Chlorzoxazone Metabolism by Porcine Cytochrome P450 Enzymes and the Effect of Cytochrome b5

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Abbreviations:
Cytochrome P450 (CYP450)
Chlorzoxazone (CLZ)
6-hydroxy-chlorzoxazone (6-OH-CLZ)
Microsomal cytochrome b5 (CYB5A)
Human Cytochrome 2E1 (hCYP2E1)
P450-oxidoreductase/CYP450 NADPH reductase (POR)
Cytochrome b5 reductase (CYB5R3)
Human embryonic kidney cells (HEK293FT)
Intrinsic clearance (C_{int})
Abstract:

Chlorzoxazone (CLZ) is a commonly used non-toxic in vivo and in vitro probe for the assessment of cytochrome P4502E1 (CYP2E1) activity. Human CYP1A1 and CYP3A4 have also been shown to contribute to CLZ metabolism. For pigs to be a potential model system for humans, it is necessary that human and pig CYP450s should have similar metabolizing capabilities. Therefore, CLZ metabolizing capabilities and specificities of porcine CYP450s were investigated. In this study, the complete coding regions of six porcine CYP450s were amplified from liver cDNA and cloned into pcDNA3.1-V5-His-TOPO vector. Expression vectors for the individual P450s and microsomal cytochrome b5 (CYB5A) were expressed in the HEK-293FT cell line to investigate their role in CLZ metabolism. As with the human enzymes, porcine CYP2E1 (K_m 290.3μM, V_max 4980 pmol/hr/mg total protein) and CYP1A1 (K_m 159.5μM, V_max 1650 pmol/hr/mg total protein) both contribute to CLZ metabolism. In addition, porcine CYP2A19 and CYP2C33v4 also metabolise the substrate, with K_m 212.1μM, V_max 6680 pmol/hr/mg total protein and K_m 126.3μM, V_max 2100 pmol/hr/mg total protein respectively, while CYP3A does not. CYB5A augmented CYP2E1 and CYP2C33v4 activity in the pig, with a significant increase in activity of 85% and 73% compared to control, respectively. Thus, CLZ should be used with caution as a probe for CYP2E1 activity in the pig. However, further information regarding the abundance of different CYP450 isoforms is needed to fully understand their contribution in microsomal, hepatocyte and in vivo systems in the pig.
Introduction

The CYP2E1 gene is the most highly conserved of all CYP450s and is found across numerous species including human, mouse, rat, pig, dog, cow, rabbit, monkey, and even fungi (Overton et al., 2008). This enzyme shows broad substrate specificity with over 80 substrates being identified, most of them being small, hydrophobic compounds including anaesthetics (Ingelman-Sundberg, 2004; Lieber, 1997). There is much interest in exploring the metabolic capabilities of human CYP2E1, as it contributes to the metabolism of many compounds ranging from therapeutics to pro-carcinogens and carcinogens (Lieber, 1997; Raucy et al., 1993). In addition, CYP2E1 expression can be altered by growth hormone, cytokines, ethanol, thyroid hormone and insulin (Waxman et al., 1989; Abdel-Razzak et al., 1993; Lieber, 1997; Peng and Coon, 1998; Emery et al., 2003). Due to differences in CYP2E1 expression, individuals may exhibit alterations in drug metabolism and efficacy when suffering from diabetes, endocrine disorders, or alcoholism (Overton et al., 2008).

Characterization of the metabolic capabilities of an enzyme usually involves the use of specific inhibitors and probe substrates as indicators of enzymatic activity. Chlorzoxazone (CLZ), a centrally acting muscle relaxant, was first shown to be metabolized by human CYP2E1 by Peter et al., (1990) and has since been used as a specific probe substrate in both in vitro and in vivo studies. One of the challenges of choosing probe substrates, apart from sensitivity and specificity, is that they must be relatively non-toxic in order to be suitable for use in in vivo studies; CLZ meets this criterion (Court et al., 1997; Ernstgård et al., 2004). However, the specificity of CLZ as a probe substrate for human CYP2E1 has been questioned, since other human CYP450s contribute to its metabolism (Carriere et al., 1993; Gorski et al., 1997). Just as
in the human, porcine CYP2E1 has also been shown to metabolize CLZ to its major metabolite, 6-OH-CLZ (Anzenbacherová et al., 2005; Baranová et al., 2005).

Pigs are often explored as potential human models in pharmaceutical studies due to their wide availability, and physiological, dietary and anatomical similarity to humans (Tsiaoussis et al., 2001; Balk, 1987; Monshouwer et al., 1998). Drug development is a complex process requiring multiple steps before reaching clinical trial testing. Individual porcine CYP450 enzymes have been studied to determine how metabolic capabilities correlate with those found in humans and for studies in veterinary pharmacology (Juskevich, 1987). Studies have demonstrated that porcine hepatocytes and microsomal systems are capable of metabolizing a wide range of human indicator substrates (Bogaards et al., 2000; Myers et al., 2001; Szotáková et al., 2004). CLZ has also been used as a probe for porcine CYP2E1 activity in pharmaceutical and agricultural studies (Lejus et al., 2002; Friis, 1995), but as is the case in humans, it is highly likely that other porcine CYP450 enzymes contribute to CLZ hydroxylation. It is important to determine which porcine enzymes may have a significant contribution to CLZ metabolism if the pig is to be an effective human model. If CLZ is used as a marker for CYP2E1 activity, the potency of CYP2E1 inhibitors may be underestimated in pharmacological studies if other CYP450s contribute to CLZ hydroxylation. Commonly used anaesthetics are metabolized by CYP2E1 and some, such as propofol, are inhibitors of CYP2E1 (Lejus et al., 2002). In addition, the microsomal hemoprotein cytochrome b5 (CYB5A) has been shown to augment human CYP2E1 activity towards CLZ hydroxylation by apparently acting as an electron donor (Yamazaki et al., 1996; Yamazaki et al., 2002).

Most studies utilize human probe substrates, inhibitors or antibodies to characterize CYP450 enzymes in other species, and this approach assumes that these compounds are also
specific to the particular porcine isoforms of the human enzymes. However, this is often not the case. Therefore, in order to definitively characterize the porcine CYP450 isoforms that may contribute to CLZ metabolism, we have cloned and individually expressed various porcine CYP450s in a cell line system, eliminating the need for inhibitors and antibodies to identify which isoforms are present. In this study, we investigated the specificity of CLZ metabolism by individually expressing porcine CYP2E1, CYP1A1, CYP2A19, CYP2C33v4, CYP2C49, and CYP3A as well as human CYP2E1 in HEK-293FT cells along with porcine P450-oxidoreductase (POR). We measured the apparent clearance efficacy of CLZ by each enzyme in our model system. We also investigated the effect of co-transfection with an expression vector for CYB5A on the rate of CLZ hydroxylation, as little is known about the effects of CYB5A on the activity of individual porcine CYP450s.
Materials and Methods:

Cloning: The expression vectors for porcine P450-oxidoreductase/CYP450 NADPH reductase (POR), cytochrome b5 reductase (CYB5R3), and CYB5A were constructed as previously described (Billen and Squires, 2009). To generate expression vectors for the different porcine CYP450s, the entire coding regions of porcine CYP1A1, CYP2A19, CYP2E1, CYP2C33v4, CYP2C49 and CYP3A were amplified from porcine liver cDNA by PCR using platinum® Taq DNA polymerase high fidelity (Invitrogen, Burlington ON). The amplicons were then T/A cloned into pcDNA3.1/V5-His TOPO (Invitrogen, Burlington ON) according to the manufacturer’s instructions. PCR conditions included an initial denaturation step for 2 min at 94 °C, 30 sec at 94 °C, annealing of primers for 15 sec at 60.1 °C with an extension for 1 min 40 sec at 68 °C for 35 cycles. A final extension of 10 min was carried out at 68 °C. Primers were designed based on their respective sequences available from NCBI; details are presented in Table 1. A plasmid containing human CYP2E1 (hCYP2E1) was obtained from Origene (Rockville, MD) and the coding region amplified and cloned into pcDNA3.1/V5-His TOPO (Invitrogen, Burlington ON). Expression vectors for V5-His tagged proteins were generated so that the levels of the expressed proteins could be estimated by Western blotting using anti-V5 antibody; the introduction of V5-His tag has been shown to not interfere with enzymatic activity (Billen and Squires, 2009; Handley-Gearhart et al., 1994). The identity of all clones was confirmed by sequencing. The amplified sequence of porcine CYP3A did not match the sequence of porcine CYP3A29 and differed by two amino acids; Asn423→His and Lys458→Arg. To ensure that this was not a cloning artefact, the sequence of CYP3A was confirmed by identifying its presence in liver cDNA prepared from three different breeds of pigs (Yorkshire, Large White and Sireline).
**Cell Culture:** Human embryonic kidney cells (HEK-293FT; Invitrogen, Burlington, ON) were cultured in 75 cm flasks (Sarstedt, Montreal, Québec) under conditions described previously (Billen and Squires, 2009). Cells were plated at $10^6$ cells per well in 6-well plates (BD Biosciences, Mississauga, ON). Confluent cells were transfected with Lipofectamine 2000 (Invitrogen, Burlington ON) 24 hrs after plating. Each transfection contained 2.35 µg of individual CYP450 expression plasmid along with 0.40 µg POR, 0.25 µg CYPB5R3, and 1µg CYB5A. Where plasmid amounts were varied, empty pcDNA3.1-V5-His-TOPO vector was used to bring the total DNA amount up to 4.0 µg for each transfection. Twenty-four hours later, transfected cells were subsequently incubated with varying amounts of CLZ (Sigma, St. Louis, MO) dissolved in DMSO; the concentration of organic solvent did not exceed 0.1% in culture. Substrate was incubated for 24 hrs and a 1 mL aliquot of media was collected for analysis; trials were performed in triplicate. Protein was precipitated from the collected media by the addition of 1/10 volume trichloroacetic acid (40% v/v) and centrifuged for 15 min; 100 µl of supernatant was then subjected to HPLC analysis. Cells were scraped into 300 µl of RIPA buffer containing 0.5% sodium deoxycholate, 1% SDS, and 1% Nonidet P-40 (Sigma, St. Louis, MO) with a Complete protease inhibitor tablet (Roche, Mississauga, Ontario), sonicated for 1min and then centrifuged to pellet cell debris. The protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Labs, Richmond, CA).

**Western Blot:** Western blot analysis was carried about as previously described (Billen and Squires, 2009) with the following modifications. Proteins were separated on sodium dodecyl sulphate-12% polyacrylamide gels and incubated with a 1:5000 dilution of primary mouse-anti-V5 antibody (Invitrogen, Burlington, ON) and a 1:24000 dilution of secondary goat-anti-mouse-HRP antibody (Sigma). The blot was visualized as described previously (Billen and Squires,
2009) and band densities were analyzed by Northern Eclipse software (Empix Imaging, Mississauga, ON). Since all proteins contained the V5-His tag, immunostaining with anti-V5 antibody allowed for the measurement of all expressed CYP450s in individual samples. In order to ensure that the activity in each sample was based on equal protein expression across samples, the enzyme activities were normalized to the protein content of the individually expressed CYP450s based on immunoblot band densities.

**HPLC Assays:** A 100 µl volume of sample was analyzed by HPLC on a C18 reverse phase column (250 x 4.6 mm, 5 µm). The solvent system consisted of buffer A: 5% acetonitrile, 95% water and 0.2% acetic acid and buffer B: 100% acetonitrile with a flow rate of 1 ml/min. The gradient used was as follows: 0 min- 80% A, 10 min-10% A, 15 min – 10% A, 15.1 min – 80% A, and 20 min – 80% A. CLZ and the 6-OH-CLZ metabolite were monitored by absorbance at 295 nm; the retention times for 6-OH-CLZ and CLZ were 6.4 min and 9.45 min, respectively.

**Data Analysis:** Statistical analysis was carried out using the SAS/STAT version 9.1 (SAS Institute Inc., Cary, NC) The CYB5A dose-response was analysed using linear regression. All kinetic parameters were determined using SigmaPlot 8.0 (SPSS Inc., Chicago, IL) with Kinetic Module software. The \( K_m \) and \( V_{\text{max}} \) parameters were determined from either the Michaelis-Menten equation (1) or the Hill equation (2); examination of the Eadie-Hofstee plot was used to determine the most appropriate model. Where a “hook”–shaped as opposed to a linear Eadie-Hofstee plot was observed, the data was fitted using the Hill equation as this is characteristic of sigmoidal data (Madan et al., 2002).

\[
 v = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \quad (1) \\
 v = \frac{V_{\text{max}} \cdot [S]^n}{K' + [S]^n} \quad (2)
\]
Results:

An intact cell system was used to assess the activity of the individual CYP450 isoforms CYP1A1, CYP2A19, CYP2E1, hCYP2E1, CYP2C33v4, CYP2C49 and CYP3A towards CLZ. Initial transfections consisted of 0.40 µg, 0.70 µg, 0.25 µg, 1 µg of POR, CYP450, CYB5R3 and CYB5A plasmid amounts respectively as similar band intensities were obtained for each protein on Western blotting. Typical western blots obtained from individual transfections are depicted in Figure 1. Pre-trials were conducted in order to determine which CYP450s were active in catalyzing CLZ hydroxylation with a CLZ concentration of 400 µM. Porcine CYP3A did not produce detectable metabolites and CYP2C49 produced 10-fold lower metabolite levels than the other CYP450s; therefore these CYP450s were not chosen for further analysis. The production of 6-OH-CLZ was linear with increasing amounts of CYP450 expression plasmids that were transfected and was linear up to 72 hours of incubation (data not shown). Control incubations of cells without CYP450 plasmid but transfected with POR, CYB5R3, and CYB5A and treated with CLZ did not produce any detectable 6-OH-CLZ metabolite (data not shown).

Chlorzoxazone was incubated at 25-700 µM, 50-900 µM and 50-700 µM, for transfections with hCYP2E1, porcine CYP2E1, and CYP1A1 respectively (Figure 2). The production of 6-OH-CLZ by these CYP450s displayed sigmoidal kinetics and the data was therefore modelled by the Hill equation. For transfections with CYP2C33v4 and CYP2A19, chlorzoxazone was incubated at 10-600 µM and 25-800 µM respectively (Figure 3. The production of 6-OH-CLZ by these CYP450s was modelled by the Michaelis-Menten equation. The kinetic parameters, $K_m$ and $V_{max}$ along with the $V_{max}/K_m$ ratio (intrinsic clearance, $C_{int}$), a measure of enzymatic efficiency towards CLZ clearance, are summarized in Table 2.
The effect of CYB5A on CLZ metabolism was next investigated by separate co-transfections of increasing amounts of CYB5A expression plasmid with expression plasmids for hCYP2E1, CYP2E1, CYP2A19, CYP2C33v4, and CYP1A1. The transfected cells were subsequently incubated with chlorzoxazone at levels on the linear part of the kinetic curve; this was 300 µM CLZ for hCYP2E1, porcine CYP2E1 and CYP2A19 and 100 µM CLZ for CYP2C33 and CYP1A1. A significant stimulatory dose response for CYB5A was found for three CYP450s (Figure 4). A 73%, 85% and >300% increase in CLZ activity was seen with CYP2C33v4, CYP2E1 and hCYP2E1 at the highest level of CYB5A transfected, compared to the control without CYB5A. There was no significant response observed by co-transfection of CYB5A with CYP2A19 or CYP1A1 (data not shown).
Discussion:

The present study investigated the specificity of CLZ as a probe substrate for porcine CYP2E1 and the effects of CYB5A on CLZ catalytic activity. We show that the metabolism of CLZ is not limited to CYP2E1 in the pig and that CYP1A1, CYP2A19, CYP2C33v4 also metabolize CLZ. We investigated hCYP2E1 along with porcine CYP2E1 in order to compare kinetic parameters for these homologues within the same system. The apparent $K_m$ for porcine CYP2E1 was more than double that of hCYP2E1 and the $V_{\text{max}}$ of hCYP2E1 was almost double that of porcine CYP2E1. Anzenbácherová et al., (2005) found similar results in microsomal systems, with CLZ hydroxylation in human greater than in conventional pig. Baranová et al., (2005) also found that CYP2E1 from minipig exhibited lower affinity for the substrate in both reconstituted and microsomal systems with $K_m$ values being ~1.2 to 4.7 fold greater compared to the human counterpart. CYP2E1 from the minipig and conventional pig are comparable with sequences differing by a single amino acid, a valine at position 346 is found in place of an aspartic acid in the conventional pig (Baranová et al., 2005). Other studies have also concluded that the enzymatic parameters for CLZ metabolism differ between species. Bogaards et al., (2000) observed a range of kinetic parameters in mouse, rat, rabbit, dog, minipig, monkey, and man with $K_m$ values ranging from 25-306 µM and $V_{\text{max}}$ ranges of 285-3184 pmol/min/mg microsomal protein. Although the magnitudes of the apparent kinetic parameters are unique to the system in which they are studied, the overall conclusion that the porcine CYP2E1 is capable of CLZ metabolism, although it is less efficient than hCYP2E1, can be drawn.

Evidence supporting the contribution of other porcine CYP450 enzymes to CLZ metabolism has been also been reported in literature. Court et al., (1997) described a two-enzyme model for the metabolism of CLZ in porcine hepatic microsomes, which suggests the
involvement of two kinetically distinct enzymes with different affinities for the same substrate (Madan et al., 2002). Skaanild and Friis (2007) argue that CLZ is a poor probe substrate for porcine CYP2E1; after treatment of porcine microsomes with anti-human CYP2A6 antibody, CLZ activity was reduced by an average of 65%, suggesting that porcine CYP2A may equally contribute to metabolism. Myers et al., (2001) treated barrows with inducers specific for the human CYP1A, CYP2B and CYP3A subfamilies, which increased apparent levels of porcine CYP1A, CYP2B, and CYP3A proteins with no apparent effect on CYP2A or CYP2E1 levels; however, CLZ activity was increased in these treated liver fractions. This suggests that porcine CYP1A, CYP2B, and CYP3A may also contribute to CLZ hydroxylation. Our study demonstrates that porcine CYP2A19 and CYP1A1, but not CYP3A are active with this substrate. It is possible, however, that other isoforms of CYP3A exist in the pig which may have CLZ hydroxylation activity.

Antibodies and inducers may not have the same specificity for CYP450 isoforms across different species, and the sources of antibodies used between experiments may yield varying results. The use of individual porcine cDNA-expressed enzymes allows for a more definitive characterization of the individual enzymes, showing which porcine CYP450 isoforms are capable of CLZ metabolism. Although we did not quantify the absolute amounts of CYP450s in our expression system, the expression level of each CYP450 was normalized based on the density of the Western blots that were immunostained for the V5 epitope on each of the enzymes and this accounted for small differences in the levels of expression of the different CYP450s.

Several human CYP450s have been shown to metabolize CLZ other than hCYP2E1. Masimirembwa et al., (1999) assessed the specificities of human CYP450 substrates using microsomal yeast extracts containing cDNA-expressed enzymes. CLZ metabolism occurred at a
high turnover with CYP1A1 in addition to CYP1A2 and CYP2E1. Carriere et al., (1993) observed an increase in CLZ metabolism in human hepatocytes after induction of CYP1A with 3-methylcholanthrene, in addition to microsomal yeast cells engineered to express CYP1A1. Carriere et al., (1993) also reported detectable CLZ hydroxylation activity by human CYP2C9 and CYP3A4 expressed in yeast microsomes; however, the turnover numbers reported were ~40-fold lower compared to CYP1A1. Yamazaki et al., (1995) demonstrated low levels of CLZ hydroxylation by human CYP3A4 in an E.coli expression system. In contrast, Gorski et al., (1997) observed a reduction in metabolism of CLZ after treatment of human microsomes with CYP3A4 inhibitors and antibodies, but observed a subsequent restoration of activity with the addition of α-naphthoflavone, a CYP3A4 inducer. CLZ metabolism was also observed with human CYP2A6 and CYP1A1 expressed in microsomes from B-lymphoblastoid cells. However, treatment of human microsomes with inhibitors specific for each CYP450 isoform did not show significant inhibition of CLZ activity. Therefore, it was concluded that CYP3A4 may make a significant contribution to CLZ metabolism in vivo where CYP2A6 and CYP1A1 likely do not.

We did not find detectable levels of 6-OH-CLZ produced with porcine CYP3A; this may be an example of a difference in metabolic profiles between species.

The effect of CYB5A on the catalytic properties of porcine CYP450 enzymes has not been investigated extensively. The ability of porcine CYB5A to modify the activity of CYP17A1 in the pig has been reported (Billen and Squires, 2009). The contribution of CYB5A to CYP450-mediated catalysis is complex and is dependent on the substrate and CYP450 isoform (Schenkman and Jansson, 2003). In some cases, CYB5A can modify the reaction by having an inhibitory or stimulatory effect and in other cases it has been shown to be obligatory. Our results show that the effects of CYB5A on CLZ hydroxylation activity are isoform specific, as it only
significantly augmented activity of porcine CYP2E1 and CYP2C33v4 and human CYP2E1. In a study by Yamazaki et al., (1996), the presence of CYB5A was shown to enhance catalytic activity of hCYP2E1 towards CLZ, with only 7% activity reported in the absence of CYB5A. Gillam et al., (1994) also found that metabolism of CLZ was higher in the presence of human CYB5A with a decrease in $K_m$ and increase in $V_{\text{max}}$. Although the exact mechanism of CYB5A in CYP450 catalysis is unknown, studies utilizing apo-CYB5A which is devoid of heme, show no enhancement of activity towards CLZ by hCYP2E1, suggesting the contribution of CYB5A is dependent on its electron-transferring properties (Yamazaki et al., 2002). CYB5A has been shown to reduce the competition for POR, at least between human CYP2A6 and CYP2E1, enhancing activity of CYP2E1, suggesting that CYB5A enhances electron transfer (Yizheng et al., 1997). Studies have reported a lack of effect by CYB5A on the activity of human CYP1A1/2 towards ethoxyresorufin, and phenacetin (Yamazaki et al., 2002), while some have observed a slight augmenting effect with 2-aminoanthracene (Duarte et al., 2007). Yamazaki et al., (2002) also reported an augmenting effect of CYB5A on CYP2A6 catalyzed coumarin and nicotine metabolism. We did not find an effect of CYB5A on CLZ metabolism with either CYP2A19 or CYP1A1. The contribution of the human CYP2C subfamily to CLZ metabolism has not been reported; Gorski et al., (1997) did not detect 6-OH-CLZ in B-lymphoblastoid microsomes expressing human CYP2C9. With the two porcine CYP2C isoforms investigated, only CYP2C33v4 was efficient at metabolizing CLZ, and CYB5A increased this activity.

The tissue content of various CYP450 isoforms and their activities determines their contribution to the metabolism of a substrate in vivo (Rendic and Carlo, 1997; Bertz and Granneman, 1997). The contribution of enzymes to reactions is determined by the $V_{\text{max}}/K_m$ ratio in addition to the levels of their expression. Our results indicate that CYP2E1, CYP1A1, and
CYP2C33v4 have a lower $V_{\text{max}}/K_m$ ratio than CYP2A19. This suggests that CYP2A19 is mostly responsible for CLZ hydroxylation; however, the relative abundance of these isoforms \textit{in vivo} must be considered. Currently, information about the abundance of various porcine CYP450 isoforms is limited. Kojima and Morozumi (2004) found that hepatic expression of CYP2C33v4 and CYP2E1 mRNA was greatest, followed by CYP2C49 > CYP1A1 and CYP2A19 > CYP2B22. Therefore, it is possible that CYP2C33v4 and CYP2E1 maybe be the main isoforms responsible for CLZ metabolism. However, Szotáková et al., (2004) found no correlation between CLZ metabolism and CYP2E1 protein from immunoblotting. Thus, conclusions about the contribution of porcine CYP450s to CLZ metabolism \textit{in vivo} are highly depend on liver abundance, which is yet to be conclusively determined.

In summary, we present definitive evidence for the contribution of four porcine CYP450s to CLZ metabolism, with novel information about the contribution of CYB5A in this metabolism for some isoforms. The porcine enzymes are similar to their human homologues in terms of CLZ metabolism and CYB5A augmentation. As in human, porcine CYP1A1, CYP2E1, and CYP2A19 are capable of CLZ hydroxylation. However, species differences are also evident as the porcine CYP3A does not metabolize CLZ. We have shown that CYP2C33v4 contributes to CLZ hydroxylation, although human CYP2C9 does not appear to metabolize CLZ; it is unclear whether other human CYP2C isoforms do. A better understanding of porcine CYP450 isoform abundance is needed before conclusions can be drawn about the use of CLZ as an \textit{in vivo} probe for porcine CYP2E1.
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*Ddrug Metab Dispos* 27: 1117-1122.


DMD # 30528


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Footnotes

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Figure legends:

**Figure 1:** Example of a typical western blot showing expressed V5-His tagged porcine CYP450s and human CYP2E1 (hCYP2E1) as visualized by staining with anti-V5 antibody. All CYP450s are co-expressed with POR, CYB5R3, and CYB5A (not shown); each well contains 20 µg of protein.

**Figure 2:** The production of 6-OH-CLZ by CYP450 isoforms that displayed sigmoidal kinetics and modelled by the Hill equation. **A.** hCYP2E1, **B.** porcine CYP2E1, **C.** CYP1A1. The corresponding Eadie-Hofstee plots displaying a “hook” shape, characteristic of sigmoidal data are depicted as inserts. Each point represents a mean of four experiments performed in triplicate (n =12) ± S.D.

**Figure 3:** The production of 6-OH-CLZ by CYP450 isoforms that were modelled by the Michaelis-Menten equation. **A.** CYP2C33v4, **B.** CYP2A19. The corresponding Eadie-Hofstee plots displaying a linear shape are depicted as inserts. Each point represents a mean of four experiments performed in triplicate (n =12) ± S.D.

**Figure 4:** The significant effect on CLZ hydroxylation by co-transfection with increasing amounts of CYB5A expression plasmid along with expression vectors for **A.** CYP2C33v4, **B.** porcine CYP2E1, **C.** hCYP2E1. There was a significant increase in CLZ hydroxylation rate by increasing levels of CYB5A transfection by linear regression analysis (P< 0.001). Data is presented as a mean of four experiments performed in triplicate (n =12) ± S.D.
Table 1: Summary of primers used for PCR amplification of CYP450 isoforms.

<table>
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<tr>
<th>Protein</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Plasmid Name</th>
<th>Reference sequence</th>
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</thead>
<tbody>
<tr>
<td>Human CYP2E1</td>
<td>gcctgtcgccctcgg gcgtc</td>
<td>tgcgcgggaatgacac agag</td>
<td>hCYP2E1</td>
<td>NM00077 3.3</td>
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<tr>
<td>Porcine CYP1A1</td>
<td>gccatgttccttgctttg gaga</td>
<td>agagegacatgcatctg gac</td>
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<td>NM214412</td>
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<tr>
<td>Porcine CYP2C49</td>
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<tr>
<td>Porcine CYP2A19</td>
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<tr>
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Forward primers were modified by adding a Kozak sequence (GCC) immediately upstream of the start codon (underlined). Reverse primers were designed to eliminate the original stop codon of the protein sequence allowing for the incorporation of the V5-His-tag.
Table 2: Summary of kinetic parameters for CLZ hydroxylation by porcine CYP450s and hCYP2E1

<table>
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<tr>
<th>CYP</th>
<th>Km (µM)</th>
<th>$V_{\text{max}}$ (pmol/hr/mg total cell protein)</th>
<th>$C_{\text{int}}$</th>
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<td>8650 ± 356</td>
<td>65.6</td>
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<td>CYP2E1</td>
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</tr>
<tr>
<td>CYP2C33</td>
<td>126.3 ± 17.9</td>
<td>2100 ± 93.3</td>
<td>16.6</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>159.5 ± 10.0</td>
<td>1650 ± 55.3</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Kinetic parameters for human CYP2E1 (hCYP2E1) porcine CYP2E1 and porcine CYP1A1 were modelled by the Hill equation, since these isoforms displayed sigmoidal kinetics. Kinetic parameters for porcine CYP2C33v4 and CYP2A19 were modelled by the Michaelis-Menten equation.
Figure 1

- POR ~ 78kDa
- CYP450 ~ 56-58kDa
- CYBR3 ~ 33kDa
Figure 3
Figure 4

(A) Metabolic Rate (pmol/hr/mg protein) vs. CYB5A (ug)

(B) Metabolic Rate (pmol/hr/mg protein) vs. CYB5A (ug)

(C) Metabolic Rate (pmol/hr/mg protein) vs. CYB5A (ug)