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# Oxygen Surrogate Systems for Supporting Human Drug-Metabolizing Cytochrome P450 Enzymes

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## ABSTRACT

**Oxygen surrogates (OSs) have been used to support cytochrome P450 (P450) enzymes for diverse purposes in drug metabolism research, including reaction phenotyping, mechanistic and inhibition studies, studies of redox partner interactions, and to avoid the need for NADPH or a redox partner. They also have been used in engineering P450s for more cost-effective, NADPH-independent biocatalysis. However, despite their broad application, little is known of the preference of individual P450s for different OSs or the substrate dependence of OS-supported activity. Furthermore, the biocatalytic potential of OSs other than cumene hydroperoxide (CuOOH) and hydrogen peroxide ($H_2O_2$) is yet to be explored. Here, we investigated the ability of the major human drug-metabolizing P450s, namely CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP1A2, to use the following OSs: $H_2O_2$, tert-butyl hydroperoxide (*tert*-BuOOH), CuOOH, (diacetoxyiodo)benzene, and bis(trifluoroacetoxy)iodobenzene. Overall, CuOOH and *tert*-BuOOH were found to be the most effective at supporting these P450s. However, the ability of P450s to be supported by OSs effectively was also found to be highly dependent on the substrate used. This suggests that the choice of OS should be tailored to both the P450 and the substrate under investigation, underscoring the need to employ screening methods that reflect the activity toward the substrate of interest to the end application.**

## SIGNIFICANCE STATEMENT

**Cytochrome P450 (P450) enzymes can be supported by different oxygen surrogates (OSs), avoiding the need for a redox partner and costly NADPH. However, few data exist comparing relative activity with different OSs and substrates. This study shows that the choice of OS used to support the major drug-metabolizing P450s influences their relative activity and regioselectivity in a substrate-specific fashion and provides a model for the more efficient use of P450s for metabolite biosynthesis.**

## Introduction

Cytochrome P450 (P450) enzymes are a superfamily of heme-containing monooxygenases capable of mediating a variety of chemically challenging reactions on a diverse set of substrates (Guengerich, 2001, 2005; Hrycay and Bandiera, 2008, 2015; de Montellano, 2015). The human xenobiotic-metabolizing P450 enzymes are collectively responsible for 80%–90% of human oxidative metabolism of xenobiotics, with the major contributors being CYP3A4 (30.2%), CYP2D6 (20%), CYP1A2 (8.9%), CYP2C19 (6.8%), and CYP2C9 (12.8%) (Zanger and Schwab, 2013). Therefore, these enzymes have been the focus of efforts to determine which forms metabolize new drugs (reaction phenotyping) and the prediction of drug-drug interactions (Zanger and Schwab, 2013). Furthermore, because of their ability to act on a remarkably broad set of substrates in an often highly stereo-, chemo-, and regioselective manner, human drug-metabolizing P450s have been used as biocatalysts for the production of authentic drug metabolites (Vail et al., 2005). More recently, they have been investigated for late-stage functionalization of existing drug scaffolds (Obach et al., 2018; Fessner, 2019) and other fine chemicals. However, their direct application in metabolite production and late-stage functionalization is hindered by their often low catalytic activity and the need for a redox partner, typically cytochrome P450 reductase (CPR), and an expensive cofactor (NADPH), limiting the economic feasibility of P450-dependent processes (Girhard et al., 2015). While whole-cell biocatalysts and in vitro cofactor regenerating systems can address the cost of cofactor provision to some degree, whole-cell systems rely on the substrate passing through the cell wall, and the reaction mixture is more complex in both cases, all of which affects subsequent product recovery.

Oxygen surrogates (OSs), such as peroxides and hypervalent iodine compounds, have been known for many years to support P450 activity in the absence of a redox partner and cofactor, by virtue of the peroxide

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**ABBREVIATIONS:** CPR, cytochrome P450 reductase; CuOOH, cumene hydroperoxide; hCPR, human NADPH-cytochrome P450 reductase; luciferin H-EGE, ethylene glycol ester of 6'-desoxyluciferin; luciferin ME-EGE, ethylene glycol ester of luciferin 6'-methyl ether; luciferin MultiCYP, methyl ester of luciferin 6'-methyl ether; Luciferin PFBE luciferin 6'-pentafluorobenzyl ether OS, oxygen surrogate; P450, cytochrome P450; *tert*-BuOOH, tert-butyl hydroperoxide.

shunt in the P450 catalytic cycle or through direct transfer of an oxygen atom to the P450 ferric heme (Hrycay and O'Brien, 1971b; Kadlubar et al., 1973; Cho et al., 2007). Various OSs have been identified to support P450 catalysis (Hrycay and O'Brien, 1971a; Hrycay et al., 1972; Kadlubar et al., 1973; Chance et al., 1979; Tan et al., 1983; Lindstrom and Aust, 1984; Hecker et al., 1987; Weiss et al., 1987; Vaz et al., 1990; Plastaras et al., 2000), including, most commonly, CuOOH, *tert*-BuOOH, $H_2O_2$, and iodosylbenzene. They have been particularly used in investigations of the interactions of P450s with their redox partners concerning potential allosteric effects, the mechanisms of P450 inhibition, the exploration of the P450-redox partner interaction surface, and other mechanistic questions (Guengerich et al., 1997; Keizers et al., 2005; Lin et al., 2012; Varfaj et al., 2014; Yoshimoto et al., 2016).

While the use of OSs in P450 research is well established, their application in biocatalysis has attracted attention more recently. Implementation of OSs could make P450 catalysis more economically viable. However, inherent drawbacks in the use of OSs are the rapid inactivation of the P450 in the presence of peroxides and other reactive OSs, and the typically low efficiency of OS-mediated monooxygenation reactions (He et al., 1998; Kumar et al., 2005, 2006). Therefore, several studies have attempted to improve the ability of P450s to use cheap OSs by directed evolution or rational engineering approaches (Joo et al., 1999; Cirino and Arnold, 2002, 2003; Keizers et al., 2005; Kumar et al., 2005, 2006; Otey et al., 2006). More recent studies have aimed to optimize conditions for using peroxides to support P450 activities, but among human drug-metabolizing P450s, only CYP3A4 and CYP2D6 have been studied in any detail. No reports have examined iodosylbenzene derivatives for biocatalytic applications with drug-metabolizing P450s (Chefson et al., 2006; Kumar et al., 2006), although they have been explored with microbial forms (Dangi et al., 2018). Therefore, we set out to perform a more systematic analysis of the five major human xenobiotic-metabolizing P450s with several different commonly available OSs to determine the most appropriate OS to use for each form for both mechanistic studies and biocatalytic applications.

## Methods and Materials

**Materials.** The pCW vector from which all expression plasmids were derived was provided by Professor F.W. Dahlquist (University of Oregon, Eugene, OR). The bicistronic expression constructs pCW/CYP1A2/hCPR, pCW/CYP2C9$^{His}_{FL}$/hCPR, pCW/2C19$^{His}_{FL}$/hCPR, pCW/2D6 (Variant DB11), and pCW/CYP3A4/hCPR were constructed as previously described (Sandhu et al., 1994; Gillam et al., 1995; Richardson et al., 1995; Parikh et al., 1997; Shukla et al., 2005). For all P450s except CYP2D6, these plasmids encode an enzyme modified to encode the peptide "MALLLAVFL" in the N-terminal in place of the native hydrophobic anchor to enable expression in bacteria. For CYP2D6, the N-terminus was replaced in the expressed enzyme with the peptide "MARQVHSSWNL" upstream of the proline-rich region that marks the start of the catalytic domain. DH5αF'IQ strain cells were obtained from Life Technologies/ThermoFisher and were pretransformed with the pGro7 plasmid, which was obtained from Dr. H. Yanagi (HSP Research Institute, Shimogyo-ku, Kyoto, Japan; Nishihara et al., 1998).

**Expression of P450s.** Expression of recombinant P450s in *Escherichia coli* and preparation of subcellular bacterial membrane fractions was performed according to established methods (Gillam et al., 1993). P450 concentrations were measured via Fe(II)-CO versus Fe(II) difference spectroscopy and hCPR concentrations were quantified using the cytochrome *c* reduction assay, both performed as described by Guengerich et al. (2009).

**Enzymatic Assays.** All assays were conducted with bacterial membrane fractions containing 0.5 μM P450 coexpressed with hCPR in 100 mM potassium phosphate buffer (pH 7.4). Reactions were initiated by the respective support system, i.e., OS or an NADPH-regenerating system [final concentrations: 100 mM potassium phosphate buffer (pH 7.4), 0.25 mM $NADP^+$, 10 mM glucose-6-phosphate, 0.5 U/ml glucose-6-phosphate dehydrogenase]. Final concentrations of OSs were 1, 0.5, 0.25, and 0.1 mM. All reactions were incubated at 37°C for the times indicated in descriptions of individual assays below, with moderate shaking to allow aeration of samples. As a control, reactions were carried out in parallel in the absence of enzyme, which resulted in no detectable product formation in all cases. All incubations were performed according to a preset plan with three independent replicates. Except for alkoxyresorufins, which were monitored continuously, reactions for all substrates were carried out at a single high-substrate concentration and terminated at a fixed time, as our purpose was to screen for general ability to use OSs to detect broad differences between P450s rather than to compare kinetics.

Dealkylation or hydroxylation of the luminogenic substrates was measured in opaque, white 384-well plates with the luciferin derivatives at the concentrations recommended by the manufacturer (50 μM luciferin MultiCYP, 6 μM luciferin 1A2, 100 μM luciferin-H, 30 μM luciferin ME-EGE, 10 μM luciferin H-EGE, and 50 μM luciferin pentafluorobenzyl ether; Promega, Madison, WI). The 20 μl incubations were quenched after 60 minutes by the addition of an equal volume of the respective luminescence detection reagent. Samples were left for 20 minutes at 25°C to allow the development of luminescence before analysis in a luminescence detector (CLARIOstar Plus Multi-Mode Microplate Reader; BMG Labtech, Ortenberg, Germany). For assays with luminogenic substrates, the luciferin detection reagent contained luciferase (all substrates), esterase (luciferin ME-EGE, luciferin H-EGE) and D-cysteine (luciferin 1A2), so the tolerance of the luciferase reaction toward applied OSs was assessed at 1 mM of each OS. The tolerance of the esterase and luciferase in the detection reagent to OSs was tested by carrying out a CYP2D6-dependent reaction supported by NADPH/hCPR, as described above, using luciferin ME-EGE as the substrate. The reaction, however, was quenched by adding an equal volume of 2 mM OS. Subsequently, an equal volume (two volumes of the reaction volume) of detection reagent was added. The result was compared with a control reaction to which 0.5 volume of 100 mM potassium phosphate buffer (pH 7.4) was added rather than OS. The same procedure was applied to test the tolerance of D-cysteine toward OSs but while using CYP1A2 and the respective substrate (luciferin-1A2) and detection reagent.

The *O*-dealkylation of 7-methoxyresorufin (5 μM) was measured in a continuous assay using a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA) as previously described (Anari et al., 1997). Resorufin fluorescence was measured every 5 seconds over a total time of 30 minutes at excitation and emission wavelengths of 530 and 582 nm, respectively. Initial rates were determined from the linear range with reference to a resorufin standard.

For the metabolism of diclofenac, the assay was performed as described previously with slight modifications (Strohmaier et al., 2019). Briefly, reactions were carried out in potassium phosphate buffer (100 mM, pH 7.4) at 37°C, using 200 μM diclofenac in a final reaction volume of 200 μl and were initiated with the respective support system as described above. Reactions were terminated after 60 minutes by transferring 200 μl to a tube containing 50 μl acetic acetonitrile [5.66% (v/v) acetic acid in acetonitrile] and 10 μl 1 mM *p*-nitrocatechol (internal standard) and were processed following the same procedure as previously described (Strohmaier et al., 2019).

Caffeine metabolism and testosterone metabolism were carried out as previously described using 100 μM the respective substrate (Strohmaier et al., 2019).

Assays with omeprazole were conducted in the dark with an initial concentration of 100 μM omeprazole in a total reaction volume of 250 μl. The reactions were quenched after 60 minutes by addition of the internal standard (25 μl of 1 mM progesterone) and thorough mixing with 1 ml ethyl acetate for 1 minute. After 10 minutes centrifugation at 18,000*g*, 800 μl of the organic phase was transferred to a clean tube and evaporated to dryness under nitrogen. The dried extracts were resuspended in 24 μl acetonitrile, followed by the addition of 76 μl highly purified water immediately prior to analysis. Samples were analyzed using a 150 mm × 5 μM C18 column (Agilent, Santa Clara, CA) at a flow rate of 1.5 ml/min using a gradient from 24% to 63% acetonitrile in water over 18 minutes. Metabolites were detected by absorbance at 302 nm.

The hydroxylation of midazolam was carried out with 300 μM midazolam in a total reaction volume of 250 μl. After 60 minutes, 25 μl 1 mM nordazepam was added, and the reactions were quenched by the addition of 1 ml ethyl acetate with subsequent extraction followed by phase separation by centrifugation at 18,000*g* for 10 minutes. High-performance liquid chromatography analysis was conducted as previously described (Hunter et al., 2011).

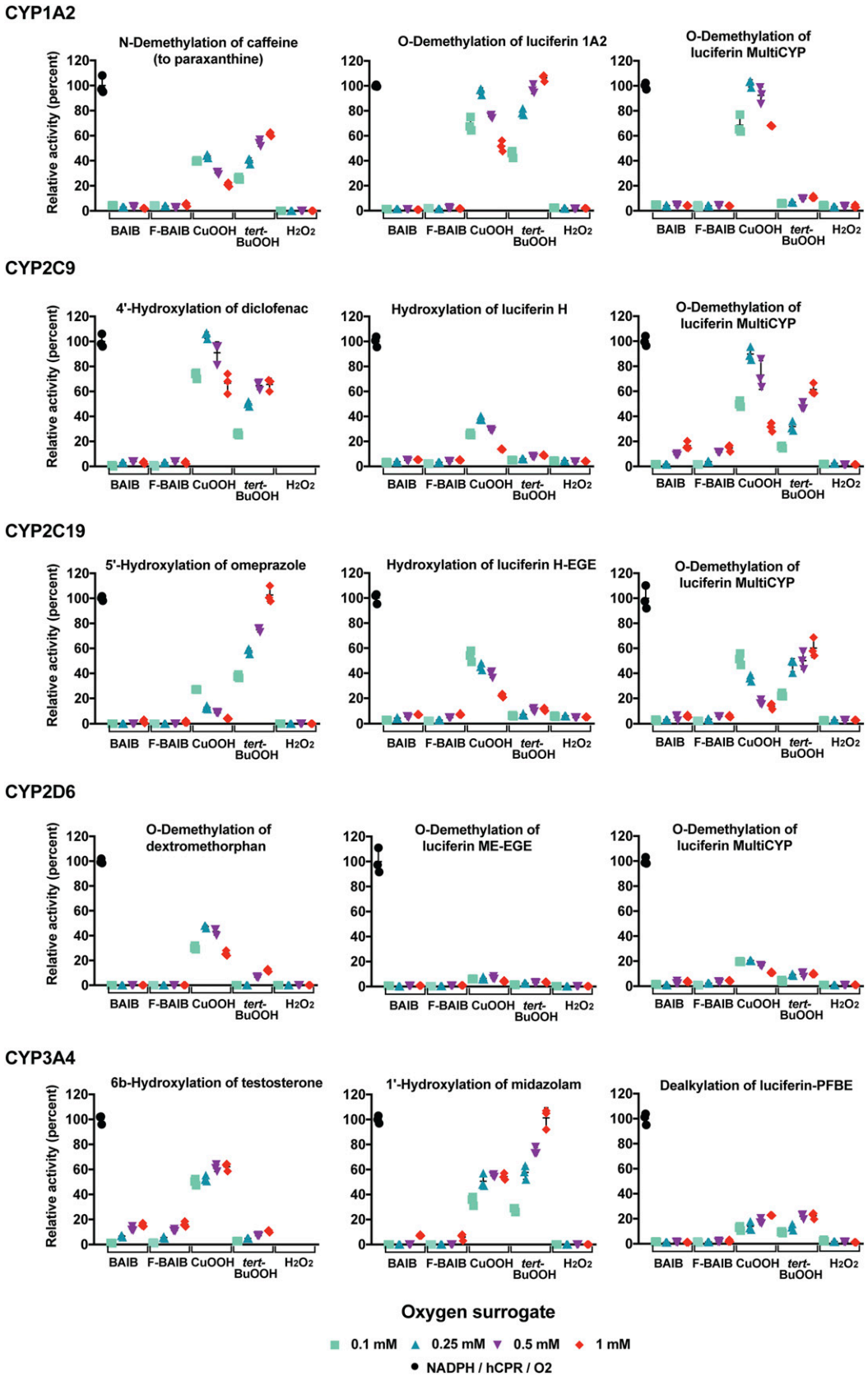


**Fig. 1.** OSs support the activity of the major drug-metabolizing P450s in a substrate and OS-specific manner. The major drug-metabolizing P450s were incubated with the substrates indicated in the presence of an NADPH-generating system (black circles) or one of the following four concentrations of each OS: 0.1 (light blue squares), 0.25 (blue triangles), 0.5 (purple inverted triangles), and 1 mM (red diamonds). Incubations were carried out for 60 minutes and at the substrate concentrations as follow: 50 μM luciferin MultiCYP, 6 μM luciferin 1A2, 100 μM luciferin-H, 30 μM luciferin ME-EGE, 10 μM luciferin H-EGE, 50 μM luciferin PFBE, 200 μM diclofenac, 100 μM caffeine, 100 μM testosterone, 100 μM omeprazole, 300 μM midazolam, and 100 μM dextromethorphan. Product formation is shown relative to the activity supported by hCPR and the NADPH generating system (set to 100%). Individual replicates are shown along with the means ± S.D. of three replicate incubations.

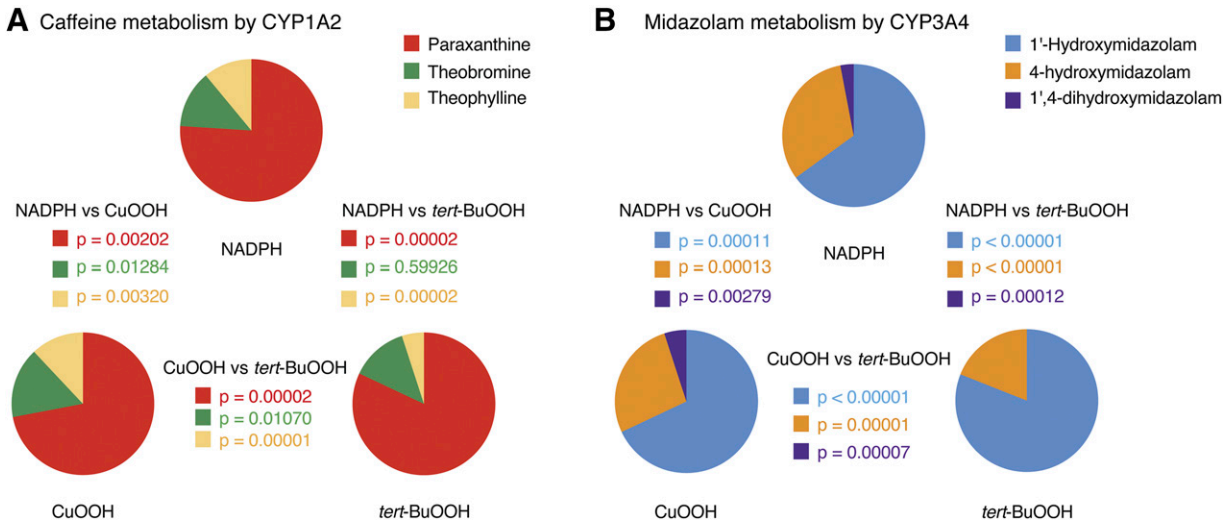


**Fig. 2.** The regioselectivity of caffeine and midazolam metabolism by CYP1A2 and CYP3A4 is influenced by the support system used. Pie charts show the relative formation of three metabolites of caffeine by CYP1A2 (A) or midazolam by CYP3A4 (B) in the presence of CuOOH, *tert*-BuOOH, or an NADPH-generating system, with oxygen and hCPR as indicated. Data represent the means of three independent incubations. *P* values indicate statistical significance (two-tailed unpaired Student's *t* test).

Dextromethorphan metabolism was assayed at 100 μM dextromethorphan. Reactions were terminated after 60 minutes by the addition of 25 μl of 1 mM *p*-nitrocatechol and thorough mixing with 1 ml ethyl acetate followed by phase separation by centrifugation at 18,000*g* for 10 minutes. Subsequent analysis was performed according to a previously described method (Gumulya et al., 2019).

## Results and Discussion

Five commercially available, inexpensive OSs were studied here: $H_2O_2$, *tert*-BuOOH, CuOOH, (diacetoxyiodo)benzene, and bis(trifluoroacetoxy)iodobenzene. Overall, organic hydroperoxides were most effective at supporting P450-mediated activity, while much lower activity, if any, was observed with $H_2O_2$ or the iodosylbenzene derivatives studied (Fig. 1). This may be due to rapid inactivation of the P450s by such OSs, as observed in other studies (Gustafsson et al., 1979; He et al., 1998; Yoshimoto et al., 2016; Albertolle et al., 2017). The optimal concentration of OS for supporting each P450 was consistent across multiple substrates. Generally, product formation was highest with the lower concentrations of CuOOH, either 0.1 mM (CYP2C19) or 0.25 mM CuOOH (CYP1A2, CYP2C9, and CYP2D6), but CYP3A4 showed a slight increase in metabolite formation at 0.5 and 1 mM. By contrast, *tert*-BuOOH–supported activity was maximal at the highest concentration tested here in all cases, suggesting a less effective association between the ferric heme and *tert*-BuOOH and/or enhanced resistance of the P450s toward oxidative damage by *tert*-BuOOH compared with CuOOH.

The activities of CYP3A4 and CYP2D6 supported by *tert*-BuOOH and CuOOH in our study were lower relative to the NADPH-supported control than those reported previously by Chefson et al. (2006). One key difference between the present and previous studies was that NADPH was used directly in Chefson et al. (2006), whereas an NADPH-regenerating system was used here. NADPH is usually rapidly converted in P450 reactions to $NADP^+$, which can inhibit CPR. Thus, this may have led to lower activity of the control in the study reported by Chefson et al. (2006). Alternatively, differences in the P450:CPR ratio or other incubation conditions between ours and the earlier study may have caused the discrepancy.

In the present study, hydrogen peroxide was the least effective OS overall, which agrees with several other studies on microsomal P450s reported previously (Kadlubar et al., 1973, 2005; Chefson et al., 2006). CYP1A2-mediated 7-methoxyresorufin *O*-demethylase activity in liver microsomes was reported to be supported effectively by $H_2O_2$ (Anari et al., 1997); however, we were unable to confirm this result using recombinant human CYP1A2 (Supplemental Fig. 1). CYP3A4-dependent N-oxide formation from quinidine was reported to be comparable between reactions supported by $H_2O_2$ and NADPH/CPR/$O_2$ (Guengerich and Johnson, 1997). Similarly, CYP2B1 and CYP2B4 metabolized *N*,*N*-dimethylaniline to the corresponding N-oxide equally effectively in the presence of $H_2O_2$ as in reactions supported by NADPH (Guengerich and Johnson, 1997). Collectively, this suggests that the ability of $H_2O_2$ to support activity may depend to some extent on the type of the reaction. Notably, no N-oxidation reactions were included among the activities studied in this work.

For most substrates, only a single significant metabolite could be detected (as shown in Fig. 1); however, for caffeine metabolism by CYP1A2 and midazolam metabolism by CYP3A4, multiple products were detected at measurable levels (Fig. 2). We cannot exclude the possibility that additional metabolites may have been formed from alkoxyresorufins and luciferin derivatives, but to our knowledge, there is no precedent for alternative metabolites not detectable by the methods used. The regioselectivity of midazolam and caffeine metabolism was altered slightly but significantly when using organic peroxides compared with NADPH/CPR-supported reactions (Fig. 2; Supplemental Tables 1 and 2). Similar observations have been made previously with various P450s and diverse substrates (Hrycay et al., 1975; Hanna et al., 2001; Yoshimoto et al., 2016; Dangi et al., 2018). This phenomenon is generally explained by a greater tendency of these OSs to undergo homolytic O-O bond scission as compared with $H_2O_2$ because of electron donating substituents (White and Coon, 1980; Barr et al., 1996; Nam et al., 2000). This may result in a mechanism in which hydrogen abstraction from the substrate is mediated by the resulting alkoxy radical rather than by compound I (White and Coon, 1980; Blake and Coon, 1981; Weiss and Estabrook, 1986). Therefore, the changes in regioselectivity observed may be related to the different positioning of the alkoxy radical and compound I relative to the substrate.

Overall, for each isoform, the relative activity supported by the OSs differed according to which substrate was used to evaluate activity. This phenomenon was particularly pronounced in case of

*tert*-BuOOH–supported metabolism. This observation that relative activity differs in a substrate-dependent fashion is consistent with a previous study in which the $K_M^{CuOOH}$ of CYP3A4 differed with different substrates (Kumar et al., 2006). The authors speculated that the active site could be masked to a greater or lesser extent, depending on the substrate, hampering access of the OS to the heme and resulting in substrate-dependent changes of the $K_M^{CuOOH}$. In such a scenario, a sterically less complex, albeit hydrophobic OS (i.e., *tert*-BuOOH), would have better access to the active site than a larger one (i.e., CuOOH). If accessibility of the OS was diminished by a larger hydroperoxide, one might expect that the relative activity with, e.g., CuOOH would be affected to a larger extent than that supported by a simpler peroxide (e.g., tert-BuOOH) in the presence of different substrates. If hydrogen abstraction from the substrate is mediated by the resulting alkoxy radical rather than by compound I, the orientations of the substrate and OS relative to the heme in the active site may determine the relative effectiveness of hydrogen abstraction by the OS-derived alkoxy radical and subsequent substrate monooxygenation by oxygen rebound from the heme. No metabolite formation was observed for any of the OSs in the absence of a P450, ruling out the possibility that purely chemical reactions contribute to the differences seen.

We also considered whether the remnant of the OS may have affected the orientation or accessibility of the substrate to the heme, leading to increased or decreased turnover. However, addition of 1 mM *tert*-butanol to the NADPH/CPR-supported incubations of CYP3A4 with testosterone and midazolam affected neither product formation nor stereoselectivity (data not shown), suggesting that such an effect is unlikely.

In conclusion, this study has revealed that the organic peroxides, CuOOH and *tert*-BuOOH, are the best starting points for supporting activity of the major drug-metabolizing P450s in the absence of NADPH, molecular oxygen, or a redox partner, but the relative activity supported by OSs is substrate-dependent. Further studies will be necessary to explore the nature of this phenomenon. The potential for alterations in regioselectivity of substrate oxidation should be considered when OSs are used for mechanistic studies. Likewise, when OSs are used to support the activity of P450s for biocatalysis, the OS used should be tailored to both the P450 and the substrate under investigation, underscoring the need to employ screening methods that reflect the activity toward the substrate of interest to the end application.

### Authorship Contributions

*Participated in research design:* Strohmaier, Gillam.

*Conducted experiments:* Strohmaier.

*Contributed new reagents or analytic tools:* De Voss, Jurva, Andersson.

*Performed data analysis:* Strohmaier, Gillam.

*Wrote or contributed to the writing of the manuscript:* Strohmaier, De Voss, Jurva, Andersson, Gillam.

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