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Published in:
Basic & Clinical Pharmacology & Toxicology

DOI:
10.1111/j.1742-7843.2008.00323.x

Publication date:
2008

Document version
Early version, also known as pre-print

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Manuscript date: Number of pages: 18
Number of tables: 2
Number of figures: 3
Number of references: 14

Abstract

The CYP2C subfamily in humans includes 4 different isoenzymes that metabolize different substrates although with some cross reactivity. Some of these substrates, e.g. diclofenac and tolbutamide, have been investigated in porcine microsomes, but without identifying the specific CYP catalyzing the reactions. The objective of this study was therefore to test some CYP2C substrates and identify the porcine CYP2C responsible for the reaction. Three substrates - paclitaxel, tolbutamide and omeprazole were chosen, as they are metabolised by 3 different CYP2C isoenzymes in humans. Microsomes isolated from 20 different pigs, 12 conventional, and 8 minipigs, were incubated with these compounds and correlations between the metabolism rates of these 3 substrates were found indicating that the reactions are catalysed by the same enzyme. Male minipigs tend to have higher average activity than females, which is in contrast to the gender dependent expression seen for other CYP isoenzymes. The metabolic activities correlated with the protein level determined in Western blotting, using anti – human CYP2C9, indicating that this enzyme is responsible for the reaction. The expression of the CYP2C enzymes was analyzed by Real-Time PCR, using a primer set that could amplify CYP2C8, CYP2C9, and CYP2C19. The melting curves (peaks) revealed that all three genes were present, showing very different expression level in the various types of pigs. The area of one of the peaks however correlated with the CYP2C9-like enzyme concentration, suggesting that this peak represent CYP2C9. Among paclitaxel, tolbutamide and omeprazole, omeprazole is the best probe of CYP2C9-like enzyme in the pig.

Introduction

The CYP2C subfamily consists of several enzymes. In humans, 4 different enzymes have been identified [1], CYP2C8, CYP2C9, CYP2C18 and CYP2C19 constituting about 20% of total P450 and metabolising 35% of drugs [2]. The various CYP2Cs show some cross reactivity towards many of the test substrates, making it difficult to study the specific CYP2C activities independently. The CYP2C substrate, tolbutamide, which is fairly specific for human CYP2C9, has been tested in pigs. The tolbutamide hydroxylation was measured in both conventional pigs and minipigs [3, 4] showing that the minipigs had lower activity compared to human activity [4]. The activity was, however, not induced in pigs treated with a combination of β-naphthoflavone, phenobarbital and dexamethasone [3], but the enzyme activity could be inhibited by about 80% with tranylcypromine, a CYP2C19 inhibitor, whereas the activity was not inhibited by sulphaphenazole, an inhibitor of human CYP2C9 [5]. The 4-hydroxylation of another human CYP2C substrate, diclofenac, has been measured in Yucatan minipigs [6] and showed low activities in pigs compared to humans. This hydroxylation of diclofenac can be inhibited 80-100% in humans by sulphaphenazole, but the porcine activity was only slightly inhibited (20%), indicating that either the substrates or the inhibitor are not specific for this enzyme in pigs, or the reaction is catalysed by another enzyme. Mephenytoin 4-hydroxylation is used as a test reaction for human CYP2C19, but no metabolite could be detected in either conventional or minipigs [3, 6, 7]. These results show that human test substrates may not always be suitable for measuring porcine CYP2C activity. The objective of this study was therefore to characterize the porcine CYP2C with regard to the presence of enzymes and selection of probes for metabolic activity. Tolbutamide and two more specific human CYP2C substrates, paclitaxel, PAC (CYP2C8 [8]) and omeprazole, OME (CYP2C19 [9]) were chosen as test substrates.

Material and 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 [7].

Chemicals. All antibodies and 6-alfa hydroxypaclitaxel were obtained from Gentest (Massachusetts, USA), paclitaxel, tolbutamide, 4-hydroxytolbutamide, and omeprazole were from Sigma and the 5-hydroxy metabolite of omeprazole was a gift from AstraZeneca. 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. In short, the liver was homogenized in a 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 4^0C for 60 min. The pellet containing the microsomes was homogenized in a storage buffer and frozen in liquid nitrogen.

Microsomal protein concentration was determined using a modified Lowrey method.

Enzyme assay. The microsomal mixture for all assays consisted of a buffer containing 32 mM K-phosphate pH 7.46, 2.5 mM $MgCl_2$, 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 37^0C before the test substrate was added. The kinetic constants were estimated by nonlinear regression using the Michaelis-Menten model.

Paclitaxel assay. The hydroxylation of paclitaxel assay was carried out according to Desai et al 1998 with some minor modifications. In short, the reaction was started by addition of paclitaxel to a final concentration of 15 µM. After 45 min the reaction stopped by addition of 1 vol. acetonitrile and the suspension was centrifuged 8500 x g for 3 min. The HPLC was carried out using 100 ul of this solution. HPLC conditions: Column: Nova-pak C_{18}, Column temperature 30^0C; Eluent: 65 % methannol: 34.8 % water: 0.2 % acetic acid; Flow rate: 1.0 ml/min; Detection: UV at 230nm. Retention times for paclitaxel and 6-hydroxypaclitaxel were 7 min and 6.2 min respectively. The hydroxypaclitaxel metabolit was quantified according to the 6-α-hydroxypaclitaxel calibration curve and the hydroxylated paclitaxel metabolit had the same retention time.

Tolbutamide assay. The hydroxylation of tolbutamide was measured according to Palamanda et al. [10] with some minor modifications. In short, the reaction was started by addition of tolbutamide to a final concentration of 1.6 mM. The reaction was incubated 45 min and then stopped by addition of 1/25 vol. 70% perchloric acid and centrifuged 8500 x g for 3 min. Of this supernatant, 100 ul was used for HPLC analysis performed under the following conditions: Column: Spherisorb C_{18},

Column temperature 30^0C; Eluent: 38 % acetonitrile: 61.6 % water: 0.4 % acetic acid; Flow rate: 1.0 ml/min; Detection: UV at 230nm. Retention times for tolbutamide and hydroxyl-tolbutamide were 16 min and 6.3 min respectively.

Omeprazole assay. The 5- hydroxylation of omeprazole was measured according to Shu et al [11] with minor modifications. In short, the reaction was initiated by addition of omeprazole giving a final incubation concentration of 50 µM. After 30 min the reaction was stopped by addition of an equal volume of methanol. The samples were centrifuged 8500 x g for 3 min where after 100 ul was used for HPLC analysis under the following conditions: Column: Waters Symmetry C_{18}; Column temperature 30^0C; Eluent: 23 % acetonitrile: 77 % Tris buffer; Flow rate: 1.2 ml/min; Detection: UV at 302nm.

Immunoblotting. Microsomes isolated from the pig liver were used for blotting according to Skaanild & Friis (1999)[7]. In short, microsomal protein was separated by PAGE SDS gel electrophoresis and blotted to Hybond-ECL nitro-cellulose membranes. Membranes were hybridized (1h) with a diluted primary anti-human CYP antibody. Antibody binding was detected by chemiluminescence, using a biotinylated secondary antibody followed by a streptavidin-horseradish peroxide conjugate. After development, the blots were exposed to HyperfilmECL and the signal was quantified using the QuantiScan software from Biosoft (Cambridge, UK).

CYP2C gene expression analysis. Total RNA was isolated from pig livers, using the Quiagen RNAesasy Kit according to kit manual. The RNA was then reverse transcribed using GE Healthcare First strand cDNA synthesis kit according to the manual. The cDNA was amplified using primers, designed from the human CYP2C sequences, with the following sequences: forward primer 5`GGC TGT GCT CCC TGC AAT GTG A `3 and the reverse primer 5`GAT GGA GTT GAA GGT AGT TTC GTG`3. The PCR reaction was set up as follows: denaturing at 94^0C, annealing 53^0C the first 4 cycles, 51^0C the next 4 cycles, 49^0C the next 4 cycles and 47^0C for the last 23 cycles, extension 72^0C.The primers were designed to hybridise to both CYP2C8, CYP2C9 and CYP2C19-like sequences giving one PCR product of about 455 bp (from base 606 to 1061 for CYP2C8 and from base 511 to 966 for CYP2C9 and CYP2C19). Restriction fragment length analysis (RFLA) was then performed, using the restriction enzymes HindIII and PstI. HindIII digest the CYP2C 9 into two fragments of 111 bp and 344 bp, whereas PstI cuts CYP2C19 into two fragment of 93 and 362 bp. The CYP2C8 gene sequence was cut by neither of the enzymes.

Real-Time PCR was also performed using Roche-Lightcycler 480 and Roche SyberGreen PCR kit, using the same PCR program as for the normal PCR. After 40 PCR cycles, melting curves were obtained in order to test the reaction specificity.

Statistics

Student's t-test for unpaired data was used for comparison of the differences between two means and P-values < 0.05 were accepted as evidence of statistically differences. Rank analysis using the Spearman method was used to perform the correlation analysis between peak area and immunochemical levels.

Results

Microsomes were incubated with different concentrations of the substrates, and their Michaelis-Menten kinetic curves are depicted in fig.1. The resulting Vmax and Km values (table 1) are lowest for paclitaxel especially in the conventional pigs. The highest Km values were found for tolbutamide, whereas the highest Vmax were measured for the omeprazole hydroxylation.

Further the capacity to metabolise these compounds was measured in microsomes from different groups of pigs (conventional males, castrates and females, minipigs males and females). Paclitaxel was metabolised to the same extent in all groups, whereas the metabolism of tolbutamide and omeprazole tended to be higher in male pigs although not significant at $p<0,05$ level (Table 2).

Correlations were found between the hydroxylation activities of these 3 substrates ($r = 0.8$ between omeprazole and tolbutamide, $r = 0.6$ between omeprazole and paclitaxel). The various CYP2C levels were determined by immunoblotting using antibodies against human CYP2C8, CYP2C9 and CYP2C19. Correlation analysis between the CYP protein levels and substrate metabolism showed that the concentration of a CYP2C9-like protein correlates with the metabolism of all three substrates, where omeprazole hydroxylation gives the best fit (Fig. 2). Negative correlations were found for the CYP2C8-like protein and the CYP2C19 like protein.

To further identify the protein catalysing the metabolism of these substrates, the expressions of the enzymes were analysed using isolated RNA from the pig livers and then reverse transcribe it to cDNA. The cDNA was amplified, using a primer set, designed from the human sequences, to hybridise to CYP2C8, CYP2C9 and CYP2C19. All three products are of the same size, but can, if they have the sequence as the human genes, be digested by different restriction enzymes, making it possible to distinguish the products from one another. The digestion with PstI showed that the PCR fragment could be digested by this restriction enzyme and for one of the female minipigs (57919); the PCR fragment was nearly totally digested. Digestion with HindIII, however, failed or gave very faint digestion band.

As the digestion of the PCR fragment did not give results for all CYP2C, Real - Time PCR was set up; ending with a melting curve program to demonstrate that the PCR fragment contained 3 different bands. Three different melting peaks (peak 1, 2, and 3) with melting temperatures 79 C, 83 C and 85 C respectively were identified. (Fig. 3). When looking at pig 57919 giving rise to the PCR fragment(s) that was digested nearly 100% with PstI, it is noted that the mRNA give rise to 2 very small peaks an a big peak with a melting temperature of 83 C. i.e. peak 2. Analysis of male minipig

PCR melting peaks revealed that the product mainly contained the band with the highest melting temperature (peak 3). Spearman ranking correlation analysis of the minipig results showed that there was correlation between the area of peak 3 and the immunoblotting levels estimated using the anti human CYP2C9 (r = 0.6) , whereas negative correlations were found between the CYP2C9 concentration and the area of peak 1 and 2 .

Discussion

The microsomal metabolism of 3 potential CYP2C substrates was measured to identify porcine CYP2C substrates. Paclitaxel, which in human microsomes is metabolised to 6-α-hydroxy paclitaxel catalysed by CYP2C8, is also metabolised by porcine microsomes. The metabolite, however, is claimed not to be the 6 hydroxypaclitaxel, but an unknown metabolite according to Vaclavikova et al.[12]. In the present study, however, the metabolite has the same retention time as the human metabolite 6-α-hydroxy paclitaxel furthermore Vmax and Km values were similar to those published for human microsomes [13]. The metabolism of tolbutamide, a substrate that is mainly metabolised by human CYP2C9 and to lesser extent by human CYP2C19, was also analysed, using porcine microsomes. In pigs tolbutamide Km values were higher than the values reported in human, whereas the Vmaxs were in the same range [9,10]. The porcine omeprazole Km and Vmax were about 10 times higher than the human high affinity counterparts, whereas the levels were the same for the low affinity reactions [11,14]. The metabolism rates of the 3 substrates measured in the 20 pigs correlated, indicating that they are metabolised by the same enzyme, in contrast to what is seen in humans. A fairly good correlation between the metabolism rates of the 3 substrates and the protein level of a CYP2C9-like enzyme, and the lack of correlation with CYP2C8-like and CYP2C19-like enzymes suggested that a CYP2C9-like enzyme is responsible for the reactions. For all test substrates it was noted that male minipigs exhibited higher catalytic activity than females.

In the expression analysis of CYP2C digestion of the PCR product(s) with Pst1 gave rise to restriction fragment of the expected size and for pig 57919 the PCR product was nearly totally digested indicating that CYP2C19 was about the only CYP2C expressed in this pig. Digestion with Hind III however failed which could be ascribed to either lack of the digestion site in the porcine sequence due to a base pair change or that CYP2C9 is barely expressed. The Real-Time PCR analysis of minipig mRNA however revealed that all 3 products are present as 3 melting curve peaks appeared. Peak 2 could be identified as CYP2C19 as this peak was about the only one found for pig 57191. The area of peak 3 was higher for male minipig than for female suggesting that this peak could represent the CYP2C9 product as the concentration of this enzyme is higher in males than females. Spearman ranking correlation analysis between the protein level and peak area supported this hypothesis.

In summary, porcine microsomes can metabolise the 3 human substrates paclitaxel, tolbutamide and omeprazole. A CYP2C9 like protein catalyses the reactions and omeprazole appears to be the best

substrate. It is noted that the male minipigs posses higher activity than the females in contrast to what have been seen for other CYP`s in minipigs, where the female catalytic activity is the highest. Based on Real - Time PCR analysis it seems all three CYP2C are present in the pig.

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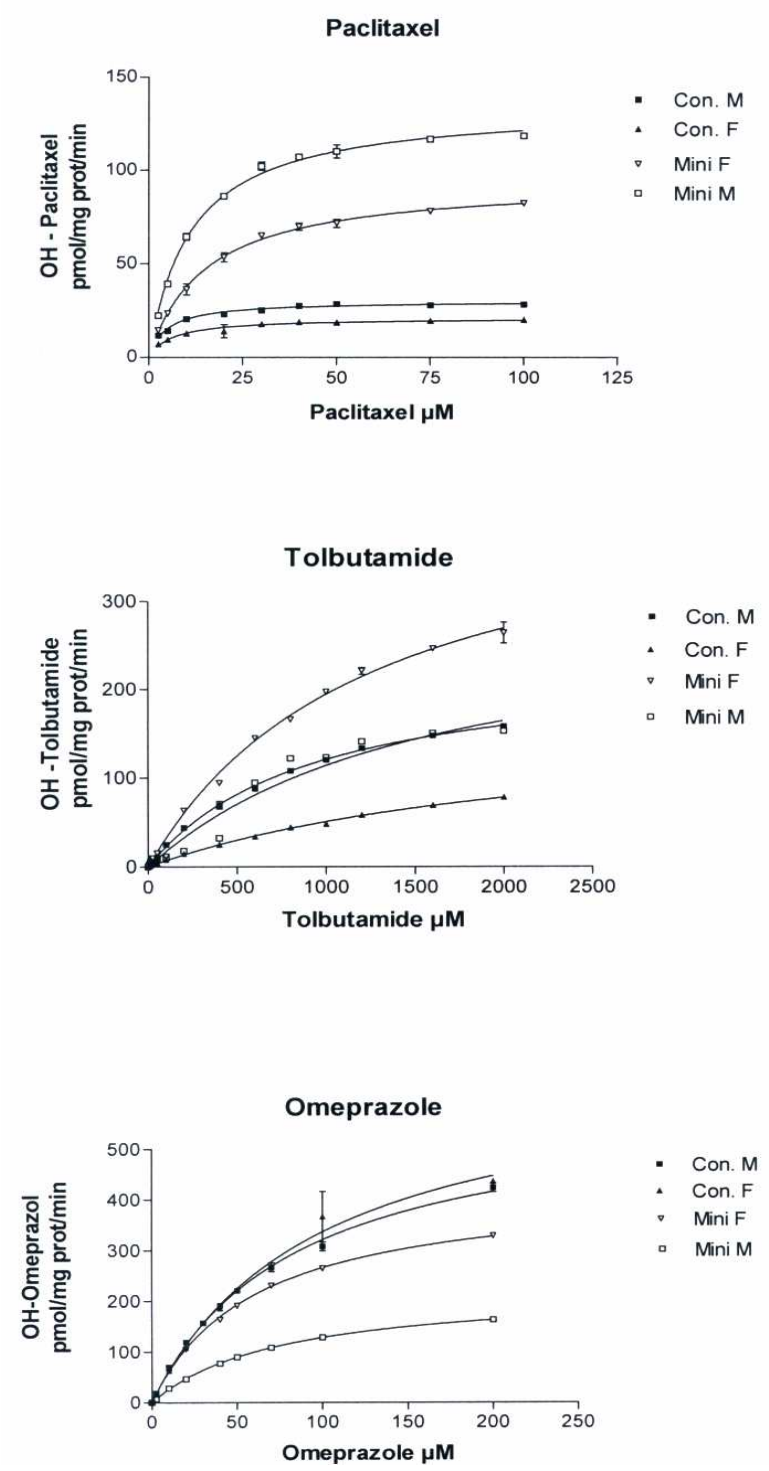

Fig. 1. The Michaelis –Menten curves for the different substrate incubations measured in 4 different pigs, conventional male and female (Con. M and F) and minipig male and female (Mini M and F)

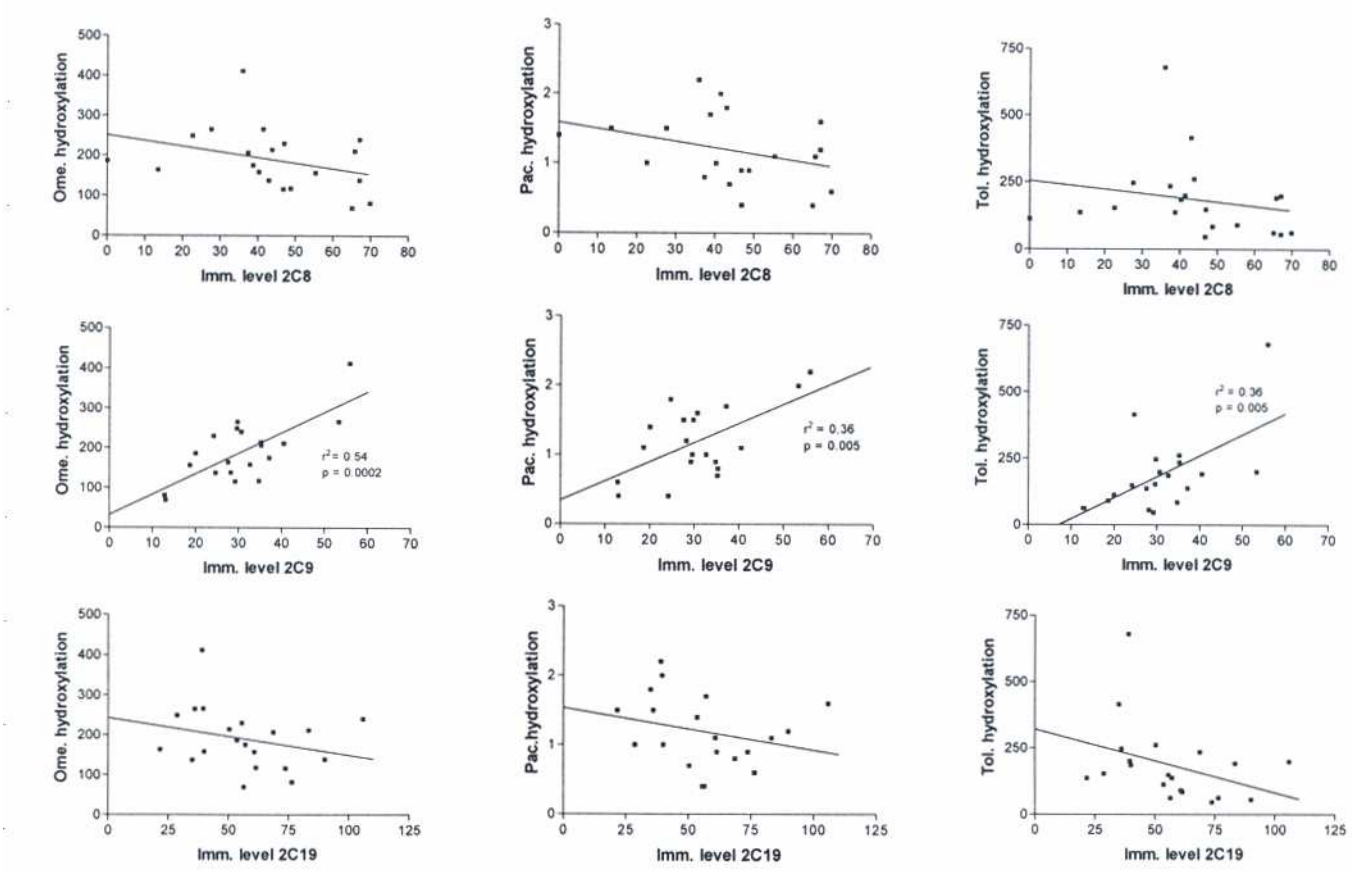

Fig. 2. Relationships between hydroxylation of omeprazole, paclitaxel and tolbutamide and immunochemical levels of enzymes determined using anti-human antibodies against CYP2C8, CYP2C9, and CYP2C19.

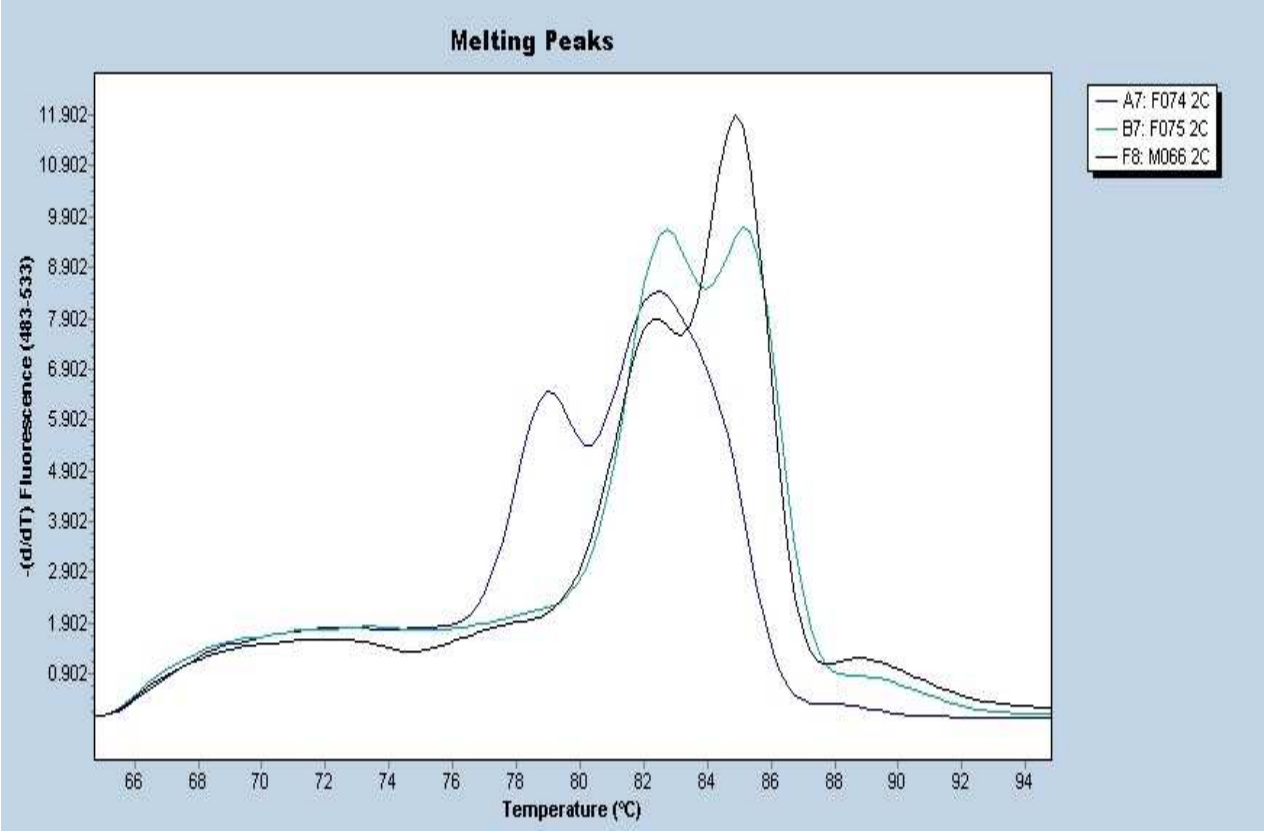

Fig.3. Melting program peaks for 3 different minipigs showing peaks for 3 different PCR products with 3 different melting temperatures.

Pigs	Substrate	Km uM	Vmax pmol/mg prot/min	Vmax/Km
Con. M	Pac.	4,8	30,1	6,3
Con. F	Pac.	6,3	21,1	3,3
Mini M	Pac.	11,2	135	12,1
Mini F	Pac.	15,5	94,7	6,1
Con. M	Tol.	930	233,4	0,3
Con. F	Tol.	1616	298	0,2
Mini M	Tol.	1265	440,9	0,3
Mini F	Tol.	2250	166	0,1
Con. M	Ome.	83,7	590,2	7,1
Con. F	Ome.	97,7	665,9	6,8
Mini M	Ome.	60,4	426,9	7,1
Mini F	Ome.	75,5	225,4	3,0

Table 1. Data for the enzyme kinetics of paclitaxel, tolbutamide, and omeprazole hydroxylations for 4 different pigs.

Pigs	OH- pac.	OH-tol. pmol/mg prot/min	OH-ome.
Castrat	1,4	113,2	425,9
Castrat	0,6	63,4	186,5
Castrat	1,1	91,2	359,4
Castrat	1,5	247,4	608,3
	1,1± 0,4	128,8 ± 81,7	395 ± 174,3
Female	1,7	137,7	399,6
Female	1,5	137,4	377,5
Female	0,4	62,1	157,7
Female	1,0	153,8	570,3
	1,1± 0,6	122,8 ± 41,1	376,3 ± 168,3
Male	0,4	149,0	525,4
Male	0,8	234,7	473,4
Male	0,9	84,9	267,6
Male	0,7	261,8	489,6
	0,7 ± 0,2	182,6 ± 81,0	439 ± 135,6
Minipigs			
Female	1,6	199,3	551,3
Female	1,1	191,8	484,5
Female	0,9	47,4	265,1
Female	1,2	56,1	316,4
	1,2 ± 0,3	123,6 ± 83,2	404,3 ± 135,6
Male	2,0	201,1	610,9
Male	2,2	680,7	1100,1
Male	1,0	185,7	363,2
Male	1,8	415,5	316,0
	1,8 ± 0,6	370.8 ± 231,7	597,6 ± 359,1

Table 2. Microsomal paclitaxel-, tolbutamide-, and omeprazole hydroxylation activities for 20 different pigs divided into groups of males, females and castrates.