Tissue distribution and phenobarbital induction of target SLC- and ABC- transporters in cattle

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TISSUE DISTRIBUTION AND PHENOBARBITAL INDUCTION OF TARGET SLC- AND ABC-TRANSPORTERS IN CATTLE

Short running title: SLC- and ABC-transporters in cattle tissues

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ABSTRACT

In veterinary pharmaco-toxicological sciences, few data about uptake and efflux drug transporters (DTs) expression and regulation phenomena have been published. In the present study, the tissue distribution and transcriptional modulation of solute carrier (SLC) and ATP-binding cassette (ABC) DTs were investigated in cattle orally administered with phenobarbital (PB) by using a quantitative Real Time RT-PCR approach. The criterion for target gene selection was the PB-responsiveness in human and rodent model species.

All target DTs were expressed in the liver. Only two out of the seven PB-responsive target DTs (SLCO1B3 and SLC10A1) were not constitutively expressed in extra-hepatic tissues. The greatest number of quantifiable DTs (SLCO2B1, ABCB1, ABCC2, ABCG2) were noticed in intestine and testis, followed by adrenal gland (SLCO2B1, ABCB1, ABCG2), lung (ABCB1, ABCG2), kidney and skeletal muscle (ABCG2). Phenobarbital administration never altered DTs mRNA levels, except for an increase of hepatic ABCC2 mRNA and a down-regulation of renal ABCG2.

Altogether, these results confirm only to some extent data obtained in humans and laboratory species; clearly, they should be considered a preliminary step for further molecular investigations about species-differences in DT gene expression and regulation as well as in DT expression and function.

KEY WORDS

SLC-transporters, ABC-transporters, cattle, phenobarbital, gene expression.

INTRODUCTION

Drug metabolizing enzymes[1] play a fundamental role in the metabolism of xenobiotics and drugs to whom the living organism is exposed (Xu et al., 2005). Recently, beyond phase I and II of drug metabolism, two additional steps (phase 0 and phase III of drug disposition) have assumed an increasing importance in pharmaco-toxicology. In phase 0, the xenobiotic uptake occurs through the action of multi-specific solute carrier[2] transporters (influx transporters), for which an ATP hydrolysis is not required. In phase III, the xenobiotic efflux is basically carried out by transporters (efflux transporters) of the multidrug resistance[3] superfamily of ATP-binding cassette[4] transporters, requiring an ATP binding and hydrolysis (Zaïr et al., 2008; Hagenbuch 2010). Drug metabolizing enzymes and drug transporters[5] show coordinated mechanisms of regulation and expression; therefore, they cooperate in detoxification and excretion of xenobiotics (Xu et al., 2005; Olinga et al., 2008).

The term induction denotes a dose-dependent increase in DMEs gene expression and function discovered more than 35 years ago, whose magnitude depends on constitutional, dietary, physiological and environmental factors. The induction of DMEs and DTs may potentially lead to changes in the bioavailability of xenobiotics or undesirable drug-drug interactions. As a consequence, studies about regulation of DMEs and DTs gene expression have gained increasing importance (Xu et al., 2005(Olinga et al., 2008; van de Kerkhof et al., 2008).

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[1] Drug metabolizing enzymes: DMEs.
[2] Solute carrier: SLC.
[3] Multidrug resistance: MDR.
[4] ATP-binding cassette: ABC.
[5] Drug transporters: DTs.

Phenobarbital[6] is a typical DMEs inducer, eliciting a number of pleiotropic effects on liver function (Handshin and Meyer, 2003; Kodama and Negishi, 2006; Lambert et al., 2009). Since the early 60's PB has been used as a prototype inducer for studies on xenobiotic drug metabolism as well as for genetic, pharmacological, and toxicological investigations (Kakizaki et al., 2003). Besides a large subsets of phase I and II DMEs, PB has been shown to modulate the expression of some DTs like the multidrug resistance-associated protein 2[7] and solute organic anion transporting polypeptides[8] (Johnson et al., 2002; Patel et al., 2003), thereby altering the glucuronidation and the following biliary excretion of endogenous substrates or, to a wider extent, of xenobiotics (Ritter et al., 1999).

In human and rodent model species an extensive literature about the expression of DTs in organs involved in xenobiotic drug metabolism or blood-tissues barriers as well as their substrates/inhibitors has been published (Faber et al., 2003; Augustine et al., 2005; Zaïr et al., 2008; Vähäkangas and Myllynen, 2009; Hagenbuch, 2010; Kim et al., 2007; Mruk et al., 2011). In veterinary pharmaco-toxicology, species-differences in the expression and substrate specificity of ABC-transporters have been recently reviewed (Martinez et al., 2008; Schrickx and Fink-Gremmels, 2008; Mealey, in press); furthermore, information about the constitutive expression of some efflux transporters in a number of species of veterinary interest (e.g., horse, trout, turkey, poultry and cattle) have been published, too (Taguchi et al., 2002; Haritova et al., 2008; Warren et al., 2009; Haritova et al., 2010; Lončar et al., 2010; Mani et al., 2010; Tydén et al., 2010). Despite this, in veterinary species little is still known about DTs tissue

[6] Phenobarbital: PB.
[7] Multidrug resistance-associated protein 2: MRP2.
[8] Organic anion transporting polypeptides: OATPs.

distribution and molecular mechanisms involved in their transcriptional regulation following the exposure to prototypical inducers, such as PB.

In this study, the constitutive expression and modulation of the solute carrier organic anion transporter[9] family 1, member 3 (SLCO1B3), SLCO2B1 and SLC10A1 as well as ABCB1, ABCB11, ABCC2 and ABCG2 have been investigated, for the first time and by using a quantitative Real Time RT-PCR[10] approach, in liver and foremost extrahepatic tissues of control and PB-treated cattle. These target genes were chosen on a literature basis, and were essentially represented by hepatic DTs known to be specifically modulated by PB in human and laboratory species (Fardel et al., 2001; Courtois et al., 2002; Patel et al., 2003; Cheng et al., 2005; Jigorel et al., 2006; Martin et al., 2008; Olinga et al., 2008; van de Kerkhof et al., 2008; Lambert et al., 2009; Le Vee et al., 2009; Martin et al., 2010). This study was part of a wider project aimed to characterize the effects of PB upon cattle DMEs, DTs and nuclear receptors[11] involved in their regulation, whose just preliminary liver post-translational data have been published so far (Cantiello et al., 2006).

MATERIALS AND METHODS

Chemicals

Phenobarbital was obtained from Sigma-Aldrich (St. Louis, MO). Chloroform, isopropyl and ethyl alcohol were obtained from Thermo Electron Corporation

[9] Solute carrier organic anion transporter: SLCO.
[10] quantitative Real Time RT-PCR: qPCR.
[11] Nuclear receptors: NRs.

(Waltham, MA), whereas TRIzol® reagent and agarose from Invitrogen (Carlsbad, CA). High Capacity cDNA Reverse Transcription Kit, RNAlater® solution and Power SYBR® Green PCR Master Mix were from Applied Biosystems (Foster City, CA). Oligonucleotide primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

Animals, treatments and tissue collection

The experiment was run in an authorized facility located nearby the Faculty of Veterinary Medicine of Turin, according to the European Community Directive 86/609, recognized and adopted by the Italian Government (DLgs 116/92). The experimental plan was approved by the Italian Ministry of Health.

Seven healthy male Friesian cattle (about 300 kg bw and 10 months old) were divided, on a weight basis, into two groups of three and four animals. The former one served as control[12], while individuals of the second group received PB[13] (18 $mg \cdot kg^{-1} \cdot body\ weight^{-1} \cdot day^{-1}$ and for 7 days) by oral gavage. Cattle were slaughtered the day after the suspension of PB administration. After exsanguination, aliquots (about 200 mg each) of liver, duodenum, kidney, lung, adrenal gland, testis and skeletal muscle were collected, immediately frozen in liquid nitrogen and, then, stored at -80°C until use. Animal carcasses were not intended to be used for human consumption and were then destroyed according to existing legislation.

[12] Control group: CTRL.
[13] Group administered with PB: PHEN.

Total RNA extraction and reverse-transcription

Total RNA was isolated by using the TRIzol® reagent, according to the manufacturer's instruction. Ribonucleic acid concentration and quality were determined by using the Nanodrop ND-1000 spectrophotometer (Labtech France, Paris, France). Its integrity was confirmed by denaturing gel electrophoresis and visualization of 18S and 28S rRNA bands.

The reverse transcription of 2 μg of RNA was performed by using the High Capacity cDNA Reverse Transcription Kit (Foster City, CA) and random primers (final assay volume of 20 μL) following the purchaser's procedure. Furthermore, the SPUD assay was carried out to detect the presence of inhibitors in cDNA generated from RNAs extracted from target tissues (Nolan et al., 2006).

Quantitative Real Time RT-PCR

Primer oligonucleotide sequences of candidate internal control genes[14] and target DTs used for qPCR are listed in Table 1 and 2, respectively. Primers were designed *ex novo* except for β-actin[15], glyceraldehydes-3-phosphate dehydrogenase[16] and glucose-6-phosphate dehydrogenase[17] (Toffolatti et al., 2006), ribosomal protein, large P0[18] and

[14] Internal control genes: ICGs.
[15] β-actin: ACTB.
[16] Glyceraldehyde-3-phosphate dehydrogenase: GAPDH.
[17] Glucose-6-phosphate dehydrogenase: G6PDH.
[18] Ribosomal protein, large P0: RPLP0.

18S rRNA (Robinson et al., 2007) as well as the TATA-box-binding protein[19] (Lisowski et al., 2008).

Bos taurus coding sequences were obtained from GenBank website [http://ncbi.nlm.nih.gov/], and best primer pairs for amplification were chosen by using the Primer Express™ Software 3.0 (Applied Biosystems, Foster City, CA, USA). As the gene annotation process of cattle genome is still in progress, only partial (for GAPDH and 18S transcripts) and predicted sequences (for ABCB1 and ABCC2) were taken into account for some target genes. The Primer Express™ Software 3.0 as well as OligoAnalyzer 3.1 [http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx] were used to confirm the absence of primer dimers and hairpin formation. The SYBR Green chemistry was used and a careful validation of primer pairs was undertaken to be sure that only the target gene sequence was amplified. Primers specificity was checked *in silico* by using the NCBI Nucleotide Basic Local Alignment Search Tool and Primer-Nucleotide Basic Local Alignment Search Tool [http://blast.ncbi.nlm.nih.gov/Blast.cgi]; furthermore, to avoid amplification of potentially contaminating genomic DNA, one of two primers was designed spanning an intron-exon junction. Finally, the presence of specific amplification products was confirmed by agarose gel electrophoresis and dissociation curve after qPCR reaction. Each primers pair was optimized to 300-900 nM range to identify primers concentration providing the highest sensitivity.

Calibration curves were obtained following the amplification of decreasing amounts of different cDNA pools. Two cDNA pools were prepared according to preliminary information about gene amplification in target tissues (data not shown). The

[19] TATA-box-binding protein: TBP.

former included liver, intestine, adrenal gland and testis, while the second one comprised kidney, lung and skeletal muscle. Just to avoid inaccurate results, tissues in which target genes were not constitutively expressed or, alternatively, showed mean cycle threshold[20] values over 30 or very close to the limit of quantification of the thermal cycler were not taken into consideration. Pooled cDNA diluted at 3- or 4-fold intervals were used to evaluate qPCR performances, such as PCR efficiency[21] determined by using the equation E_x = 10(-1/slope) -1), sensitivity assay and test linearity correlation. Only E_x values comprised between 1.9 (90% of efficiency) and 2.1 (110% of efficiency) as well as high correlation coefficients ($0.9846 \leq r \leq 0.9998$) were accepted.

The qPCR was performed on 5µL of cDNA in a final volume of 20 µL, by using Power SYBR®Green PCR Master Mix and an ABI-Prism 7000 thermal cycler (Applied Biosystems, Foster City, CA) under standard qPCR conditions taken from the Power SYBR® Green RT-PCR Reagents Kit User Guide. These ones were represented by a first step of activation for the AmpliTaq Gold® Polymerase activation (10 min at 95°C), followed by denaturation (40 cycles of 15 sec at 95°C), annealing and extension (1 min at 60°C) steps. Samples without reverse transcriptase (RT *minus*) and template were used as controls. The $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) was then used to analyze data. Target genes constitutive expression in extra-hepatic tissues and their modulation following PB administration were expressed as n-fold change, that is normalized to the $\Delta\Delta C_t$ mean values of liver and CTRL group, respectively, to whom an arbitrary value of 1 were assigned.

[20] Cycle threshold: C_t.
[21] PCR efficiency: E_x.

Statistical analysis

Open source geNorm^{PLUS} (Vandesompele et al., 2002) and NormFinder version 0.953 (Andersen et al., 2004) algorithms were used to identify the two most stably expressed ICGs. Differences in DT mRNA levels between CTRL and PHEN were analyzed by using the Student's *t*-test (GraphPad InStat 3, San Diego, California, USA). A p value < 0.05 was considered as statistically significant. Data were expressed as mean arbitrary units[22] ± standard deviation[23].

RESULTS

Validation of qPCR assays and selection of the best ICGs

A relatively "pure" RNA (260 and 280 nm absorbance ratios were about 1.9 - 2.0) without co-purified contaminants (absorbance ratios at 260 and 230 nm comprised between 1.8 - 2.2) was obtained from each tissue aliquot. All qPCR primers allowed the obtainment of specific amplicons, distinct dissociation peaks and a single band of the expected size in agarose gel electrophoresis.

To identify the best ICG to be used for data normalization, mRNA levels of eight candidate genes (ACTB, B2M: β_2-microglobulin[24], GAPDH, G6PDH, cyclophilin A[25], RPLP0, TBP and 18S) were measured in target tissues. Thereafter, a pre-selection was made by using the mean C_t value obtained from pooled cDNA samples as a criterion.

[22] Arbitrary units: a.u.
[23] Standard deviation: SD.
[24] β_2-microglobulin: B2M.
[25] Cyclophilin A: PPIA.

The 18S gene was excluded as a result of its high constitutive expression in target tissues (C_t~18). On the contrary, GAPDH as well as G6PDH and TBP were not taken into account as they were shown to be poorly expressed (C_t > 29) in the lung (the former one) and kidney, lung and skeletal muscle (the two other ones). Once verified the absence of significant differences between CTRL and PHEN, the amplification data of the remaining candidate ICGs (ACTB, B2M, PPIA and RPLP0) were submitted to geNorm^{PLUS} [http://www.biogazelle.com/mybiogazelle/] and NormFinder [http://www.mdl.dk/Files/NormFinder-HowTo%20v18.pdf] algorithms to identify the most stable couple of ICGs. In both analyses, ACTB was proved as the best ICG, with the lowest NormFinder stability and geNorm^{PLUS} M values (0.078 and 0.99, respectively). The NormFinder software identified ACTB and B2M as the best combination of two ICGs, with a stability value of 0.058. On the other hand, geNorm^{PLUS} recognized ACTB, B2M and RPLP0 as the three reference genes with the lowest M value. However, the variability value among sequential normalization factors (based on the n and n+1 least variable targets) was considered greater than expected (the threshold value is set at 0.15) by the software. In this case, the use of n-1 reference targets showing the lowest M value is recommended; therefore, ACTB, B2M and RPLP0 were used as ICGs in the present study.

The main features of each qPCR assay (4 ICGs, 7 DTs), namely slope, E_x, *y*-intercept, correlation coefficients[26] and linear dynamic range are reported in Tables 3-4.

Constitutive expression of DTs in target tissues

[26] Correlation coefficients: R^2.

The constitutive expression of PB-responsive SLC- and ABC-transporters in target tissues is reported in Figures 1 and 2. To help the reader's understanding, gene expression profiles were compared with liver ones, in which all these DTs were proved to be expressed. On the whole, lower amounts of DT mRNAs were found in extrahepatic tissues, except for ABCB1. Sometimes, target genes were expressed at negligible levels (i.e., SLCO1B3, SLC10A1, ABCB11). Only three out of seven candidate DTs (ABCG2, ABCB1 and, to a lower extent, SLCO2B1) were expressed in half or more target tissues. Apart from the liver, the intestine and testis were the tissues in which the larger number of DTs (SLCO2B1, ABCB1, ABCC2, ABCG2) were found, followed by adrenal gland (SLCO2B1, ABCB1, ABCG2), the lung (ABCB1 and ABCG2) and, finally, kidney and skeletal muscle (only ABCG2 for both).

Modulation of DTs gene expression by PB

Whole results on the effects of PB upon DT mRNAs in cattle target tissues are reported in Tables 5-6. No transcriptional modulation of target DTs was ever noticed, except for an increase ($p<0.05$) of ABCC2 mRNA in the liver (see Figure 3A) and a down-regulation ($p<0.05$) of ABCG2 gene in the kidney (see Figure 3B).

DISCUSSION

In pharmaco-toxicological sciences, induction is a phenomenon in which a xenobiotic up-regulates the expression (gene-protein) and function of DMEs. The concept of induction has been known from many years, and nowadays it is well established that a

wide number of drugs, pesticides, food additives, industrial chemicals, natural products and environmental pollutants are likely to induce DMEs. What's more, recent advances in DMEs molecular biology have brought in new information about the molecular mechanisms regulating the expression of DMEs in the presence of an inducer. Most of DMEs are induced by a receptor-mediated increase of gene transcription, and members of the NR superfamily of transcription factors have been shown to be the key mediators of most transcriptional changes occurring in target genes (Xu et al., 2005; Graham and Lake, 2008).

Phenobarbital is an early example of a known inducer. It is considered a prototype of a large group of structurally unrelated chemicals (PB-*like* compounds) that induce a large number of hepatic DMEs (i.e., cytochromes P450[27] 2B, 2C and 3A and transferases). This process is part of several pleiotropic effects elicited by PB in the living organism. Induction occurs predominantly in the liver, intestine and, to a lower extent, in other extrahepatic tissues (e.g., kidney and lung: Denison and Whitlock, 1995; Kakizaki et al., 2003; Goriya et al., 2005). What's more, target gene up-regulation is mostly driven by NRs (NR1I2 and NR1I3: Kodama and Negishi, 2006). Thus, PB was (Dupuy et al., 2001) and it is still used as a prototypical inducer useful to characterize DMEs expression (Longo et al., 2004), NR-dependant regulation phenomena (Tamasi et al., 2009), as well as potential drug-drug interactions (Ballent et al., 2010). By contrast, few papers investigating the *in vivo* effects of PB upon DMEs gene expression have been published so far in veterinary species, and none in cattle (Kawalek et al., 2003; Chirulli et al., 2005; Goriya et l., 2005; Marini et al., 2007; Makino et al., 2009).

[27] Cytochromes P450: CYPs.

In the past two decades the characterization of DTs distribution, their substrate specificities and modulation by prototypical DMEs inducers have provided outstanding information about molecular mechanisms governing the disposition of both endogenous compounds and xenobiotics in humans and model species (Jigorel et al., 2006; Martin et al., 2008; Zolk and Fromm, 2011). Drug transporters are of extremely importance also in veterinary medicine, as many drugs commonly used in veterinary chemotherapy are substrates for one or more DTs (Schrickx and Fink-Gremmels, 2008); despite this, few data are actually available about their expression and modulation. The goal of the present study was to provide a first overview about tissue distribution of PB-responsive SLC- and ABC-transporters in cattle and their modulation following a PB oral administration at a dose most probably able to induce DMEs.

First of all, the chosen protocol was successful. Although PB did not elicit a transcriptional effect upon NRs, hepatic CYP2B22, 2C31, 2C87 and 3A, glucuronosyltransferase 1A1-*like* and glutathione S-transferase A1-*like* mRNA levels were significantly up-regulated; what's more, immunoblotting investigations and relative enzymatic catalytic activities, measured by using prototypical marker substrates, confirmed transcriptional results (Cantiello et al., 2006; Zancanella et al., personal data). On a second instance, it should be underlined that partial or predicted sequences were considered for some ICGs and DTs (GAPDH, 18S, ABCB1 and ABCC2), as cattle genome annotation process is still in progress. Therefore, some attention should be still given in the extrapolation of present results, cause sequences generated from incomplete data or automated computational analysis might contain errors or gaps. A critical discussion of obtained results, for each subset of target genes (ICGs, SLC- and ABC-transporters) is hereby reported.

Identification of the best ICG

A reliable normalization is a fundamental prerequisite for gene expression data analysis, and such an approach requires the measurement of an ICG. In the present study, geNorm^{PLUS} was chosen, among the different free algorithms taken into account, to identify the best ICG. Such a choice was justified by the fact that both algorithms indicated ACTB and B2M as the two most stable genes, but geNorm^{PLUS} provided an additional information about the optimal number of ICGs to be used in the present experiment. In this respect the use of a third gene, besides ACTB and B2M, was suggested for a more accurate data normalization, and RPLP0 was identified by using geNorm^{PLUS}.

Solute carrier transporters

Among PB-responsive SLC-transporters, the SLCO1B3 gene encodes for OATP1B3, a protein mostly expressed at the basolateral membrane of human hepatocytes (Hagenbuch, 2010); moreover, SLCO1B3 is also expressed in placenta (Briz et al., 2003), prostate (Hamada et al., 2008), colon (Ballestero et al., 2006) and gastrointestinal tumors (Hagenbuch and Meier, 2004). In cattle, SLCO1B3 was for the most part expressed in the liver (likewise to humans), whereas poor mRNA amounts were detected in kidney, testis, adrenal gland and skeletal muscle. Phenobarbital did not affect SLCO1B3 gene expression in cattle, but this same influx transporter did not unequivocally respond to PB in humans: in fact, a gene down-regulation was noticed in

primary hepatocytes (Jigorel et al., 2006), while SLCO1B3 gene expression was either unchanged or induced in liver slices (Olinga et al., 2008).

If compared with SLCO1B3, the SLCO2B1 gene (coding for OATP2B1 protein) presents a wider tissue distribution, as it is constitutively expressed in cells of excretory organs and in a number of blood/organ barriers (Hagenbuch, 2010; Meyer zu Schwabedissen and Kim, 2009). In cattle, the SLCO2B1 gene was expressed in all the analyzed tissues, and comparable mRNA amounts were found in liver and intestine as well as in testis and adrenal gland. In human primary hepatocytes, the SLCO2B1 gene transcription was suppressed after 72 hrs of incubation with PB (Jigorel et al., 2006); what's more, PB has been shown to significantly down-regulate murine OATP2B1 gene expression *in vivo*, too (Cheng et al., 2005). By contrast, cattle SLCO2B1 mRNA levels were never affected by PB in liver and in extra-hepatic tissues as well.

Among SLC-transporters a key role is played by SLC10A1, the foremost uptake system for conjugated bile salts; this gene encodes for NCTP, a protein exclusively expressed at the hepatocyte basolateral membrane (Hagenbuch, 2010). Likewise to humans, measurable amounts of SLC10A1 mRNA were just found in cattle liver, but PB did not affect SLC10A1 gene expression. Nevertheless, conflicting results have been obtained in previous comparative *in vitro*/*in vivo* studies. A reduction of SLC10A1 mRNA was observed in PB-exposed human primary hepatocytes (Jigorel et al., 2006); dose-dependent effects (up-regulation at μM concentrations, down-regulation at mM ones) were observed in human liver slices and HepaRG cells (Olinga et al., 2008; Lambert et al., 2009); finally, SLC10A1 gene expression was not significantly affected at both transcriptional and post-translational levels in rats and mice intraperitoneally

injected with PB (Hagenbuch et al., 2001, Wagner et al., 2005). Thus, definitive conclusions cannot still be drawn.

ATP-binding cassette transporters

Among members of the ABC-transporter gene superfamily believed responsive to PB there is ABCB1, encoding for MDR1 and mdr1a/1b proteins. In cattle, this gene was expressed in most of tissues subject of investigation (adrenal gland>intestine>testis>lung>liver), likewise to humans and model species (Brady et al., 2002; Nishimura and Naito, 2005; Nishimura et al., 2009). Phenobarbital has been recognized as a *p*-glycoprotein[28] substrate in humans (Luna-Tortós et al., 2008; Zhang et al., 2010; Chan et al., 2011); moreover, it has been shown to increase P-gp protein expression and transport activity in mouse brain capillaries (Abbott et al., 2010; Wang et al., 2010) as well as in other *in vitro* models, such as human hepatocytes (Jigorel et al., 2006), liver and intestinal slices (Olinga et al., 2008; van de Kerkhof et al., 2008), and HepG2, LS180 and Caco-2 cell lines (Martin et al., 2008; Schuetz et al., 1996). In the present study, PB up-regulated liver ABCB1 mRNA (250% *vs* CTRL), and the lack of a statistical significance might be essentially attributed to the high biological variability noticed within PHEN group.

In cattle, quantifiable amounts of ABCB11 mRNA were found exclusively in the liver, thereby confirming its predominant hepatic localization. Based on raw C_t values information, the ABCB11 gene was also expressed (but not at quantifiable levels) in testis (data not shown). These data agree with humans ones (Nishimura and Naito,

[28] *p*-glycoprotein: P-gp.

2005). Phenobarbital did not affect ABCB11 mRNA in cattle, but in humans distinct transcriptional effects (up- or down-regulation, a biphasic increase and following decrease, dose- and time-dependent modulation) have been observed *in vitro* (Jigorel et al., 2006; Olinga et al., 2008; Lambert et al., 2009).

In human and laboratory species, the ABCC2 gene is predominantly expressed in liver, kidney, small intestine and, to a lower extent, in testis, lung and adrenal gland (Nishimura and Naito, 2005; Kim et al., 2007; Nishimura et al., 2010). Except for the kidney, a similar pattern of gene expression was noticed in cattle tissues. This would suggest that ABCB1 and ABCC2, as well as P-gp and MDR2 proteins, might play a fundamental role in intestinal drug efflux mechanisms not only in humans but also in cattle. Phenobarbital significantly up-regulated ABCC2 gene expression in cattle, according to previously published comparative *in vitro* data (Jigorel et al., 2006; Martin et al., 2008; Olinga et al., 2008; Lambert et al., 2009).

The gene (ABCG2) coding for breast cancer resistance protein[29] is expressed in a number of mammalian tissues, and particularly in those having important barrier function: intestine, liver, brain, placenta, kidney and mammary gland (Jonker et al., 2005; Han and Sugiyama, 2006; Huls et al., 2009). In cattle, the ABCG2 gene was the DT greatly more expressed in target tissues (liver≈intestine>adrenal gland>lung>kidney≈skeletal muscle). Usually PB is considered neither a BCRP substrate nor an inhibitor (Cerveny et al., 2006); on the other hand, the ABCG2 gene has been shown to be up-regulated by PB in HepaRG cell line, hepatocyte primary cultures and rodent brain capillaries (Jigorel et al., 2006; Lambert et al., 2009; Wang et

[29] Breast cancer resistance protein: BCRP.

al., 2010). In the present experiment, a significant down-regulation of ABCG2 gene was noticed in cattle kidney, a result actually with no possible explanation.

Collectively, present results consent to draw some general considerations. To date, DTs are believed to play a significant role not only in multi-drug resistance phenomena, but also in kinetic behavior (i.e., absorption and distribution) of endogenous compounds as well as of xenobiotics (Xu et al., 2005; International Transporter Consortium, 2010; Zolk and Fromm, 2011). Knowledge about the impact of DTs in veterinary pharmaco-toxicology is still limited, though some progress has recently been done (Martinez et al., 2008; Schrickx and Fink-Gremmels, 2008; Haritova et al., 2008; Haritova et al., 2010; Mealey, in press). The major drawback is the lack of systematic data about DTs physiological levels in animal tissues and information about molecular mechanisms involved in their transcriptional regulation. To the best of our knowledge, this is the first study providing new information about the mRNA tissue distribution and transcriptional modulation of DTs in cattle orally administered with PB at inducing purposes. As a whole, cattle DTs tissue distribution was similar to that observed in humans and rodent model species; in particular, liver, duodenum and testis were confirmed as tissues mostly endowed of DTs and, therefore, regularly involved in cattle transport activity.

Since the 60's PB is known as a general prototypical inducer of DMEs, but it affects DTs gene/protein expression, too (Johnson et al., 2002; Patel et al., 2003; Xu et al., 2005); in this respect, it is believed that xenobiotics up-regulating DT proteins might be general substrates for these latter ones; a clear example in this respect is represented by PB and its interaction with P-gp (Yang and Liu, 2008). Unlike data of tissue distribution, contrasting results about the transcriptional effects of PB on target DTs

were obtained. Nonetheless, these outcomes were not astonishing. Drug transporters and DMEs share common mechanisms of transcriptional activation. Nuclear receptor 1I2 and NR1I3 transactivate CYP2B, 2C and 3A promoters following the exposure to PB and PB-*like* compounds (Xu et al., 2005; Bell and Michalopoulos, 2003; Chen and Goldstein, 2009); furthermore, CYP2B induction in liver and extrahepatic tissues is strictly related to the corresponding amount of NR1I3 mRNA (Pustylnyak et al., 2009). These same transcription factors are actively involved in the regulation of DTs (Wagner et al., 2005; Xu et al., 2005; Wang et al., 2010; Chan et al., 2011). Thus, it should be conceivable to trace back present conflicting results to (a) tissue-differences in the regulation of DTs (likewise to DMEs: Marini et al., 2007) or, alternatively, to (b) a dissimilar constitutive expression of these crucial NRs in cattle tissues, with resultant species-differences in the animal response to prototypical inducers such as PB (Graham and Lake, 2008). Species-differences in CYP2B response to PB and PB-*like* compounds may occur through variations in NR ligand binding domain sequences or in NR1I2/NR1I3-dependent transcriptional activation of target genes (Kiyosawa et al., 2008; Pustylnyak et al., 2009; Kojima et al., 2009); in this respect, neither NR1I2 nor NR1I3 gene were ever modulated by PB in present cattle tissues (Zancanella et al., personal data). Another attractive subject of discussion is whether differences in the transcriptional response of candidate DTs could be somewhat attributed to PB dosage protocol. It's known that DTs (and DMEs as well) are transcriptionally modulated by PB in a dose- and time-dependent way (Hagenbuch et al., 2001; Wagner et al., 2005; Olinga et al., 2008; Lambert et al., 2009), even though most of these studies have been made *in vitro* (e.g., by using primary hepatocytes, liver slices or established cell lines), in laboratory species (i.e., Jones et al., 1998; Zheng et al., 2011) and, seldom, in the dog

(Kawalek et al., 2003; Fukunaga et al., 2009). On the contrary, a single-dose protocol was used in those *in vivo* studies where the effects of PB were investigated in veterinary species (Dupuy et al., 2001; Chirulli et al., 2005; Goriya et l., 2005; Marini et al., 2007). Altogether, as different PB concentrations, routes of administration and protocol duration were used, a clear-cut comparison among species cannot be done; nevertheless, the presence of low PB concentrations in cattle tissues might hypothetically be offered as a further explanation for present conflicting results.

Presented data suggest that a closer molecular approach about localization, regulation and function of DTs not only in cattle but also in other veterinary species and, within a species, in target tissues is nowadays needed. Several *in vitro* and *ex vivo* procedures such as organ primary cultures, tissue slices, transfected cell lines and reporter gene constructs, might be used for this purpose (Graham and Lake, 2008). These tools, together with emerging new *in silico* models, *omic* and next generation sequencing methodologies, will allow veterinary pharmaco-toxicologists to predict the clinical relevance of drug-drug interactions and the development of new drugs tailored to reach faster and effectively the target tissue (Schrickx and Fink-Gremmels, 2008).

In conclusion, in the present study the distribution and transcriptional modulation of PB-responsive DTs have been investigated for the first time in liver and extrahepatic tissues of control and PB-treated cattle. Drug transporters tissue distribution substantially confirm previously published comparative data, while PB up-regulated only hepatic ABCC2 and decreased renal ABCG2 mRNA levels. Presented data should be considered as a starting point for further molecular studies in cattle, designed to investigate more in depth the presence of species-differences in drug transporter gene expression and regulation as well as in drug transporter protein expression and function,

which might ultimately result in a different kinetic behavior and/or in dangerous drug-drug interactions.

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LEGENDS TO TABLES 1-6 AND FIGURES 1-3.

Table 1. Candidate internal control genes: GenBank accession numbers, oligonucleotide sequences, references and amplicon sizes.

Table 2. Target drug transporters: GenBank accession numbers, oligonucleotide sequences, references and amplicon sizes.

Table 3. Main features (slope, E_x, *y*-intercept, R^2 and linear dynamic range) of best internal control gene qPCR assays.

Table 4. Main features (slope, E_x, *y*-intercept, R^2 and linear dynamic range) of target drug transporters qPCR assays.

Table 5. Relative abundances of SLC-transporters mRNA in cattle orally administered with PB. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of CTRL, to whom an arbitrary value of 1 was assigned.

Table 6. Relative abundances of ABC-transporters mRNA in cattle orally administered with phenobarbital. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of CTRL, to whom an arbitrary value of 1 was assigned.

Fig. 1. Constitutive expression of SLC- drug transporters in cattle tissues. Messenger RNA was extracted from cattle tissue aliquots and drug transporter mRNA levels were measured by a qPCR approach. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of liver tissue, to whom an arbitrary value of 1 was assigned.

Fig. 2. Constitutive expression of ABC- drug transporters in cattle tissues. Messenger RNA was extracted from cattle tissue aliquots and drug transporter mRNA levels were measured by a qPCR approach. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of liver tissue, to whom an arbitrary value of 1 was assigned.

Fig. 3. Effect of PB on hepatic ABCC2 and renal ABCG2 gene expression.

Messenger RNA was extracted from tissue aliquots obtained from CTRL and PHEN animals, and target drug transporter mRNA levels were measured by a qPCR approach. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of CTRL, to whom an arbitrary value of 1 was assigned.

Table 1. Candidate internal control genes: GenBank accession numbers, oligonucleotide sequences, references and amplicon sizes

gene acronym	GenBank ID	primer sequence 5' → 3'	reference	amplicon size (bp)
ACTB	NM173979	F: GTCATCACCATCGGCAATGAG R: AATGCCGCAGGATTCCATG	Toffolatti et al., 2006	84
B2M	NM173893	F: TCGTGGCCTTGGTCCTTCT R: AATCTTTGGAGGACGCTGGAT	designed *ex novo*	71
GAPDH	U85042	F: ACACCCTCAAGATTGTCAGCAA R: TCATAAGTCCCTCCACGATGC	Toffolatti et al., 2006	102
G6PDH	NM001244135	F: GCAAAGAGATGGTCCAGAACC R: TGTCCCGGTTCCAAATGG	Toffolatti et al., 2006	75
PPIA	NM178320	F: CTCTTTTGAGCTGTTTGCAGACA R: CCAAATCCTTTCTCTCCAGTGCT	designed *ex novo*	81
RPLP0	NM001012682	F: CAACCCTGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA	Robinson et al., 2007	227
TBP	NM001075742	F: ACAACAGCCTCCCACCCTATGC R: GTGGAGTCAGTCCTGTGCCGTAA	Lisowski et al., 2008	111
18S	DQ222453	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	Robinson et al., 2007	152

bp, base pairs.

Table 2. Target drug transporters: GenBank accession numbers, oligonucleotide sequences, references and amplicon sizes

gene acronym	GenBank ID	primer sequence 5' → 3'	reference	amplicon size (bp)
SLCO1B3	NM205804	F: CACTAACTATTCGAACGCTAGGAGG R: AGTTGTTGATGGACCACTTCATACA	designed *ex novo*	93
SLCO2B1	NM174843	F: GTGTGGAATACATCACGCCCT R: TTGGTGTAGAAGACCTGGCTTTT	designed *ex novo*	88
SLC10A1	NM001046339	F: GCTTCTCCTTGTTGCCATCTTTAG R: AGGTCATTTTTGTGTCATCTCTGG	designed *ex novo*	71
ABCB1	XM590317	F: GGAAGAGCACAGTCGTCCA R: CCTTGCCATCGATTAACACTG	designed *ex novo*	75
ABCB11	NM001192703	F: CAGCCATCATTGGTCTGAGTGT R: GACTTCATCAGCAACTGAGCCA	designed *ex novo*	86
ABCC2	XM599177	F: TCTGTCCAATGCACTTAATATCACA R: TCGCTCAACAGCCACAATGT	designed *ex novo*	91
ABCG2	NM001037478	F: CCCCATGAGGATGTTACCAAGTA R: CCTTTGGCTTCAGTCCTAACAGA	designed *ex novo*	71

bp, base pairs.

Table 3. Main features (slope, E_x, *y*-intercept, R^2 and linear dynamic range) of best internal control gene qPCR assays.

gene acronym	cDNA pool	slope	E_x (%)	*y*-intercept	R^2	dynamic range (C_t)
ACTB	liver. intestine. testis. adrenal	-3.39	97.2	27.63	0.9934	18.20 - 26.17
	lung. kidney. muscle	-3.51	92.7	30.53	0.9949	23.24 - 28.45
B2M	liver. intestine. testis. adrenal	-3.32	100.1	26.55	0.9935	18.61 - 25.07
	lung. kidney. muscle	-3.32	100.1	29.26	0.9850	23.36 - 29.43
PPIA	liver. intestine. testis. adrenal	-3.46	94.5	29.12	0.9956	20.73 - 27.44
	lung. kidney. muscle	-3.31	100.5	31.31	0.9943	24.50 - 30.41
RPLP0	liver. intestine. testis. adrenal	-3.35	98.8	26.42	0.9898	19.61 - 26.76
	lung. kidney. muscle	-3.28	101.8	28.08	0.9872	21.53 - 28.46

Table 4. Main features (slope, E_x, *y*-intercept, R^2 and linear dynamic range) of target drug transporters qPCR assays.

gene acronym	cDNA pool	slope	E_x (%)	*y*-intercept	R^2	dynamic range (C_t)
SLCO1B3	liver	-3.36	98.4	32.17	0.9998	20.05 - 26.13
SLCO2B1	liver, intestine, testis, adrenal	-3.27	102.2	36.51	0.9846	24.60 - 30.39
SLC10A1	liver	-3.33	99.7	32.08	0.9986	20.13 - 26.13
ABCB1	liver, intestine, testis, adrenal	-3.49	93.4	33.50	0.9860	21.12 - 29.24
	lung	-3.25	103.1	37.81	0.9901	28.20 - 34.05
ABCB11	liver	-3.36	98.4	35.65	0.9911	23.40 - 31.81
ABCC2	liver, intestine, testis	-3.49	93.4	36.76	0.9944	24.20 - 32.79
ABCG2	liver, intestine, testis, adrenal	-3.48	93.8	34.74	0.9947	24.26 - 28.45
	lung, kidney, muscle	-3.34	99.3	38.38	0.9922	28.32 - 34.38

Table 5. Relative abundances of SLC-transporters mRNA in cattle orally administered with PB. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of CTRL, to whom an arbitrary value of 1 was assigned.

	n-fold change (a.u.) ± SD					
	SLCO1B3		SLCO2B1		SLC10A1	
tissue	CTRL	PHEN	CTRL	PHEN	CTRL	PHEN
liver	1.00 ± 0.34	1.49 ± 0.43	1.00 ± 0.05	1.31 ± 0.36	1.00 ± 0.45	0.92 ± 0.32
intestine	ND		1.00 ± 0.45	1.46 ± 0.19	ND	
kidney	DNQ		DNQ		ND	
lung	ND		DNQ		DNQ	
testis	DNQ		1.00 ± 0.54	1.45 ± 0.95	DNQ	
adrenal	DNQ		1.00 ± 0.74	0.97 ± 0.59	ND	
muscle	DNQ		DNQ		DNQ	

DNQ, detected but not quantifiable; ND, not detected.

Table 6. Relative abundances of ABC-transporters mRNA in cattle orally administered with PB. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of CTRL, to whom an arbitrary value of 1 was assigned.

	n-fold change (a.u.) ± SD							
tissue	ABCB1		ABCB11		ABCC2		ABCG2	
	CTRL	PHEN	CTRL	PHEN	CTRL	PHEN	CTRL	PHEN
liver	1.00 ± 0.54	2.29 ± 0.86	1.00 ± 0.45	1.11 ± 0.40	1.00 ± 0.31	2.00 ± 0.55*	1.00 ± 0.32	1.36 ± 0.13
intestine	1.00 ± 0.90	0.62 ± 0.27	DNQ		1.00 ± 1.17	0.67 ± 0.96	1.00 ± 0.75	0.43 ± 0.26
kidney	DNQ		DNQ		DNQ		1.00 ± 0.32	0.29 ± 0.23*
lung	1.00 ± 0.77	1.86 ± 1.71	DNQ		DNQ		1.00 ± 0.61	1.24 ± 0.47
testis	1.00 ± 1.55	0.22 ± 0.18	DNQ		1.00 ± 0.71	1.07 ± 1.14	1.00 ± 0.65	0.97 ± 0.71
adrenal	1.00 ± 1.01	0.63 ± 0.83	DNQ		DNQ		1.00 ± 0.69	0.55 ± 0.20
Muscle	DNQ		DNQ		DNQ		1.00 ± 0.35	0.64 ± 0.28

DNQ, detected but not quantifiable; *: significant differences ($p<0.05$) *vs* CTRL.

Fig. 1

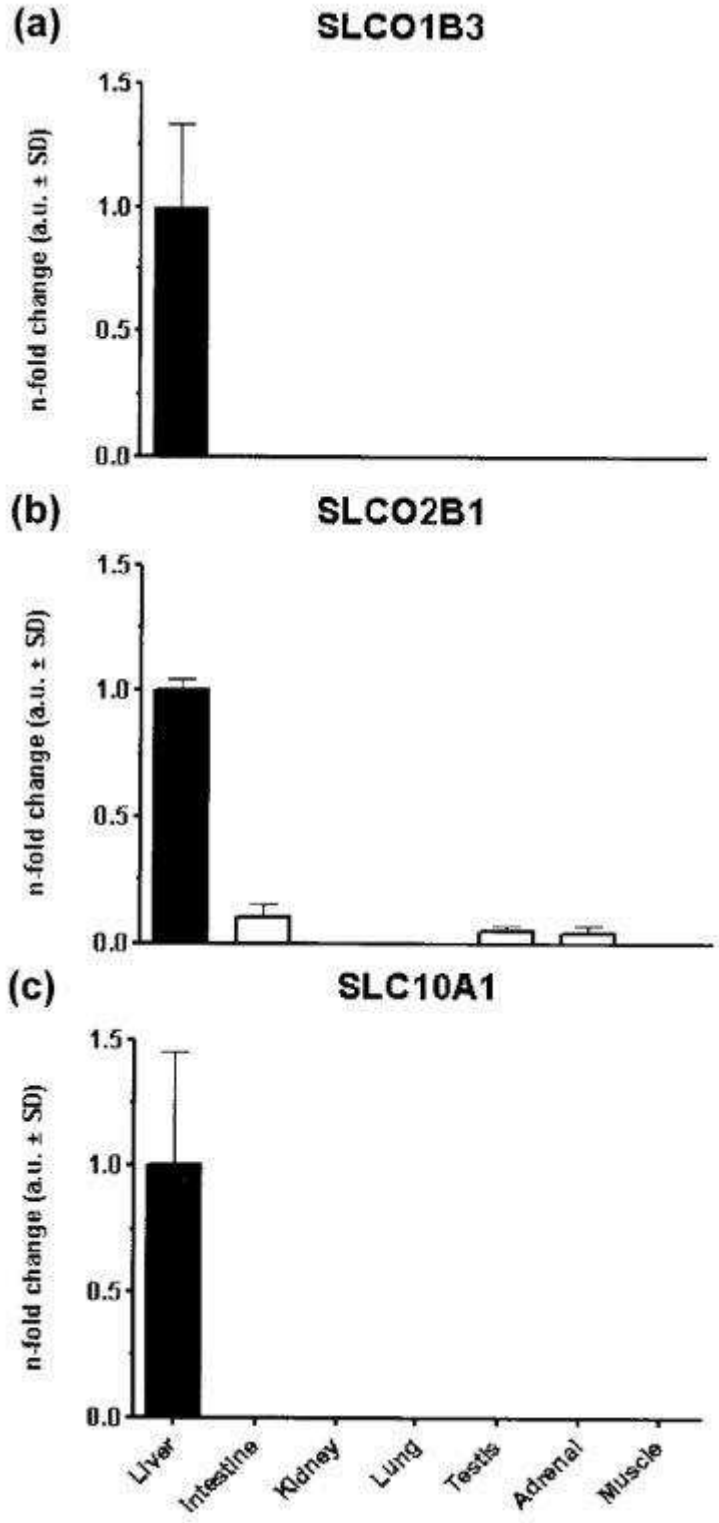

(a)
SLCO1B3
n-fold change (a.u. ± SD)
1.5
1.0
0.5
0.0
(b)
SLCO2B1
n-fold change (a.u. ± SD)
1.5
1.0
0.5
0.0
(c)
SLC10A1
n-fold change (a.u. ± SD)
1.5
1.0
0.5
0.0
Liver
Intestine
Kidney
Lung
Testis
Adrenal
Muscle

Fig.2

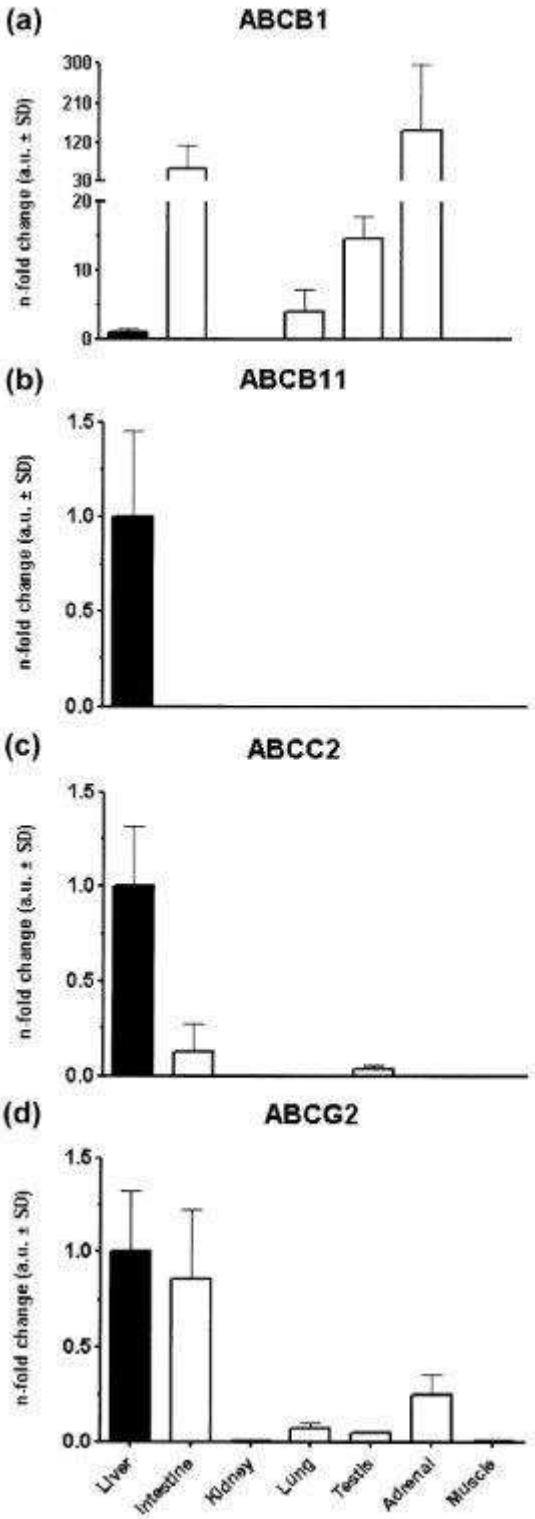
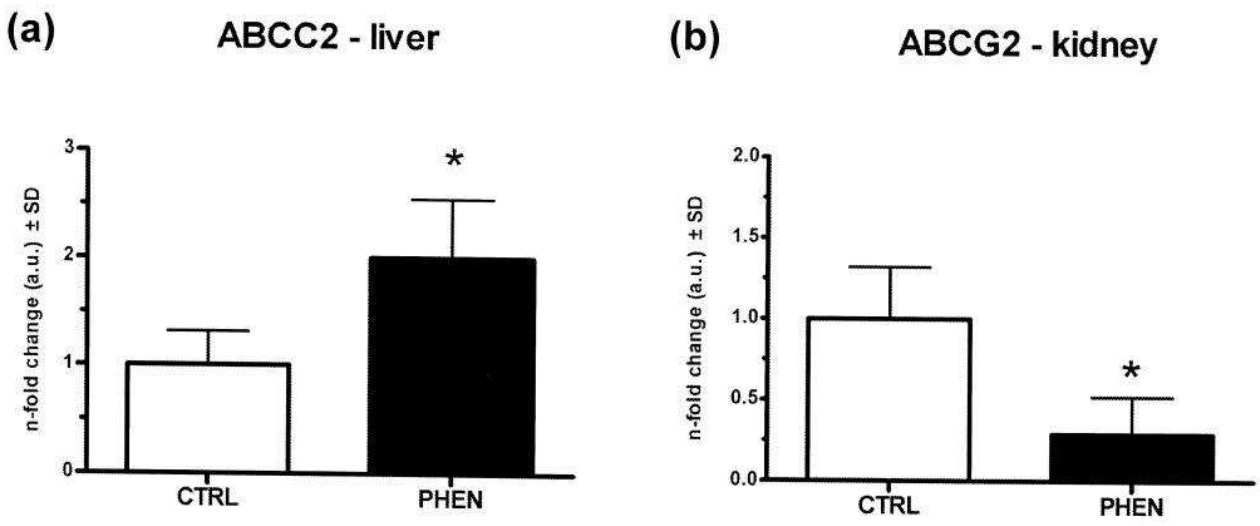

(a)
ABCB1
n-fold change (a.u. ± SD)
300
210
120
30
20
10
0
(b)
ABCB11
n-fold change (a.u. ± SD)
1.5
1.0
0.5
0.0
(c)
ABCC2
n-fold change (a.u. ± SD)
1.5
1.0
0.5
0.0
(d)
ABCG2
n-fold change (a.u. ± SD)
1.5
1.0
0.5
0.0
Liver
Intestine
Kidney
Lung
Testis
Adrenal
Muscle

Fig. 3


(a)
ABCC2 - liver
n-fold change (a.u.) ± SD
3
2
1
0
*
CTRL
PHEN
(b)
ABCG2 - kidney
n-fold change (a.u.) ± SD
2.0
1.5
1.0
0.5
0.0
*
CTRL
PHEN