Endogenous gene and protein expression of drug transporting proteins in cell lines routinely used in drug discovery programs.

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Gene and protein expression of transporters in cells

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Abbreviations

ABC, ATP binding cassette transporter; HPR, human proteome resource; IHC, immunohistochemistry; msAb, monospecific polyclonal antibodies; PrEST, protein epitope signature tags; RT-PCR, real-time polymerase chain reaction; SLC, solute carrier transporter.
Abstract

The aim of this study was to investigate the gene and protein expression profiles of important drug transporting proteins in human cell lines commonly used for studies of drug transport mechanisms. Human cell lines used to transiently or stably express single transporters (HeLa, HEK293) and leukaemia cell lines used to study drug resistance by ABC-transporters (HL-60, K562) were investigated, and compared with organotypic cell lines (HepG2, Saos-2, Caco-2 and Caco-2 TC7). For gene expression studies, real-time PCR was used, while monospecific polyclonal antibodies were generated and used to investigate protein expression by immunohistochemistry. Thirty-six transporters were studied for gene expression and nine for protein expression. The antibodies were validated using expression patterns in human tissues. Finally, the function of one ubiquitously expressed transporter, MCT1; SLC16A1 was investigated using $^{14}$C-lactic acid as a substrate. In general, the adherent cell lines (HeLa, HEK293) displayed low transporter expression and the expression patterns were barely affected by transfection. The leukaemia cell lines (K562, HL-60) and Saos-2 also had low endogenous transporter expression, while the organotypic cell lines (HepG2 and Caco-2) showed higher expression of some transporters. Comparison of gene and protein expression profiles gave poor correlations, but better agreement was obtained for antibodies with a good validation score, indicating that antibody quality was a significant variable. Importantly, the monocarboxylic acid transporting protein MCT1 was significantly expressed in all, and functional in most of the cell lines, indicating that MCT1 may be a confounding factor when the transport of small anionic drugs is investigated.
Introduction

One problem when using cell lines in studies of a specific transporter function is that the results may be confounded by endogenous transporter activity (Goh et al., 2002). Only in rare cases, has the background expression of endogenous transporters been investigated in such cell lines (Hilgendorf et al., 2007). Further, since many traditionally used cell lines are of a polyclonal origin, variations in gene expression and regulation may be a problem (Hayeshi et al., 2008). Another limitation is that most investigations of transporter expression have been conducted at the mRNA level. Although such studies may give a hint of protein expression, in particular for highly expressed transporter genes (Landowski et al., 2004), posttranscriptional regulatory mechanisms and variations in mRNA and protein stability will result in discrepancies between gene and protein expression, e.g. (Tian et al., 2004). To the best of our knowledge, studies on protein expression patterns of transporters in cell lines have not yet been investigated, although techniques that, eventually, will allow such studies are becoming available, e.g. (Kamiie et al., 2008). Western blotting will only mirror the functional transporter as long as it is correctly folded and sorted to the plasma membrane. For instance, in some cases the ABC-transporter MRP2 (ABCC2) will not be sorted to the cell membrane in HEK293 cells in sufficient amounts (Keitel et al., 2003), which is a normal mechanism for short-term regulation of MRP2 (Sekine et al., 2008). The membrane constitution may also affect the function of the transporter, e.g. BCRP (ABCG2) activity differs in insect Sf9 cells that lack cholesterol and eukaryotic HEK293 cells (Pal et al., 2007). Only in a few cases, has the transporter gene or protein expression been correlated to the transporter function, e.g. (Taipalensuu et al., 2004).

Transformed human cell lines are widely used tools in research on drug transport mechanisms. Common adherent cell lines that are easy to maintain, such as the human
embryonic kidney cell line HEK293 generated through transformation with adenovirus type 5 (Graham et al., 1977) and the human cervix epitheloid carcinoma HeLa (Gey, 1952) cells can easily be transiently or stably transfected with an uptake or efflux transporter of interest, as exemplified by selected references (Zhang et al., 1998; Ahlin et al., 2008). Suspension cell lines, often originating from various tumours of the immune system, such as K562 from chronic myeloic leukaemia (Gahmberg and Andersson, 1981) and HL-60 from acute promyelocytic leukaemia (Gallagher et al., 1979) are used to study drug resistance mechanisms mediated by ABC-transporters (Puhlmann et al., 2005; Assef et al., 2009). Further, the differentiated cell lines, such as the colorectal carcinoma cell line Caco-2 (Fogh et al., 1977), the hepatocellular carcinoma cell line HepG2 (Aden et al., 1979) and the human osteosarcoma cell line Saos-2 (Fogh et al., 1977) are used as organotypic cell models for the intestinal epithelium, hepatocytes and osteoblasts, respectively (Rodan et al., 1987; Artursson and Karlsson, 1991; Maruyama et al., 2007).

In this study, we quantified the gene expression for 36 transporters (10 ABC, 25 SLC and the human peptide transporter 1, HPT1; CDH17) in eight cell lines commonly used in research on transport mechanisms. We also investigated how environmental differences in, e.g., culture conditions, and transient and stable over-expression of a transporter influenced the gene expression. In parallel, as a part of the Human Proteome Resource (HPR) Project (Uhlen et al., 2005), we generated monospecific polyclonal antibodies (msAb) for nine transporters, which we validated in human tissues and used with the intention of examining expression in six of the cell lines. Finally, we investigated the function of the highly ubiquitously expressed MCT1 in these cell lines.
Methods

Cell lines

Human cell lines were used throughout this study. The adherent cell lines, HeLa derived from an individual with cervical cancer (Gey, 1952), HEK293 derived from human embryonic kidney cells (Graham et al., 1977), the suspension grown cell lines HL-60, derived from leukocytes of an individual with acute promyelocytic leukaemia (Gallagher et al., 1979), and K562, derived from a chronic myeloid leukaemia (Gahmberg and Andersson, 1981), were included in the study. Four differentiated cell lines were also studied. The Saos-2 cell line was derived from osteosarcoma in human bone (Fogh et al., 1977) and is used to study osteoblastic properties and for transfection purposes (Rodan et al., 1987; Matsson et al., 2007). The gene expression data used for Saos-2 empty vector cells have earlier been published by our group (Matsson et al., 2007). Caco-2 and Caco-2 TC7 cell lines were derived from human colon cancer (Fogh et al., 1977) and are extensively used as in vitro models for the human intestine and used to predict drug uptake over the intestinal barrier (Artursson and Karlsson, 1991). HepG2 cells were derived from hepatocellular carcinoma and are extensively investigated for its hepatocyte-like characteristics e.g. (Maruyama et al., 2007). The gene expression data used for HepG2 cell have earlier been published by Hilgendorf and co-workers (Hilgendorf et al., 2007).

Culture conditions

mRNA and function

The trypsin solutions contained 0.05% trypsin (Gibco, Paisley, Scotland) and 0.002-0.02% EDTA, (MP Biomedicals, Irvine, CA). The adherent cell lines, unless otherwise stated, were harvested at 70-90% confluence on plastic support. The suspension cells (HL-60 and K562) were harvested directly from suspension after 72 hours in culture. The cells used for RT-PCR
and functional studies were cultured in normal cell culture flasks according to protocols from respective distributors.

Protein
The cells used for immunohistochemistry (IHC) were cultured in normal cell culture flasks according to protocols from respective distributors. The cells were harvested and agarose cell gels were prepared for IHC (Andersson et al., 2006).

Gene expression profiles
The cell lines were harvested and RNA was isolated from the samples using the RNeasy mini kit (QIAGEN, Hilden, Germany) according to the protocol provided from the manufacturer, with the addition of an extra on-column DNase step (QIAGEN, Hilden, Germany). RNA concentration was measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Wilmington, DE) and the RNA quality was examined using a Bioanalyser (Agilent, Palo Alto, CA). RNA was converted into cDNA using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). 500 ng of the total RNA samples were added to a master mixture containing 10 µl 10×RT Buffer, 4 µl 25×dNTPs, 10 µl 10× random primers, 5 µl Multiscribe RTase 50 U/µl and 21 µl nuclease free water. The reverse transcriptase PCR mixture was incubated at 25°C for 10 minutes and at 37°C for 120 minutes in a Mastercycler gradient system (Eppendorf, Hamburg, Germany). The output cDNA (5 ng/µl) was stored at -70°C pending TaqMan analysis. Analyses of the cDNA samples were performed as previously described (Hilgendorf et al., 2007). The real-time-PCR (RT-PCR) analysis was performed in at least duplicates with 1-2 ng cDNA per well.

Relative Expression Analysis:
The relative expression analyses were performed as described previously (Hilgendorf et al., 2007). To obtain a better overview of the gene expression and simplify comparison with protein expression data, the relative expression levels were classified into four groups (negative, weak, moderate, strong), as described previously (Hilgendorf et al., 2007). Owing to the very low relative expression of many transporters, all genes not reaching the set threshold level later than 35 cycles were said to be negative.

**Immunohistological screening of human drug transporters**

The protein expression data for the transporters was generated as a part of the large HPR project, with the aim of mapping protein expression of the whole human genome in human tissues, tumours and cell lines (Uhlen et al., 2005). Briefly, first two Protein Epitope Signature Tags (PrEST), consisting of a 50-150 amino acid sequence unique to the specific protein, were identified and expressed as recombinant proteins as described previously (Nilsson et al., 2005; Ponten et al., 2008). Each PrEST was injected subcutaneously in New Zealand rabbits to produce an immune response. The resulting antibodies were affinity purified from serum by depletion of tag specific antibodies, followed by purification of monospecific antibodies using affinity columns loaded with the protein specific PrESTs (Nilsson et al., 2005). Quality assurance was performed by i) sequence verification of the PrEST clone ii) analysing the size of resulting recombinant protein to assure that the correct antigen has been produced and purified iii) and checking the antibodies for cross-reactivity to PrESTs spotted on protein arrays (Ponten et al., 2008).

Internal validation of the antibodies was performed by i) comparing IHC staining patterns of the msAb with the staining patterns of commercial antibodies and/or with experimental/bioinformatics data for a subset of 13 different normal tissues, four different tumours tissues and eight cell lines ii) investigating the consistency of immunofluorescence
images with previously published protein localisation and/or bioinformatics data

iii) investigating the presence, size and strength of bands on western blot gels (Berglund et al., 2008; Ponten et al., 2008). An external validation step was performed, where tissue staining patterns were compared to data from the literature on transporter expression and localisation. To allow a high-throughput staining, the cell lines were subcultured and agarose cell gels were prepared, which were used to produce tissue microarrays containing approximately 450 cells each. The cell microarrays were IHC stained in duplicates (Andersson et al., 2006) and the resulting images were annotated using an automated image-analysis application (Stromberg et al., 2007). The staining patterns were divided into five groups (not representative, negative, weak, moderate and strong expression) depending on the intensity of the staining and the number of cells stained. The localisation of the transporters was assessed by ocular examination of stained images.

With this approach, staining patterns for nine msAb were generated (MDR1, MRP1, MRP2, MRP4; IBAT, PEPT1, MCT1, OCT2 and OATP1B3) (Uhlen et al., 2005). During the course of this study, three additional antibodies (BSEP, OAT4 and OATP2B1) were generated, but no staining of the cell lines has yet been performed.

**Functional studies**

The function of MCT1 was investigated to assess its potency as a confounding factor in transporter experiments. The functionality was investigated using a known MCT1 substrate, $^{14}$C-lactic acid (15.3 µM), and a MCT1 inhibitor, quercetin (100 µM) (Broer et al., 1997; Shim et al., 2007).

The four adherent cells lines (HeLa, HEK293, Caco-2 and HepG2) were seeded into 96-well plates (at 50000-70000 cells per well) two days prior to the experiment. Before the incubation the cell monolayers were washed twice with 200 µl Hank Balanced Salt Solution (HBSS).
The uptake of lactic acid was studied for 5 minutes and the transport process was terminated by washing the cell monolayers five times with 200 µl ice-cold HBSS. Thereafter, 50 µl trypsin solution was added to detach the cells from the surface and 200 µl 1M NaOH was added for at least one hour to lyse the cells. This was followed by neutralising the solution with 1M HCl and 100 µl of the solutions were added to scintillation vials and supplemented with 3 ml of Ultima Gold scintillation cocktail (PerkinElmer, Shelton, CT). The radioactivity was measured in a Beckman LS6000IC liquid scintillation counter (Beckman Coulter, Fullerton, CA). The two suspension cell lines (HL-60 and K562) were incubated in 96 well plates (at 100 000 cells per well) using the same solutions and incubation times as for adherent cells. The cell solutions were added to a filter plate (Millipore, Billerica, MA) and the incubation solution was removed. The transport processes were terminated by washing the cells five times with ice-cold HBSS. The filters were removed and placed in scintillation vials and allowed to dry for 30 minutes in 37°C. 3 ml of Ultima Gold scintillation cocktail (PerkinElmer, Shelton, CT) was added to the vials and the samples were analysed in a Beckman LS6000IC liquid scintillation counter (Beckman Coulter, Fullerton, CA). The scintillation data was normalized to the protein content of each well using a BCA protein assay reagent kit (Pierce biotechnology, Rockford, IL).

The function of the MCT1 was investigated by comparing the transport in the presence (Uptake\textsubscript{+inhibitor}) and absence (Uptake\textsubscript{-inhibitor}) of inhibitor. MCT1 was classed as functional if the lactic acid uptake was significantly inhibited by quercetin (using Equation 1 and two-sided paired t-test). Cell lines showing non significant inhibition or no substrate uptake were defined as non-functional.

Equation 1: \[
\text{Inhibition\%} = 100 \times \left(1 - \frac{\text{Uptake}_{-\text{inhibitor}}}{\text{Uptake}_{+\text{inhibitor}}} \right)
\]
Results

Gene expression patterns

The relative gene expression patterns for the cell lines are summarized in Figures 1 and 2; see also supplemental data Figure S1. HeLa cells expressed 21 of the 36 investigated transporters, 20 of which displayed only weak expression, with the only highly expressed transporter being MCT1. Similarly, HEK293 cells expressed 21 transporters, of which 16 had a low expression, and again, MCT1 showed a higher expression. HL-60 cells expressed 16 (6 ABC and 10 SLC) transporters with MRP1, MCT1, OATP4A1 and OATP4C1 at significant levels, while K562 cells expressed 20 transporters with only MCT1 at a pronounced level. The osteosarcoma cell line Saos-2 expressed 14 transporters, 12 of which had weak expression. Of the efflux transporters, MRP4 expression was significant, while MCT1 was the uptake transporter with the highest expression. The gene expression of Caco-2 was comparable to previous publications (Seithel et al., 2006; Hilgendorf et al., 2007). The expression of MCT5 was higher than that of MCT1, which is in agreement with the small intestinal-like properties of Caco-2 cells, that also express other SLC-transporters present in the small intestine such as PEPT1 and IBAT, Figure 2 (Hilgendorf et al., 2007). In HepG2, a high level of expression of MRP2 was observed. In summary, with a few exceptions, the endogenous gene expression in the cell lines was low, the general exception being MCT1, Figure 1.

Ubiquitously expressed transporters

Apart from MCT1, eight of the 36 transporters investigated (three ABC (MRP1, 4 and 5) and five SLC (PEPT2, MCT5, OCT1, OCTN1 and OCTN2)) were expressed in all investigated cell lines, supplemental data Figure S1. Most of these were expressed at low levels, however
MCT1 showed higher expression in all cell lines except for Caco-2, where MCT5 substituted for MCT1 as the most highly expressed monocarboxylate transporter.

**The effect of culturing conditions and the transfection process**

Next, we compared how the cell culture conditions affect the gene expression of transporters. Figure 2. The cell membranes are often reversibly modified by a transfection agent to facilitate the uptake of the transgene to obtain a high transient or stable expression of a gene in a cell line. Moreover, an excess of foreign genetic material is introduced into the cell. Thus, the transfection process creates a cellular stress reaction that may alter its gene expression profile compared with that of the untransfected “control” cells. Therefore, empty vector-transfected cells are usually used as controls. However, to our knowledge, the effect of the transfection procedure on the background expression of drug transporting proteins has not been investigated. We therefore studied whether stable transfection, using the methods previously described by Lohmann and co-workers (Lohmann et al., 2007) with each of four different transporters (BCRP, OAT3, OCT1, OATP1B1), Figure 2a, and transient transfection using polyethyleneimine as transfection agent of HEK293 cells with OCT1, data not shown, affected the endogenous gene expression of transporters. As expected, the expression levels of each of the introduced transporter proteins were well above those of the endogenous transporters, Figure 2a. The endogenous expression of some transporters was slightly increased by the transfection process. However, since these transporters were expressed at very low levels these changes were insignificant.

HEK293 cells were cultured both adherently and in suspension for transport studies, and we wondered whether the different conditions significantly alter transporter expression. As can be seen in Figure 2b, HEK293 cells grown in suspension and adherent cultures exhibited
similar expression patterns, with the exception of some ABC-transporters, and MCT1 that had a slightly higher expression in the suspension cells, Figure 2b.

Recent studies showed large inter-laboratory differences in transporter expression when different Caco-2 clones were cultivated under various conditions according to local traditions (Hayashi et al., 2008). Here, we investigated the variability in transporter expression for two commonly used Caco-2 clones, Caco-2 (obtained from ATCC) and its subclone Caco-2 TC7, using the same cell culture conditions. Caco-2 TC7 cells had a slightly higher gene expression of the SLC transporters, but the differences between the two cell lines was marginal, suggesting that comparable culture conditions may reduce variability in Caco-2 cells, Figure 2c. When used as an intestinal in vitro model, the Caco-2 cells are grown on filters for 21 days and, during this period, the gene expression of many drug transporters gradually increases (Seithel et al., 2006). In analogy, we assumed that Caco-2 cells grown on plastic for 4 days only, would have a lower expression of transporters than cells grown on filters for 21 days. The results in Figure 2d confirm this assumption, but as for the two Caco-2 clones in Figure 2c, the differences were not very large.

In conclusion, in our hands, changes in culture conditions only resulted in small differences in the gene expression of transporters, indicating that experiments performed under such different conditions in the same laboratory ought not to result in large differences in transporter expression. Cellular stress from the introduction and expression of a transgene resulted in increased expression levels of some endogenous transporters. However, these transporters had very low expression levels and, therefore, the differences are considered to be negligible in comparison to the high expression level of the transgene, Figure 2a.

**Antibody validation in human tissues**
We were able to generate antibodies that met the release criteria from the Human Proteome Resource for nine of the transporters, which is a normal success rate (Berglund et al., 2008). About 50 human organs from three individuals were stained with each of the antibodies, and we compared the results from IHC with data from the literature to learn about the specificity of each of the nine transporter-specific antibodies, Figure 3. The references used for this external validation are given in Table 1. This external validation, in combination with the internal HPR validation, indicated that four of the antibodies displayed an unspecific and partly atypical staining pattern, in comparison with what is presented in the literature.

Notably, some of these antibodies gave the postulated staining pattern in tissues where their expression is expected, but also stained tissues where no staining had been observed previously. Thus, as anticipated, the peptide transporter, PEPT1, stained the apical side of the intestinal epithelium, Figure 3o, and the renal tubuli (data not shown), but it also stained many other tissues, e.g. the cerebral cortex, for which negative results have been published in the literature, Table 1 and Figure 3p. Similarly, according to literature, the ileal bile acid transporter, IBAT, and the cation transporter, OCT2, are mainly restricted to the intestine and kidney, respectively, and while our antibodies stained these tissues, Figure 3m and q, they also exhibited broader staining patterns than expected and stained many tissues that had been expected to give negative results, e.g. Figure 3n and 4r (Table 1). Finally, MRP4 showed poor correlation between the antibody staining and the wide tissue distribution reported in the literature, Figure 3k-l (Table 1).

In contrast, the five remaining antibodies, gave typical staining patterns, with specific staining in the tissues where it had been expected. Thus, the MDR1 stained many tissues as anticipated, including the apical surface of intestinal epithelial cells, bile canaliculi, renal tubuli and placenta, e.g. in Figure 3a. Further, tissues lacking MDR1, such as skeletal muscle, did not generally show any antibody staining, Table 1 and e.g. Figure 3b. The MRP1
was expressed in many tissues, which is consistent with the literature with staining being detected in, e.g. kidney, Figure 3c, and testes, and as anticipated no staining in liver, Figure 3d and Table 1. As expected, the MRP2 antibody stained the bile canaliculi in hepatocytes, Figure 3e, duodenum and kidney and the tissue staining pattern corresponded well with data published earlier, e.g. no staining of heart tissue, Figure 3f and Table 1. MCT1 displayed ubiquitous expression, as anticipated from the literature, e.g. Figure 3g-h, and the liver specific OATP1B3 stained the hepatocytes, but was absent in other organs, Figure 3i-j and Table 1.

**Protein expression and comparison with gene expression**

As a part of the HPR project, the protein expression is analysed by IHC in a panel of 47 cell lines (Stromberg et al., 2007), including 6 of the 8 cell lines included in the present study, Table 2b and Figure 4. In general, the results confirmed the unspecific reactivity of antibodies with poor validation. For instance, all cell lines were stained for the ileal bile acid transporter, IBAT, while the corresponding gene expression was found only in the cell line of colonic origin, which is known to express IBAT (Hilgendorf et al., 2007), Table 2b. Similarly, all six cell lines were moderately or strongly stained by the small intestinal peptide transporter PEPT1, which have shown weak expression levels in earlier studies, e.g. (Bleasby et al., 2006). The five msAb that displayed the expected tissue expression patterns were assumed to give more reliable results, Table 2b. Examples of IHC for the cell lines are shown in Figure 4. Similar to the gene expression data, the MDR1 antibody stained Caco-2 and HepG2 cells at moderate levels and HeLa and HEK293 at weak levels, but did not stain HL-60 and K562 cells which were expected to express this protein, Table 2. A clear membrane localization of MDR1 was observed in Caco-2, HepG2 and HeLa cells. The msAb for MRP1 stained HL-60 and Caco-2 weakly, whereas the other four cell lines were moderately stained. This is
generally consistent with the gene expression, with the exception of HeLa and HL-60, Table 2c. For MRP2, HeLa, HEK293 and HL-60 displayed weak, Caco-2 and HepG2 moderate and K562 strong staining. This was comparable to gene expression, except for K562, where no gene expression for MRP2 was observed at the selected time point, Table 2a. Both MRP1 and especially MRP2 showed intracellular staining patterns in most of the cell lines, which is in agreement with earlier findings, Figure 4 (Keitel et al., 2003). Consistent with the gene expression data, MCT1 showed pronounced typical membrane staining in all six cell lines, whereas the protein expression for OATP1B3 was negative in all cell lines investigated, Table 2 and Figure 4. The overlap between gene and protein expression in the cell lines was investigated for all nine transporters using four expression levels, Table 2c (negative, weak, moderate and strong). The expression levels were in agreement in 43% of the cases. When the five transporters with good validation scores were considered, the overlap increased to 67% and, in most of the remaining cases, the gene and protein expression were comparable. For the four antibodies with poorer validation scores, the agreement was 13 %, i.e. much lower than for the transporters with better validation scores, Table 2c.

**MCT1 function**

Since both gene and protein expression of MCT1 was significant, we reasoned that this ubiquitously expressed transporter could, if functional, be a confounding factor in transport experiments, in particular if small drugs with monocarboxylic acid moieties are studied (Meredith and Christian, 2008). Using the endogenous MCT1-substrate ¹⁴C-lactic acid, a significant uptake that could be completely or partly inhibited by quercetin was observed in five out of the six cell lines, Figure 5.
Discussion

In this contribution, we used RT-PCR to assess the endogenous gene expression levels of 36 transporters in human cell lines commonly used in drug transport studies, and investigated the protein expression of nine of these transporters, using a new type of monospecific polyclonal antibodies, produced as a part of a Human Proteome Resource (Uhlen et al., 2005).

The low endogenous transporter expression in HEK293 and HeLa cells, Figure 1; supplemental data Figure S1, supports their suitability as systems for the overexpression of human transport proteins (Zhang et al., 1998; Ahlin et al., 2008). IHC showed that some ABC transporters, especially MRP2, are partially located intracellularly in all cell lines, Figure 4. These results are in agreement with studies indicating that MRP2 overexpressed in HEK293 cells may remain intracellular and not be functional at the plasma membrane (Sekine et al., 2008), but whether MRP2 is functional or not, and a potential confounding factor, remains to be investigated. On the other hand, a clear membrane localisation was observed for MCT1 in all investigated cell lines, Figure 4. This suggested that MCT1 might be functional in these cell lines and that, based on subcellular localization, HEK293 and HeLa cells are a more attractive alternative for overexpression of SLC-transporters than ABC-transporters.

The two leukaemia cell lines K562 and HL-60 have been extensively used in studies of multi-drug resistance mediated by ABC-transporters (Baran et al., 2007; Assef et al., 2009). In many studies, K562 displayed resistance to MDR1 substrates, while MRP1 is known to be a prominent contributor to drug resistance in HL-60 cells, e.g. (Baran et al., 2007; Assef et al., 2009). Surprisingly, in our hands, these cell lines exhibited low levels of or no gene and protein expression of MDR1, Table 2. Since similar results have been observed by others (Baran et al., 2007; Assef et al., 2009), it is possible that our K562 and HL-60 cells have not been exposed to selection pressure from cytostatics for a long time, and therefore lost the
expression of MDR1. However, the two cell lines expressed several other multi-drug resistance proteins of the MRP family, Figure 1; supplemental data Figure S1, which is consistent with the literature (Gillet et al., 2004; Johnsson et al., 2005). Since the K562 and HL-60 cell lines are used to overexpress individual ABC-transporters (Baran et al., 2007; Assef et al., 2009), our results propose that the endogenous expression of ABC-transporters should be monitored in order to avoid biased conclusions regarding drug efflux mechanisms. An alternative for expression of ABC-transporters is Saos-2 cells, which also have a low background expression in general and of ABC-transporters in particular, Figure 1; supplemental data Figure S1. Saos-2 cells have previously been successfully used to overexpress single ABC-transporters (Wierdl et al., 2003). The gene expression data obtained with the previously studied cell line Caco-2, Figure 1; supplemental data Figure S1, were in agreement with the literature, indicating that our results are reproducible, over longer time periods (Hilgendorf et al., 2007; Hayeshi et al., 2008).

The endogenously expressed transport protein of potential concern in all these cell lines was MCT1, which displayed a comparably high gene and protein expression and also was found to be functional in five out of six investigated cell lines; Table 2, Figure 5. MCT1 is one of several ubiquitous transporters found in the expression analysis, but was the only one that reached what could be considered as high expression levels. It transports lactic and pyruvic acid and, hence, is of importance in glycolysis and gluconeogenesis. In general, MCT1 accepts short chain monocarboxylates and small drugs with carboxylate groups such as salicylate as substrates, suggesting that care should be exercised to avoid biased results, at least when investigating small monocarboxylate compounds. Fortunately, very few drug substrates have been identified for MCT1 (Meredith and Christian, 2008).

Other complicating factors in cell culture studies are the variability introduced by lab-to-lab differences in cell culture procedures, cellular stress (as exemplified here by the introduction
of a transgene) and clonal variation. Recently, a large lab-to-lab variation in the mRNA expression and function of transport proteins was observed for Caco-2 cells (Hayeshi et al., 2008). Since, in our experience, the time dependent variability inside our laboratory - where the same culture procedures are used over time - is much smaller than that observed between laboratories, we investigated how transporter expression varied between suspension and adherent cultures, plastic and filter grown cells, transfection procedure and cell clones with somewhat different properties. Our results indicated that, although differences could be observed they were generally smaller than expected and in most cases probably of limited significance, Figure 2. We conclude that maintenance of good, reproducible cell handling and cell culture conditions will reduce the variability in transporter expression.

The unspecific reactivity observed, in particular, for the msAb with poor validation scores was a point of concern, but may also be an inherent property of these antibodies (Berglund et al., 2008; Ponten et al., 2008). In contrast to monoclonal antibodies that react with a single epitope on the antigen, msAb have the potential to react with several epitopes and, hence, obtain a broader reactivity (Nilsson et al., 2005). Thus, in theory, these antibodies may maintain reactivity to their antigen in tissues of poorer quality, where the antigen may be partly denatured, such as human brain tissues obtained post mortem. Therefore, it can not be excluded that, at least partially, the atypical reactivity observed with the poorly validated antibodies is real but has not been observed previously, Table 2b. According to our own and the internal HPR validation approach (Berglund et al., 2008; Ponten et al., 2008), the msAb, with good correlation between the literature and the tissue staining patterns observed in this study, also gave a good correlation between gene and protein data for the cell lines, Table 2c. This suggests that a thorough antibody validation process is essential to get protein expressional data of high quality. We emphasize that the extensive validation of the msAbs in this study is unique, and has barely been achieved for the commercial monoclonal antibodies.
used in many published studies. The validation showed that a tissue-specific staining pattern in a single or just a few tissues does not assure the absence of non-specific staining in a multitude of other tissues.

In conclusion, this is the first study that compares the gene and protein expression in human cell lines for a broad range of transporters. The results indicate that, in general, the gene and protein expression were both low in the investigated cell lines. Independently of the antibody quality, specific staining in the expected tissues was obtained, but for the poorly validated antibodies, additional unspecific staining of those tissues that were expected to be negative was observed, underscoring the importance of thorough validation of the antibodies. We also showed, for the first time, that the monocarboxylic acid transporter MCT1 is ubiquitously expressed and functional in human cell lines, and we suggest that this should be considered when transport of small monocarboxylic drugs is investigated.
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References


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Footnotes

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b. Part of this work was presented at a poster at the AAPS annual meeting, 2008 November 16-20, Atlanta, USA.
Legends for figures

Figure 1: Relative gene expression of ABC (a) and SLC+HPT1 (b) transporters in HeLa, HEK293, HL-60, K562, Saos-2, Caco-2 and HepG2. The bars represent the mean relative expression levels for each transporter.

Figure 2: The effect of the transfection process and culture conditions on the relative gene expression profile. (a) The effect of stable transfection on the transfected protein and endogenous transporters. (b) Gene expression of HEK293 cells in suspension (dark grey bars) and adherent (light grey bars) culture. (c) Gene expression of Caco-2 TC7 (dark grey bars) and Caco-2 (light grey bars) cells cultured in a configuration used for transport studies, see the methods section. (d) Gene expression of Caco-2 cultured on a plastic support for 4 days (dark grey bars) and Caco-2 cells cultured on a filter support for 21 days (light grey bars).

Figure 3: Examples of human tissues stained with msAb of human transporters. Brown-black staining is specific antibody-specific staining and the tissue sections are counterstained with hematoxylin (blue staining) to enable visualization of microscopical features. See results section for details.

Figure 4: Localisation of the five transporters, showing good validation scores, in HEK293 and Caco-2 cells. Arrows indicate intracellular staining in MRP1 and 2. Brown-black staining is antibody-specific, and the tissue section is counterstained with hematoxylin (blue staining) to enable visualization of microscopical features. Images were annotated using an automated image-analysis application. Staining levels: Red = strong, Orange = moderate, Yellow = weak and White = absent. See results section for details.
Figure 5: MCT1 function in six cell lines. The cells were incubated with the MCT1 substrate 14C-lactic acid only (dark grey bars) and with 14C-lactic acid together with the MCT1 inhibitor quercetin (light grey bars).

Figure S1: Qualitative gene expression data of 36 transporters, based on the relative gene expression levels in Figure 1, in all cell lines investigated.

Expression levels: Black = strong, Dark grey = moderate, Light grey = weak and White = absent.
Table 1: Antibody validation from tissue staining patterns

<table>
<thead>
<tr>
<th>Transport proteins</th>
<th>HPR validation(^a)</th>
<th>Literature validation(^b)</th>
<th>Validation Score(^c)</th>
<th>References(^d)</th>
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                      |                       |                             |                          | Tanabe et al. (2001) *J Pharmacol Exp Ther* 297:1137  
                      |                       |                             |                          | 1143. |
                      |                       |                             |                          | Nishimura et al. (2005) *Drug Metab Pharmacokinetics* 20:452-477.  
                      |                       |                             |                          | 170.  
| OATP1B3            | Medium                | Typical                      | Good                     |                             |
| MRP4               | Very low              | Contradictive                | Poor                     |                             |
| IBAT               | Low                   | Contradictive                | Poor                     |                             |
| PEPT1              | Very low              | Contradictive                | Poor                     |                             |
| OCT2               | Low                   | Contradictive                | Poor                     |                             |

\(^a\) HPR=Human Proteome Resource. The validation was performed by i) comparing IHC staining patterns with staining patterns of commercial antibodies and/or with experimental/bioinformatics data for a subset of 13 different normal tissues, four different tumours tissues and eight cell lines ii) investigating the consistency of our IHC staining patterns with previously published protein localisation and/or bioinformatics data

\(^b\) Literature validation was performed by comparing our IHC staining patterns with data from the literature on transporter expression and localisation

\(^c\) A weighted validation score, based on the HPR and literature validation

\(^d\) References used for literature validation
Table 2: Qualitative gene (a) and protein (b) expression data for the nine transporters investigated using immunohistochemistry. Black = Strong expression, Dark grey = Moderate expression, Light grey = Weak expression and White = absent. (c) The overlap between gene and protein expression for the transporters. White= overlapping expression, Light grey = adjacent expression levels, Dark grey = a difference of two expression levels and Black = a difference of three expression levels.

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<th>MRP1</th>
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Figure 1
Figure 5

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