Minireview for special section entitled "Drug metabolism: a half-century plus of progress, continued need, and new opportunities"

Species specificity and selection of models for drug oxidations mediated by polymorphic human enzymes

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Running Title Page

Running title: Roles of P450 and FMO enzymes

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Abbreviations: FMO, flavin-containing monooxygenase; GSH, glutathione; P450, cytochrome P450.

Abstract

Many drug oxygenations are mainly mediated by polymorphic cytochromes P450 (P450s) and also by flavin-containing monooxygenases (FMOs). More than 50 years of research on P450/FMO-mediated drug oxygenations have clarified their catalytic roles. The natural product coumarin causes hepatotoxicity in rats via the reactive coumarin 3,4-epoxide, a reaction catalyzed by P450 1A2; however, coumarin undergoes rapid 7-hydroxylation by polymorphic P450 2A6 in humans. The primary oxidation product of the teratogen thalidomide in rats is deactivated 5′-hydroxythalidomide plus sulfate and glucuronide conjugates; however, similar 5′-hydroxythalidomide and 5-hydroxythalidomide are formed in rabbits *in vivo*. Thalidomide causes human P450 3A enzyme induction in liver (and placenta) and is also activated *in vitro* and *in vivo* by P450 3A through the primary human metabolite 5-hydroxythalidomide (leading to conjugation with glutathione/nonspecific proteins). Species differences exist in terms of drug metabolism in rodents and humans, and such differences can be very important when determining the contributions of individual enzymes. The approaches used for investigating the roles of human P450 and FMO enzymes in understanding drug oxidations and clinical therapy have not yet reached maturity and still require further development.

Significance Statement

Drug oxidations in animals and humans mediated by P450s and FMOs are important for understanding the pharmacological properties of drugs, such as the species-dependent teratogenicity of the reactive metabolites of thalidomide and the metabolism of food-derived odorous trimethylamine to non-odorous (but proatherogenic) trimethylamine *N*-oxide. Recognized differences exist in terms of drug metabolism between rodents, non-human primates, and humans, and such differences are important when determining individual liver enzyme contributions with substrates in *in vitro* and *in vivo* systems.

Introduction

Many drug metabolism reactions are mediated by cytochromes P450 (P450s, EC 1.14.14.1). The first article on P450 was published in 1962 (Omura and Sato, 1962). In 2022, we celebrate 60 years of research on the many forms of P450 that exist in animals and humans. P450 research has come a long way from early studies in the 1960s to the current era of personalized medicine in individual patients. Drug oxidation reactions are also catalyzed by several non-P450 enzymes in humans (Rendic and Guengerich, 2015, Rendic et al., 2022), e.g., flavin-containing monooxygenases (FMOs) [previous referred to as liver microsomal mixed-function amine oxidases (Masters and Ziegler, 1971) or flavin adenine dinucleotide (FAD)-containing monooxygenase (Poulsen and Ziegler, 1979)]. Both P450s and FMOs require NADPH and oxygen for the oxygenation of substrates (Ziegler, 1988). FMOs (EC 1.14.13.8) are a family of NADPH-dependent enzymes that oxygenate a range of heteroatom-containing substances (Krueger and Williams, 2005; Cashman and Zhang, 2006; Phillips and Shephard, 2020), including benzydamine and trimethylamine. Many drug-drug interactions are pharmacokinetic in nature and caused by modifications of drug metabolism, generally through enzyme inhibition or enzyme induction (Rendic and Guengerich, 2010). *In vitro* determinations of the roles of the enzymes primarily responsible for specific drug oxidation reactions generally use human liver preparations and constitute a key method for estimating and understanding a variety of drug interactions (Brown et al., 2005; Shimizu et al., 2021b). To address the inhibitory potential of a drug, it is important to determine inhibition parameters in *in vitro* systems (Ito et al., 1998) and to elucidate the individual enzyme contributions *in vitro* (i.e., fraction metabolized by each enzyme, f_m) to substrate oxidations in livers (Youdim et al., 2008).

Rodents are often used as animal models in drug development; however, it is well known that

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species differences exist in terms of drug metabolism between rodent and human cytochrome P450s (Kazuki et al., 2016; Yamazaki et al., 2016b). The relevance and limitations of animal models used in non-clinical safety assessments of investigational products should be carefully considered. For example, coumarin (**Fig. 1A**), a naturally occurring food flavoring (Lake, 1999; Abraham et al., 2010), has been reported to cause hepatotoxicity in rats via the reactive metabolite coumarin 3,4-epoxide, which is important because coumarin is a structural base for a family of anticoagulant rodenticides (Vassallo et al., 2003; Rietjens et al., 2010). Coumarin has also been used as a medicine in humans and has a tolerable daily intake of 0.1 mg/kg body weight (Abraham et al., 2010). Apparent species differences should be noted between the rapid coumarin 7-hydroxylation seen in humans (Yamazaki et al., 1994; Kiyotani et al., 2003) and the slow coumarin 7-hydroxylation in rats (Murayama and Yamazaki, 2021). Thalidomide is another substrate with notable species differences in terms of metabolism. Thalidomide is teratogenic in non-human primates and rabbits (Calabrese and Resztak, 1998) but not in rodents (Kim and Scialli, 2011). Among a range of animal species, thalidomide is differentially oxidized to either 5-hydroxythalidomide or 5′-hydroxythalidomide (Nishiyama et al., 2015) (**Fig. 1B**).

Applications of P450 research in drug metabolism studies continue to expand and develop. Further, interest has also been focused on a member of the flavin monooxygenase monooxygenase (FMO) family, namely FMO3, because it is responsible for *N*- and *S*-oxygenations of various drugs and recently FMO5 has also been shown to catalyze Bäyer-Villiger reaction leading to the formation of esters (Rendic et al., 2022). FMO3 is of particular interest because genetic polymorphism in the human *FMO3* gene can affect its catalytic efficiency with respect to typical medicinal substrates (Catucci et al., 2012; Nakamaru et al., 2014) and thereby lead to drug interactions (Yamazaki and Shimizu, 2013). Phenotype–gene analyses have revealed impaired FMO3 variants associated with the metabolic disorder trimethylaminuria, a condition that causes an unpleasant fishy smell

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(Humbert et al., 1970). FMO3 extensively catalyzes trimethylamine *N*-oxygenation to produce non-odorous (Shephard et al., 2012) but proatherogenic (Hartiala et al., 2014) trimethylamine *N*-oxide.

Research on P450s in non-human primates (Uno et al., 2022c) and in animal models with introduced human *P450 (CYP)* genes or transplanted with human liver cells (Uehara et al., 2022) has extended into different fields, from molecular to *in vivo* research. The present minireview summarizes recent findings on polymorphic P450- and FMO-mediated oxidation and bioactivation reactions. This research has furthered our understanding of the pharmacological properties of drugs such as teratogenic thalidomide and food-derived trimethylamine and provides important fundamental information for future investigations of species specificity and selection of models for drug oxidations mediated by polymorphic human enzymes.

Brief historical perspective

The extensive contributions of scientists throughout the world to the field of P450 research over past six decades is noteworthy. The success of P450 research has had implications in disciplines such as drug-drug interactions and pharmacogenetics.

Because thalidomide was previously considered to undergo very little metabolism by the P450 system, drug interactions between thalidomide and hormonal contraceptives have been considered unlikely (Trapnell et al., 1998; Teo et al., 2000). However, this was recently shown not to be the case. The ubiquitously expressed E3 ligase protein cereblon is reportedly a common direct protein target explaining the antiproliferative activities of thalidomide, lenalidomide, and pomalidomide (Ito et al., 2010; Lopez-Girona et al., 2012); however, this target also exists in rodents, which show

no thalidomide teratogenicity (Kim and Scialli, 2011). Its teratogenic effects notwithstanding, thalidomide was subsequently approved for the clinical treatment of multiple myeloma (Palumbo et al., 2008; Nakamura et al., 2013), at which point the effects of thalidomide on P450 activities were investigated again in more detail. Thalidomide enhanced P450 3A4/5-dependent midazolam hydroxylation and cyclosporine A clearance at clinically relevant concentrations (Okada et al., 2009) and induced human P450 3A enzymes through the pregnane X receptor (PXR) (Murayama et al., 2014). Thalidomide was shown to be hydroxylated by human liver microsomal P450 3A4/5 (Chowdhury et al., 2010). Thalidomide is metabolized by two major pathways (**Fig. 1B**) by various P450s, as previously investigated (Ando et al., 2002; Lu et al., 2004). Furthermore, human P450 3A4 and 3A5 also oxidize thalidomide to form 5-hydroxy and dihydroxy metabolites (Chowdhury et al., 2010; Yamazaki et al., 2011; Yamazaki et al., 2012). The secondary oxidation of 5-hydoxythalidomide is faster than the primary thalidomide 5-hydoxylation mediated by recombinant human P450 3A4/5 in *in vitro* systems (Chowdhury et al., 2014; Yamazaki et al., 2016a). The second oxidation step in the human P450 3A4 pathway generates a reactive intermediate, possibly an arene oxide, as was suggested earlier (Gordon et al., 1981). Such intermediates can be trapped by glutathione (GSH) to give GSH adducts (Yamazaki et al., 2016a) that have been confirmed *in vivo* in humanized-liver mouse models after oral doses of 100 mg/kg thalidomide (Yamazaki et al., 2013). Although five control mice had no apparent adverse effects after an oral dose of 270 mg/kg (~1 mmol) thalidomide/kg, an oral LD_{50} of 270 mg/kg was observed for humanized-liver mice (two of five animals died after 2 days) (Miura et al., 2021b). Differences in species susceptibility to thalidomide teratogenicity may result from differences in the primary biotransformation of the compound by drug-metabolizing enzymes.

One historical perspective on the catalytic mechanism of P450 involves electron transport (**Fig. 2A**), mediated by a multicomponent monooxygenase system based on NADPH (Yamazaki et al.,

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1996; Yamazaki et al., 2001). Microsomal P450s receive electrons from NADPH-cytochrome P450 reductase. The catalytic cycle of P450 involves the activation of molecular oxygen to a reactive form (**Fig. 2A**). In contrast, FMOs are characterized by a stable 4a-flavin hydroperoxide intermediate capable of oxygenating both nucleophiles and electrophiles in its catalytic cycle (**Fig. 2B**), formed even in the absence of a substrate (Jones and Ballou, 1986). The initiation of drug oxidation reactions is generally done with the addition of NADPH when investigating typical P450-mediated reactions. However, with FMOs, the absence of NADPH in the preincubation period decreases FMO-mediated drug oxygenation activities (Taniguchi-Takizawa et al., 2015). NADPH stabilizes of FAD in FMO enzymes, which have a higher pH optimum (8.4) (Nagashima et al., 2009) in the FMO catalytic cycle.

Historical findings in P450 research should not be neglected when planning new studies. For example, human drug-metabolizing P450s (in phospholipid membranes) require an additional protein, NADPH-cytochrome P450 reductase, in an environment with appropriate ionic strength to catalyze aerobic reactions in the presence of NADPH (Yamazaki et al., 1995). Because benzydamine *N*-oxygenation and *N*-demethylation are mediated by FMO1/3 and P450 2D6/3A4, respectively, benzydamine can serve as a suitable probe substrate for both FMOs and P450s for human drug interaction studies. Dimethyl sulfoxide is a widely used organic solvent in biochemical analysis (Hickman et al., 1998; Busby et al., 1999), but a high concentration of dimethyl sulfoxide (more than 4%, v/v) prevents the mutagenic activation of some procarcinogens by P450 enzymes in preincubation procedures, thereby indicating that dimethyl sulfoxide can interfere with P450 metabolism (Mori et al., 1985). P450 2E1 reactions are especially prone to inhibition by solvents, in that most of these are substrates (and a concentration of 1% (v/v) is approximately 10 mM). Low concentrations of dimethyl sulfoxide (0.1% and 0.5%, v/v) inhibited FMO3 enzyme activities in human liver microsomes and recombinant systems, respectively (Taniguchi-Takizawa et al., 2015).

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Dimethyl sulfoxide was suggested to be an FMO substrate in humans as a result of *in vivo* urinary excretion (of dimethyl sulfone (Hucker et al., 1967)) and of its inhibitory effects on *in vitro* FMO3-dependent oxygenations in human liver microsomes (Taniguchi-Takizawa et al., 2015). The contribution of FMO to the metabolic clearance of new drug candidates could be underestimated under some current high-throughput experimental conditions that employ dimethyl sulfoxide for use with P450 enzymes.

FMOs constitute an enzyme system complementary to the cytochrome P450 (P450) enzyme family, which should be taken into account during drug development (Cashman, 2008). Because FMOs are not readily induced or inhibited, it has been suggested that there may be advantages in designing drugs that are partly metabolized by FMOs, i.e., drugs that are not metabolized exclusively by P450s (Krueger and Williams, 2005; Cashman, 2008). However, the contributions of FMOs to the metabolic elimination of new drug candidates may be underestimated under the usual experimental and physiological conditions of pH 7.4, which are more suited to P450 enzymes initiated by the addition of NADPH to reaction mixtures containing drug candidates with classic dissociation constants (pK_a base) of >8.4 (Taniguchi-Takizawa et al., 2021)

Utility of non-human primates in drug development because of their physiological and genetic similarities to humans has been separately reviewed with information on the major multiple forms of drug-metabolizing P450 enzymes and FMO3 having generally similar substrate selectivities to those of human P450s and FMO3 (Uno et al., 2016; Uno et al., 2018; Uehara et al., 2020; Uno et al., 2022c).

Some key recent advances

Thalidomide causes human P450 3A enzyme induction/cooperativity in liver (and placenta) and is also activated by P450 3A through its human primary metabolite (5-hydroxy thalidomide) *in vitro* and *in vivo* (Murayama et al., 2017). Screening for immunomodulatory drugs that possess less teratogenicity/toxicity is a strategic pathway toward the development of new anti-cancer drugs. Lenalidomide (Kim and Schmidt-Wolf, 2015; Chen et al., 2017), a second-generation immunomodulatory analog of thalidomide in which one keto group has been removed (plus the addition of an amino group) has more potent anti-tumor activity than thalidomide (Zeldis et al., 2013). Thalidomide is well distributed to the brain in rodents (with a brain/plasma ratio of 0.89) (Huang et al., 2005) and to cerebrospinal fluid in non-human primates (with a cerebrospinal fluid/plasma ratio of 0.42) (Muscal et al., 2012). In contrast, lenalidomide distribution to the brain in rodents is low (Rozewski et al., 2012). These facts notwithstanding, a clinical response to lenalidomide has been observed in cerebrospinal tumors (Warren et al., 2011). In contrast to the metabolites identified for the first-generation drug thalidomide and the second-generation drug pomalidomide (Shimizu et al., 2017), no significant hydroxylated metabolites of lenalidomide (Murayama et al., 2018a) have been detected in *in vitro* or *in vivo* assays (Chowdhury et al., 2014).

Non-human primates, cynomolgus monkeys and common marmosets, are generally expected to show similar drug-metabolizing properties to humans. Reported areas under the plasma concentration curves after oral administrations of the five typical human P450 cocktail probes (caffeine, warfarin, omeprazole, metoprolol, and midazolam) (Turpault et al., 2009) were generally similar among humans, monkeys, and marmosets after normalization by doses per body weights (Koyanagi et al., 2015; Toda et al., 2018). However, caffeine is metabolized by human P450 1A2 to paraxanthine (N_3-demethylation) (Turpault et al., 2009), by monkey P450 2C9 to theophylline

(N_7-demethylation) (Utoh et al., 2016), and by marmoset P450 1A2 and 3A4 to N_3-demethylated and 8-hydroxylated metabolites, respectively (Uehara et al., 2016). Some attention should be paid to the differences even in non-human primates and humans.

The fraction of victim drug metabolized by an enzyme (f_m) *in vitro* is one of the parameters used to estimate *in vivo* drug–drug interactions. The f_m value is usually determined by comparing the intrinsic clearances (CL_{int}) of victim drugs for selectively inhibited and uninhibited hepatic microsomes *in vitro*. Pooled human liver microsomes in which one P450 enzyme has been selectively inactivated by a suitable mechanism-based inhibitor using preincubation methods has been commercially introduced by the pharmaceutical industry (Murayama et al., 2018b; Parmentier et al., 2019), effectively yielding *in vitro* "knock-outs." Furafylline, a 1,3,8-trisubstituted xanthine, results in the loss of human P450 1A2 activity (Racha *et al.*, 1998). Coumarin oxidations investigated were 3,4-epoxidation, ultimately leading to the formation of *o*-hydroxyphenylacetic acid, and 7-hydroxylation in humanized-liver mice treated with furafylline, a mechanism-based inhibitor of P450 1A2 (Racha *et al.*, 1998). The strategy of using suppressed liver P450 1A2 activities in humanized-liver mice was introduced to elucidate the roles of the enzymes in the *in vivo* activation of coumarin to its primary reactive 3,4-epoxide (Miura et al., 2021a). Using the mechanism-based inactivation of P450 2C9 by tienilic acid (Hutzler et al., 2009), we could precisely assess the oxidation activities of selectively inactivated human liver microsomes to determine the *in vitro* contributions of P450 2C9 and 3A enzymes to the metabolism of diclofenac at different ionic strengths of incubation buffers (Miura et al., 2020). We also compared the roles of P450 2C9 and 3A in metabolite formation from *S*-warfarin *in vivo* using humanized-liver mice. A modified strategy using the selective *in vivo* inactivation of liver P450 2C9 in humanized-liver mice was developed to provide important information on the differences between *in vitro* and *in vivo* drug metabolite formation from diclofenac, a typical P450 2C9 probe substrate used in *in vitro*

experiments (Miura et al., 2020).

Traditional urinary screening tests for the metabolic capacity of FMO3 in individual humans have also been carried out (Yamazaki and Shimizu, 2007; Yamazaki and Shimizu, 2013). Some updated findings for the metabolic capacity of FMO3 screened on the basis of urine testing of trimethylamine *N*-oxide levels are shown in **Fig. 3.** The variability in *in vivo* activities was primarily attributed to FMO3 variants. Increasing numbers of single-nucleotide substitutions of the human *FMO3* gene are being recorded in mega-databases (Shimizu et al., 2019). A series of reliable FMO3 genotyping confirmation methods has been assembled and developed for ~40 impaired FMO3 variants (Shimizu et al., 2021a). This series of systems should facilitate the easy detection in the clinical setting of FMO3 variants in subjects susceptible to low drug clearances or drug interactions possibly caused by impaired FMO3 function.

Current challenges and knowledge gaps

The success of research on drug metabolizing enzymes and its application in drug discovery and development has been remarkable. Because most of the major recombinant human drug-metabolizing P450s are now commercially available as enzyme sources, it might be thought that adequate procedures for determining the roles of human P450 enzymes in new drug oxidations have been developed and have reached maturity, but this is not necessarily the case. For example, the P450 forms responsible for hydroxylation of the antihistamine drug ebastine could not be previously identified using the recombinant P450 enzymes commercially available. Using more traditional inhibition studies, human intestinal P450 2J2 and 4F12 were found to metabolize endogenous substrates and also to be involved in the metabolism of ebastine (Hashizume et al., 2002). Information from package inserts for some clinically important medicines, such as the

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antibiotic linezolid, indicates that no roles of major human P450 enzymes have been identified in liver microsomal drug oxidations, although those studies did not rule out the role of currently uncharacterized P450s (Wynalda et al., 2000). Similarly, recent findings from *in vitro* assays for linezolid have shown contribution of P450 2J2 (Obach, 2022), an enzyme not generally included in testing at the preclinical drug development stage.

The relative levels of P450 expressions in livers show similarities in humans (Shimada et al., 1994), monkeys (Uehara et al., 2011), and marmosets (Shimizu et al., 2014). Although it should be noted that expression levels are not always comparable between mRNA and protein levels, P450 3A expression is most abundant and P450 2C expression is moderately abundant in human and monkey livers in terms of protein levels and in marmoset livers in terms of mRNA levels. In addition, monkey P450 2As (Uehara et al., 2011) and marmoset P450 2Ds (Shimizu et al., 2014) are more abundant, but monkey and marmoset P450 1As are less abundant compared with their human P450 counterparts (Shimada et al., 1994).

Most major drug metabolizing enzymes are known to be polymorphic, or strictly speaking "variant" (polymorphism is defined as an incidence ≥1%) . Although the molecular structure of the P450 2C9.2 variant has been solved using X-ray crystallography (Parikh et al., 2020), it is not be realistic to evaluate the functional activity of minor variant forms of P450 enzymes in *in vitro* systems, only some major ones (e.g. 2C9.2 and 2C9.3). Examples of application of knowledge of P450 variants to clinical practice are still few. Digital simulations using physiologically based pharmacokinetic (PBPK) models may be useful additions to the estimations of drug concentrations in human blood in advance of pre-clinical and clinical studies (Ota et al., 2019; Notsu et al., 2020). Pharmacokinetic modeling of *in vivo* drug concentrations could serve as a useful guide when assessing and setting treatments for clinical patients with polymorphic variations of major drug-metabolizing enzymes

(Ota et al., 2019; Notsu et al., 2020). A major problem is that coding-region variants have different effects with individual substrates (Yamazaki et al., 1998).

Perspective on future directions

The *in vitro* methods used to assess the inhibitory potential of drugs and the individual contributions of enzymes to substrate oxidation in human liver vary in different laboratories. Consequently, more standardized methods are required. The application of human liver microsomes with specifically inactivated P450 enzymes can provide information about the development of inactivated-P450 human hepatocytes before transplantation into immunodeficient mice, thereby creating a standard animal model for investigating the contributions of specific P450s in humans (Miura et al., 2020; Miura et al., 2021a). However, these will not be stable mouse lines, in that they are not genetically altered.

To facilitate extrapolation of the *in vivo* pharmacokinetics of drugs from data obtained in non-human primates to humans, polymorphic drug oxygenation enzymes were investigated in monkeys and marmosets (Uno et al., 2016; Uno et al., 2018; Uehara et al., 2020; Uno et al., 2022c). Veterinary applications for dogs, pigs, and other animals can be anticipated not only for human model animals but also for pets and domestic farm animals as clinical targets with human medicines. For instance, comparisons of human enzymes with those of animal species used in preclinical studies have shown that dogs exhibited hepatic metabolic activities toward human P450 3A substrates erythromycin and nifedipine at levels similar to those of humans, whereas cynomolgus macaques, guinea pigs, and rat enzymes showed substantially greater activities (Shimada et al., 1997). A newly identified dog P450 3A98, a testosterone 6β- and estradiol 16α-hydroxylase, was abundantly expressed in small intestine and is likely the major P450 3A enzyme in small intestine in

combination with dog liver-specific P450 3A12 (Uno et al., 2022a). Roles of new dog P450 2J2, cat P450 2J2, and pig P450 2J33, 2J34, and 2J35 were recently investigated (Uno et al., 2022b). Liver microsomal FMO1/3 were functionally characterized in monkeys, marmosets, dogs, minipigs, and rats *in vivo* and/or *in vitro* (Yamazaki et al., 2014; Taniguchi-Takizawa et al., 2015; Uehara et al., 2017; Shimizu et al., 2020).

Because human P450 2A6 mediates nicotine oxidation and also the metabolic activation of tobacco-related procarcinogens (Yamazaki et al., 1992; Shimada et al., 2016), it has been postulated that polymorphic P450 2A6 is involved in tumor initiation (Fujieda et al., 2004). Human biomonitoring studies have suggested that heterocyclic amines and *N*-nitrosamines in meat-derived products may play important roles in colorectal carcinogenesis (Wu et al., 2006). A chemopreventive effect of low-dose aspirin against colorectal tumors was previously found in patients, especially in non-smokers, who had been treated for colorectal adenomas or adenocarcinomas or who had more than 100 adenomatous polyps (Ishikawa et al., 2014; Ishikawa et al., 2021). P450 2A6 is not the enzyme responsible for aspirin metabolism, in which the major human P450 involved in both 3- and 5-hydroxylation of salicylic acid is P450 2E1 (Bojic et al., 2015). However, the *CYP2A6* wild-type allele could be a candidate biomarker for reduced chemopreventive effects of daily aspirin, due to unknown mechanisms, and this hypothesis could be applicable to future personalized treatments (Yamazaki et al., 2021). The methods used to investigate the roles of human P450 enzymes in drug oxidations and clinical treatments have not yet reached maturity and require further development.

Conclusions:

This minireview was developed for the special section of drug metabolism: a half-century plus of

progress, continued need, and new opportunities. P450 research has come a long way from early studies in the 1960s to the current era of personalized medicine (especially with respect to drug metabolism mediated by polymorphic P450s) in individual patients including children and the elderly. P450s have been the focus of attention in many pharmaceutical companies and industrial applications. Research on P450s using non-human primates and animal models with introduced human *P450 (CYP)* genes or with transplanted human hepatocytes has attracted biochemists, pharmacologists, and toxicologists and the scope has broadened from molecular research to *in vivo* investigations. P450/FMO research developed from early studies with animal liver to now include personalized medicine informed by studies of polymorphic P450s/FMO3 in individual patients, including children. The success of drug oxidation research has had implications in fields such as herbal medicine, drug-drug interactions, pharmacogenetics, and physiologically based pharmacokinetic modeling. The extensive contributions of scientists throughout the world to the field of drug oxygenation research over a half-century plus should be recognized. The techniques used for investigating the roles of human P450/FMO enzymes in drug oxidations and clinical treatments have not yet reached maturity and require further development. We encourage more young scientists to join the important and exciting world of basic and advanced research on drug oxidation and to both become familiar with traditional techniques and strive to develop new methods and approaches.

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Authorship contribution

Participated in research design: Yamazaki

Conducted experiments: N/A

Contributed new reagents or analytic tools: N/A

Performed data interpretation: Yamazaki, Shimizu

Contributed to the writing of the manuscript: Yamazaki

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Footnotes

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Declaration of Interest

The authors declare that they have no conflicts of interest with respect to the contents of this article.

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Legends for figures

Fig. 1. Oxidation of coumarin to *o*-hydroxyphenylacetic acid and 7-hydroxycoumarin (**A**) (Miura et al., 2021a) and of thalidomide to 5′-hydroxythalidomide and 5-hydroxythalidomide (**B**) (Kuwagata et al., 2021; Miura et al., 2021b).

Fig. 2. Catalytic cycles of P450s (**A**) and FMOs (**B**). As indicated in the catalytic cycle, ferrous P450 can bind substrate (Yamazaki et al., 1996; Yamazaki et al., 2001), whereas for FMOs, only in the third step is the flavin hydroperoxide intermediate capable of oxygenating substrates (Ziegler, 2002).

Fig. 3. Metabolic capacity of FMO3 screened on the basis of urine testing of trimethylamine *N*-oxide levels. Japanese volunteers with self-reported malodor who responded to an Internet article for screening were investigated, and the data were updated from those of our previous study (Yamazaki and Shimizu, 2007). The frequency of individuals with <40% of wild-type FMO3 metabolic capacity in the self-reported population was 1.5% in this Japanese cohort.

Fig. 1
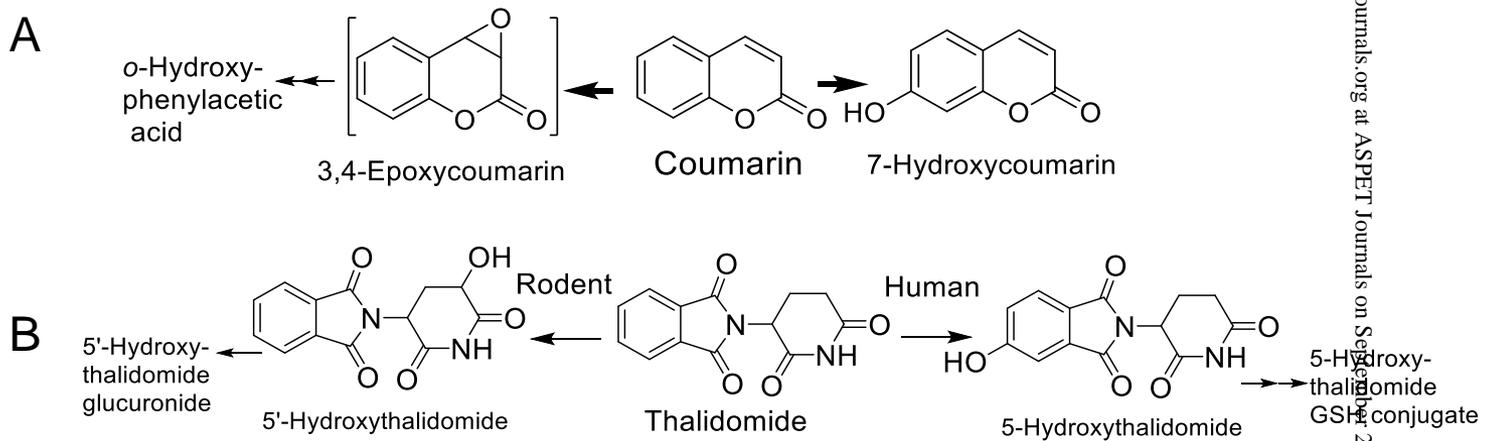
A

o-Hydroxy-
phenylacetic
acid

3,4-Epoxycoumarin   Coumarin   7-Hydroxycoumarin

B

5'-Hydroxy-
thalidomide
glucuronide

5'-Hydroxythalidomide   Rodent   Thalidomide   Human   5-Hydroxythalidomide   5-Hydroxy-
thalidomide
GSH conjugate

Fig. 2

(A)

P450

ROH → Fe^{3+} ← RH

(6)   (1)

Fe^{3+}ROH   Fe^{3+} RH

H$_2$O   (5)   (2) | e$^-$

H$^+$ Fe^{2+}-O$_2^-$RH   Fe^{2+} RH

(4)   (3)

e$^-$ O$_2$

Fe^{2+}-O$_2$RH

(B)

FMO

NADP$^+$ (FAD)   NADPH + H$^+$

NADP$^+$ (6)   (1)   NADP$^+$
(FAD)   (FADH$_2$)

H$_2$O   (5)   (2)   O$_2$
R$_3$N→O

NADP$^+$ NADP$^+$
(FADHOH)   (FADHO$_2$H)
R$_3$N→O   (4)   (3)   R$_3$N

NADP$^+$
(FADHO$_2$H)
R$_3$N

Fig.3

(A) Self-reported malodor

(B) Healthy volunteers

n = 5,416
Mean, 92%
Median, 96%

n = 151
Mean, 97%
Median, 98%

Metabolic capacity of FMO3
(% of urinary trimethylamine *N*-oxide/trimethylamine plus its *N*-oxide)