Expression of four porcine CYPs in *E. coli* and development of fluorogenic CYP inhibition assays .

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While the pathways of xenobiotic metabolism in humans are well characterized, they are less so in the animal models used during drug development and in animals of commercial importance. With a view to providing tools for use in helping to select the appropriate animal model and developing veterinary treatments, we have expressed four porcine (Sus scrofa) CYPs, CYP1A2, CYP2C49, CYP3A39 and CYP2E1 in E. coli along with porcine NADPH P450 reductase for the first time. The activity of these enzymes has been investigated using substrates routinely used in human CYP in vitro work and we have developed a quick and simple, fluorescence based assay to look at the potential for compounds to inhibit the four porcine CYPs. This assay is based on Cypex's fluorogenic inhibition screen for human CYPs and allows comparisons to be made between human and porcine CYPs. Substrates tested included diethoxyfluorescein, 7-ethoxyresorufin, 7-benzyloxyquinoline, 7-methoxy-4-aminomethylcoumarin, 7-methoxy-4-trifluoromethylcoumarin and 3-cyano-7-ethoxycoumarin. For each CYP a panel of eleven known inhibitors of human CYPs was assayed and, for each compound, the IC_{50} was compared to that obtained with human and canine CYPs.

Results

The four CYPs, chosen based on their abundance in porcine liver (Achour B. et al (2011)), were expressed in *E. coli* together with porcine NADPH P450 reductase, table 1. The activity of the recombinant CYPs was assayed with 7-ethoxycoumarin (7-EC), 7-ethoxyresorufin (7-ER) (CYP1A2), diclofenac, tolbutamide (CYP2C49), testosterone (CYP3A39) and chlorzoxazone (CYP2E1). With CYP1A2 the Km for 7-EC was <0.01 µM which proved too low to make it practical to develop the assay further. 7-ER was used as an alternative substrate giving a Km 10 fold lower than with human CYP1A2 (table 1). Incubating CYP2C49 with diclofenac did not yield 4'-hydroxydiclofenac but other unidentified diclofenac metabolites were seen on HPLC so tolbutamide was used as an alternative substrate for CYP2C49. When CYP2E1 and CYP3A39 were first co-expressed with P450 reductase the enzyme activity was low (below the limit of detection for CYP2E1). In order to increase activity a second 'high reductase' co-expression plasmid was made with both the CYP and NADPH P450 reductase being expressed from pCW. CYP2E1 and CYP3A39 were also supplemented with human cytochrome b_5 at a molar ratio of 5:1 to the CYP to further increase the activity. Kinetic parameters for each enzyme are shown in table 1.

Plate reader assays were set up for the four CYPs with a range of fluorogenic substrates being tested; CYP1A1 with 7-ethoxyresorufin (7-ER), CYP2C49 with 7-methoxy-4-trifluoromethylcoumarin (MFC), diethoxyfluorescein (DEF) and 3-cyano-7-ethoxycoumarin (CEC), CYP3A39 with 7-benzyloxyquinoline (7-BQ), DEF and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) and CYP2E1 with 7-ER, MFC, BFC, 7-ethoxyfluorocoumarin, 7-BQ, 7-methoxy-4-aminomethylcoumarin, DEF and CEC. The substrates selected for each CYP are shown in table 2. These assays were then used to screen 11 compounds for their ability to inhibit the recombinant CYPs.

The IC_{50} values obtained for each compound with CYP1A2, CYP2C49 and CYP3A39, compared to those obtained for human and canine CYP orthologs, are shown in fig. 1. In general the inhibition of the porcine CYPs mirrored that seen with human and canine CYPs although there were some differences. Porcine CYP1A2 was not inhibited by paroxetine and porcine CYP3A39 was inhibited by ticlopidine much more than human and canine CYP3A. Where there were differences between human and canine CYP inhibition e.g. CYP1A2 inhibition by montelukast, the porcine isoform tended to mirror the human equivalent. CYP2E1 was only inhibited by montelukast (IC_{50} 19 µM) although the values for montelukast were found to be somewhat variable, presumably due to montelukast's poor solubility.

Conclusion

Pigs are increasingly being developed as an alternative non-rodent model species for *in vivo* drug development studies however the drug metabolizing enzymes in porcine liver are relatively poorly characterized. Here we have described the expression of recombinant CYPs from porcine liver which allows some comparisons to be made *in vitro* between pig and other species. In the main the activity of the pig enzymes behaved in similar ways to their human and canine orthologs although there were a few differences which, may, if unaccounted for, lead to unexpected results in *in vivo* studies. Clearly other CYP isoforms in pig liver may mimic their human equivalents more closely (e.g. CYP2C33, CYP3A29) but the isoforms that have been expressed here are the most abundant members of their family in porcine liver and so would be expected to have the greatest contribution to porcine drug metabolism. This is a preliminary study and the cloning and expression work is ongoing to express additional porcine liver enzymes in order that more complete comparisons can be made.

Refs

Pritchard M. P., Ossetian, R., Dongtao N. L., Henderson C. J., Burchell, B., Wolf C. R. & Friedberg, T. (1997) Arch. Biochem. Biophys. 345 (2) 342 - 354

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Methods

cDNAs coding for porcine CYP1A2, CYP2C49, CYP3A39 and CYP2E1 were cloned into pCW with an ompA leader (Pritchard *et al* (1997)). Porcine NADPH P450 reductase was cloned into a pACYC184 based expression plasmid with an N terminal pelB leader. *E. coli* JM109 was used for protein expression, carried out as previously described (Pritchard *et al* (1997)). CYP levels were determined spectrophotometrically, the NADPH P450 reductase was quantified by determining the NADPH cytochrome *c* reductase activity.

Enzyme assays were carried out at 37°C in 50 mM potassium phosphate pH7.4, (CYP1A2, CYP2C49) or 100 mM potassium phosphate pH7.4 (CYP3A39, CYP2E1), with 5 mM MgCl2. Assays were started by the addition of NADPH generating system and stopped after 5 min. Samples were analysed by HPLC.

Plate reader assays were carried out at 37°C in 96 well plates in 50 mM potassium phosphate pH 7.4, 5 mM $MgCl_2$ with a final protein concentration of 0.1 mg/ml. After a 10 min preincubation at 37°C the reaction was started by the addition of NADPH generating system and the fluorescence was monitored approximately every 90 seconds over a period of 30 min. The rate of increase of fluorescence was plotted in Excel. Potential inhibitors were dissolved in DMSO (final concentration 1.5% v/v). IC_{50} values were calculated in Excel using XLfit software (IDBS).

Table 1, Expression levels and recombinant porcine CYP activities

	P450 content (pmol/mg)	Substrate	Km (µM) (Values for human in brackets)	Vmax (pmol/min/pmol) (Values for human in brackets)
CYP1A2 low reductase	500	7-ER	0.03 (0.3)	0.6 (1.1)
CYP2C49 low reductase	500	Tolbutamide	500 (150, CYP2C9)	0.2 (2.5, CYP2C9)
CYP3A39 low reductase	250	Testosterone	50 (20, CYP3A4)	0.4 (14, CYP3A4)
CYP3A39 high reductase + cyt b_5	200	Testosterone	40 (25, CYP3A4)	20 (75, CYP3A4)
CYP2E1 high reductase + cyt b_5	500	Chlorzoxazone	120 (45)	4 (10)

Table 2, Plate reader assay substrates

	Substrate	Km (µM)	Concentration in assay (µM)	Detection Wavelengths
CYP1A2	7-ER	0.03	0.025	λ_{ex} 572 nm λ_{em} 604 nm
CYP2C49	DEF	2.00	1.500	λ_{ex} 485 nm λ_{em} 530 nm
CYP3A39	DEF	1.20	1.000	λ_{ex} 485 nm λ_{em} 530 nm
CYP2E1	CEC	10.00	10.000	λ_{ex} 410 nm λ_{em} 460 nm

Fig 1, IC_{50} Values for CYP1A2, CYP2C49 and 3A39 compared to human and canine CYPs