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Endogenous drug transporters in *in vitro* and *in vivo* models for the prediction of drug disposition in man

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Abstract

The epithelial canine and porcine kidney cell lines MDCK, MDCKII and LLC-PK1, respectively are employed to establish recombinant models of drug transport. Endogenous drug carriers in these cells may contribute to the activities of recombinant drug transporters, thus making it difficult to assess their properties. We analysed the expression of endogenous transporters in these cell lines by RT-PCR and by determining drug transporter activities. Concerning drug efflux, multidrug resistance protein 1 (MDR1) and MRP1 mRNAs were found in all lines. MRP2 mRNA was expressed in all cell lines except MDCK. Transepithelial transport of vinblastine and its modulation by a MDR1-specific inhibitor or by the MDR1- and MRP-inhibitor verapamil, indicated that MDCKII cells have, in comparisons to the other cell lines, relatively high levels of functional MDR1 while vinblastine transport in MDCK cells is likely to be mediated more by MRP1. Notably, LLC-PK1 cells displayed little activity attributable to either MDR1 and MRP1, thus making them suitable for the expression of these efflux pumps. Of the drug uptake carriers, OATP-A mRNA was only expressed in MDCK cells. OATP-C mRNA was barely detectable in MDCK cells and absent in MDCKII and LLC-PK1 cells. In agreement with transcriptional profiling, the OATP-mediated uptake of either estradiol-glucuronide or estrone-sulfate was either absent or barely detectable in all cell lines thus implying that they are suitable to establish recombinant models for human OATP's. Transcriptional profiling was also performed on porcine and canine tissues and revealed that MRP1 was expressed in canine but not in human or porcine liver, whereas surprisingly OATP-C was expressed in canine kidney but only in human and porcine liver. The findings presented are relevant to the use of porcine and canine models for drug disposition.

Keywords: Drug disposition; Cell lines; Recombinant models; Expression profiling

1. Introduction

Carrier-mediated drug transport is an important pathway of drug disposition. The key human hepatic and renal membrane drug efflux transporters are MDR1, MRP1 (ABCC1) and MRP2 (ABCC2) (reviewed by [1]). Although substrates of MRP1 and MRP2 are mainly bulky, conjugated anions, these proteins also transport some non-conjugated drugs that are substrates of MDR1. For example, vinblastine, a well-known substrate of MDR1 is also transported by MRP1 [2] and MRP2 [3].

Human extrahepatic OATP-A (SLC21A3) and hepatic OATP-C (SLC21A6) are proposed to be two of the key drug uptake transporters [4]. They mediate Na^+-independent, saturable transport of bulky (type II) organic cations and anion [5,6], possibly through an exchange for HCO_3^- or by using GSH efflux as a driving force. Their substrates include differently charged amphipathic compounds such as bile acids, BSP, unconjugated and conjugated steroid hormones such as E_2 17βG, as well as xenobiotics [6,7]. Drugs such as the anti-histamine fexofenadine and cholesterol-lowering pravastatin are transported by OATP-A and -C, respectively.

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Abbreviations: BSP, bromosulphophthalein; E_2 17βG, estradiol 17β-glucuronide; FBS, foetal bovine serum; HBSS, Hanks' balanced salt solution; MDCK, Mardin–Darby canine kidney cells; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; OATP, organic anion transporting polypeptide; RT-PCR, reverse transcription-polymerase chain reaction.

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Recently, recombinant cellular *in vitro* drug transport models have become important tools for drug development to predict drug disposition in man. New drug transporter genes are rapidly being identified, cloned and stably expressed in cell lines to enable investigations of their drug transport properties.

Such cellular models have facilitated the determination of substrate specificities and the kinetics of hepatic and renal drug transporters. Models have been established using polarised epithelial-derived cell lines such as the porcine kidney-derived LLC-PK1 and the canine kidney-derived MDCK cells, which enable directional drug transport studies and simulate hepatic and renal drug transport. Examples where these cell lines have been employed include the expression of recombinant human MDR1 in LLC-PK1 [8] and MDCK cells [9], expression of recombinant human multidrug resistance-associated protein 2 (MRP2) in LLC-PK1 [10], MDCK [11] and MDCKII cells [12], and expression of the recombinant rat organic anion transporting polypeptide 1 (OATP1) in LLC-PK1 cells [13] and human OATP8 in MDCKII cells [6].

Endogenous expression of drug transporters in the parental cell lines have been a concern as they may interfere with the activities mediated by the recombinant protein. However, little information is available on the expression of endogenous drug transporters in these cell lines. The main objective of this study was to provide this information. The transporters determined included: MDR1, MRP1, MRP2, OATP-A and -C. Drug transporters were also profiled in porcine and canine liver and kidney tissues for comparison between *in vitro* and *in vivo* drug transport systems. Given that dog is an important species for predicting drug metabolism and disposition in man [14] and pig hepatocytes are currently investigated as components for bioartificial livers [15], information on the expression of drug carriers in these species is of interest to drug development and biotherapy.

An initial obstacle in characterising the endogenous mRNA levels of these drug transporters in the canine and porcine tissues and cell lines *via* RT-PCRs, was the limited genetic information on drug transporter proteins of the porcine and canine species at that time. This also prevented quantitative RT-PCR analysis. To resolve this problem, primers were designed based on sequences relatively conserved for the protein in different species. As a result, novel partial cDNA sequences of the canine and porcine drug transporters have also been obtained in this study.

2. Materials and methods

2.1. Radiolabelled substrates

17β-Estradiol-glucuronide, [^{3}H]E_2 17βG (44 Ci/mmol) and [^{3}H]estrone-sulfate (43.1 Ci/mmol) was purchased from NEN Life Sciences Products Inc., while [^{3}H]vinblastine sulphate (12.7 Ci/mmol) was obtained from Amersham Pharmacia Biotechnology.

2.2. Animals and cell cultures

RNA was isolated from porcine (female Cotswald) and canine (male Beagle) tissues, one animal each. MDCK (obtained from the ATCC) and MDCKII cells (provided by Jan Wijnholds, University of Amsterdam) were cultured in DMEM (Gibco-BRL) supplemented with 10% (v/v) FBS, 50 units/mL of penicillin, and 50 μg/mL streptomycin, at 5% CO_2 and 37°. LLC-PK1 cells (from ATCC) were cultured under similar condition but using Medium 199 (Gibco-BRL) supplemented with 10% (v/v) FBS, 50 units/mL of penicillin, and 50 μg/mL streptomycin. Cells were harvested using 0.25% (w/v) trypsin and incubated at 37° for 10 min.

2.3. Establishment of recombinant cell lines for OATP-C

As an antibody directed against OATP-C was not available, OATP-C modified with a short peptide sequence derived from hemeagglutinin (HA) was expressed. 3′-HA-tagged OATP-C cDNA was obtained by modification of the OATP-C cDNA (GenBank accession no. AF060500) using PCR. The HA-tag comprising 27 nucleotides (5′-TAC CCA TAC GAC GTA CCA GAC TAC GCA-3′) that would encode the amino acids YPYDVPDYA, was fused by PCR in frame with the C-terminal end of OATP-C, while simultaneously two linkers, *Xho*I and *Xba*I sites, were incorporated at the 5′- and 3′-ends of the cDNA, respectively. This cDNA was cloned into the pcDNAzeo3.1 expression vector (Invitrogen BV) at the *Xho*I and *Xba*I sites, and the construct transfected into LLC-PK1 cells by calcium phosphate coprecipitation. G-418-resistant clones were isolated and analysed for the expression of OATP-C, initially by immunoblotting employing antibodies directed against the HA-tag. Subsequently, cell lines that express OATP-C homogeneously were identified by immunofluorescence microscopy and subsequently used for drug uptake studies.

2.4. Primer design for RT-PCR

The full length cDNA sequences of human MDR1, MRP1 to MRP6, OATP-A and -C were employed to identify in cDNA data bases orthologous sequences in hamster, rat and mouse. Primers were designed against regions that were conserved between the human transporter cDNAs and these orthologous forms in at one or more of the rodent species, employing the GeneWorks Version 2.5 program (Oxford Molecular Ltd.). It was further verified that none of the primer sequences for a given transporter was identical to the other human transporter cDNAs listed above and therefore also not identical to any of the rodent orthologous forms identified in our search. The primer

Table 1
Primers employed for RT-PCR and their annealing temperatures (T_m)

Product	List of primers	Sense and antisense primer pairs (5′ → 3′)	Size (bp)	T_m (°)
MDR1	R1–1 (2282–2302) R1–2 (2758–2783)	GGCATTTACTTCAAACTTGTC GCTTGGTGAGGATCTCTCCAGCTTTG	502	45
MRP1	P1–7 (3804–3825) P1–8 (4178–4194)	TGGAGTGTGTGGCAACTGCAT TTCCCAGCTCCCGTCCG	391	55
MRP2	P2–3 (3620–3639) P2–4 (3940–3963)	AAATGTGTCTTTTCCTGG TAAGATTCTGAAGAGGCGATTT	325	45
OATP-A	A1–3 (178–201) A1–4 (678–697)	CCATGCTCACACAAATAGAGAGAC GATGCCAACAAAAGTCCAAT	520	40
OATP-C	C1–5 (364–381) C1–6 (1087–1107)	AGGTATTCTAAAGAAACT CTGACCATACTGTTGCTCTAC	744	45
β-Actin	F1 (249–255) R1 (338–344)	ACTATCGGCAATGAGCGGTTC AGAGCCACCAATCCACACAGA	288	50

Positions where the primers are found in the respective human cDNA sequences have been indicated in parentheses. Sizes of the PCR products given are those predicted from the respective human genes.

pairs were also designed to yield a PCR amplified product of around 0.5–1 kb. Sequences of these primers have been listed in Table 1.

It was ensured that the primers would give different sized products for mRNA and genomic DNA, respectively, to exclude the possibility that the signal obtained by RT-PCR could have been due to DNA contamination. Comparison with published genomic organisation of human MDR1 [16], revealed that the MDR1primers, would have amplified from exon 18 to exon 22 of the human MDR1, across four intronic sequences, had genomic DNA been present in the PCR templates. Similarly, the MRP1 primers would have amplified across exons 26–29 of the human MRP1 [17], while the MRP2 primers would amplify between exons 25 and 28 of the human MRP2 [18]. As for the OATPs, the OATP-A primers would have amplified across exons 1 and 2, while the OATP-C primers would have amplified across exons 1–4, based on the OATPs' genomic organisation [6].

The identity of the PCR products obtained was also confirmed by sequencing and comparison to sequences deposited in the gene-bank.

2.5. RT-PCR

Total RNA was isolated from frozen tissues using TRIzol Reagent (Gibco-BRL), and isolated from the cell lines MDCK, MDCKII and LLC-PK1, using the RNeasy method from Qiagen, according to the manufacturers' instructions. Subsequently, single-stranded cDNAs were synthesised from 3 μg total RNA using 0.2 μg of random hexamers and 50 U MMLV reverse transcriptase (Amersham Pharmacia), in a buffer containing 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM of each dNTP, 20–40 U/mL RNase inhibitor (Amersham Pharmacia), and 0.1 mg/mL bovine serum albumin, in a total volume of 35 μL for each reaction. Reverse transcriptions were performed at 37° for 1 hr. PCR reactions were performed using 5 μL of the RT products, 2.9 U *Taq* DNA polymerase (Promega Ltd.,), 5 μL of 10× Mg^{2+}-free PCR buffer (100 mM Tris–HCl (pH 9), 500 mM KCl, and 1% (v/v) Triton-X 100), 1.5 mM MgCl, and 50 pmol of each primer, made up to a final volume of 50 μL with water. The concentration of Mg^{2+} used was increased to 4 mM for amplification of OATP-C transcripts. Using the DNA Engine Tetrad Thermal Cycler (MJ Research Inc.), each sample was amplified for 35 cycles at 95° for 4 min, at the respective annealing temperature (see Table 1) for 2 min, and at 68° for 2 min, followed by one cycle at 68° for 10 min. Each PCR reaction included a negative control without any cDNA template. The PCR products were separated by 1.2% (w/v) agarose electrophoresis and visualised by UV in the presence of ethidium bromide.

2.6. Transepithelial transport assay for vinblastine

Cells were cultured for 5 days at an initial density of 2×10^6 cells per Transwell permeable growth support (3 μm pore size, 4.7 cm^2 clear polyester filter cup insert) from Costar. LLC-PK1 cells were grown in Medium 199 (Gibco-BRL) whereas MDCK cells were grown in DMEM (Gibco-BRL, Paisley, UK) with both media containing 10% FBS. The assay was performed as described using the cell culture medium, but without the serum supplement. Briefly, [^{3}H]vinblastine was diluted with unlabelled vinblastine, to give an activity of 0.13 Ci/mmol and a final concentration of 0.5 μM in the assay. For measurement of [^{3}H]vinblastine transport from the apical side to the basal side, basal medium was replaced with 2 mL of 0.5 μM of non-radioactive vinblastine, while the apical medium was replaced with 0.5 μM of [^{3}H]vinblastine, and the cells incubated at 37°. At each time interval of 0.5 and 1 hr, 25-μL aliquots of medium were taken from both the apical

and basal sides. Each aliquot was added with 3 mL of scintillant and the radioactivity quantified. The radioactive vinblastine stock was determined in duplicate as a standard. Vectorial transport of [^{3}H]vinblastine sulphate was investigated with the various cell lines, in the absence or presence of either verapamil or LY335979 (Eli Lilly & Co.), a potent inhibitor of P-glycoprotein.

2.7. Drug uptake assays

Cells were grown to confluency in Transwells as described for the transport assay with vinblastine. Subsequently, medium was removed and the cells were washed with HBSS (Hanks' balance salt solution without phenol red, pH 7.4, Gibco-BRL). For the transport assays, the cells were placed in HBSS. Serum was omitted as described [19] as it may sequester the substrate. Estradiol-glucuronide (1 μM final, 2.025 Ci/mmol) or estrone-sulfate (1 μM final, 2.16 Ci/mol) was added either to the apical or the basolateral side, while unlabelled substrate was added to the opposite side. The incubation was terminated after 3 min by washing cells three times with ice-cold HBSS. Subsequently, the cells were lysed with 500 μL 1% Triton-X 100 and the lysate was counted in scintillation vials in the presence of scintillant.

3. Statistics

The one-tailed *t*-test was employed to evaluate if known inhibitors of drug transporters altered their activity compared to the activity obtained in absence of inhibitors.

4. Results

4.1. Control RT-PCRs

PCR analyses carried out on the RNAs without using a reverse transcriptase (Fig. 1), demonstrated that the total RNAs prepared were essentially free of genomic DNA. Amplifications of β-actin from the various total RNAs were also carried out for standardisation (Fig. 1). The rat β-actin primers amplified human, canine and porcine actin mRNA, generating the 0.3-kb bands expected and confirmed that approximately equal amounts of total transcripts had been used in each PCR. Negative controls in lanes 5 and 11 show no DNA contamination in the reaction medium. Such controls were included in each of the PCRs performed subsequently.

4.2. Transcriptional profiling for drug efflux pumps in cell lines and tissues

Fig. 1 shows that MDR1 cDNA fragments of the expected size, 0.5 kb, were amplified from all tissues and cell lines sampled (lanes 1–4 and 6–10). The primers for both MRP1 and MRP2 were also able to amplify across species. Again, amplicons of the expected size, 0.5 kb, were obtained from all tissues for both drug transporters except importantly for the absence of MRP1 mRNAs in the canine liver (lane 1). As for the cell lines, MRP1 mRNA was clearly detected in RNA isolated from MDCK, MDCKII and LLC-PK1 cells (lanes 4, 10 and 9, respectively), while MRP2 transcripts were amplified from MDCKII and LLC-PK1 (lanes 10 and 9, respectively) but not MDCK RNAs (lane 4). The results of transcriptional profiling are summarised in Table 2.

4.3. Transcriptional profiling for mRNAs encoding drug uptake proteins in cell lines and tissues

Primers for OATP-A yielded an amplicon of the correct size, 0.5 kb, in all tissues tested (Fig. 1), with little being detected in porcine kidney sample (Fig. 1). OATP-A mRNA was also present in MDCK cells, but absent from MDCKII and LLC-PK1. Results with OATP-C were slightly different. OATP-C's transcripts were detected not only in the human, canine and porcine liver samples, but surprisingly also in the canine kidney sample (lane 2). OATP-C mRNA was also found in canine kidney MDCK cells (Fig. 1, lane 4). However, this mRNA was undetectable in MDCKII or LLC-PK1 cells. The results of transcriptional profiling are summarised in Table 2.

4.4. Nucleotide and amino acid sequences of orthologous transporters

Identities of the putative canine and porcine partial cDNAs were verified by DNA sequencing and the genebank accession number of these sequences is given in

Table 2
Summary of mRNA expression profiles of drug transporters across human, canine and porcine tissues and cell lines

	Human liver	Canine liver	Canine kidney	MDCK	MDCKII	LLC-PK1	Porcine liver	Porcine kidney
MDR1	✓	✓	✓	✓	✓	✓	✓	✓
MRP1	✓	×	✓	✓	✓	✓	✓	✓
MRP2	✓	✓	✓	×	✓	✓	✓	✓
OATP-A	✓	✓	✓	✓	×	×	✓	+/−
OATP-C	✓	✓	✓	+/−	×	×	+/−	×

"✓" denotes that the corresponding mRNAs yielded a strong signal in RT-PCR analysis from the respective tissues or cell lines; "×" indicates that the corresponding mRNAs were not detectable *via* RT-PCR; "+/−" implies that the corresponding mRNAs were just detectable after 35 cycles of PCR.

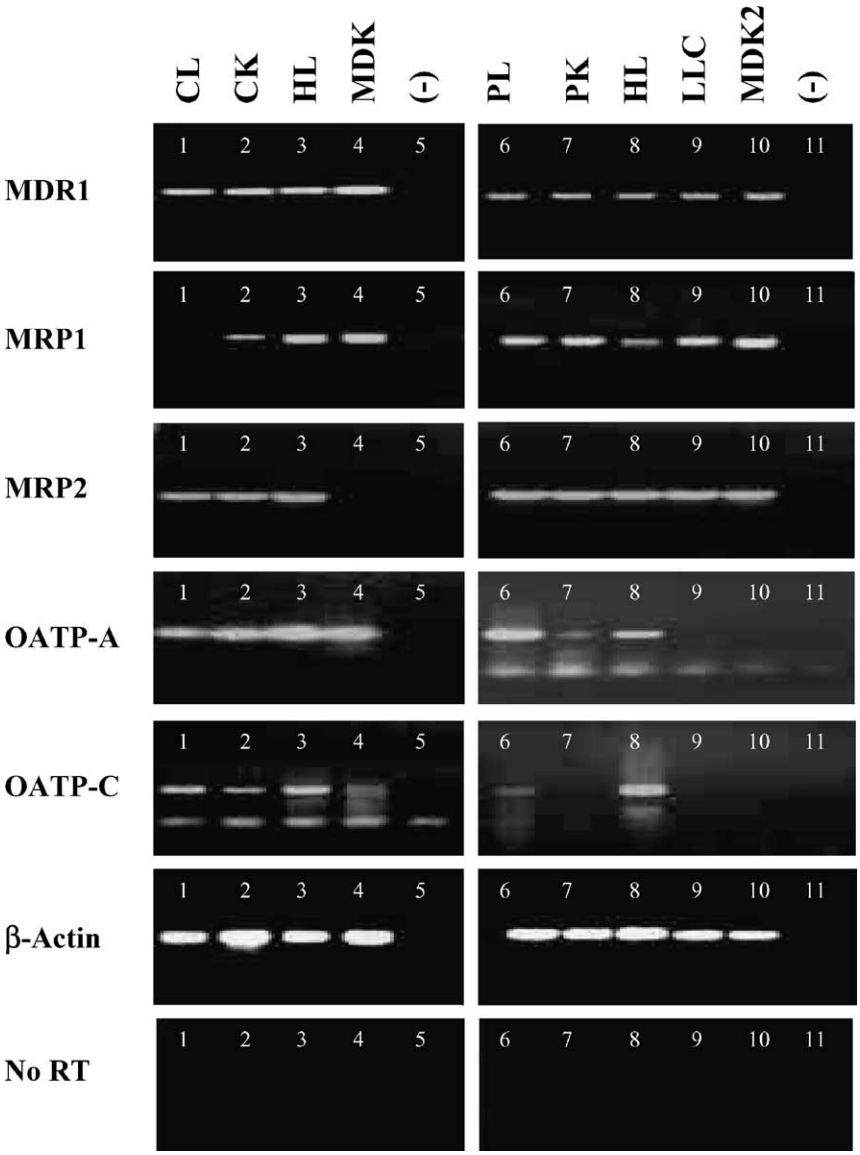

Fig. 1. Qualitative transcriptional profiling of MDR1, MRP1, MRP2, OATP-A and -C in human, canine and porcine tissues and cell lines. Sizes of the PCR products have been listed in Table 1, column 4. Respective specific primers from conserved regions across species were used to detect each mRNA across human, canine and porcine tissues and cell lines, as described under ''Materials and methods''. (1) Canine liver (CL); (2) canine kidney (CK); (3 and 8) human liver (HL); (4) MDCK (MDK); (5 and 11), negative control without sample from RT reaction (−); (6) porcine liver (PL); (7) porcine kidney (PK); (9) LLC-PK1 cells (LLC); (10) MDCKII (MDK2). Low molecular weight bands seen in some panels are primers and can also be seen in control reactions.

Table 3. The partial cDNA sequences were translated into peptide sequences which were aligned against human MDR1 and MRP1 to MRP6. Partial canine and porcine sequences which yielded the highest similarity to a given human transporter were thought to represent its orthologous forms. It was found that the canine MDR1 shares 78% amino acid identity with the human MDR1, while the porcine MDR1 shares 66% amino acid identity with the human MDR1, over the regions analysed. Similarly, canine MRP1 was found to share over 80% amino acid identity with human MRP1, while porcine MRP1 was found to exhibit only 43% amino acid identity with human MRP1, over the partial segments isolated. Canine MRP2 displayed 91% amino acid identity with human MRP2, while porcine MRP2 is 76% identical to the human MRP2 protein, over the segment analysed.

The putative OATP-A PCR fragments from the canine and porcine samples were sequenced, and confirmed to be likely canine and porcine OATP-As, respectively (Tables 3 and 4). Canine OATP-A exhibits approximately 84% of nucleotide similarity and the same amino acid identity to the human OATP-A, while porcine OATP-A demonstrates 92% nucleotide similarity and 85% amino acid identity to the human OATP-A, across the regions isolated (Table 4). The amino acid identities of either canine or porcine OATP-A to the other members of the OATP family were much lower. On the protein level, partial canine OATP-C peptide was 73% identical to the human OATP-C whereas

Table 3
List of GenBank accession numbers for the novel canine and porcine partial cDNA sequences

Transporter	GenBank accession no.	Size (bp)	Approximate nucleotide position with respect to the human orthologue
Canine MDR1	AF403240	404	2318–2721
Canine MRP1	AF403241	392	3848–4194
Canine MRP2	AF403242	431	3664–4049
Canine OATP-A	AF403243	468	177–644
Canine OATP-C	AF403244	583	515–1097
Porcine MDR1	AF403245	371	2371–2741
Porcine MRP1	AF403246	404	3848–4194
Porcine MRP2	AF403247	314	3667–3930
Porcine OATP-A	AF403248	485	177–660

The sizes of the partial cDNAs isolated and the approximate positions to which they correspond in the human orthologues are indicated in columns 3 and 4, respectively. Insufficient amounts of RT-PCR product for sequencing were obtained for porcine OATP-C.

its similarity to the other member of the OATP family was distinctly lower.

4.5. Characterisation of drug efflux activities in cell lines

Vectorial transport of [^{3}H]vinblastine sulphate was investigated with the various cell lines, in the absence or presence of either verapamil (20 μM) or LY335979 (0.5 μM). LY335979 (at 1 μM) specifically inhibits MDR1 but not MRP1 or MRP2-mediated transport [20], whereas verapamil (at 10 μM) inhibits both MDR1 [21]and MRP1 [2], but not MRP2-mediated transport [22]. Results in Fig. 2 demonstrate that in all three cell lines the basolateral-to-apical transport was higher than that in the opposite direction. Verapamil inhibited the transport in this direction only in MDCK and MDCKII cells by more than 2-fold. The MDR1-specific inhibitor LY335979 reduced the basolateral-to-apical flux of vinblastine in MDCK and MDCKII cells less than did verapamil and had little effect on this parameter in LLC-PK1 cells. Treatment with verapamil slightly increased the transport in the reverse orientation in MDCKII and LLC-PK1 cells, however, this effect was not significant.

Table 4
Percentage homologies between peptide sequences of the canine and porcine OATP-A and -C, and members of the human OATP family

Human	Canine OATP-A (%)	Porcine OATP-A (%)	Canine OATP-C (%)
OATP-A [U21943]	***84***	85	30
OATP-B [AB026256]	12	12	12
OATP-C [AF060500]	42	39	73
OATP-D [AB031050]	19	8	34
OATP-E [AB031051]	55	53	9
OATP8 [AJ400763]	40	39	40

The partial cDNAs obtained during transcriptional profiling as described for the experiment displayed in Fig. 1 were sequenced and compared to human OATP sequences deposited in the gene-bank after translation into protein. The highest score of similarity are bold and italicised. The respective GenBank accession numbers are in square parentheses. Insufficient amounts of RT-PCR product for sequencing were obtained for porcine OATP-C.

4.6. Characterisation of OATP-mediated uptake in cell lines

E_2 17βG has been known to be a better substrate for OATP-C than for OATP-A whereas estrone 3-sulfate is a broad spectrum substrate for all known OATPs with OATP-A and -C having similar activities towards this compound [5,7]. Both activities are known to be inhibited by BSP. Uptake of E_2 17βG and estrone-sulfate in all three parental cell lines was 2–3-fold lower than that seen with LLC-PK1 cells which expressed OATP-C. BSP treatment lead to a strong reduction in the uptake of both substrates in the latter cell line. Some slight, but significant, inhibition was also seen with MDCKII and LLC-PK1 cells, employing E_2 17βG. With the broad spectrum substrate estrone-sulfate a small but significant inhibition was only seen with LLC-PK1 cells. It should be noted that basolateral-to-apical transepithelial transport of E_2 17βG was very low in MDCK, MDCKII and LLC-PK1 cells as well as in LLC-PK1 that expressed recombinant human OATP-C but not recombinant MRP2 (data not shown). LLC-PK1 cells that expressed both human transporters displayed a marked basolateral to apical transport (manuscript in preparation).

5. Discussion

Quantitative RT-PCR would have been most appropriate to characterise the levels of transporter mRNAs in the various cell lines, however for many canine and porcine transporters no sequence information to design Taqman primers, which need to be identical to their target sequence, was available. Primary structures within a transporter family tend to be highly conserved [23]. Therefore, alignments of sequences within the same transporter family across species yield high similarities. Based on these observations, we devised a strategy to detect canine and porcine transporter mRNAs for which the sequence was unknown. Initially, DNA sequence alignments were performed across species for the respective drug transporter proteins. Conserved regions were used to design primers for RT-PCR. The primers were also chosen to amplify across introns to distinguish between mRNA and genomic DNA, which may be present as a contaminant in the RNA preparation. The primers successfully amplified partial cDNAs of MDR1, MRP1, MRP2, OATP-A and -C, from the human, the canine and the porcine species (Fig. 1, Tables 3 and 4). Hitherto unknown partial sequences for canine and porcine drug transporters were obtained and have been deposited in the GenBank. The amount of

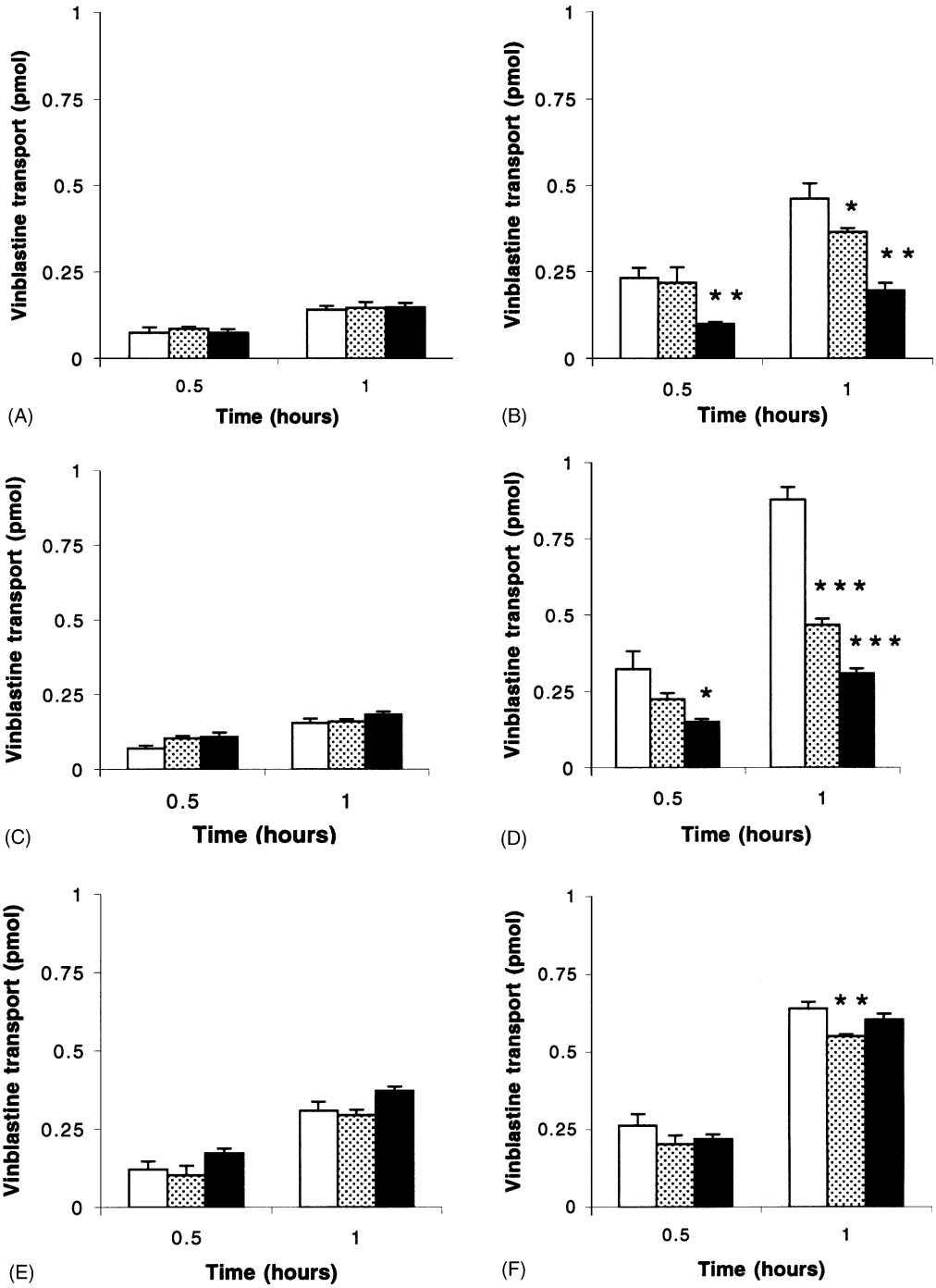

Fig. 2. Transepithelial transport of [^{3}H]vinblastine sulphate in MDCK, MDCKII and LLC-PK1 cell lines. The experiment was carried out at 37° on semi-permeable Transwell inserts as described in "Materials and methods". Cells were incubated with 0.5 μM of [^{3}H]vinblastine, in the absence (open bars) or presence of 0.5 μM of LY335979 (stippled bars) or 20 μM of verapamil (filled bars). Three aliquots of 25 μL were drawn from each well for scintillation counting after 30 and 60 min of incubation. Panels A, C and E represent the apical-to-basolateral transport; and panels B, D and E the transport in the reverse orientation. The data presented are representative of three individual experiments for MDCK (A, B), MDCKII (C, D) and LLC-PK1 (E, F) cells. (*) Significantly different from control ($P < 0.05$); (**) significantly different from control ($P < 0.01$); (***) significantly different from control ($P \leq 0.001$).

RT-PCR product obtained for porcine OATP-C was however insufficient for sequencing.

While this work was in progress, the sequences of full length canine MDR1 and MPP2 cDNA were published [24]. It was found that the partial canine MRP2 cDNA sequence was completely identical to the full length MRP2 cDNA sequence, at nucleotide positions 1194–1336 with respect to the full-length canine MRP2. The partial canine MDR1 cDNA was identical to nucleotides at positions 1966–2369 with respect to the full-length canine MDR1,

except at two positions, 2148 and 2152 [24]. Only the first one introduced an amino acid change from ^{695}Pro (our sequence) → ^{695}Ser (full-length sequence). Assuming no sequencing errors in both, this may represent a polymorphic site of the canine MDR1. It is important to note that the RT-PCR analyses displayed in Fig. 1 was specific for OATP-A and -C in the porcine and canine tissues, because the relevant PCR products showed the highest sequence similarity to human OATP-A and -C, respectively, whereas the similarity to other members of the OATP family was distinctly lower (Table 4). To our knowledge this is the first time that cDNA sequences of porcine and caninine OATPs have been characterised.

Overall, the human liver, canine kidney and porcine liver samples were found to contain mRNAs of MDR1, MRP1, MRP2, OATP-A, except for the absence of MRP1 mRNA in the canine liver (Table 2). This correlated well with reports indicating the presence of these ABC-transporters in human liver and kidney [25]. However, while this work was in progress, Conrad *et al*. [24] reported that transcripts of MRP1 were strongly detected in the canine liver sample, but only weakly detectable on Western blots using an anti-human MRP1 antibody, MRPm6. In the human liver, MRP1 has not been detected by RNase protection assays [26], but similar to our findings reported to be present in hepatocytes at a very low level (reviewed in [25]).

OATP-C was detected in all liver samples but unexpectedly also in canine kidney and in the cell lines derived from it (Table 1). It is unusual that OATP-C was expressed in the canine kidney, as the transporter protein was discovered to be liver-specific in the human [27], rat and mouse [28]. Nevertheless, the levels of functional OATP-C in canine kidney may still be low and need to be confirmed by immunohistochemical staining. It is unlikely that the transcript amplified from the canine kidney encodes a different member of the OATP family, because the partial OATP-C cDNA sequences obtained from the PCR products derived from canine liver and canine kidney RNAs were identical, and share 73% identity at the amino acid level with the human OATP-C, in the corresponding regions.

Even though the information gained in this report on the sequence of various canine and porcine transporters would have made it now feasible to perform quantitative PCR, we decided to focus instead on the functional characterisation of drug uptake in efflux transporters in the various cell lines. In the case of OATPs this approach has the advantage that the functional assays would also identify uptake by OATPs other than those characterised here.

To evaluate the presence of drug efflux activities, we assayed the transepithelial transport of vinblastine in the cell lines. To distinguish between MDR1- and MRP1- or MRP2-mediated transport, the assay was performed in the presence of the MDR1-specific inhibitor LY335979 [20] or in the presence of verapamil that is an inhibitor of MDR1 and MRP1 [2,21] (Fig. 2). LY335979 showed the strongest inhibition of basolateral-to-apical transport in MDCKII cells, thus indicating that they contain the highest level of MDR1. Both verapamil and LY335979 had only a slight inhibitory effect on this parameter in LLC-PK1 cells, indicating low levels of endogenous MDR1 and MRP1. However, it should be noted that whereas RT-PCR analyses yielded similar signals for MDR1 and MRP1 in all cell lines, functional analysis clearly detected differences. This may be due to non-linearity of the RT-PCR reaction and/or due to poor correlation between mRNA and functional protein levels.

Results concerning MDR1-mediated transport of vinblastine in all three cell lines agree with those presented in literature [29–31]. MDR1 activity in MDCK and MDCKII cell lines has been documented, while LLC-PK1 exhibits little or no endogenous MDR1 activity. MRP1 was also reported to be absent in the latter cell line, using MRPr1 antibody for Western blotting [12]. In addition, very low levels of endogenous MRP1- and MRP2-mediated efflux activities in LLC-PK1 cells were demonstrated in this report. Absence of functional MRP2 in these cells was also suggested by immunological studies [10].

Concerning drug uptake little is known about the activity of OATPs in the three cell lines. RT-PCR analyses suggested that MDCK cells expressed OATP-A and little OATP-C whereas MDCKII and LLC-PK1 cells did not express either of these two drug uptake transporters.

Functional analyses revealed that uptake of E_2 17βG and estrone-sulfate in all three cell lines was much lower than in a recombinant cell line that expressed human OATP-C (Figs. 3 and 4). Transport of these steroid-conjugates into the recombinant cell line was also strongly inhibited by BSP. This compound showed a weak inhibition of E_2 17βG transport into MDCKII and LLC-PK1 cells, with only the latter cell line showing a small but significant reduction of uptake of estrone-sulfate by BSP. We are not entirely sure if this small inhibition was due to the presence of OATPs other than OATP-A or -C (both transporters were not detected by RT-PCR) or due to the ability of BSP to reduce uptake *via* a non-transporter-mediated pathway. The presence of other OATPs could also explain that the uptake of E_2 17βG in all three cell lines was rather similar, even though only MDCK cells expressed OATP-A and barely detectable levels of OATP-C as evidenced by RT-PCR.

In conclusion, the data presented provide for the first time partial cDNA sequences for porcine MRPs and canine and porcine OATPs. They also show that even though RT-PCR revealed OATP-A expression in MDCK cells, but not in the other two cell lines, little OATP-mediated uptake was present in all three cell lines analysed. Therefore, all three cell lines are suitable for the expression of recombinant OATPs to establish cellular models for drug uptake. However, the data also indicate that coexpression of drug uptake and efflux transporters will be necessary in these cells to establish recombinant models for transepithelial transport [19]. Furthermore, our results indicate differences in the

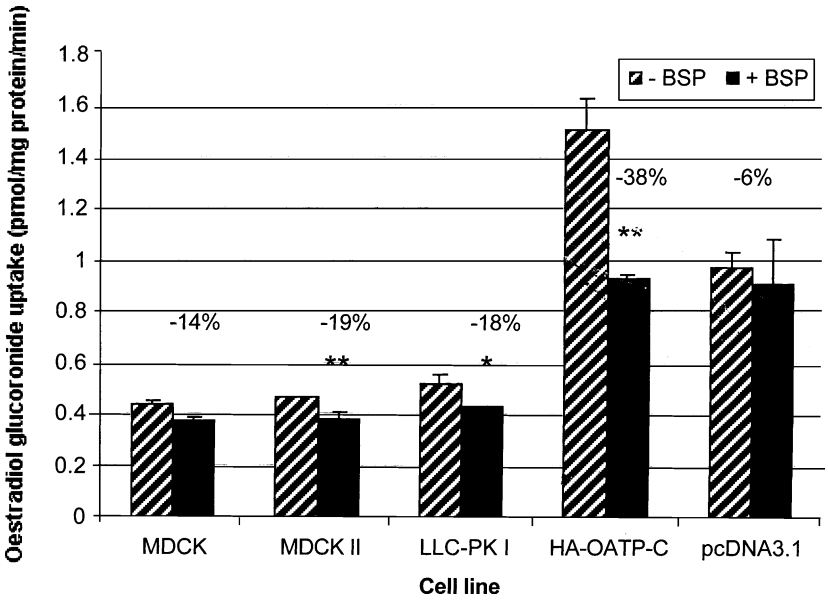

Fig. 3. Uptake of estradiol 17β-glucuronide into cell lines: The uptake of E_2 17βG was determined as described in "Materials and methods" in the absence (striped bars) or presence (filled bars) of bromosulphophthalein (BSP, 100 μM). Cultured cells were pre-exposed to BSP for 5 min before addition of tritium-labelled estradiol-glucuronide [final concentration 1 μM]. The following cell lines were analysed: MDCK, MDCKII, LLC-PK1, LLC-PK1 transfected with HA-OATP-C (HA-OATP-C) and LLC-PK1 cells transfected with the empty mammalian expression vector pcDNA3.1 (pcDNA3.1). Uptake was stopped after 3 min. Data [mean ± standard error for triplicate determinations (three separate wells of cells) are expressed as pmol uptake/mg cellular protein per min. Significantly different from the uptake determined in the absence of BSP; (**) $P < 0.01$; (*) $P < 0.05$.

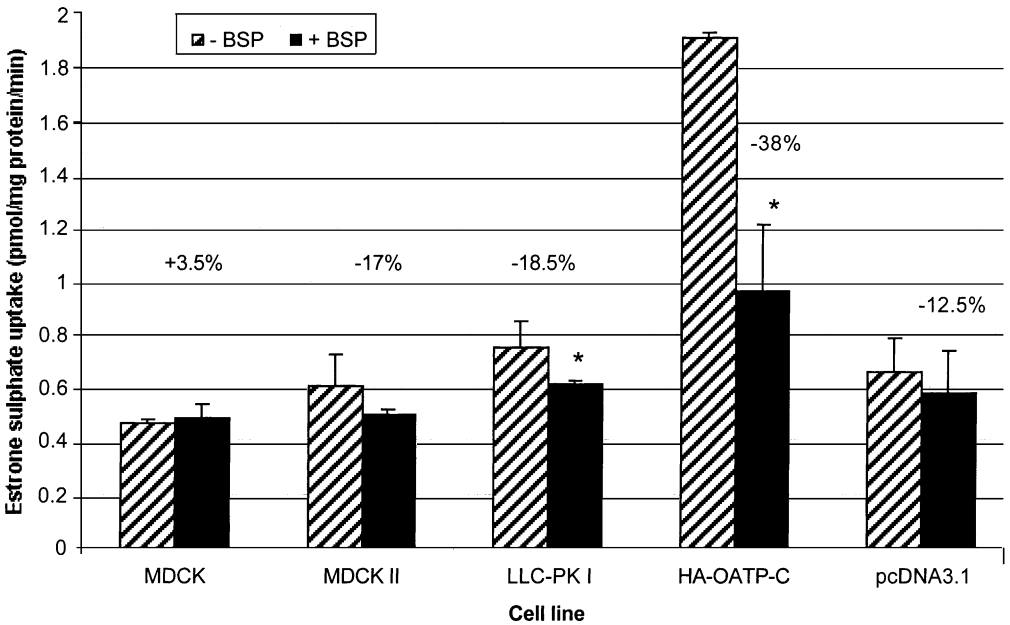

Fig. 4. Uptake of estrone-sulfate into cell lines: The uptake of estrone-sulfate was determined as described in "Materials and methods" in the absence (striped bars) and presence (filled bars) of bromosulphophthalein (BSP, 100 μM). Cultured cells were pre-exposed to BSP for 5 min before addition of tritium-labelled estrone-sulfate [final concentration 1 mM]. The following cell lines were analysed: MDCK, MDCKII, LLC-PK1, LLC-PK1 transfected with HA-OATP-C (HA-OATP-C) and LLC-PK1 cells transfected with the empty mammalian expression vector pcDNA3.1 (pcDNA3.1). Uptake was stopped after 3 min. Data [mean ± standard error for triplicate determinations] (three separate wells of cells) are expressed as pmol uptake/mg cellular protein per min. Significantly different from the uptake determined in the absence of BSP; (**) ($P < 0.01$); (*) ($P < 0.05$).

tissue-specific expression of drug transporters in different species. If confirmed by protein analysis, this observation is likely to be relevant for drug disposition studies employing these species

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