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

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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.

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Research is underway in the Gillam group to engineer thermostable P450 enzymes as biocatalysts for application in drug discovery and development and fine chemical synthesis. Enzymes developed in the course of this research have been licensed for commercial distribution under the tradename "CYPerior."

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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.

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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 9000*g* 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

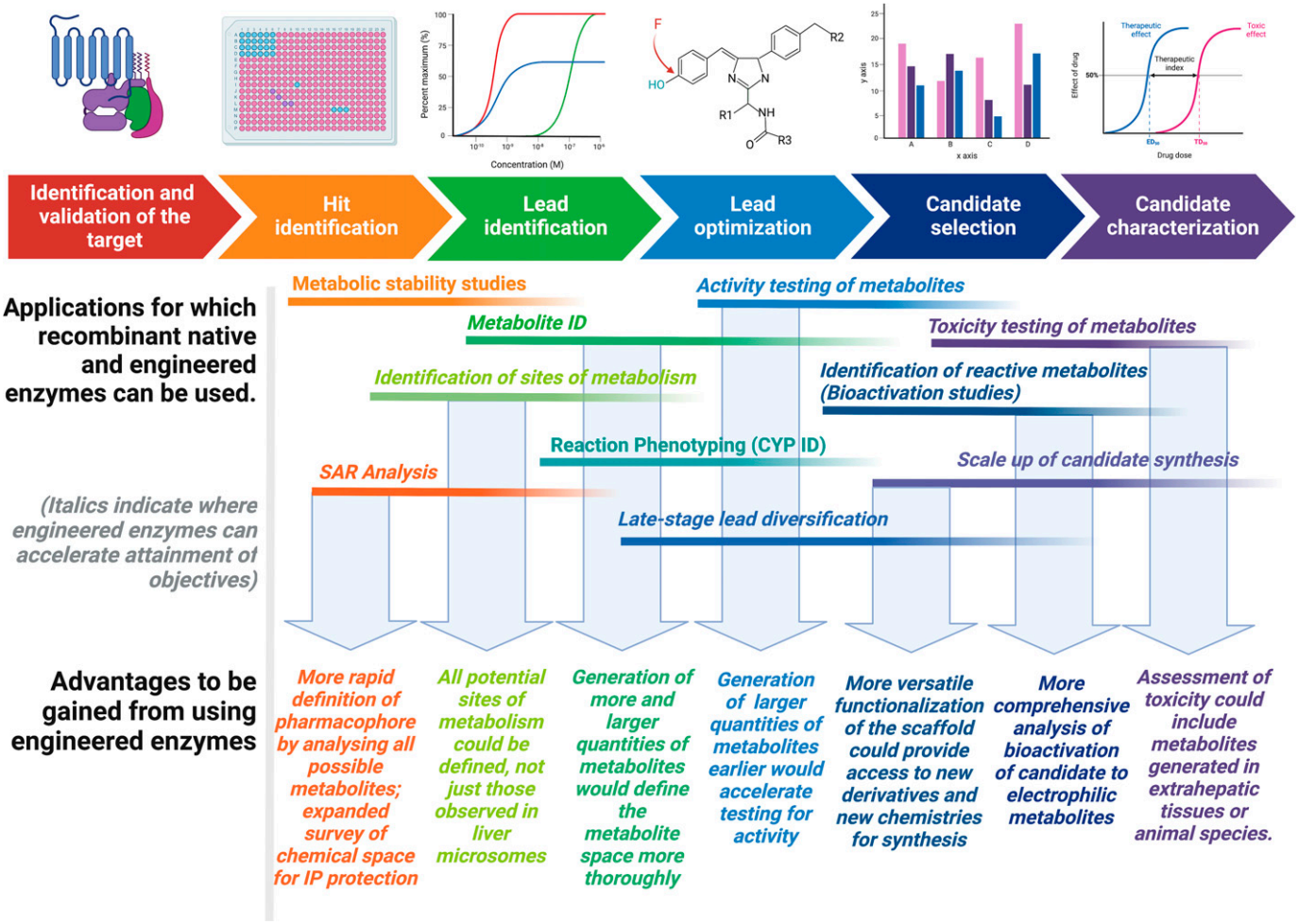

Fig. 1. Current and possible future opportunities for improving the efficiency of DDD by using recombinant and engineered enzymes.

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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.

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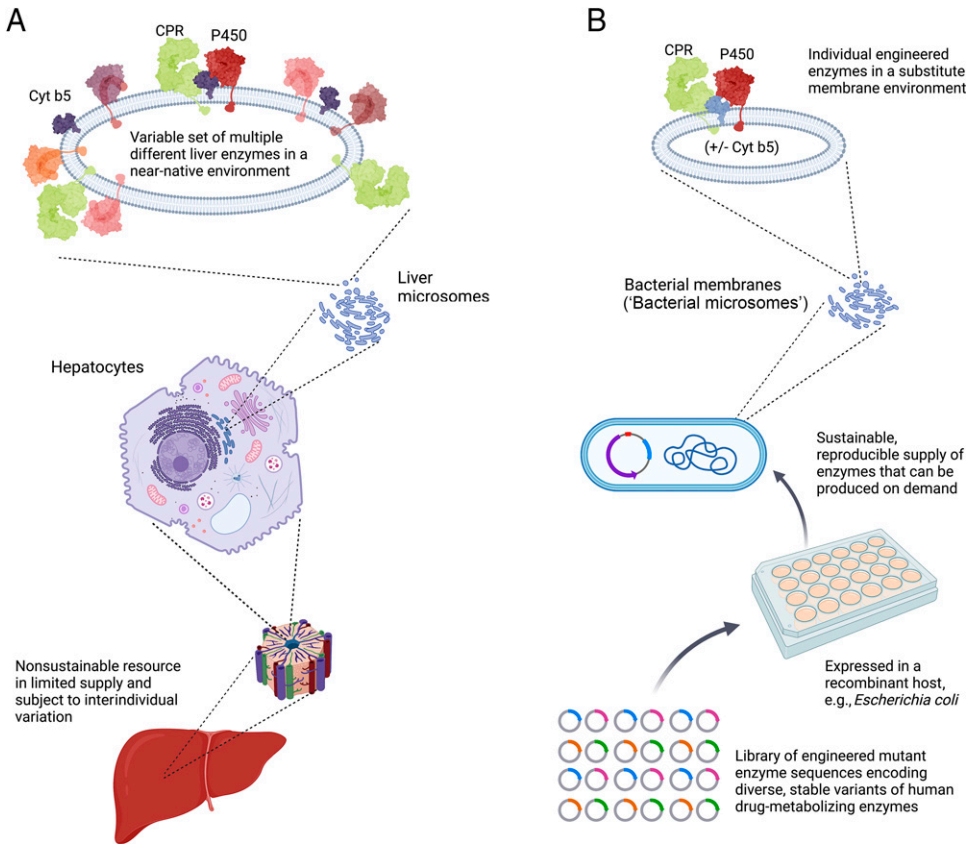

Fig. 2. Comparison of native and engineered metabolic systems. Hepatocytes and subcellular fractions, such as microsomes prepared from liver tissue (A), represent a metabolic system that better reflects enzyme activity in human or animal liver but which is a non-sustainable resource and subject to interindividual 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.

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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).

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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).

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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.,

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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.

Authorship Contributions

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

References

Agematu H, Matsumoto N, Fujii Y, Kabumoto H, Doi S, Machida K, Ishikawa J, and Arisawa A (2006) Hydroxylation of testosterone by bacterial cytochromes P450 using the Escherichia coli expression system. *Biosci Biotechnol Biochem* **70**:307–311.

Arnold FH (2018) Directed Evolution: Bringing New Chemistry to Life. *Angew Chem Int Ed Engl* **57**:4143–4148.

Bakkes PJ, Biemann S, Bokel A, Eickholt M, Girhard M, and Urlacher VB (2015) Design and improvement of artificial redox modules by molecular fusion of flavodoxin and flavodoxin reductase from Escherichia coli. *Sci Rep* **5**:12158.

Bakkes PJ, Riehm JL, Sagadin T, Rühlmann A, Schubert P, Biemann S, Girhard M, Hutter MC, Bernhardt R, and Urlacher VB (2017) Engineering of versatile redox partner fusions that support monooxygenase activity of functionally diverse cytochrome P450s. *Sci Rep* **7**:9570.

Balani SK, Miwa GT, Gan LS, Wu JT, and Lee FW (2005) Strategy of utilizing in vitro and in vivo ADME tools for lead optimization and drug candidate selection. *Curr Top Med Chem* **5**:1033–1038.

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Barnes HJ, Arlotto MP, and Waterman MR (1991) Expression and enzymatic activity of recombinant cytochrome P450 17 alpha-hydroxylase in Escherichia coli. *Proc Natl Acad Sci USA* **88**:5597–5601.
Behrendorff JBYH, Johnston WA, and Gillam EMJ (2013) DNA shuffling of cytochrome P450 enzymes. *Methods Mol Biol* **987**:177–188.
Beyer N, Kulig JK, Fraaije MW, Hayes MA, and Janssen DB (2018) Exploring PTDH-P450BM3 Variants for the Synthesis of Drug Metabolites. *ChemBioChem* **19**:326–337.
Biggs BW, Lim CG, Sagliani K, Shankar S, Stephanopoulos G, De Mey M, and Ajikumar PK (2016) Overcoming heterologous protein interdependency to optimize P450-mediated Taxol precursor synthesis in Escherichia coli. *Proc Natl Acad Sci USA* **113**:3209–3214.
Bisterfeld C, Holec C, Böse D, Marx P, and Pietruszka J (2017) Chemoenzymatic Total Synthesis of the Proposed Structures of Putaminoxins B and D. *J Nat Prod* **80**:1563–1574.
Boström J, Brown DG, Young RJ, and Keserü GM (2018) Expanding the medicinal chemistry synthetic toolbox. *Nat Rev Drug Discov* **17**:709–727.
Bracco P, Janssen DB, and Schallmey A (2013) Selective steroid oxyfunctionalisation by CYP154C5, a bacterial cytochrome P450. *Microb Cell Fact* **12**:95.
Cali JJ, Ma D, Sobol M, Simpson DJ, Frackman S, Good TD, Daily WJ, and Liu D (2006) Luminogenic cytochrome P450 assays. *Expert Opin Drug Metab Toxicol* **2**:629–645.
Cali JJ, Ma D, Wood MG, Meisenheimer PL, and Klaubert DH (2012) Bioluminescent assays for ADME evaluation: dialing in CYP selectivity with luminogenic substrates. *Expert Opin Drug Metab Toxicol* **8**:1115–1130.
Cao NT, Nguyen NA, Park CM, Cha GS, Park KD, and Yun CH (2021) A Novel Statin Compound from Monacolin J Produced Using CYP102A1-Catalyzed Regioselective C-Hydroxylation. *Pharmaceuticals (Basel)* **14**:981.
Cha GS, Ryu SH, Ahn T, and Yun CH (2014) Regioselective hydroxylation of 17β-estradiol by mutants of CYP102A1 from Bacillus megaterium. *Biotechnol Lett* **36**:2501–2506.
Charlton SN and Hayes MA (2022) Oxygenating Biocatalysts for Hydroxyl Functionalisation in Drug Discovery and Development. *ChemMedChem* **17**:e202200115.
Choi KY, Jung E, Yun H, Yang YH, and Kim BG (2014) Engineering class I cytochrome P450 by gene fusion with NADPH-dependent reductase and S. avermitilis host development for daidzein biotransformation. *Appl Microbiol Biotechnol* **98**:8191–8200.
Chougnet A, Grinkova Y, Ricard D, Sligar S, and Woggon WD (2007) Fluorescent probes for rapid screening of potential drug-drug interactions at the CYP3A4 level. *ChemMedChem* **2**:717–724.
Chu LL, Pandey RP, Jung N, Jung HJ, Kim EH, and Sohng JK (2016) Hydroxylation of diverse flavonoids by CYP450 BM3 variants: biosynthesis of eriodictyol from naringenin in whole cells and its biological activities. *Microb Cell Fact* **15**:135.
Chun Y-J, Shimada T, and Guengerich FP (1996) Construction of a human cytochrome P450 1A1: rat NADPH-cytochrome P450 reductase fusion protein cDNA and expression in Escherichia coli, purification, and catalytic properties of the enzyme in bacterial cells and after purification. *Arch Biochem Biophys* **330**:48–58.
Chun YJ, Jeong TC, Roh JK, and Guengerich FP (1997) Characterization of a fusion protein between human cytochrome P450 1A1 and rat NADPH-P450 oxidoreductase in Escherichia coli. *Biochem Biophys Res Commun* **230**:211–214.
Corcos C, Brey J, and Corcos L (2002) The nuclear receptors CAR and PXR control induction of cytochromes P450 by phenobarbital. *Med Sci* **18**:429–437.
Crespi CL and Penman BW (1997) Use of cDNA-expressed human cytochrome P450 enzymes to study potential drug-drug interactions. *Adv Pharmacol* **43**:171–188.
Crespi CL (1991) Expression of cytochrome P450 cDNAs in human B lymphoblastoid cells: applications to toxicology and metabolite analysis. *Methods Enzymol* **206**:123–129.
Crespi CL, Langenbach R, and Penman BW (1993) Human cell lines, derived from AHH-1 TK+/- human lymphoblasts, genetically engineered for expression of cytochromes P450. *Toxicology* **82**:89–104.
Crespi CL, Penman BW, Steimel DT, Gelboin HV, and Gonzalez FJ (1991) The development of a human cell line stably expressing human CYP3A4: role in the metabolic activation of aflatoxin B1 and comparison to CYP1A2 and CYP2A3. *Carcinogenesis* **12**:355–359.
Crespi CL, Steimel DT, Aoyama T, Gelboin HV, and Gonzalez FJ (1990) Stable expression of human cytochrome P450IA2 cDNA in a human lymphoblastoid cell line: role of the enzyme in the metabolic activation of aflatoxin B1. *Mol Carcinog* **3**:5–8.
Crespi CL and Stresser DM (2000) Fluorometric screening for metabolism-based drug–drug interactions. *J Pharmacol Toxicol Methods* **44**:325–331.
Cusack KP, Koolman HF, Lange UEW, Peltier HM, Piel I, and Vasudevan A (2013) Emerging technologies for metabolite generation and structural diversification. *Bioorg Med Chem Lett* **23**:5471–5483.
Davies M, Jones RDO, Grime K, Jansson-Löfmark R, Fretland AJ, Winiwarter S, Morgan P, and McGinnity DF (2020) Improving the Accuracy of Predicted Human Pharmacokinetics: Lessons Learned from the AstraZeneca Drug Pipeline Over Two Decades. *Trends Pharmacol Sci* **41**:390–408.
Degregorio D, Sadeghi S, and Gilardi G (2011a) Towards improved catalytic efficiency in engineered human cytochrome P450 3A4-BMR. *FEBS J* **278**:101–102.
Degregorio D, Sadeghi SJ, Di Nardo G, Gilardi G, and Solinas SP (2011b) Understanding uncoupling in the multiredox centre P450 3A4-BMR model system. *J Biol Inorg Chem* **16**:109–116.
Dennig A, Busto E, Kroutil W, and Faber K (2015) Biocatalytic one-pot synthesis of l-tyrosine derivatives from monosubstituted benzenes, pyruvate, and ammonia. *ACS Catal* **5**:7503–7506.
Di Nardo G, Dell'Angelo V, Catucci G, Sadeghi SJ, and Gilardi G (2016) Subtle structural changes in the Asp251Gly/Gln307His P450 BM3 mutant responsible for new activity toward diclofenac, tolbutamide and ibuprofen. *Arch Biochem Biophys* **602**:106–115.
Di Nardo G and Gilardi G (2020) Natural Compounds as Pharmaceuticals: The Key Role of Cytochromes P450 Reactivity. *Trends Biochem Sci* **45**:511–525.
Dodhia VR, Fantuzzi A, and Gilardi G (2006) Engineering human cytochrome P450 enzymes into catalytically self-sufficient chimeras using molecular Lego. *J Biol Inorg Chem* **11**:903–916.
Fairhead M, Giannini S, Gillam EMJ, and Gilardi G (2005) Functional characterisation of an engineered multidomain human P450 2E1 by molecular Lego. *J Biol Inorg Chem* **10**:842–853.
Fasan R (2012) Tuning P450 Enzymes as Oxidation Catalysts. *ACS Catal* **2**:647–666.
Fessner ND (2019) P450 Monooxygenases Enable Rapid Late-Stage Diversification of Natural Products *via* C-H Bond Activation. *ChemCatChem* **11**:2226–2242.
Fessner ND, Badenhorst CPS, and Bornscheuer UT (2022) Enzyme Kits to Facilitate the Integration of Biocatalysis into Organic Chemistry - First Aid for Synthetic Chemists. *ChemCatChem* **14**, e202200156.
Fessner ND, Srdic M, Weber H, Schmid C, Schonauer D, Schwaneberg U, and Glieder A (2020) Preparative-Scale Production of Testosterone Metabolites by Human Liver Cytochrome P450 Enzyme 3A4. *Adv Synth Catal* **362**:2725–2738.
Fisher CW, Caudle DL, Martin-Wixtrom C, Quattrochi LC, Tukey RH, Waterman MR, and Estabrook RW (1992a) High-level expression of functional human cytochrome P450 1A2 in Escherichia coli. *FASEB J* **6**:759–764.
Fisher CW, Shet MS, Caudle DL, Martin-Wixtrom CA, and Estabrook RW (1992b) High-level expression in Escherichia coli of enzymatically active fusion proteins containing the domains of mammalian cytochromes P450 and NADPH-P450 reductase flavoprotein. *Proc Natl Acad Sci USA* **89**:10817–10821.
Fredenhagen A, Schroer K, Schröder H, Hoepfner D, Ligibel M, Porchet Zemp L, Radoch C, Freund E, and Meishammer A (2019) Cladosporin derivatives obtained by biotransformation provide guidance for the focused derivatization of this antimalarial lead compound. *ChemBioChem* **20**:650–654.
Fura A, Shu Y-Z, Zhu M, Hanson RL, Roongta V, and Humphreys WG (2004) Discovering drugs through biological transformation: role of pharmacologically active metabolites in drug discovery. *J Med Chem* **47**:4339–4351.
Gillam EMJ, Baba T, Kim B-R, Ohmori S, and Guengerich FP (1993) Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme. *Arch Biochem Biophys* **305**:123–131.
Gillam EMJ, Guo Z, Martin MV, Jenkins CM, and Guengerich FP (1995) Expression of cytochrome P450 2D6 in Escherichia coli, purification, and spectral and catalytic characterization. *Arch Biochem Biophys* **319**:540–550.
Gillam EMJ and Hayes MA (2013) The evolution of cytochrome P450 enzymes as biocatalysts in drug discovery and development. *Curr Top Med Chem* **13**:2254–2280.
Goodwin B, Gauthier KC, Umetani M, Watson MA, Lochansky MI, Collins JL, Leitersdorf E, Mangelsdorf DJ, Kliewer SA, and Repa JJ (2003) Identification of bile acid precursors as endogenous ligands for the nuclear xenobiotic pregnane X receptor. *Proc Natl Acad Sci USA* **100**:223–228.
Griffiths DA, Best DJ, and Jezequel SG (1991) The screening of selected microorganisms for use as models of mammalian drug metabolism. *Appl Microbiol Biotechnol* **35**:373–381.
Guillemard L, Kaplaneris N, Ackermann L, and Johansson MJ (2021) Late-stage C–H functionalization offers new opportunities in drug discovery. *Nat Rev Chem* **5**:522–545.
Gumulya Y, Baek J-M, Wun S-J, Thomson RES, Harris KL, Hunter DJB, Behrendorff JBYH, Kulig J, Zheng S, Wu X, et al. (2018) Engineering highly functional thermostable proteins using ancestral sequence reconstruction. *Nat Catal* **1**:878–888.
Gumulya Y, Huang W, D'Cunha SA, Richards KE, Thomson RES, Hunter DJB, Baek J-M, Harris KL, Boden M, De Voss JJ, et al. (2019) Engineering thermostable CYP2D enzymes for biocatalysis using combinatorial libraries of ancestors for directed evolution (CLADE). *ChemCatChem* **11**:841–850.
Harris KL, Thomson RES, Gumulya Y, Foley G, Carrera-Pacheco SE, Syed P, Janosik T, Sandinge A-S, Andersson S, Jurva U, et al. (2022) Ancestral sequence reconstruction of a cytochrome P450 family involved in chemical defence reveals the functional evolution of a promiscuous, xenobiotic-metabolizing enzyme in vertebrates. *Mol Biol Evol* **39**:msac116.
Hilberath T, Windeln LM, Decembrino D, Le-Huu P, Bilsing FL, and Urlacher VB (2020) Two-step Screening for Identification of Drug-metabolizing Bacterial Cytochromes P450 with Diversified Selectivity. *ChemCatChem* **12**:1710–1719.
Hogg JA (1992) Steroids, the steroid community, and Upjohn in perspective: a profile of innovation. *Steroids* **57**:593–616.
Huang W, Johnston WA, Hayes MA, De Voss JJ, and Gillam EMJ (2007) A shuffled CYP2C library with a high degree of structural integrity and functional versatility. *Arch Biochem Biophys* **467**:193–205.
Humphreys WG (2022) Biosynthesis using cytochrome P450 enzymes: Focus on synthesis of drug metabolites. *Adv Pharmacol* **95**:177–194.
Humphreys WG and Unger SE (2006) Safety assessment of drug metabolites: Characterization of chemically stable metabolites. *Chem Res Toxicol* **19**:1564–1569.
Hunter DJB, Behrendorff JBYH, Johnston WA, Hayes PY, Huang W, Bonn B, Hayes MA, De Voss JJ, and Gillam EMJ (2011) Facile production of minor metabolites for drug development using a CYP3A shuffled library. *Metab Eng* **13**:682–693.
Ivanov YD, Taldaev A, Lisitsa AV, Ponomarenko EA, and Archakov AI (2022) Prediction of Monomeric and Dimeric Structures of CYP102A1 Using AlphaFold2 and AlphaFold Multimer and Assessment of Point Mutation Effect on the Efficiency of Intra- and Interprotein Electron Transfer. *Molecules* **27**:1386.
Johnston WA, Huang W, De Voss JJ, Hayes MA, and Gillam EMJ (2007) A shuffled CYP1A library shows both structural integrity and functional diversity. *Drug Metab Dispos* **35**:2177–2185.
Joo H, Lin Z, and Arnold FH (1999) Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. *Nature* **399**:670–673.
Josephy DP, Guengerich FP, and Miners JO (2005) "Phase I and Phase II" drug metabolism: terminology that we should phase out? *Drug Metab Rev* **37**:575–580.
Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, et al. (2021) Highly accurate protein structure prediction with AlphaFold. *Nature* **596**:583–589.
Jung ST, Lauchli R, and Arnold FH (2011) Cytochrome P450: taming a wild type enzyme. *Curr Opin Biotechnol* **22**:809–817.
Kang JY, Ryu SH, Park SH, Cha GS, Kim DH, Kim KH, Hong AW, Ahn T, Pan JG, Joung YH, et al. (2014) Chimeric cytochromes P450 engineered by domain swapping and random mutagenesis for producing human metabolites of drugs. *Biotechnol Bioeng* **111**:1313–1322.
Kinner A, Nerke P, Siedentop R, Steinmetz T, Classen T, Rosenthal K, Nett M, Pietruszka J, and Lütz S (2022) Recent advances in biocatalysis for drug synthesis. *Biomedicines* **10**:31.
Klenk JM, Nebel BA, Porter JL, Kulig JK, Hussain SA, Richter SM, Tavanti M, Turner NJ, Hayes MA, Hauer B, et al. (2017) The self-sufficient P450 RhF expressed in a whole cell system selectively catalyses the 5-hydroxylation of diclofenac. *Biotechnol J* **12**:8.
Kliewer SA (2003) The nuclear pregnane X receptor regulates xenobiotic detoxification. *J Nutr* **133**(7 Suppl)2444S–2447S.
Kola I and Landis J (2004) Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* **3**:711–715.
Kumar S (2010) Engineering cytochrome P450 biocatalysts for biotechnology, medicine and bioremediation. *Expert Opin Drug Metab Toxicol* **6**:115–131.
Kumar S, Chen CS, Waxman DJ, and Halpert JR (2005) Directed evolution of mammalian cytochrome P450 2B1: mutations outside of the active site enhance the metabolism of several

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substrates, including the anticancer prodrugs cyclophosphamide and ifosfamide. *J Biol Chem* **280**:19569–19575.
Kumar S, Liu H, and Halpert JR (2006a) Engineering of cytochrome P450 3A4 for enhanced peroxide-mediated substrate oxidation using directed evolution and site-directed mutagenesis. *Drug Metab Dispos* **34**:1958–1965.
Kumar S, Sun L, Liu H, Muralidhara BK, and Halpert JR (2006b) Engineering mammalian cytochrome P450 2B1 by directed evolution for enhanced catalytic tolerance to temperature and dimethyl sulfoxide. *Protein Eng Des Sel* **19**:547–554.
Lall MS, Bassyouni A, Bradow J, Brown M, Bundesmann M, Chen J, Ciszewski G, Hagen AE, Hyek D, Jenkinson S, et al. (2020) Late-Stage Lead Diversification Coupled with Quantitative Nuclear Magnetic Resonance Spectroscopy to Identify New Structure-Activity Relationship Vectors at Nanomole-Scale Synthesis: Application to Loratadine, a Human Histamine H_1 Receptor Inverse Agonist. *J Med Chem* **63**:7268–7292.
Le TK, Cha GS, Jang HH, Nguyen THH, Doan TTM, Lee YJ, Park KD, Shin Y, Kim DH, and Yun CH (2019) Regioselective hydroxylation pathway of tenatoprazole to produce human metabolites by Bacillus megaterium CYP102A1. *Process Biochem* **87**:95–104.
Le-Huu P, Petrović D, Strodel B, and Urlacher VB (2016) One-pot, two-step hydroxylation of the macrocyclic diterpenoid β-cembrenediol catalyzed by P450 BM3 mutants. *ChemCatChem* **8**:3755–3761.
Lee GY, Kim DH, Kim D, Ahn T, and Yun CH (2015) Functional characterization of steroid hydroxylase CYP106A1 derived from Bacillus megaterium. *Arch Pharm Res* **38**:98–107.
Lewis JC, Mantovani SM, Fu Y, Snow CD, Komor RS, Wong CH, and Arnold FH (2010) Combinatorial alanine substitution enables rapid optimization of cytochrome P450BM3 for selective hydroxylation of large substrates. *ChemBioChem* **11**:2502–2505.
Li J, Li F, King-Smith E, and Renata H (2020) Merging chemoenzymatic and radical-based retrosynthetic logic for rapid and modular synthesis of oxidized meroterpenoids. *Nat Chem* **12**:173–179.
Li S, Podust LM, and Sherman DH (2007a) Engineering and analysis of a self-sufficient biosynthetic cytochrome P450 PikC fused to the RhFRED reductase domain. *J Am Chem Soc* **129**:12940–12941.
Li W, Josephs JL, Skiles GL, and Humphreys WG (2008) Metabolite generation via microbial biotransformations with Actinomycetes: rapid screening for active strains and biosynthesis of important human metabolites of two development-stage compounds, 5-[(5S,9R)-9-(4-cyanophenyl)-3-(3,5-dichlorophenyl)-1-methyl-2,4-dioxo-1,3,7-triazaspiro[4.4]non7-yl-methyl]-3-thiophenecarboxylic acid (BMS-587101) and dasatinib. *Drug Metab Dispos* **36**:721–730.
Li Y, Drummond DA, Sawayama AM, Snow CD, Bloom JD, and Arnold FH (2007b) A diverse family of thermostable cytochrome P450s created by recombination of stabilizing fragments. *Nat Biotechnol* **25**:1051–1056.
Li Y and Wong LL (2019) Multi-functional oxidase activity of CYP102A1 (P450BM3) in the oxidation of quinolines and tetrahydroquinolines. *Angew Chem Int Ed Engl* **58**:9551–9555.
Loskot SA, Romney DK, Arnold FH, and Stoltz BM (2017) Enantioselective total synthesis of nigelladine A via late-stage C–H oxidation enabled by an engineered P450 enzyme. *J Am Chem Soc* **139**:10196–10199.
McLean KJ, Hans M, Meijrink B, van Scheppingen WB, Vollebregt A, Tee KL, van der Laan JM, Leys D, Munro AW, and van den Berg MA (2015) Single-step fermentative production of the cholesterol-lowering drug pravastatin via reprogramming of Penicillium chrysogenum. *Proc Natl Acad Sci USA* **112**:2847–2852.
Moore LB, Maglich JM, McKee DD, Wisely B, Willson TM, Kliewer SA, Lambert MH, and Moore JT (2002) Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol Endocrinol* **16**:977–986.
Munday SD, Dezvarei S, Lau ICK, and Bell SG (2017) Examination of Selectivity in the Oxidation of ortho- and meta-Disubstituted Benzenes by CYP102A1 (P450BM3) Variants. *ChemCatChem* **9**:2512–2522.
Murakami H, Yabusaki Y, Sakaki T, Shibata M, and Ohkawa H (1987) A genetically engineered P450 monooxygenase: construction of the functional fused enzyme between rat cytochrome P450c and NADPH-cytochrome P450 reductase. *DNA* **6**:189–197.
Narhi LO and Fulco AJ (1986) Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in Bacillus megaterium. *J Biol Chem* **261**:7160–7169.
Nguyen THH, Yeom SJ, and Yun CH (2021) Production of a human metabolite of atorvastatin by bacterial CYP102A1 peroxygenase. *Appl Sci (Basel)* **11**:13.
Nodate M, Kubota M, and Misawa N (2006) Functional expression system for cytochrome P450 genes using the reductase domain of self-sufficient P450RhF from Rhodococcus sp. NCIMB 9784. *Appl Microbiol Biotechnol* **71**:455–462.
O'Hanlon JA, Ren X, Morris M, Wong LL, and Robertson J (2017) Hydroxylation of anilides by engineered cytochrome $P450_{BM3}$. *Org Biomol Chem* **15**:8780–8787.
Obach RS, Walker GS, and Brodney MA (2016) Biosynthesis of fluorinated analogs of drugs using human cytochrome P450 enzymes followed by deoxyfluorination and quantitative nuclear magnetic resonance spectroscopy to improve metabolic stability. *Drug Metab Dispos* **44**:634–646.
Obach RS, Walker GS, Sharma R, Jenkinson S, Tran TP, and Stepan AF (2018) Lead diversification at the nanomole scale using liver microsomes and quantitative nuclear magnetic resonance spectroscopy: application to phosphodiesterase 2 inhibitors. *J Med Chem* **61**:3626–3640.
Ortega Ugalde S, Luirink RA, Geerke DP, Vermeulen NPE, Bitter W, and Commandeur JNM (2018) Engineering a self-sufficient Mycobacterium tuberculosis CYP130 by gene fusion with the reductase-domain of CYP102A1 from Bacillus megaterium. *J Inorg Biochem* **180**:47–53.
Otey CR, Bandara G, Lalonde J, Takahashi K, and Arnold FH (2006) Preparation of human metabolites of propranolol using laboratory-evolved bacterial cytochromes P450. *Biotechnol Bioeng* **93**:494–499.
Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, et al. (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* **496**:528–532.
Palmer-Brown W, Miranda-CasoLuengo R, Wolfe KH, Byrne KP, and Murphy CD (2019) The CYPome of the model xenobiotic-biotransforming fungus Cunninghamella elegans. *Sci Rep* **9**:9240.
Park SH, Kang JY, Kim DH, Ahn T, and Yun CH (2012) The Flavin-Containing Reductase Domain of Cytochrome P450 BM3 Acts as a Surrogate for Mammalian NADPH-P450 Reductase. *Biomol Ther (Seoul)* **20**:562–568.
Penman BW, Chen L, Gelboin HV, Gonzalez FJ, and Crespi CL (1994) Development of a human lymphoblastoid cell line constitutively expressing human CYP1A1 cDNA: substrate specificity with model substrates and promutagens. *Carcinogenesis* **15**:1931–1937.
Penman BW, Reece J, Smith T, Yang CS, Gelboin HV, Gonzalez FJ, and Crespi CL (1993) Characterization of a human cell line expressing high levels of cDNA-derived CYP2D6. *Pharmacogenetics* **3**:28–39.
Persson KP, Ekehed S, Otter C, Lutz ESM, McPheat J, Masimirembwa CM, and Andersson TB (2006) Evaluation of human liver slices and reporter gene assays as systems for predicting the cytochrome p450 induction potential of drugs in vivo in humans. *Pharm Res* **23**:56–69.
Quinn L, Dempsey R, Casey E, Kane A, and Murphy CD (2015) Production of drug metabolites by immobilised Cunninghamella elegans: from screening to scale up. *J Ind Microbiol Biotechnol* **42**:799–806.
Raucy JL (2003) Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. *Drug Metab Dispos* **31**:533–539.
Rea V, Falck D, Kool J, de Kanter FJJ, Commandeur JNM, Vermeulen NPE, Niessen WMA, and Honing M (2013) Combination of biotransformation by P450 BM3 mutants with on-line post-column bioaffinity and mass spectrometric profiling as a novel strategy to diversify and characterize p38 alpha kinase inhibitors. *MedChemComm* **4**:371–377.
Rentmeister A, Arnold FH, and Fasan R (2009) Chemo-enzymatic fluorination of unactivated organic compounds. *Nat Chem Biol* **5**:26–28.
Rentmeister A, Brown TR, Snow CD, Carbone MN, and Arnold FH (2011) Engineered bacterial mimics of human drug metabolizing enzyme CYP2C9. *ChemCatChem* **3**:1065–1071.
Richardson TH, Jung F, Griffin KJ, Wester M, Raucy JL, Kemper B, Bornheim LM, Hassett C, Omiecinski CJ, and Johnson EF (1995) A universal approach to the expression of human and rabbit cytochrome P450s of the 2C subfamily in Escherichia coli. *Arch Biochem Biophys* **323**:87–96.
Ringle M, Khatri Y, Zapp J, Hannemann F, and Bernhardt R (2013) Application of a new versatile electron transfer system for cytochrome P450-based Escherichia coli whole-cell bioconversions. *Appl Microbiol Biotechnol* **97**:7741–7754.
Romero PA, Krause A, and Arnold FH (2013) Navigating the protein fitness landscape with Gaussian processes. *Proc Natl Acad Sci USA* **110**:E193–E201.
Rudolf JD, Chang CY, Ma M, and Shen B (2017) Cytochromes P450 for natural product biosynthesis in Streptomyces: sequence, structure, and function. *Nat Prod Rep* **34**:1141–1172.
Rushmore TH, Reider PJ, Slaughter D, Assang C, and Shou M (2000) Bioreactor systems in drug metabolism: synthesis of cytochrome P450-generated metabolites. *Metab Eng* **2**:115–125.
Ryu SH, Park BY, Kim SY, Park SH, Jung HJ, Park M, Park KD, Ahn T, Kang HS, and Yun CH (2014) Regioselective hydroxylation of omeprazole enantiomers by bacterial CYP102A1 mutants. *Drug Metab Dispos* **42**:1493–1497.
Sabbadin F, Hyde R, Robin A, Hilgarth EM, Delenne M, Flitsch S, Turner N, Grogan G, and Bruce NC (2010) LICRED: a versatile drop-in vector for rapid generation of redox-self-sufficient cytochrome P450s. *ChemBioChem* **11**:987–994.
Sagadin T, Riehm J, Putkaradze N, Hutter MC, and Bernhardt R (2019) Novel approach to improve progesterone hydroxylation selectivity by CYP106A2 via rational design of adrenodoxin binding. *FEBS J* **286**:1240–1249.
Sagadin T, Riehm JL, Milhim M, Hutter MC, and Bernhardt R (2018) Binding modes of CYP106A2 redox partners determine differences in progesterone hydroxylation product patterns. *Commun Biol* **1**:99.
Sakaki T (2012) Practical application of cytochrome P450. *Biol Pharm Bull* **35**:844–849.
Sakaki T, Kominami S, Takemori S, Ohkawa H, Akiyoshi-Shibata M, and Yabusaki Y (1994) Kinetic studies on a genetically engineered fused enzyme between rat cytochrome P4501A1 and yeast NADPH-P450 reductase. *Biochemistry* **33**:4933–4939.
Salazar O, Cirino PC, and Arnold FH (2003) Thermostabilization of a cytochrome p450 peroxygenase. *ChemBioChem* **4**:891–893.
Salter R, Beshore DC, Colletti SL, Evans L, Gong Y, Helmy R, Liu Y, Maciolek CM, Martin G, Pajkovic N, et al. (2019) Microbial biotransformation - an important tool for the study of drug metabolism. *Xenobiotica* **49**:877–886.
Sandhu P, Baba T, and Guengerich FP (1993) Expression of modified cytochrome P450 2C10 (2C9) in Escherichia coli, purification, and reconstitution of catalytic activity. *Arch Biochem Biophys* **306**:443–450.
Saravanakumar A, Sadighi A, Ryu R, and Akhlaghi F (2019) Physicochemical properties, biotransformation, and transport pathways of established and newly approved medications: a systematic review of the top 200 most prescribed drugs vs. the FDA-approved drugs between 2005 and 2016. *Clin Pharmacokinet* **58**:1281–1294.
Sawayama AM, Chen MMY, Kulanthaivel P, Kuo MS, Hemmerle H, and Arnold FH (2009) A panel of cytochrome P450 BM3 variants to produce drug metabolites and diversify lead compounds. *Chemistry* **15**:11723–11729.
Schmitz D, Janocha S, Kiss FM, and Bernhardt R (2018) CYP106A2-A versatile biocatalyst with high potential for biotechnological production of selectively hydroxylated steroid and terpenoid compounds. *Biochim Biophys Acta Proteins Proteomics* **1866**:11–22.
Schmitz D, Zapp J, and Bernhardt R (2012) Hydroxylation of the triterpenoid dipterocarpol with CYP106A2 from Bacillus megaterium. *FEBS J* **279**:1663–1674.
Schmitz LM, Hageneier F, Rosenthal K, Busche T, Brandt D, Kalinowski J, and Lütz S (2021) Recombinant expression and characterization of novel P450s from Actinosynnema mirum. *Bioorg Med Chem* **42**:116241.
Schmitz LM, Schäper J, Rosenthal K, and Lütz S (2019) Accessing the Biocatalytic Potential for C−H-Activation by Targeted Genome Mining and Screening. *ChemCatChem* **11**:5766–5777.
Schroer K, Kittelmann M, and Lütz S (2010) Recombinant human cytochrome P450 monooxygenases for drug metabolite synthesis. *Biotechnol Bioeng* **106**:699–706.
Shet MS, Fisher CW, Arlotto MP, Shackleton CHL, Holmans PL, Martin-Wixtrom CA, Saeki Y, and Estabrook RW (1994) Purification and enzymatic properties of a recombinant fusion protein expressed in Escherichia coli containing the domains of bovine P450 17A and rat NADPH-P450 reductase. *Arch Biochem Biophys* **311**:402–417.
Shiota N, Nagasawa A, Sakaki T, Yabusaki Y, and Ohkawa H (1994) Herbicide-resistant tobacco plants expressing the fused enzyme between rat cytochrome P4501A1 (CYP1A1) and yeast NADPH-cytochrome P450 oxidoreductase. *Plant Physiol* **106**:17–23.
Simić S, Zukić E, Schmermund L, Faber K, Winkler CK, and Kroutil W (2022) Shortening synthetic routes to small molecule active pharmaceutical ingredients employing biocatalytic methods. *Chem Rev* **122**:1052–1126.
Stepan AF, Tran TP, Helal CJ, Brown MS, Chang C, O'Connor RE, De Vivo M, Doran SD, Fisher EL, Jenkinson S, et al. (2018) Late-stage microsomal oxidation reduces drug–drug

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interaction and identifies phosphodiesterase 2A inhibitor PF-06815189. *ACS Med Chem Lett* **9**:68–72.
Stresser DM, Turner SD, Blanchard AP, Miller VP, and Crespi CL (2002) Cytochrome P450 fluorometric substrates: identification of isoform-selective probes for rat CYP2D2 and human CYP3A4. *Drug Metab Dispos* **30**:845–852.
Strohmaier SJ, Baek JM, De Voss JJ, Jurva U, Andersson S, and Gillam EMJ (2020) An inexpensive, efficient alternative to NADPH to support catalysis by thermostable cytochrome P450 senzymes. *ChemCatChem* **12**:1750–1761.
Sueyoshi T and Negishi M (2001) Phenobarbital response elements of cytochrome P450 genes and nuclear receptors. *Annu Rev Pharmacol Toxicol* **41**:123–143.
Szczebara FM, Chandelier C, Villeret C, Masurel A, Bourot S, Duport C, Blanchard S, Groisillier A, Testet E, Costaglioli P, et al. (2003) Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nat Biotechnol* **21**:143–149.
Thistlethwaite S, Jeffreys LN, Girvan HM, McLean KJ, and Munro AW (2021) A promiscuous bacterial P450: The unparalleled diversity of BM3 in pharmaceutical metabolism. *Int J Mol Sci* **22**:23.
Trubetskoy OV, Gibson JR, and Marks BD (2005) Highly miniaturized formats for in vitro drug metabolism assays using vivid fluorescent substrates and recombinant human cytochrome P450 enzymes. *J Biomol Screen* **10**:56–66.
Vail RB, Homann MJ, Hanna I, and Zaks A (2005) Preparative synthesis of drug metabolites using human cytochrome P450s 3A4, 2C9 and 1A2 with NADPH-P450 reductase expressed in Escherichia coli. *J Ind Microbiol Biotechnol* **32**:67–74.
van der Meer JR and Belkin S (2010) Where microbiology meets microengineering: design and applications of reporter bacteria. *Nat Rev Microbiol* **8**:511–522.
Venkataraman H, Verkade-Vreeker MCA, Capoferri L, Geerke DP, Vermeulen NPE, and Commandeur JNM (2014) Application of engineered cytochrome P450 mutants as biocatalysts for the synthesis of benzylic and aromatic metabolites of fenamic acid NSAIDs. *Bioorg Med Chem* **22**:5613–5620.
Virus C, Lisurek M, Simgen B, Hannemann F, and Bernhardt R (2006) Function and engineering of the 15beta-hydroxylase CYP106A2. *Biochem Soc Trans* **34**:1215–1218.
Wang ZJ, Renata H, Peck NE, Farwell CC, Coelho PS, and Arnold FH (2014) Improved cyclopropanation activity of histidine-ligated cytochrome P450 enables the enantioselective formal synthesis of levomilnacipran. *Angew Chem Int Ed Engl* **53**:6810–6813.
Watanabe I, Nara F, and Serizawa N (1995) Cloning, characterization and expression of the gene encoding cytochrome P-450sca-2 from Streptomyces carbophilus involved in production of pravastatin, a specific HMG-CoA reductase inhibitor. *Gene* **163**:81–85.
Weis R, Winkler M, Schittmayer M, Kambourakis S, Vink M, Rozzell JD, and Glieder A (2009) A diversified library of bacterial and fungal bifunctional cytochrome P450 enzymes for drug metabolite synthesis. *Adv Synth Catal* **351**:2140–2146.
Wong TS, Arnold FH, and Schwaneberg U (2004) Laboratory evolution of cytochrome p450 BM-3 monooxygenase for organic cosolvents. *Biotechnol Bioeng* **85**:351–358.
Xue Y and Sherman DH (2001) Biosynthesis and combinatorial biosynthesis of pikromycin-related macrolides in Streptomyces venezuelae. *Metab Eng* **3**:15–26.
Yang Y and Arnold FH (2021) Navigating the unnatural reaction space: directed evolution of heme proteins for selective carbene and nitrene transfer. *Acc Chem Res* **54**:1209–1225.
Zhang K, El Damaty S, and Fasan R (2011) P450 fingerprinting method for rapid discovery of terpene hydroxylating P450 catalysts with diversified regioselectivity. *J Am Chem Soc* **133**:3242–3245.
Zuo R, Zhang Y, Jiang C, Hackett JC, Loria R, Bruner SD, and Ding Y (2017) Engineered P450 biocatalysts show improved activity and regio-promiscuity in aromatic nitration. *Sci Rep* **7**:842.

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