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Expression of the drug transporters *MDR1/ABCB1*, *MRP1/ABCC1*, *MRP2/ABCC2*, *BCRP/ABCG2*, and *PXR* in peripheral blood mononuclear cells and their relationship with the expression in intestine and liver

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Abstract

ATP binding cassette (ABC)-transporters like P-glycoprotein (multidrug resistance (*MDR)1/ABCB1*), the multidrug resistance associated proteins 1 and 2 (*MRP1/ABCC1* and *MRP2/ABCC2*), and the breast cancer resistance protein (*BCRP/ABCG2*) have a large impact on the pharmacokinetics of numerous drugs and may also modulate the effectiveness of drug therapy. Prediction of a patient's susceptibility to xenobiotics and individualization of drug therapy would become possible, if a simple test were available for an easy screening of transporter expression. This study quantified the mRNA expression of the four ABC-transporters and of the pregnane X receptor (PXR), a key regulator in drug metabolism and efflux, in peripheral blood mononuclear cells (PBMCs), and corresponding liver or small intestine samples of humans by real-time reverse transcription-polymerase chain reaction (RT-PCR). The results obtained prove the absence of a correlation between the expression of four major ABC-transporters in PBMCs and in the intestine or liver. For all transporters (except *MRP1/ABCC1* in the intestine), mRNA amount of the ABC-transporters was positively correlated with PXR expression in PBMCs and intestine. In conclusion, the study suggests that basal expression levels of the transporters are directly influenced by PXR expression in liver and PBMCs and demonstrates that PBMCs do not qualify as surrogate tissue for the expression of the four ABC-transporters in small intestine and liver. However, the transporter status in PBMCs remains important for drugs, whose primary site of therapeutic action is the lymphocyte and which are known substrates of the transporters.

Keywords: MDR1/ABCB1; MRP1/ABCC1; MRP2/ABCC2; ABCG2/BCRP; PXR; PBMC

1. Introduction

ATP binding cassette (ABC)-transporters represent a large family of transmembrane proteins that bind ATP and use the energy of ATP hydrolysis to transport various compounds across cell membranes [1]. ABC drug transporters in the intestine, liver, and kidney have a large impact on the pharmacokinetics of numerous drugs. In particular, they may not only restrict absorption in the gut and thus reduce bioavailability but also substantially determine drug elimination into bile, urine, and even feces. Moreover, because ABC-transporters actively restrict drug distribution to the

Abbreviations: ABC, ATP binding cassette; BCRP, breast cancer resistance protein; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hanks balanced salt solution; HHBSS, with HEPES supplemented HBSS; HIV, human immunodeficiency virus; LY335979, zosuquidar; MDR, multidrug resistance; β2mg, β2-microglobulin; MRP, multidrug resistance associated protein; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; PE, phycoerythrin; P-gp, P-glycoprotein; PXR, pregnane X receptor; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcription PCR; S.D., standard deviation

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site of action (e.g. at the blood–brain barrier), they may also modulate the effectiveness of drug therapy.

Activity of ABC-transporters is highly variable and may be altered within hours by inhibitors and within days by inducing agents. Also, genetic influences [2] or endogenous factors like sex-hormones [3] or cholesterol [4] might influence transporter activity. Moreover, for multidrug resistance (*MDR*)*1*/*ABCB1* and multidrug resistance associated protein (*MRP*)*2*/*ABCC2*, it is well known that the orphan receptor pregnane X receptor (PXR), which is a key regulator in drug metabolism and efflux, induces *MDR1*/*ABCB1* and *MRP2*/*ABCC2* when activated by PXR ligands like rifampin [5,6]. In contrast, for *MRP1*/*ABCC1*, there are only few studies on this topic, which are furthermore controversial [7,8], and for breast cancer resistance protein (*BCRP*)/*ABCG2*, no data exist so far.

Just because their activity is variable and also because the individual extent of drug–drug interactions at this level is almost unpredictable, a surrogate marker for the activity of the most important drug transporting systems would be very helpful.

Peripheral blood mononuclear cells (PBMCs) express many of the ABC-transporters important for drug transport which might restrict drug access to this important site of action [1,9–12]. Moreover, PBMCs are readily available, a prerequisite for a suitable surrogate marker. However, PBMCs only qualify as surrogate, if their transporter expression correlates with that of other tissues, especially with the intestine, whose transporter equipment appears to have the largest impact on the pharmacokinetics of numerous drugs.

We therefore investigated in humans by means of RT-PCR, whether the mRNA expression of *MDR1*/*ABCB1*, *MRP1*/*ABCC1*, *MRP2*/*ABCC2*, and *BCRP*/*ABCG2* in PBMCs correlates with that in intestine and liver. For P-glycoprotein (P-gp), we also studied the influence of the G2677T and the C3435T polymorphism in the *MDR1*/*ABCB1* gene on the expression and we evaluated the association between expression and function of P-gp as measured by rhodamine123 efflux from PBMCs. Finally, we quantified the mRNA expression of PXR in the different tissues and examined a possible correlation between PXR and transporter expression.

2. Materials and methods

2.1. Materials

Medium (RPMI), medium supplements, PBS, and Hanks balanced salt solution (HBSS) were purchased from Invitrogen (Karlsruhe, Germany), fetal calf serum (FCS) from Biochrom AG (Berlin, Germany), and rhodamine123 from Calbiochem (Bad Soden, Germany). Vacutainer®CPT™ were purchased from Becton Dickinson (Heidelberg, Germany). K/EDTA monovettes® were from Sarstedt (Nümbrecht, Germany). DMSO was from AppliChem (Darmstadt, Germany). Ficoll-Paque™Plus was purchased from Amersham Biosciences (Freiburg, Germany). High Pure RNA Isolation Kit, First Strand cDNA Synthesis Kit for PCR (AMV), RNA/DNA Stabilization Reagent for Blood/Bone Marrow, LightCycler-FastStart DNA Master SYBR Green I Kit, LightCycler-FastStart DNA Master^{PLUS} SYBR Greeen I Kit, and LightCycler-FastStart DNA Master Hybridization Probes Kit were purchased from Roche Applied Science (Mannheim, Germany). The RNeasy Mini Kit was obtained from Qiagen (Hilden, Germany) and the NucleoSpin® Blood Quick Pure Kit from Macherey-Nagel (Düren, Germany). Zosuquidar (LY335979) was obtained from Eli Lilly Company (Bad Homburg, Germany).

2.2. Tissue and blood samples

The study was approved by the Ethics Committee of the Medical Faculty of the University of Heidelberg and was conducted in accordance with all local legal requirements, the Declaration of Helsinki as amended in Somerset West 1996. All participants provided written informed consent before inclusion in the study. Hepatic samples were obtained from nine patients with hepatocellular or bile duct carcinoma ($n = 2$) or hepatic metastases from gastrointestinal tumors ($n = 7$). Intestinal samples were resected from the duodenum ($n = 8$) or ileum ($n = 14$) from patients with malignant gastrointestinal tumors ($n = 8$), pancreatic carcinoma ($n = 6$), precanceroses ($n = 5$), or inflammatory or infectious diseases of abdominal organs ($n = 3$). In all of these and 13 additional similar patients with gastrointestinal tumors, tumors of the pancreas or bile duct, or with echinococcosis PBMC samples were obtained.

Altogether, 44 patients (18 females, mean age ± standard deviation (S.D.): 48 ± 19 years; 26 males, mean age: 58 ± 13 years) were included. None of the patients received chemotherapy in the last 4 weeks before surgery or drugs with known inducing effects on ABC-transporters.

Only pieces of macroscopically healthy tissue were used for the investigations. Tissue samples were frozen in liquid nitrogen immediately after removal and stored at −80 °C until preparation of RNA. Blood samples for isolation of PBMCs were taken in Vacutainer®CPT™ and for isolation of RNA in K/EDTA monovettes®.

2.3. Isolation and cryoconservation of PBMCs

For isolation of PBMCs Vacutainer®CPT™ was centrifuged (1700 × *g*, 20 min at room temperature). After centrifugation, PBMCs were separated from erythrocytes and granulocytes by the polyester gel in the Vacutainer®CPT™ and were resuspended into the plasma by inverting the tube. PBMCs were then washed twice with

PBS at room temperature and cryoconservated in 90% FCS and 10% DMSO in liquid nitrogen.

Control PBMCs were isolated from one healthy volunteer by density centrifugation with Ficoll-Paque™Plus and cryoconservated in 90% FCS and 10% DMSO.

2.4. Rhodamine123 efflux

For rhodamine123 efflux, PBMCs were quickly thawed, washed, and resuspended in RPMI medium. For adaptation, cells were incubated in RPMI medium for 60 min at 37 °C. The PBMCs were then incubated with rhodamine123 at a concentration of 0.4 μM on a rotary shaker (30 min, 37 °C, 450 rpm). Subsequently, cells were washed with precooled RPMI medium (4 °C) and incubated another 50 min at 37 °C in rhodamine123 free medium (with or without P-gp inhibitor) to allow rhodamine123 efflux. After washing the cells with precooled RPMI (4 °C), they were stained with phycoerythrin (PE)-labeled CD8 antibody (15 min on ice in darkness). As a control, all assays were also performed in control PBMCs to account for inter-day variability.

For analysis, a Becton Dickinson FACS Calibur with a 488 nm argon laser was used. Rhodamine123 fluorescence was measured using a 530 bandpass filter and PE fluorescence with a 585 bandpass filter. In each sample, 1500 $CD8^+$ cells or about 5000 lymphocytes were counted.

Median rhodamine123 fluorescence was determined in $CD8^+$ cells or gated lymphocytes after the efflux period and compared to median rhodamine123 fluorescence of $CD8^+$ cells or gated lymphocytes from the same individual which were treated with the specific P-gp inhibitor LY335979 during the efflux period. The individual P-gp activity was then calculated as ratio of the median fluorescence of the cells incubated with or without LY335979 and normalized to the ratio of control $CD8^+$ cells or lymphocytes, which were treated identically.

The validation of the rhodamine123 efflux for measuring P-gp activity in PBMCs and the rationale for using $CD8^+$ cells have been reported previously [4,13].

2.5. MDR1/ABCB1 genotyping

To exclude possible influences of the G2677T and the C3435T polymorphisms in the *MDR1/ABCB1* gene on the expression or function of P-gp, the corresponding genotypes were determined for all participants. DNA was isolated from whole blood using the NucleoSpin® Blood Quick Pure Kit (Macherey-Nagel) according to the manufacturer's instructions. The C3435T polymorphisms in exon 26 of the *MDR1/ABCB1* gene was detected by rapid-cycle polymerase chain reaction (PCR) and fluorescent melting point analysis using fluorogenic hybridization probes on the LightCycler™ (Roche Applied Sciences) [14]. The G2677T polymorphism in exon 21 of the *MDR1/ABCB1* gene was genotyped by PCR-restriction fragment length polymorphism (RFLP) according to Cascorbi et al. [15].

2.6. Isolation of RNA and cDNA synthesis

RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Applied Science) was added to the blood samples immediately after withdrawal to prevent RNA from degradation. RNA was isolated from whole blood using the High Pure RNA Isolation Kit (Roche Applied Science) and from tissues using the RNeasy Mini Kit (Qiagen). Quality and purity of RNA were verified by gel electrophoresis, concentration was measured spectrophotometrically, and isolated RNA was stored at −80 °C until analysis. cDNA was synthesized with the First Strand cDNA Synthesis Kit for PCR (AMV) (Roche Applied Science).

2.7. Quantification of mRNA expression by real-time reverse transcription (RT)-PCR

Expressions of mRNAs of the *MDR1/ABCB1*, *MRP1/ABCC1*, *MRP2/ABCC2*, *BCRP/ABCG2*, and of the *PXR* gene were quantified by real-time RT-PCR with the LightCycler™ technology using *β2-microglobulin* (*β2mg*) as housekeeping gene. All primer pairs were synthesized by MWG Biotech (Ebersberg, Germany) and were designed to span intron/exon junctions or to yield larger products with DNA. Therefore, possibly contaminating genomic DNA was either not amplified or could be detected by additional peaks during melting point analysis. Primer sequences and references are specified in Table 1.

PCR amplification was carried out in 20 μl reaction volume containing 5 μl 1:10 diluted cDNA, 1× LightCycler-FastStart DNA Master SYBR Green I (LightCycler-FastStart DNA Master^{PLUS} SYBR Green I for

Table 1
Primers used for real-time RT-PCR

Gene	Forward primer 5′–3′	Reverse primer 5′–3′	Product (bp)	Design
MDR1/ABCB1	CCCATCATTGCAATAGCAGG	TGTTCAAACTTCTGCTCCTGA	158	This paper
MRP1/ABCC1	ATGTCACGTGGAATACCAGC	GAAGACTGAACTCCCTTCCT	348	[35]
MRP2/ABCC2	ACAGAGGCTGGTGGCAACC	ACCATTACCTTGTCACTGTCCATGA	226	[36]
BCRP/ABCG2	AGATGGGTTTCCAAGCGTTCAT	CCAGTCCCAGTACGACTGTGACA	91	[37]
PXR (tissue)	CAAGCGGAAGAAAAGTGAACG	CACAGATCTTTCCGGACCTG	246	[16]
PXR (PBMCs)	GACTTGCCCATCGAGG	AGAGTAATGGCGAATTGC	323	This paper
β2mg	CCAGCAGAGAATGGAAAGTC	CATGTCTCGATCCCACTTAAC	258	[24]

amplification of PXR) (Roche Applied Science), 0.5 μM of each primer, and between 3 and 4 mM $MgCl_2$.

Standard curves were prepared for each target and reference gene. Data were evaluated by calibrator-normalized relative quantification with efficiency correction using the RelQuant Software Version 1.01 (Roche Applied Science). The software calculates the relative amount of the target gene and the reference gene (housekeeping gene) based on the crossing point (Cp). Results are expressed as the target/reference ratio divided by the target/reference ratio of the calibrator and are therefore corrected for sample inhomogenities and detection-caused variance. The normalized ratio was calculated according to the following equation:

$$(\text{median}(\text{target}_{\text{sample}})/\text{median}(\text{reference}_{\text{sample}}))/(\text{median}(\text{target}_{\text{calibrator}})/\text{median}(\text{reference}_{\text{calibrator}}))$$

All samples were amplified at least in triplicate on different days.

2.8. Validation of the real-time RT-PCR methods

To further validate the suitability of the newly designed primers (for *MDR1/ABCB1* and *PXR*), the PCR product (20 ng) was sequenced using the dideoxy chain termination method (DYEnamic ET Kit, Amersham Biosciences) on a MegaBACE500 sequencer (Amersham Biosciences).

2.9. Statistical analysis

Data were analyzed using GraphPad Prism® Version 4.0 (GraphPad Software, San Diego, CA, USA). For comparison of the mRNA expression between individuals with different genotypes, the Kruskal–Wallis test was used. Associations were assessed by Spearman rank correlation and expressed by the corresponding correlation coefficient r_S. A p-value < 0.05 was considered significant.

3. Results

3.1. Quantification of mRNA expression by real-time RT-PCR

For the mRNA expression of the target genes (*MDR1/ABCB1*, *MRP1/ABCC1*, *MRP2/ABCC2*, *BCRP/ABCG2*, and *PXR*) and the housekeeping gene *β2mg*, a reliable and reproducible relative quantification method based on LightCycler™ technology was established and validated. The amplification curves, melting point analysis, and standard curves are exemplified for *MDR1/ABCB1* and *β2mg* in Fig. 1. The specificity of the PCR products was verified by agarose gel electrophoresis and for the newly designed primers also by sequencing of the PCR products. All PCR products were of correct size and the sequences

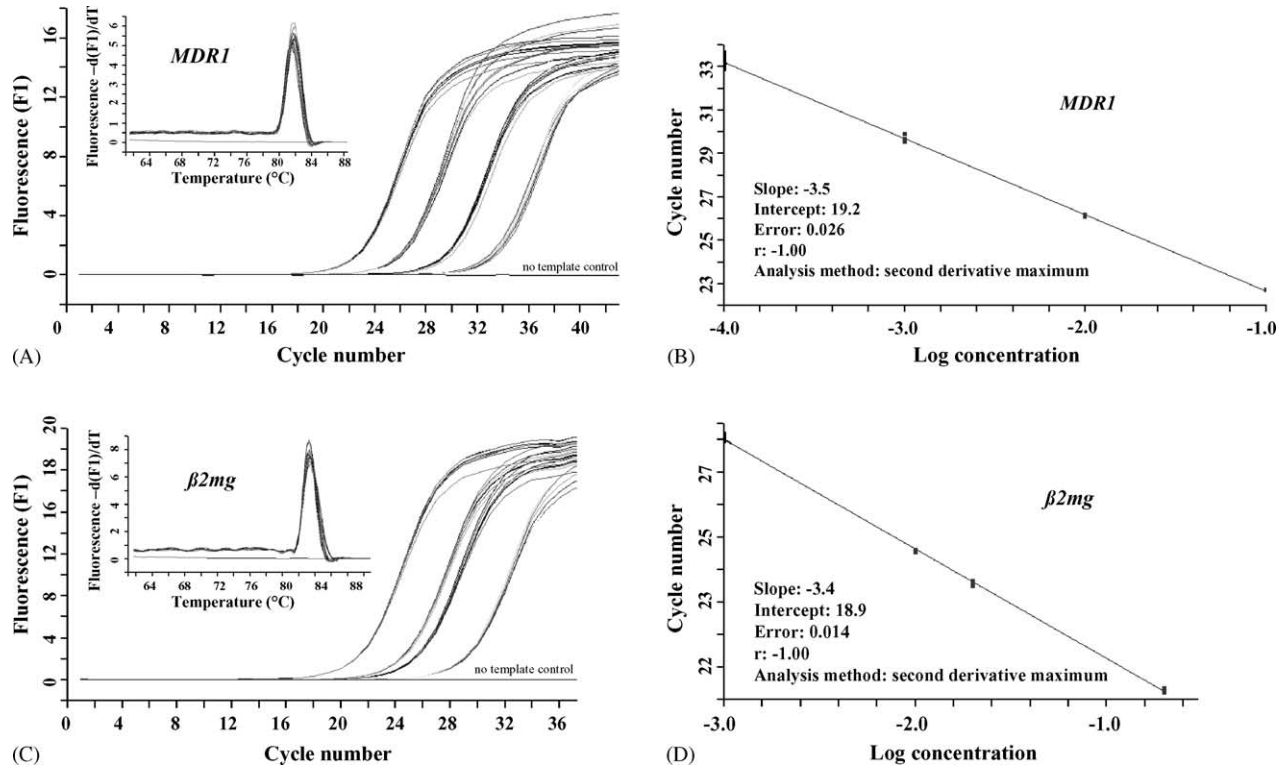

Fig. 1. Real-time RT-PCR for quantification of *MDR1/ABCB1* (A and B) and *β2mg* mRNA (C and D) with amplification curves (A and C) and melting point analysis (insert) of serial dilutions of calibrator cDNA. Standard curves (B and D) were generated by plotting the Cp value vs. the log of the dilution of the calibrator cDNA.

Table 2
Inter-individual differences in mRNA expression calculated as ratio between target gene/*β2mg*

	All patients ($n = 44$)		Patients with small intestine samples ($n = 22$)				Patients with liver samples ($n = 9$)			
	PBMCs		PBMCs		Small intestine		PBMCs		Liver	
	Mean ± S.D.	Ratio	Mean ± S.D.	Ratio	Mean ± S.D.	Ratio	Mean ± S.D.	Ratio	Mean ± S.D.	Ratio
MDR1/ABCB1	0.004 ± 0.004	66	0.003 ± 0.002	28	0.03 ± 0.02	74	0.004 ± 0.003	44	0.02 ± 0.01	4
MRP1/ABCC1	0.09 ± 0.05	13	0.07 ± 0.04	9	0.02 ± 0.04	76	0.1 ± 0.06	10	0.007 ± 0.005	6
MRP2/ABCC2	0.0006 ± 0.0003	8	0.0005 ± 0.0002	5	0.01 ± 0.02	947	0.0005 ± 0.0004	9	0.05 ± 0.02	4
BCRP/ABCG2	0.06 ± 0.06	65	0.06 ± 0.07	65	0.9 ± 0.5	94	0.03 ± 0.02	8	0.3 ± 0.1	4
PXR	0.7 ± 0.6	45	0.7 ± 0.7	34	0.6 ± 0.4	66	0.7 ± 0.6	28	1.4 ± 0.4	4

Ratio: maximum/minimum expression found in the respective tissue; *n*: sample size; S.D.: standard deviation.

determined matched the sequences published in the NCBI GenBank, confirming the specificity of the PCR assays.

Quantification of PXR in tissue and PBMCs required two different PCR reactions because the primer pair published by Chang et al. [16] did not allow reliable quantification of PXR in PBMC samples with very low PXR expression. We therefore established and validated an alternative method suitable for PXR quantification in PBMCs. Association analysis of the 29 samples, which could be quantified with both primer pairs, demonstrated a highly significant correlation ($r_S = 0.69$; $p < 0.0001$) between both methods.

Inter-individual differences in mRNA expression of the ABC-transporters and *PXR* were most prominent in the intestine followed by PBMCs and expression was rather stable in the liver samples (Table 2). Comparison of the expression in the respective intestinal segments revealed greater inter-individual differences in the duodenum compared to the ileum (Table 3), whereas the mean expression was similar except for *MRP2/ABCC2*, whose expression was about five times lower in the ileum compared to the duodenum ($p < 0.05$, Mann–Whitney test). Evaluation of ABC-transporter expression in PBMCs revealed the following order of expression: *MRP1/ABCC1* > *BCRP/ABCG2* > *MDR1/ABCB1* > *MRP2/ABCC2*. In contrast, in the liver, the most abundant ABC-transporter was *BCRP/ABCG2* followed by *MRP2/ABCC2*, *MDR1/ABCB1*, and *MRP1/ABCC1*. In the intestine, *BCRP/ABCG2* was also highly expressed followed by the other ABC-transporters, which were expressed to similar amounts.

Table 3
Inter-individual differences in mRNA expression calculated as ratio between target gene/*β2mg*

	Duodenum ($n = 8$)		Ileum ($n = 14$)	
	Mean ± S.D.	Ratio	Mean ± S.D.	Ratio
MDR1/ABCB1	0.02 ± 0.01	42	0.03 ± 0.02	10
MRP1/ABCC1	0.009 ± 0.005	8	0.03 ± 0.04	47
MRP2/ABCC2	0.02 ± 0.03	947	0.005 ± 0.007	157
BCRP/ABCG2	0.8 ± 0.5	90	0.9 ± 0.5	11
PXR	0.6 ± 0.4	49	0.6 ± 0.4	16

Due to potentially variable expression levels of *β2mg* in different tissues, comparison of ABC-transporter expression in different tissues can only be semiquantitative. However, the obtained data suggest that *MRP2/ABCC2* expression in PBMCs is about 50 times and *MDR1/ABCB1* and *BCRP/ABCG2* are about 10 times smaller than in small intestine and liver. In contrast, expression of *MRP1/ABCC1* was highest in PBMCs.

3.2. Correlation of mRNA expression of ABC-transporters in PBMCs and tissues

Analysis with Spearman rank correlation revealed no relationship between mRNA expression in PBMCs and liver or PBMCs and intestine for all ABC-transporters except *MRP2/ABCC2*, for which mRNA expression in PBMCs and intestine was significantly correlated (Table 4 and Fig. 2).

Table 4
Correlation analysis of mRNA expression

Correlation between	r_S	p
MDR1/ABCB1 in small intestine and PBMCs	−0.01	0.98
MDR1/ABCB1 in liver and PBMCs	0.13	0.74
MRP1/ABCC1 in small intestine and PBMCs	0.10	0.66
MRP1/ABCC1 in liver and PBMCs	−0.12	0.78
MRP2/ABCC2 in small intestine and PBMCs	**−0.56**	**0.005**
MRP2/ABCC2 in liver and PBMCs	0.18	0.64
BCRP/ABCG2 in small intestine and PBMCs	−0.40	0.06
BCRP/ABCG2 in liver and PBMCs	0.47	0.21
MDR1/ABCB1 and *PXR* in PBMCs	**0.96**	**<0.0001**
MDR1/ABCB1 and *PXR* in small intestine	**0.66**	**0.0008**
MDR1/ABCB1 and *PXR* in liver	0.03	0.95
MRP1/ABCC1 and *PXR* in PBMCs	**0.79**	**<0.0001**
MRP1/ABCC1 and *PXR* in small intestine	−0.17	0.45
MRP1/ABCC1 and *PXR* in liver	−0.58	0.11
MRP2/ABCC2 and *PXR* in PBMCs	**0.58**	**<0.0001**
MRP2/ABCC2 and *PXR* in small intestine	**0.61**	**0.003**
MRP2/ABCC2 and *PXR* in liver	0.25	0.52
BCRP/ABCG2 and *PXR* in PBMCs	**0.39**	**0.009**
BCRP/ABCG2 and *PXR* in small intestine	**0.61**	**0.002**
BCRP/ABCG2 and *PXR* in liver	0.45	0.23

r_S: Spearman rank correlation coefficient; significant correlations are highlighted in bold.

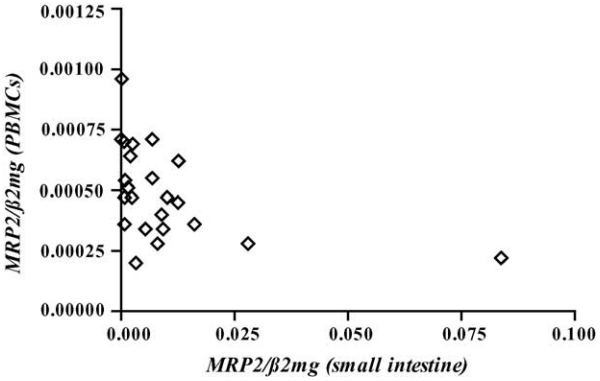

Fig. 2. Significant correlation between *MRP2/ABCC2* expression in PBMCs and small intestine ($n = 22$) expressed as ratio *MRP2/β2mg* and *PXR/β2mg*.

3.3. Effect of MDR1 polymorphisms on MDR1 expression and P-gp function

Genotype distribution in the 44 patients was 32% GG, 45% GT, and 23% TT for the G2677T polymorphism and 23% CC, 45% CT, and 14% TT for the C3435T polymorphism. For none of the tested tissues, a significant association between the G2677T or the C3435T polymorphism in the *MDR1/ABCB1* gene and the mRNA expression was present. Neither was there an influence of the polymorphisms on P-gp function in PBMCs.

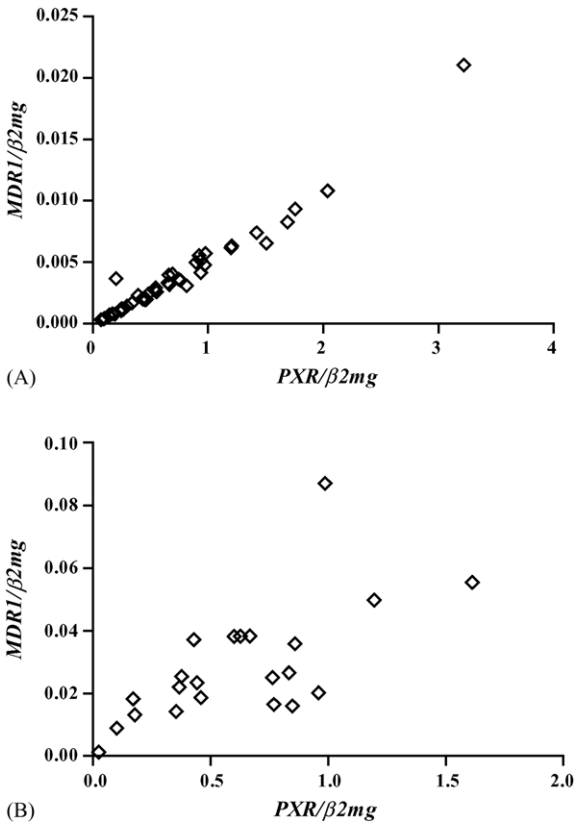

Fig. 3. Significant correlation between *PXR* and *MDR1/ABCB1* mRNA in PBMCs (A; $n = 44$) and small intestine (B; $n = 22$) expressed as ratio *MDR1/β2mg* and *PXR/β2mg*.

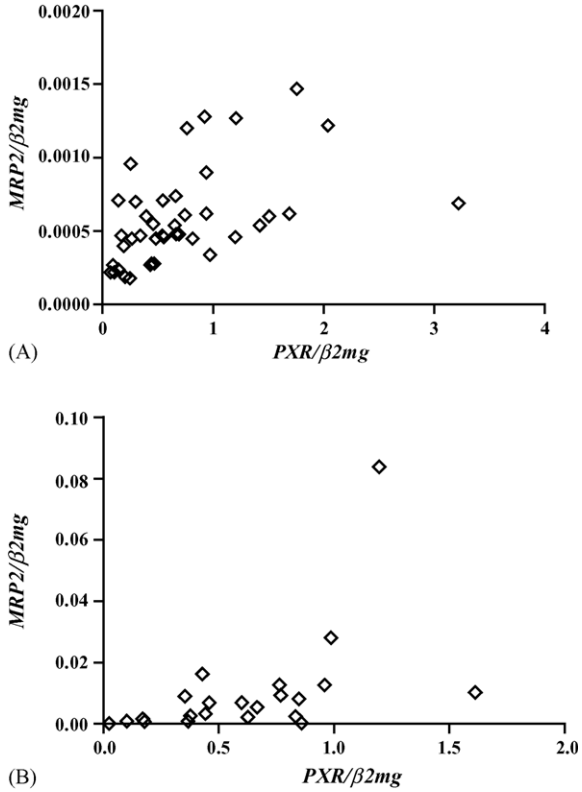

Fig. 4. Significant correlation between *PXR* and *MRP2/ABCC2* mRNA in PBMCs (A; $n = 44$) and small intestine (B; $n = 22$) expressed as ratio *MRP2/β2mg* and *PXR/β2mg*.

3.4. Correlation between mRNA expression of PXR and ABC-transporters

Analysis of PXR expression revealed several significant correlations between the amount of PXR and ABC-transporter mRNA. Figs. 3–5 show the significant relationships between *MDR1/ABCB1*, *MRP2/ABCC2*, and *BCRP/ABCG2* and *PXR* expression in PBMCs (Figs. 3A, 4A, and 5A) and small intestine (Figs. 3B, 4B, and 5B). In contrast, *MRP1/ABCC1* mRNA was only in PBMCs correlated with *PXR* (Fig. 6). In liver samples, PXR expression was not correlated with any of the transporters.

3.5. Correlation between mRNA expression of MDR1/ABCB1 and P-gp function

The function in the total fraction of all lymphocytes and the subpopulation of $CD8^+$ was significantly correlated (Fig. 7) demonstrating that both populations are suitable for investigation of P-gp activity. In contrast to the mRNA expression of P-gp, its function in PBMCs only varied by factor 6 and mRNA expression in PBMCs was not correlated with P-gp function in lymphocytes (Fig. 8).

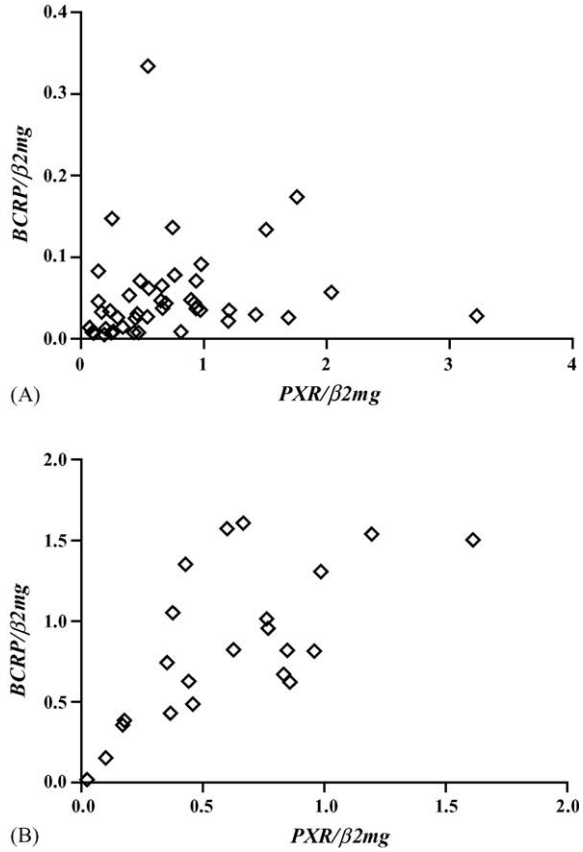

Fig. 5. Significant correlation between *PXR* and *BCRP/ABCG2* mRNA in PBMCs (A; $n = 44$) and small intestine (B; $n = 22$) expressed as ratio *BCRP/β2mg* and *PXR/β2mg*.

4. Discussion

The ABC-transporters *MDR1/ABCB1*, *MRP1/ABCC1*, *MRP2/ABCC2*, and *BCRP/ABCG2* may substantially impact the pharmacokinetic properties of many drugs and endogenous substrates, may confer the development of drug resistance, and may be a critical site of drug–drug interactions. Indeed, their activity is highly variable and changes in the in vivo activity of ABC-transporters have not altered drug elimination alone but also substantially modified drug distribution [17]. Hence, optimum doses of ABC-transporter substrates may vary greatly between patients and dose adjustment may be required even within a patient to maintain effectiveness and avoid toxicity. Prediction of a patient's susceptibility to xenobiotics and individualization of drug therapy would become possible, if a simple test were available for an easy and rapid screening of transporter expression and/or activity. In this regard, PBMCs fulfill two important criteria: they are easily obtainable and they express these four transporters. Hence, for drugs that act in lymphocytes (e.g. antiretroviral drugs), quantification of transporter expression in PBMCs may provide supportive information for drug dosing as has been shown for P-gp and MRP1 [12,18]. However, for other, more distant sites of drug action, PBMCs are only suitable as surrogate markers if ABC-transporter expression closely reflects the expression in the tissues with relevance to drug dosing.

Our objective was therefore to establish robust methods to quantify ABC-transporter expression in PMBCs and to assess its relationship to that in gut and liver, two organs

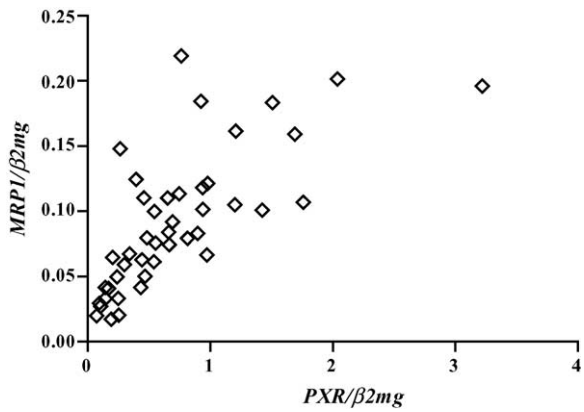

Fig. 6. Significant correlation between *PXR* and *MRP1/ABCC1* mRNA in PBMCs ($n = 44$) expressed as ratio *MRP1/β2mg* and *PXR/β2mg*.

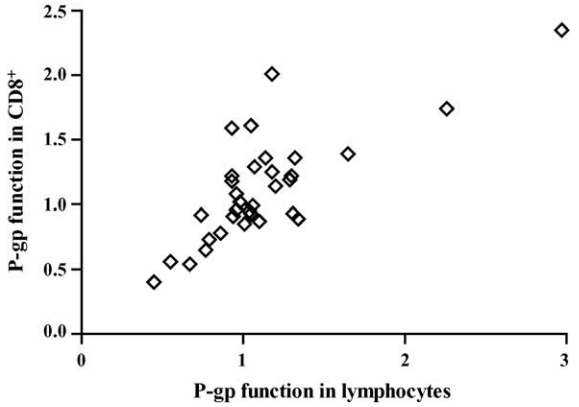

Fig. 7. P-gp function in lymphocytes and $CD8^+$ cells measured by rhodamine123 efflux (flow cytometry) calculated as ratio activity of the sample/activity of the control ($n = 34$; $r_S = 0.64$; $p < 0.0001$).

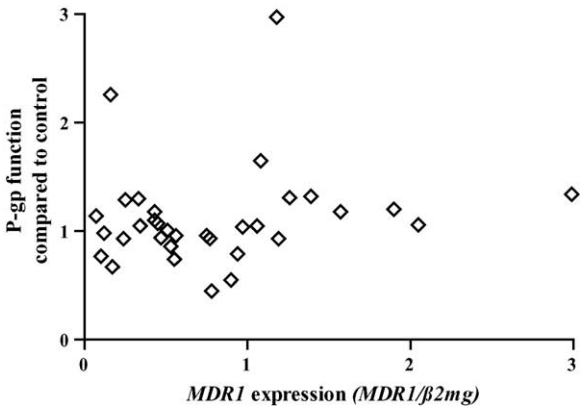

Fig. 8. Lack of relationship between mRNA expression of *MDR1/ABCB1* in PBMCs and P-gp function in lymphocytes measured by flow cytometry ($n = 34$; $r_S = 0.23$; $p = 0.20$).

being crucial for drug absorption and excretion. For quantification of mRNA expression of the four ABC-transporters and of PXR, we have established reliable and reproducible real-time RT-PCR methods. For exact quantification, it is crucial to use an appropriate reference gene, which is expressed constantly and independently of the experimental conditions. Recently, for many ''conventional'' reference genes, the suitability has been challenged, because their transcription is significantly regulated and their expression thus not independent of the experimental setting. This particularly applies for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β-actin* [19–22] which are the most often used housekeeping genes. Using a reference gene whose transcription is not constant will produce incorrect or ambiguous results. We therefore selected *β2mg*, which is stably expressed under various conditions [22,23] and has already been successfully used for quantification of *MDR1/ABCB1* mRNA [24,25].

Our data prove the absence of a correlation between the expression of four major ABC-transporters in PBMCs and in the intestine or liver except for *MRP2/ABCC2*, whose mRNA expression in PBMCs was correlated with that in the small intestine (Table 4 and Fig. 2). However, this correlation is presumably of little relevance, because the slope of the regression line is very flat and variability is large suggesting that this correlation will only explain a small part of *MRP2/ABCC2* expression in small intestine. Altogether, the data obtained demonstrate that expression in PBMCs is not a useful surrogate marker for the expression of *MDR1/ABCB1*, *MRP1/ABCC1*, *BCRP/ABCG2*, and most likely neither for *MRP2/ABCC2*.

In contrast to the three other ABC-transporters, P-gp has been investigated earlier and indeed our results related to P-gp are in accordance with several studies, which demonstrated a discordance between P-gp expression and/or activity in the intestine and PBMCs [25–27]. Also, when the relationship between intestinal and hepatic expression of P-gp was studied, no correlation was found [28]. Hence, current evidence suggests that the expression of ABC-transporters in different tissues is not co-ordinately regulated or that direct influences of xenobiotics and food constituents, to which every tissue is differently exposed, provoke differences in ABC-transporter expression making it impossible to use one tissue as a surrogate for another.

However, while of no value as a surrogate marker for two key organs in the absorption and elimination of xenobiotics, the transporter status in PBMCs remains important for drugs like antiretroviral or chemotherapeutic agents, whose primary site of therapeutic action is the lymphocyte and which are known substrates of P-gp, MRP1, MRP2, and/or BCRP. Hence, in human immunodeficiency virus (HIV) or lymphoma therapy, quantitative information on the expression of these transporters might open new strategies to adapt dosage or switch to a compound, which is not substrate of transporters with high expression levels in a given patient. For P-gp and MRP1, it has already been demonstrated that expression influences intracellular accumulation of HIV protease inhibitors and thus possibly therapeutic effectiveness [12,18].

We also addressed the regulation of the four ABC-transporters by analyzing the relationship between *PXR* expression and mRNA amount of the ABC-transporters. Interestingly, for all transporters (except *MRP1/ABCC1* in the intestine), we found a positive correlation with PXR expression in PBMCs and intestine. These findings confirm recent results on P-gp expression in PBMCs [29] and extend this correlation to the intestine and *MRP1/ABCC1*, *MRP2/ABCC2*, and *BCRP/ABCG2*.

In contrast, in hepatic tissue, no relation with PXR expression was found confirming the data on P-gp obtained in a larger cohort of 94 liver samples [30] and extending knowledge to 3 further transport systems with moderate to high hepatic expression. In our study, we were unable to dissect the underlying reasons for this discrepancy to PBMCs and intestine.

While data on the involvement of PXR in the induction process of *MRP1/ABCC1* are controversial [7,8], it is well known that PXR induces *MDR1/ABCB1* and *MRP2/ABCC2* by binding to responsive elements in the gene when activated by PXR ligands like rifampin [5,6,31]. Our data suggest that PXR in PBMCs and in the intestine may not only be involved in the induction of ABC-transporters by exogenous ligands, but also in xenobiotic-independent regulation of basal expression as Wolbold et al. have hypothesized for the expression of other PXR-inducible proteins like cytochrome P450 3A4 (CYP3A4) [30]. While we cannot exclude that these correlations are caused by a co-ordinated regulation of the expression of ABC-transporters and PXR by other factors, these data may also suggest that inter-individual differences in ABC-transporter expression are influenced by the individual abundance of PXR.

Thus far, no information on the possible influence of PXR on the transcriptional regulation of *BCRP/ABCG2* has been published. The correlation found in this study suggests that PXR also participates in the regulation of mRNA transcription of this transporter. Induction and/or luciferase reporter assays with PXR ligands may help to elucidate the role of PXR in the regulation of the expression of *MRP1/ABCC1* and *BCRP/ABCG2* and to assess the causality of the observed relationship.

The influence of the G2677T and the C3435T polymorphism on P-gp expression and function is still controversial. Our data support the hypothesis that these two polymorphisms do not influence mRNA expression and function of P-gp [24,25], because no association between the polymorphisms and P-gp expression or function was present. Also, no relationship was found between *MDR1/ABCB1* mRNA expression and P-gp function in PBMCs. The reason for this discrepancy is unknown, but it confirms

earlier results demonstrating significant dissociation between P-gp expression level and activity [32–34]. Future studies will have to evaluate whether P-gp activity or expression correlates with intracellular drug disposition and which parameter will more closely predict drug concentrations at the site of action and ultimately effectiveness of a drug. For measuring P-gp activity, the whole fraction of lymphocytes as well as the subpopulation of $CD8^+$ cells appears suitable, because the function in both fractions was closely correlated confirming previously obtained results [13].

In conclusion, our data demonstrate that quantification of ABC-transporter mRNA in PBMCs is not a suitable surrogate for expression in small intestine and liver. Similarly, P-gp function in lymphocytes does not reflect the P-gp status in intestine or liver precluding the possibility to monitor hepatic or intestinal P-gp activity using blood samples as an easily obtainable source. However, the large variability of P-gp function in PBMCs suggests that P-gp substrates will access this sanctuary site only to a highly variable extent. Functional testing may therefore be a promising way to optimize therapy with drugs primarily acting in lymphocytes. Similarly, the even larger variability of BCRP expression in PBMCs and the high expression level of this transporter and of MRP1 in this compartment may make a functional evaluation even more important for substrates of BCRP and MRP1.

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References

[1] Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev 2003;55:3–29.

[2] Hoffmeyer S, Burk O, von Richter O, Arnold HP, Brockmoller J, Johne A, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci USA 2000;97:3473–8.

[3] Cummins CL, Wu CY, Benet LZ. Sex-related differences in the clearance of cytochrome P450 3A4 substrates may be caused by P-glycoprotein. Clin Pharmacol Ther 2002;72:474–89.

[4] Troost J, Albermann N, Haefeli WE, Weiss J. Cholesterol modulates P-glycoprotein activity in human peripheral blood mononuclear cells. Biochem Biophys Res Commun 2004;316:705–11.

[5] Synold TW, Dussault I, Forman BM. The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. Nat Med 2001;7:584–90.

[6] Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, et al. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J Biol Chem 2002; 277:2908–15.

[7] Kauffmann HM, Pfannschmidt S, Zoller H, Benz A, Vorderstemann B, Webster JI, et al. Influence of redox-active compounds and PXR-activators on human MRP1 and MRP2 gene expression. Toxicology 2002;171:137–46.

[8] Magnarin M, Morelli M, Rosati A, Bartoli F, Candussio L, Giraldi T, et al. Induction of proteins involved in multidrug resistance (P-glycoprotein, MRP1, MRP2 LRP) and of CYP 3A4 by rifampicin in LLC-PK1 cells. Eur J Pharmacol 2004;483:19–28.

[9] Chaudhary PM, Mechetner EB, Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. Blood 1992;80:2735–9.

[10] Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood 2002;99:507–12.

[11] Steinbach D, Sell W, Voigt A, Hermann J, Zintl F, Sauerbrey A. BCRP gene expression is associated with a poor response to remission induction in therapy in childhood acute myeloid leukemia. Leukemia 2002;16:1443–7.

[12] Meaden ER, Hoggard PG, Newton P, Tjia JF, Aldam D, Cornforth D, et al. P-glycoprotein and MRP1 expression and reduced ritonavir and saquinavir accumulation in HIV-infected individuals. J Antimicrob Chemother 2002;50:583–8.

[13] Fröhlich M, Albermann N, Sauer A, Walter-Sack I, Haefeli WE, Weiss J. In vitro and ex vivo evidence for modulation of P-glycoprotein activity by progestins. Biochem Pharmacol 2004;68:2409–16.

[14] Nauck M, Stein U, von Karger S, März W, Wieland H. Rapid detection of the C3435T polymorphism of multidrug resistance gene 1 using fluorogenic hybridization probes. Clin Chem 2000;46:1995–7.

[15] Cascorbi I, Gerloff T, Johne A, Meisel C, Hoffmeyer S, Schwab M, et al. Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in White subjects. Clin Pharmacol Ther 2001;69:169–74.

[16] Chang TK, Bandiera SM, Chen J. Constitutive androstane receptor and pregnane X receptor gene expression in human liver: interindividual variability and correlation with CYP2B6 mRNA levels. Drug Metab Dispos 2003;31:7–10.

[17] Ding R, Tayrouz Y, Riedel K-D, Burhenne J, Weiss J, Mikus G, et al. Substantial pharmacokinetic interaction between digoxin and ritonavir in healthy volunteers. Clin Pharmacol Ther 2004;76:73–84.

[18] Jones K, Bray PG, Khoo SH, Davey RA, Meaden ER, Ward SA, et al. P-glycoprotein and transporter MRP1 reduce HIV protease inhibitor uptake in CD4 cells: potential for accelerated viral drug resistance? AIDS 2001;27:1353–8.

[19] Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 2004;313:856–62.

[20] Hamalainen HK, Tubman JC, Vikman S, Kyrola T, Ylikoski E, Warrington JA, et al. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. Anal Biochem 2001;299:63–70.

[21] Zhong H, Simons JW. Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 1999; 259:523–6.

[22] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 2000;46:69–81.
[23] Lupberger J, Kreuzer KA, Baskaynak G, Peters UR, le Coutre P, Schmidt CA. Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and their comparison as control transcripts for RT-PCR. Mol Cell Probes 2002;16:25–30.
[24] Oselin K, Nowakowksi-Gashaw I, Mrozikiewicz PM, Wolbergs D, Pähkla R, Roots I. Quantitative determination of MDR1 mRNA expression in peripheral blood lymphocytes: a possible role of genetic polymorphisms in the MDR1 gene. Eur J Clin Invest 2003;33: 261–7.
[25] Oselin K, Gerloff T, Mrozikiewicz PM, Pahkla R, Roots I. MDR1 polymorphisms G2677T in exon 21 and C3435T in exon 26 fail to affect rhodamine123 efflux in peripheral blood lymphocytes. Fundam Clin Pharmacol 2003;17:463–9.
[26] Ashgar A, Gorski JC, Haehner-Daniels B, Hall SD. Induction of multidrug resistance-1 and cytochrome P450 mRNAs in human mononuclear cells by rifampin. Drug Metab Dispos 2002;30:20–6.
[27] Becquemont L, Camus M, Eschwege V, Barbu V, Rey E, Funck-Brentano C, et al. Lymphocyte P-glycoprotein expression and activity before and after rifampicin in man. Fundam Clin Pharmacol 2000; 14:519–25.
[28] von Richter O, Burk O, Fromm MF, Thon KP, Eichelbaum M, Kivistö KT. Cytochrome P450 3A4 and P-glycoprotein expression in human small intestinal enterocytes and hepatocytes: a comparative analysis in paired tissue specimens. Clin Pharmacol Ther 2004; 75:172–83.
[29] Owen A, Chandler B, Back DJ, Khoo SH. Expression of pregnane-X-receptor transcript in peripheral blood mononuclear cells and correlation with MDR1 mRNA. Antivir Ther 2004;9:819–21.
[30] Wolbold R, Klein K, Burk O, Nussler AK, Neuhaus P, Eichelbaum M, et al. Sex is a major determinant of CYP3A4 expression in human liver. Hepatology 2003;38:978–88.
[31] Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J Biol Chem 2001;276:14581–7.
[32] Meaden ER, Hoggard PG, Khoo SH, Back DJ. Determination of P-gp and MRP1 expression and function in peripheral blood mononuclear cells in vivo. J Immunol Methods 2002;262:159–65.
[33] De Moerloose B, Dhooge C, Laureys G, Benoit Y, Demarche M, Devalck C, et al. Discrepant flow cytometric expression and function of P-glycoprotein in neuroblastic tumors. Cytometry 1999;37:125–32.
[34] De Moerloose B, Dhooge C, Philippe J. Discordance of P-glycoprotein expression and function in acute leukemia. Adv Exp Med Biol 1999; 457:107–18.
[35] Gutmann H, Fricker G, Török M, Michael S, Beglinger C, Drewe J. Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake. Pharm Res 1999;16:402–7.
[36] Pascolo L, Fernetti C, Pirulli D, Crovella S, Amoroso A, Tiribielli C. Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells. Biochem Biophys Res Commun 2003;28:259–65.
[37] Sauerbrey A, Sell W, Steinbach D, Voigt A, Zintl F. Expression of the BCRP gene (ABCG2/MXR/ABCP) in childhood acute lymphoblastic leukaemia. Br J Haematol 2002;118:147–50.