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Is bupropion a more specific substrate for porcine CYP2E than chlorzoxazone and \( p \)-nitrophenol?

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Running title: Is BUP a more specific substrate for porcine CYP2E than CLX and PNP?

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Abbreviations: CLX chlorzoxazone, PNP para-nitrophenol, BUP bupropion, DEDC diethyldithiocarbamate, TROL troleandomycine
ABSTRACT
Porcine microsomes are able to hydroxylate chlorzoxazone (CLX) and p-nitrophenol (PNP), the most commonly used human test substrates for CYP2E1. However, in pigs, CYP2E appears not to be the only enzyme involved in the hydroxylation of CLX and PNP, as the enzyme capacity and immunochemical level of the apoprotein do not correlate. The present study shows that the hydroxylation of CLX and PNP is inhibited 50-65 % by anti-human CYP2A6, suggesting that these substrates are metabolized almost equally well by CYP2A and CYP2E in pigs. To find an alternative probe to porcine CYP2E, bupropion, another human substrate, was examined. Incubation with bupropion (BUP) concentrations ranging from 0.005 to 7 mM and with various inhibitors revealed that this substrate is metabolized by both CYP2A and CYP2E, as the reaction is inhibited by both DEDC and anti CYP2A6. At the high substrate concentration (5mM), however, the CYP2A6 inhibition decreased compared to inhibition percentages found using the low substrate concentration (0.5mM). The opposite was found for CYP2E, as inhibition studies with antibodies and DEDC indicate that it catalysed a negligible part of the reaction at the low substrate concentration and up to 84 % at the high concentration. Thus hydroxylation of bupropion is following the same pattern in pigs as in humans and the activity measured in pigs is comparable with the human counterpart. Furthermore BUP is a more specific substrate for CYP2E than CLX and PNP although not perfect.
INTRODUCTION

Human CYP2E1 is involved in metabolism of a number of chemicals, including carcinogens and a variety of endogenous compounds and is induced by alcohol, fasting, diabetes, and obesity. (Rendic, 2002; Lieber, 1997). The activity of CYP2E1 is usually quantified by measuring the 6-OH hydroxylation of chlorzoxazone (CLX) (Peter R et al, 1990) or hydroxylation of p-nitrophenol (PNP) (Koop et al.,1989). Pigs are often used as appropriate animal models in pharmacological and toxicological testing and CLX and PNP have been used as prototypical substrates of CYP2E in this specie as well (Meyers et al., 2001; Nebbia et al., 2003; Bogaard et al., 2000; Skaanild and Friis, 1999; Desille et al., 1999; Baranova et al., 2005; Lejus et al., 2002). However, it has never been verified that CLX or PNP are selective probes for a CYP2E1 like enzyme in pigs, and a number of observations indicate that they are metabolized by other porcine CYPs. Bogaard et al., (2000) found that the metabolism rate of CLX in micropig microsomes was half of that reported for humans, and in contrast to the human reaction the porcine reaction could not be inhibited with anti-rat CYP2E1. In rats, these substrates are metabolized not only by CYP2E, but also to a lesser extent by CYP1A2 and CYP3A1 (Kobayashi et al., 2002). We have shown that the 6-OH hydroxylation of CLX neither correlates with the immunochemical level of CYP2E nor with the mRNA level (Skaanild and Friis, 1999). In contrast, it shows the same gender related pattern as the CYP2A activity and the immunochemical level for CYP2A. In human microsomes it has been reported that CYP2A6 is involved in the PNP hydroxylation, as the reaction was inhibited by 8-methoxypsoralen, an inhibitor of human CYP2A6 (Monostory et al., 2004). In another study the induction of the metabolism of the two substrates was not followed by an induction in the immunochemical level of CYP2E confirming the lack of correlation (Meyers et al., 2001). However, Baranova et al. (2005) conclude that there is similarity between human and minipig CYP2E based on inhibition studies with diethyldithiocarbamate (DED). Both the CLX hydroxylation catalysed by the cloned cDNA-expressed protein and by porcine microsomes was inhibited giving rise to different but comparable Ki values.

As the data from the majority of these studies suggest that the CYP2E might not be the only enzyme involved in hydroxylation of CLX and PNP, the objective of this study was to confirm this hypothesis and examine bupropion as a prototype substrate for porcine CYP2E. Bupropion is metabolised by CYP2E1 and CYP2B6 in humans depending on the concentration of the substrate (Faucette et al., 2000).
METHODS

Animals. Eight Göttingen minipigs (4 female, 4 male, age 4 months) and 12 conventional pigs (4 female, 4 male, 4 castrates, age 3.5 months) have previously been described. (Skaanild and Friis, 1999)

Chemicals. All antibodies were obtained from Gentest (Massachusetts, USA), hydroxybupropion was from BD Biociences (Bedford, USA) and all other chemicals were of analytical grade obtained either from GE Healthcare or Sigma (St. Louia, USA).

Isolation of liver microsomes. Isolation of microsomes was performed according to Olsen et al. 1997. Briefly the liver was homogenized in 50 mM Tris-HCl buffer containing 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged and the supernatant was transferred to new tubes and centrifuged once more at 105,000xg at 4°C for 60 min. The pellet containing the microsomes was homogenized in storage buffer and frozen in liquid nitrogen.

Microsomal protein concentration was determined using a modified Lowrey method (Petterson, 1977).

Enzyme assay. The microsomal mixture for all assays consisted of a buffer containing 32 mM K-phosphate pH 7.46, 2.5 mM MgCl₂, 15 mM glucose-6-phosphate, 10 U glucose-6-P-dehydrogenase/ml, 1.1 mM NADP and 0.91 mg microsomal protein in a total volume of 1.075 ml. The mixture was pre-incubated for 5 min at 37°C before the test substrate was added. The kinetic constants were estimated by nonlinear regression using the Michaelis-Menten model.

Chlorzoxazone hydroxylase assay. The reaction was initiated by addition of 50 ul of a 3.7 uM chlorzoxazone solution (54 mCi/mmol; final concentration 0.17 uM). After 15 min incubation the reaction was stopped by addition of an equal volume of methanol. The samples were analyzed by HPLC as described earlier (Skaanild and Friis 1997)

p-nitrophenol hydroxylase assay. The reaction was initiated by addition of PNP to a final concentration of 200 uM. After 15 min incubation the reaction was stopped by addition of 1/16 volume of 50% CCL₃COOH. After centrifugation for 5 min at 10,000 x g 100 ul of the supernatant was aspirated for HPLC analysis under the following conditions: Column: Novapak C18 (5u, 15 cm); Column temperature: 30°C; Eluent: 75% water: 25% acetonitrile: 0.1% triflouroacetic acid; flow 1ml/min.

Bupropion hydroxylase assay. To initiate the reaction bupropion (BUP) was added giving final concentrations from 0.05-7 mM. After 1h incubation the reaction was stopped by adding 0.5 vol of
70% perchloracid and then the reaction was centrifuged for 3 min at 10,000 x g and 50 ul of the supernatant was injected. The sample was analysed by HPLC under the following conditions: Column: Nova-Pak C₁₈ (300 x 3.9 W31981B); Column temperature: 40°C; Eluent: 30% acetonitrile: 0.3 % trifluoroacetic acid: 69.7% water; Flow: 0.8ml/min; UV 210nm.

Coumarin hydroxylase assay. To initiate the reaction 50 µl of a 2.2 mM coumarin solution in water was added and the reaction was incubated for further 10 min. then stopped by addition of 1.1 ml methanol. The samples were analyzed by HPLC as described earlier (Skaanild and Friis, 1999).

Nifedipine oxidase assay. The reaction was started by the addition of 15 ul of a 14.66 mM nifedipine solution in acetone. The samples were incubated for 10 min and then further treated as described earlier (Skaanild and Friis, 1999).

Inhibition assay. Inhibitory polyclonal anti-human CYP2A6 was added to the microsome solution (20 µl/mg prot.) and pre-incubated for 5 min at 37°C, and then the reactions were started by adding substrates. Chemical inhibition was performed using diethyldithiocarbamate (DEDC), an inhibitor of human CYP2E1 and CYP2A6, and troleandomycine (TROL) a potent inhibitor of human CYP3A4. The chemical inhibitors were also added to the incubation solution before addition of the substrate. The final concentrations of DEDC and TROL were 0.3 mM and 1.0 mM respectively.
RESULTS

Inhibition studies using anti-human CYP2A6 revealed that CLX and PNP hydroxylation could be inhibited 70% and 50% respectively, suggesting that neither CLX nor PNP are very useful substrates for CYP2E (Fig.1).

The hydroxylation of bupropion was therefore investigated and preliminary experiments using a substrate concentration of 0.5 mM bupropion showed that the formation of hydroxybupropion was linear up to 90 min. incubation time (Fig.2.). Porcine microsomes were then incubated with different concentrations of bupropion from 0.05 to 7mM giving a Vmax of 0.2 nmol/mg prot./min and Km = 0.52 mM for the minipig and a Vmax = 0.14 nmol/mg prot./min and Km = 0.21 mM for the conventional pig (Fig.2, Table 1.). The influence of CYP2A was tested using the same bupropion concentrations together with anti-human CYP2A6 antibody. This slightly reduced the Vmax to 0.18 nmol/mg prot/min and 0.11 nmol/mg prot/min in the two strains, indicating that CYP2A only play a minor role. The reactions were markedly inhibited with DEDC, an inhibitor of human CYP2E1 and CYP2A6, leading to Vmax of 0.04 nmol/ mg prot /min and 0.05 nmol/mg prot. Two (2) substrate concentrations were chosen for further inhibition studies, a low of 0.5mM (Vmax for the DEDC inhibited curve), and a high of 5mM (Vmax for the uninhibited reaction). The mean formation of hydroxybupropion measured in microsomes from 20 pigs was 135 ± 125 pmol/mg prot/ min and 152 ± 113 pmol/mg prot/min using test substrate concentrations of 0.5mM and 5mM respectively. Inhibition experiments were conducted with microsomes from 4 different pigs, and the following inhibitors were used, anti-human CYP2A6, troleandomycine (TROL), an inhibitor of CYP3A4 and DEDC (Fig.4). Anti-human CYP2A inhibited the reactions on average 50 % and 13 % respectively. Chemical inhibition with TROL does not have any effect at either concentration. DEDC inhibited both reactions to the same extent, 77 % and 78 % respectively. In table 2 the inhibition percentages (5mM substrate concentration) of CYP2A6 and DEDC are shown for each pig. The results show that the inhibitory antibody against CYP2A6 exhibit different effects in the different pigs from 0 % to 24 % inhibition. The DEDC inhibition percentages also vary (62 %-88 %).

To verify that TROL can inhibit the porcine CYP3A, a test reaction with nifedipine, a human and porcine test substrate for CYP3A, was carried out. TROL inhibited the nifedipine oxidation by 80%. DEDC’s inhibitory effects on the CYP2A coumarin hydroxylation was also tested. DEDC inhibited this reaction 98 %, confirming that DEDC is a potent inhibitor of porcine CYP2A.
DISCUSSION

CLX and PNP are considered classical prototype drugs for human CYP2E1 (Peter et al., 1990; Koop et al., 1989). Therefore these drugs have also been used as test substrates for the porcine CYP2E (Meyers et al., 2001; Nebbia et al., 2003; Bogaard et al., 2000; Skaanild and Friis, 1999, Desille et al., 1999; Baranova et al., 2005; Lejus et al., 2002). The present study clearly demonstrates that porcine liver microsomes are able to catalyse the hydroxylation of both CLX and PNP and that CYP2E is not the only enzyme involved in these processes as anti-human CYP2A6 could inhibit the reaction. This inhibition is not due to a cross reaction between anti-human CYP2A and CYP2E as an inhibition of 0% was obtained for one of the pigs. The CLX hydroxylation has been analyzed by Baranova et al., (2005) using inhibition with DEDC and they found different Ki levels for the cDNA expressed enzymes compared to the microsomal Ki level. A plausible explanation is that the Ki observed for the cDNA expressed enzyme is the real Ki for this enzyme. However, the Ki found using microsomes is the combined Ki for CYP2A and CYP2E enzymes as the present study shows that DEDC is also a potent inhibitor of CYP2A. The differences in enzyme specificity (Vmax/Km) between microsomes and cDNA expressed enzymes may also be caused by CYP2A activity, because the specificity of this enzyme toward CLX is probably different from that of CYP2E.

Bupropion was chosen as a possible alternative to CLX and PNP due to the fact that this compound is metabolized by human CYP2E at high concentrations. The control reaction set up with substrate concentrations from 0.005 to 7.0mM showed that Km values and Vmax values in pigs are comparable to the ones found for humans (Faucette et al., 2000). The reaction without inhibitor reached Vmax at a higher substrate concentration than the DEDC inhibited reaction indicating that they are catalysed by different enzymes. The last reaction is, as in humans, probably catalysed by CYP2B as DEDC inhibits both the CYP2A and the CYP2E catalyzed part of the reaction.

The inhibition experiments using two substrate concentrations, a low (0.5mM) and a high (5mM), showed that CYP3A is not involved as TROL could not inhibit the reactions. Both CYP2A and CYP2E are involved, however, CYP2A to a minor extent at the high concentration, as the inhibition percentages were low. CYP2E, on the other hand, must be the predominant enzyme at the high concentration, as DEDC inhibits the two reactions equally well. At the high concentration it was furthermore seen that the anti-CYP2A could not inhibit the reaction at all in one of the pigs, showing that this antibody does not cross-react with any of the other isoenzymes involved in this reaction. It can be concluded from this study that 1. CLX and PNP are poor substrates for
measuring CYP2E activity in porcine microsomes and 2. the porcine microsomal metabolism of bupropion resemble that of human, where the reaction mainly is catalysed by CYP2E at high substrate concentration (5 mM).
REFERENCES


Fig. 1. Time graph for OH-bupropion formation using a substrate concentration of 0.5 mM.

Fig. 2. Mean inhibition of chlorzoxazone and $p$-nitrophenol hydroxylation using anti-human CYP2A6 ($n = 4$).

Fig. 3. The rate of hydroxybupropion formation with and without inhibitors versus the bupropion concentration.

Fig. 4. Average inhibition of BUP hydroxylation by different inhibitors anti-human CYP2A, troleandomycine (1mM) and diethyldithiocarbamate (0.3mM) at two different substrate concentrations ($n=4$).

Table 1. Data for the enzyme kinetics of BUP hydroxylation without and with inhibition for minipig and conventional pig liver microsomes

Table 2. The inhibition percentage measured for 4 pigs using anti-human CYP2A6 and DEDC at a substrate concentration of 5mM.