the reductase domain of P450RhF (RhFRed) (Nodate et al., 2006; Li et al., 2007a; Sabbadin et al., 2010), and the reductase domain of CYP102D1 (Choi et al., 2014). The linker joining the two domains is often tweaked (Zuo et al., 2017), and in some studies, the redox partner's interaction face has also been engineered (Sagadin et al., 2019). Fusions have also been explored with cofactor-recycling systems, e.g., phosphite dehydrogenase (Beyer et al., 2018).

Opportunities in Preclinical Drug Development Presented by the Use of Engineered Enzymes

The overarching benefit of engineering enzymes is the ability to change their properties and, particularly, to create *diversity* in the catalysts in terms of their catalytic activities: substrate specificity, regio- and stereoselectivity, and degree of promiscuity (Table 1). The trend is toward small, focused libraries of more or less promiscuous catalysts that are small enough to be screened, yet have a high likelihood of yielding interesting catalytic profiles toward any given drug or pharmaceutical intermediate (Zhang et al., 2011).

Libraries of engineered enzymes allow many of the aims of preclinical DDD to be addressed in a more rapid and systematic fashion. This is because a library of engineered enzymes with diverse substrate and reaction specificity is likely to generate a wider, more comprehensive set of potential metabolites than would be accessible with human P450s or within any animal species (Figs. 1 and 2). Such a library could capture metabolites that were produced by animal P450s, human extrahepatic forms or pharmacogenetic variants that are not routinely studied in Met ID or reaction phenotyping. If this were done for Met ID at the start of the lead characterization, the full range of metabolites that could be found subsequently in both in vitro studies with human enzymes or in vivo in animal models could be explored. This approach would highlight the possible existence of, and provide a means to characterize and generate, metabolites at an earlier stage of development. Importantly, within a library of enzymes, it is likely that some mutants would produce metabolites at significant levels that are "minor" in incubations with human hepatic P450s and which could then be used to produce such metabolites in quantity for structural identification or functional characterization (Fig. 1). However, it is essential to identify exactly which are the relevant metabolites in humans, e.g., which isomer is relevant where there is a chiral center.

While it may be too expensive currently to identify all possible metabolites using a library screen, so inevitably, the focus is on only metabolites mandated by the Metabolites in Safety Testing guidance, the cost/benefit ratio shifts if a comprehensive analysis becomes inexpensive. It is becoming increasingly easier to source and express enzyme diversity, which should translate to greater availability of enzyme libraries and (in theory) more competitive pricing, assuming there is a market for such enzymes. Miniaturization of incubations, along with NMR structural analysis (Obach et al., 2018) and the use of in-line microfluidic systems for parallel analysis, also offers advantages here (Rea et al., 2013).

Likewise, the analysis of metabolic soft spots could be done with a library containing more catalytic potential to rapidly reveal all *possible* sites of metabolism, including those that might only be exploited by extrahepatic forms or in animals used for safety testing (Fig. 1). In the case where significant extrahepatic metabolism is occurring in vivo in humans, these metabolic soft spots could then be addressed by, for example, chemoenzymatic fluorination strategies, in which a hydroxyl introduced by an engineered P450 could be exploited to fluorinate the lead candidate (Rentmeister et al., 2009).

Libraries of engineered enzymes could also facilitate toxicity testing. For the same reasons as outlined above, identification of reactive metabolites would be accelerated, especially for those intermediates that may be found only in particular species or tissues, e.g., reactive metabolites not produced in liver microsomes or by the "big five" P450 forms. Incubations with libraries of engineered enzymes could be miniaturized for high-throughput format (Obach et al., 2018) and combined with other tests such as mutagenicity studies (van der Meer and Belkin, 2010) or assays for glutathione adduct formation (Fig. 1).

There is an inevitable "activation energy barrier" associated with the adoption of new technology; DMPK teams have limited resources and expertise and will not necessarily have skills in biotechnology or enzymology to establish novel operating procedures. Therefore, factors such as ease of use need to be considered for implementation of engineered enzymes. Just as to fully exploit the diversity inherent in microbial enzymes, it is necessary to put them into a recombinant platform so that they can be easily screened, so too, engineered enzymes need to be easily incorporated into existing protocols. The approach taken in the author's laboratory is to develop small, focused libraries of well characterized, thermostable enzyme preparations with overlapping specificity that can be used interchangeably with liver microsomes and commercial recombinant enzyme preparations as a form of "bacterial microsomes" (Fig. 2).

Opportunities for Accelerating Drug Discovery by the Use of Engineered Enzymes

On the discovery side, the chemical diversity produced by libraries of engineered enzymes could be used for lead diversification (Fessner et al., 2022). Libraries of enzymes could be incubated with the lead and incubation extracts screened for pharmacological activity in high-throughput fashion to identify both active and inactive metabolites (Fura et al., 2004). In contrast to traditional medicinal chemistry, the nature of the modification introduced to the molecule would not need to be known at the start of the analysis. "Hits" from such a screen could be investigated in further detail to determine the change introduced and, where necessary, determine which of multiple metabolites produced was responsible for the activity. Such approaches to probe the chemical space around a lead candidate would accelerate analysis of SARs and especially facilitate identification of active metabolites for structural identification and intellectual property protection. Importantly, if active metabolites were found, the libraries would also provide a biocatalyst for subsequent scale up of production of the candidate (Fura et al.,

2004), as exemplified in (Lewis et al., 2010). In addition, prodrug strategies could be proposed based on this information.

LSF, or the introduction of a transformation on a complex molecule, has several applications, including exploration of SARs and the ability to access derivatives that may possess superior properties such as improved metabolic stability and ligand-lipophilicity efficiency. Leveraging engineered enzymes to access chemical space in a complementary manner to chemical synthesis can be a powerful way to generate chemical libraries and accessing multiple derivatives in parallel (Boström et al., 2018), and thereby expand the medicinal chemist's toolbox.

Importantly, engineered enzymes offer a wider range of possible functionalization chemistries and greater compatibility with chemoenzymatic approaches (including one-pot reactions). The use of P450BM3 mutants in chemoenzymatic cascades has been reviewed recently (Thistlethwaite et al., 2021), and many examples exist now (Bisterfeld et al., 2017; Loskot et al., 2017; Li et al., 2020). Where the end objective merits an investment of resources (e.g., to improve the large-scale production of a valuable drug), a biocatalyst could be further engineered by rational or evolutionary methods to increase catalytic efficiency. Successful integration of an efficient biocatalytic step to replace a synthetically challenging chemical transformation could reduce the time and steps required for synthesis and enhance atom economy.

A particularly exciting development is the use of P450s as templates for engineering catalysts of non-natural chemistry. Pioneering work from the Arnold laboratory has demonstrated that derivatives of P450s can catalyze the insertion of atoms other than oxygen, given a suitable donor. In particular, mutation of the Cys responsible for providing the fifth ligand to the heme iron, which anchors the prosthetic group to the protein, to Ser or His, followed by directed evolution, yielded catalysts of carbene and nitrene transfer, amidation, aminohydroxylation, and fluoroalkylation, among other reactions (reviewed in Yang and Arnold, 2021). The relevance of these reactions to drug synthesis was demonstrated in the enantioselective synthesis of levomilnacipran (Wang et al., 2014). Other exotic chemistry may also be possible, such as C-Si and C-B bond formation, as demonstrated with other hemoproteins (Arnold, 2018).

Perspective on Future Directions

Libraries of engineered recombinant drug-metabolizing enzymes represent a versatile tool for accelerating DDD by allowing rapid exploration of sequence space around lead candidates to access possible metabolites that may be produced and also diversify and functionalize structures (Fig. 1). They can be produced sustainably for those applications that do not require exact replication of human metabolic profiles but rather an examination of the metabolic possibilities of a compound. Considerable sequence and, therefore, functional diversity can be explored by rational mutagenesis, directed evolution, and mining of natural sequences; all that is required is a sequence of an enzyme and a means to express it in a heterologous host (Table 1).

In terms of drug development per se, applying enzyme libraries can survey the metabolic space in a more comprehensive and systematic manner than done currently with tissue fractions and recombinant human enzymes. The ability to access more possible metabolites should reveal toxic, reactive, or pharmacologically active metabolites earlier in DDD so as to better anticipate possible problems or opportunities from the start. A DDD pipeline could then start to resemble a more regular funnel leading more directly to an outcome, rather than a tube with bends where unexpected results necessitate deviations from the projected path.

The scope for developing engineered enzymes is very wide, but resources that can be used for screening catalyst libraries are finite; therefore, some way is needed to prioritize enzymes for screening (Zhang et al., 2011). Small, diverse libraries are needed that are rich in robust, functionally useful catalysts and available in a format that can be rapidly screened. To fully exploit the catalytic potential in engineered enzymes, however, will require a way to efficiently optimize or evolve the initial catalysts found by screening (e.g., to enhance the regioselectivity toward production of particular metabolites over others or to increase product yields overall). Rational (re)design of catalysts would require structures to be routinely available for the forms screened. Although experimental structure determination (e.g., by X-ray diffraction) is a long way from being routine, artificial intelligence methods such as AlphaFold may fill the gap (Jumper et al., 2021).

An exciting but ambitious objective would be to undertake "combinatorial medicinal chemistry" by combining DNA-coded compound libraries in aqueous media with enzyme libraries and high-throughput screening. This parallels a shift in medicinal chemistry toward large-scale, automated drug discovery using multiple permutations of chemistries at once. This will require highly robust enzymes that can be used as off-the-shelf reagents (Boström et al., 2018), along with possible immobilization of libraries, microfluidics, and integrated coupling to high-throughput screens for biologic effects.

Fessner et al. (2022) have advocated using commercial libraries of biocatalysts as "enzymatic first aid kits" for medicinal chemistry to address challenges in synthetic chemistry that are too challenging or time consuming to do by chemocatalysis. Enhanced lead diversification and LSF are straightforward applications to imagine, as long as the "activation energy" associated with adopting a new technology can be addressed. They point out that chemists are not trained in working with unstable biological molecules, but biotechnologists who have the skills to express and use enzymes are not usually educated in the principles of chemical synthesis. There is a clear need for interdisciplinary training or greater diversity in medicinal chemistry teams (Fessner et al., 2022). However, the format in which enzymes are provided will also be key to implementation. Again, thermostable enzymes that can be used as robust off-the-shelf reagents and integrated easily into existing protocols are more likely to gain acceptance faster. Better sharing of expertise from biotransformation and preclinical drug development teams, who are used to using recombinant enzymes with medicinal chemistry teams, will facilitate the use of more biosynthetic approaches across DDD. Ultimately, however, the turning point will be when the advantages of using enzyme libraries are consistently demonstrated in real examples where the benefit in time, resources, and, therefore, money saved in DDD can be analyzed and documented.

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Wrote or contributed to the writing of the manuscript: Gillam, Kramlinger.

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Address correspondence to: Elizabeth M.J. Gillam, School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, Brisbane, 4072 Australia. E-mail: e.gillam@uq.edu.au