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II Gerloff T, Schaefer M, Johne A, **Oselin K**, Meisel C, Cascorbi I, Roots I. MDR1 genotype do not discriminate between absorptive pharmacokinetic parameters of a single oral dose of 1 mg digoxin in healthy white males. British Journal of Clinical Pharmacology 2002;54:610–616.

III **Oselin K**, Nowakowski-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. European Journal of Clinical Investigation 2003;33:261–267.

IV **Oselin K**, Mrozikiewicz PM, Pähkla R, Roots I. Quantitative determination of the human MRP1 and MRP2 mRNA expression in FACS-sorted peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells. European Journal of Haematology 2003;71:119–123.

V **Oselin K**, Mrozikiewicz PM, Gaikovitch E, Pähkla R, Roots I. Frequency of *MRP1* genetic polymorphisms and their functional significance in Caucasians: detection of a novel mutation G816A in the human *MRP1* gene. European Journal of Clinical Pharmacology 2003;59:347–350.

ABBREVIATIONS

ABC	ATP-binding cassette
APC	allophycocyanin
AUC	area under the plasma concentration-time curve
bp	base pairs
BSA	bovine serum albumin
CD	cluster of differentiation
$CD4^+$	T helper cells
$CD8^+$	T cytotoxic cells
$CD19^+$	B lymphocytes
$CD56^+$	natural killer cells
95% CI	95% confidence interval
C_{max}	maximum plasma drug concentration
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
LC Red640	LightCycler Red-640-N-hydroxy-succinimide ester
LC Red705	LightCycler Red-705-Phosphoramidite
MDR	multidrug resistance
MDR1	multidrug resistance gene 1
$MgCl_2$	magnesium chloride
M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger ribonucleic acid
MRP1 and 2	multridrug resistance-associated protein 1 and 2
OR	odds ratio
P	probability
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP-Cy5.5	peridinin chlorophyll protein cyanine 5 conjugate
Pgp	P-glycoprotein
Rh123	rhodamine 123
RFLP	restriction fragment length polymorphism
RT-PCR	reverse transcription — polymerase chain reaction
SD	standard deviation
SNP	single nucleotide polymorphism
t_{max}	time of occurrence of maximum plasma drug concentration

INTRODUCTION

The transport of various molecules across lipid membranes is an essential function of cells in all living organisms and a large number of specific proteins have evolved to carry out this function. The largest transporter family is the superfamily of ATP-binding cassette (ABC) transporters (Dean *et al.* 2001). These proteins bind ATP and use the phosphate bond energy to drive the transport.

ABC transporters comprise of membrane proteins that extrude a wide variety of endogenous and exogenous compounds across the plasma membrane as well as intracellular membranes of the endoplasmic reticulum, peroxisome, and mitochondria. ABC genes typically encode 4 domains that include 2 ATP-binding domains and 2 domains with multiple transmembrane segments. The ATP-binding domains of the ABC genes contain motifs of characteristic conserved residues: Walker A and Walker B motif, found in all ATP-binding proteins; and the C motif, specific to ABC transporters that distinguishes them from other ATP-binding proteins (Dean *et al.* 2001). The superfamily of ABC transporters is divided into seven subfamilies based on organization of domains and amino acid homology. In humans, 49 ABC transporters have been identified (http://www.humanabc.org).

ABC transporters are expressed throughout many tissues of the body and they are essential in many processes in cells. Mutations in these genes cause or contribute to several human genetic disorders including cystic fibrosis and retinal degeneration; cholesterol, bile salts, and bilirubin transport defects; insulin secretion and anaemia. Transporters of the subfamilies B (ABCB/MDR/TAP) and C (ABCC/MRP/CFTR), namely P-glycoprotein (ABCB1), MRP1 (ABCC1), and MRP2 (ABCC2), appear to be most relevant for the absorption, distribution, metabolism, and excretion of drugs in humans. The present study deals with these ABC transporters.

REVIEW OF THE LITERATURE

1. Contribution of P-glycoprotein (Pgp) to physiology and drug disposition

1.1. Physiological role of Pgp

Pgp (ABCB1/MDR1) was the first human ABC transporter identified and cloned through its ability to confer a multidrug resistance (MDR) phenotype in cancer cells (Juliano and Ling 1976). The phenomenon of MDR was characterized by a transport of various anticancer agents out of the cells and decreased intracellular access to these agents resulted in a resistance to chemotherapeutics. It is now known that Pgp expression is not limited to cancer cells, but it is ubiquitously expressed throughout the body (Thiebaut *et al.* 1987; Cordon-Cardo *et al.* 1989), where it extrudes a wide variety of structurally and chemically unrelated compounds.

High level of Pgp has been found in the apical membrane of small and large intestinal epithelium (Thiebaut *et al.* 1987), which suggests Pgp function to prevent uptake of substrates across the gastrointestinal tract. Pgp is very abundant in the bile canalicular membrane of hepatocytes and on the brush border of renal proximal tubules (Thiebaut *et al.* 1987). The function of Pgp in these locations is extrusion of substrates from the hepatocytes into the bile, and in kidney from blood into the urine. This data strongly suggests Pgp role in absorption and excretion mechanisms.

In several tissues, Pgp functions as a barrier protecting cells from potential cytotoxic effects of xenobiotics. Pgp has been localized on the luminal surface of capillary endothelial cells in the brain and testis where it is considered to be a part of the blood-brain and blood-testis barrier (Cordon-Cardo *et al.* 1989). When substrates enter the endothelial cells from the blood, Pgp pumps these compounds back into the blood, reducing their distribution into the brain and into the testis. In *mdr1a* knockout mice, the mouse has two members of Pgp encoded by *mdr1a* and *mdr1b* which are expressed in tissue specific manner, the penetration of substrate agents from the blood into the brain tissue has been found to be dramatically increased up to 100 fold (Lin *et al.* 2003).

Pgp is functional in the placental syncytiotrophoblasts, facing the maternal blood compartment (Cordon-Cardo *et al.* 1990; MacFarland *et al.* 1994). The function of Pgp here appears to be analogous to that in the blood-brain barrier, protecting fetus from toxic xenobiotics and drugs, present in the maternal circulation, by active back-transport. As found by Lankas *et al.* (1998), *mdr1a* knockout mice, lacking placental Pgp, have an increased fetal susceptibility to avermectin induced teratogenicity.

Human adrenal gland is rich in Pgp, where it is expressed on the surface of cells in both the cortex and medulla (Thiebaut *et al.* 1987). It has been supposed

that Pgp might be involved in secretion of steroids, or in protection of the steroid secreting cells from the toxic effects of high steroid concentrations. *In vitro* studies have revealed that cortisol and aldosterone were transported by epithelial monolayers expressing human Pgp (Ueda *et al.* 1992).

Within haematopoietic cells, Pgp has been found to be expressed and functionally active in all subsets of peripheral blood cells, except in granulocytes (Drach *et al.* 1992). The expression and function of Pgp appeared to be the highest in $CD56^+$ natural killer and $CD8^+$ T cells. In several studies, Pgp pharmacological inhibitors and anti-Pgp monoclonal antibodies inhibited natural killer and T cell mediated cytotoxicity (Gupta *et al.* 1992; Klimecki *et al.* 1995). In human T lymphocytes, Pgp has been reported to participate in the transport of cytokines, including IL-2, IL-4 and IFN-γ (Drach *et al.* 1996; Raghu *et al.* 1996). These findings indicate that Pgp may play a role in specific immunological functions.

Preferential expression of Pgp in the apical membrane of epithelial cells suggests that the main function of Pgp is to protect cells from potential cytotoxic effects of xenobiotics. However, the physiologic function of Pgp is still not fully understood. Rodents have two *mdr1* genes, termed *mdr1a* and *mdr1b*. Loss of either or both genes has no effect on viability, fertility, or life span of mice (Schinkel *et al.* 1997), indicating that Pgp has no essential physiologic function.

1.2. Pgp role in drug disposition

The anatomical localization of Pgp, consistent with its function as an efflux pump, suggests that Pgp may play an important role in the absorption, distribution, metabolism, and excretion of drugs in humans. First, it was evidenced that Pgp conferred resistance to anticancer agents. It is now known that Pgp substrates comprise a number of drugs applied for a wide range of therapeutic applications (Table 1). Pgp transports compounds with diverse chemical structure, but not all members of a therapeutic class interact in the same manner with Pgp.

Evidence of the involvement of Pgp in drug absorption and secretion has been demonstrated *in vitro* in cell lines, in which Pgp is highly expressed. The human colon carcinoma Caco-2 cells are commonly used for studying intestinal drug transport. Pgp expression in the apical membrane of Caco-2 cells leads to a considerably higher transport of Pgp substrates in the basal-to-apical direction (corresponding to drug secretion into the gut lumen) in comparison with the apical-to-basal direction. For instance, the basal-to-apical transport of vinblastine and docetaxel in these cells was more than 10 fold greater than the apical-to-basal transport (Hunter *et al.* 1993; Wils *et al.* 1994). In the presence of Pgp inhibitors, the apical-to-basal transport of vinblastine and docetaxel was significantly enhanced. Wetterich *et al.* (1996) found that the transport rates of talinolol across Caco-2 monolayers were significantly higher in the secretory direction as

compared to the absorptive direction. In the presence of Pgp inhibitor, verapamil, the secretory pathway was inhibited, leading to very similar transport rates of the compound in both directions, presumably mainly by a passive diffusion mechanism. Similar results have been obtained using the kidney cell lines. Basal-to-apical transport of digoxin and vinblastine across human *MDR1* gene transfected porcine and canine kidney epithelial cell monolayers, used as a model for renal secretion, was much greater than the apical-to-basal transport, and Pgp inhibitors cyclosporine A, verapamil, and quinidine inhibited the basal-to-apical transport (Tanigawara *et al.* 1992; Okamura *et al.* 1993; Horio *et al.* 1989).

Table 1. Some substrates, inhibitors, and inducers of human Pgp.

Substrates	Inhibitors	Inducers
Anticancer agents	cyclosporine A	dexamethasone
actinomycin D	ketoconazole	rifampin
daunorubicin	quinidine	St. John's wort
doxorubicin	ritonavir	
etoposide	valspodar (PSC833)	
paclitaxel	verapamil	
vinblastine		
vincristine		
Immunosupressants		
cyclosporine A		
tacrolimus		
Steroids		
dexamethasone		
corticosterone		
cortisol		
Cardiac drugs		
digitoxin		
digoxin		
diltiazem		
quinidine		
verapamil		
talinolol		
H_1 antihistamines		
fexofenadine		
HIV protease inhibitors		
amprenavir		
indinavir		
nelfinavir		
ritonavir		
saquinavir		
Fluorescent dye		
rhodamine 123 (Rh123)		

Modified from Schwab *et al.* 2003; Fromm 2002; Kim 2002; Schinkel and Jonker 2003.

The development of transgenic *mdr1* knockout mice has proven to be a useful tool for studying the role of Pgp in drug disposition. In *mdr1a*(-/-) mice, the brain concentration of HIV protease inhibitors indinavir, saquinavir, and nelfinavir has been found to be higher compared with *mdr1a*(+/+) mice by 7 to 36 fold (Kim *et al.* 1998). After oral administration of indinavir, saquinavir, and nelfinavir plasma concentration was 2 to 5 fold higher in pgp knockout mice in comparison with the control animals. Markedly higher digoxin and cyclosporine A brain levels after intravenous administration have been observed in *mdr1a*(-/-) than in *mdr1a*(+/+) mice (Schinkel *et al.* 1995). *In vitro* studies with human *MDR1* transfected cells demonstrated that the tested HIV protease inhibitors, digoxin, and cyclosporine A were also good substrates for human Pgp. Consistent with Pgp expression in hepatocytes and in renal tubular cells, Pgp contributes to the excretion of unchanged drugs into the bile and urine. The biliary clearance of digoxin and doxorubicin was significantly greater in *mdr1a*(+/+) than in *mdr1a*(-/-) mice (Schinkel *et al.* 1997; van Asperen *et al.* 2000). The renal secretion of digoxin was markedly decreased in *mdr1a/1b*(-/-) mice in comparison with *mdr1a/1b*(+/+) mice (Tsuruoka *et al.* 2001).

In humans, a good correlation between the intestinal Pgp expression and the pharmacokinetics of Pgp substrates tacrolimus, cyclosporine A, and talinolol has been observed (Masuda *et al.* 2000; Lown *et al.* 1997; Westphal *et al.* 2000). These data also indicate that interindividual variability in the expression of intestinal Pgp contributes to the interindividual variation in drug disposition.

It was recognized that certain compounds inhibited the transport of Pgp substrates (Tsuruo *et al.* 1981). These agents, so-called Pgp inhibitors or MDR reversal agents, were initially focused on reversing MDR in chemotherapy resistant cancer cells. Later insights indicated that the concomitant use of Pgp inhibitors and Pgp substrates might result in drug-drug interactions. In clinical practice, drug-drug interactions of digoxin with other cardiac drugs, such as verapamil and quinidine, have been well documented (Gordon and Goldenberg 1986; Mordel *et al.* 1993; Verschraagen *et al.* 1999). In healthy volunteers, a daily dose of 240 mg verapamil caused a significant increase in digoxin steady state and peak plasma concentrations due to Pgp inhibition and increased absorption from intestine (Rodin *et al.* 1988). Coadministration of a single oral dose of Pgp inhibitor valspodar with steady state digoxin yielded an average 76% increase in digoxin area under the plasma concentration-time curve (AUC) and a 62% decrease in digoxin renal clearance (Kovarik *et al.* 1999). After a 5 day coadministration period, digoxin AUC increased by an average 211% and apparent total body clearance was decreased by 67% compared with steady state administration of digoxin alone. The inhibition of Pgp in tumors with MDR phenotype has been used to improve cytotoxic drug delivery to cancer cells. A significant increase in systemic exposure of paclitaxel, daunorubicin, doxorubicin, etoposide, and mitoxantrone in cancer patients has been observed when used in combination with Pgp inhibitors valspodar, GF120918, or cyclosporine A (Fracasso *et al.* 2000; Tidefelt *et al.* 2000; Minami *et al.* 2001 Lacayo *et al.*

2002; Malingre *et al.* 2001). However, the increase in AUC of anticancer drug led also to a substantial increase in haematological and central nervous system toxicity.

Experimental evidence obtained from human colon carcinoma cell lines indicated that Pgp expression was upregulated by rifampin (Schuetz *et al.* 1996). Greiner *et al.* (1999) detected increased Pgp levels in enterocytes from healthy volunteers during rifampin administration (600 mg/day for 10 days). Concomitantly, the oral bioavailability of a single dose of 1 mg digoxin decreased 30%. During rifampin treatment (600 mg/day for 9 days), the AUC of intravenous and oral talinolol was decreased 21% and 35%, respectively, and total clearance of talinolol was increased by 30% after intravenous administration of the drug (Westphal *et al.* 2000). A significant increase in the expression of duodenal Pgp and MDR1 mRNA expression indicated that the observed talinolol-rifampin interaction could partially be explained by a decrease in drug absorption. Increased Pgp expression after administration of St. John's wort has been evidenced in healthy volunteers (Hennessy *et al.* 2002). Coadministration of St. John's wort (900 mg/day for 11 days) resulted in a 1.6 fold and 1.9 fold increase of the oral clearances of cyclosporine A and fexofenadine, respectively (Dresser *et al.* 2003).

Most drug-drug interactions have been attributed to inhibition or induction of drug metabolising enzymes. It is now established that modification of Pgp function by concomitantly administered drugs is another important mechanism of drug-drug interactions (Table 1). The substrate binding sites and ATP-binding domains of Pgp interact cooperatively as a single functional unit. Inhibition of Pgp by inhibitors could result from either the competition in drug binding or by interrupting ATP hydrolysis, while the induction of Pgp occurs through increased protein synthesis and expression. Experimental data suggest that the induction of Pgp is likely to be regulated by pregnane X receptor activation (Geick *et al.* 2001).

1.3. Biochemical aspects of Pgp

The 1280 amino acids of Pgp are organized in two homologous halves of 610 amino acids that are joined by a linker region of about 60 amino acids (Fig. 1) (Ambudkar *et al.* 1999). Each half contains six transmembrane domains and an ATP-binding domain. Deletion from the central core of the linker region resulted in a protein that was expressed at the cell surface in levels similar to the wild type protein, but both ATP hydrolysis and drug transport activities of the mutant were completely diminished (Hrycyna *et al.* 1998). These data suggest that the two halves of Pgp interact cooperatively. Data from site-directed mutagenesis suggest that the major substrate binding domains include residues from transmembrane domains 6 and 12 (Hrycyna 2001). It is also clear that the

interaction between the ATP-binding sites and the substrate binding domains is essential to the transport.

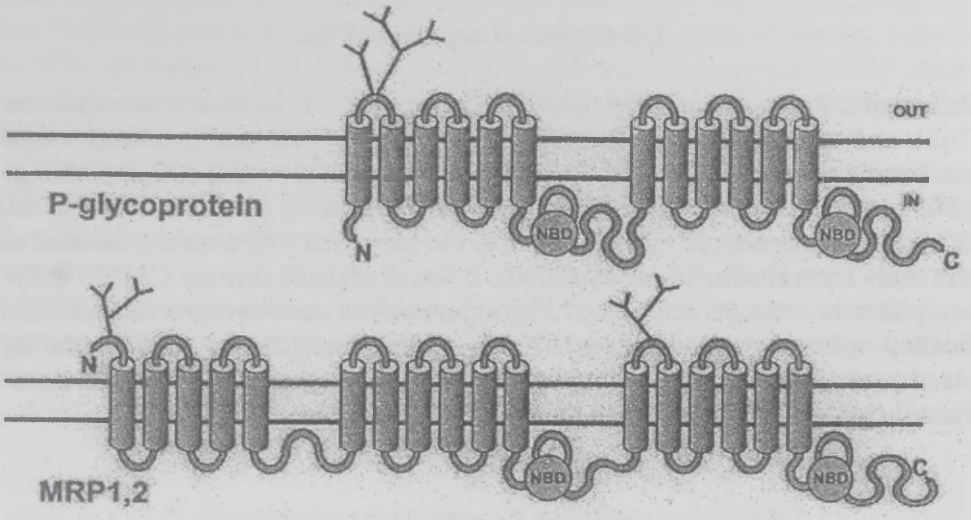


Figure 1. Predicted secondary structure of Pgp, MRP1, and MRP2. Branches indicate putative N-glycosylation sites; N and C denote N-terminal and C-terminal ends of the protein, respectively; IN and OUT indicate cytoplasmic and extracellular side of the membrane; NBD — nucleotide binding domains. Adapted from Schinkel and Jonker 2003.

The majority of experimental data support the fact that Pgp functions as a primary pump wherein the energy from the ATP hydrolysis is used for the transport of substrate, which is not linked to co- or countertransport of anions or cations. In the absence of any known substrate, Pgp exhibits a basal ATPase activity, which is unusual for an active transporter. It has been suggested that this Pgp basal activity reflects the presence of an unknown endogenous substrate. Recently, Garrigues *et al.* (2002) showed that the basal ATPase activity is tightly dependent on the presence of cholesterol in the membrane. Various compounds, known to be transported by Pgp, stimulate Pgp ATPase activity.

Full-length Pgp contains N-linked glycosylation sites (Ambudkar *et al.* 1999). All three sites are located within the first extracellular loop at amino acid residues 91, 94, and 99 (Fig. 1). The phosphorylation sites of Pgp locate within the linker region. Both, the glycosylation and phosphorylation of Pgp, are posttranslational modifications. Studies with the mutant, glycosylation deficient Pgp, demonstrated that N-linked glycosylation was not required for the function of Pgp, although it appeared to affect protein level at the cell surface (Schinkel

et al. 1993; Gribar *et al.* 2000). Similarly, Germann *et al.* (1996) showed that the phosphorylation of Pgp was not essential for its drug efflux activity.

1.4. Genetical aspects of Pgp

In humans, Pgp is encoded by the *MDR1* gene, which is located in chromosome 7q21 and consists of 28 exons (Chen *et al.* 1990). Recently, several single nucleotide polymorphisms (SNPs) in the *MDR1* gene were identified (Table 2) (Mickley *et al.* 1998; Hoffmeyer *et al.* 2000; Kim *et al.* 2001; Saito *et al.* 2002a). The phenotypic consequences of the identified SNPs were evaluated in the study from Hoffmeyer *et al.* (2000). It was evidenced that the C3435T polymorphism in exon 26 determined Pgp expression in duodenal enterocytes from healthy volunteers. Comparison of C_{max} values for 0.25 mg digoxin during steady state conditions revealed that subjects homozygous for TT had significantly higher C_{max} values than those with CC genotype.

Table 2. SNPs identified in the coding region of the human *MDR1*.

Position	SNP*	NCBI SNP rs	Effect	Location	Mutant allele frequency in Caucasians (%)	Reference
exon 2	A61G		Asn21Asp	N-terminus	11.2	Cascorbi *et al.* 2001
exon 7	A548G		Asn183Ser	1 intracellular loop	1.4	Kim *et al.* 2001
exon 11	G1199A	2229109	Ser400Asn	linker region	5.5	Cascorbi *et al.* 2001
exon 12	C1236T	1128503	Gly412Gly	linker region	41.0	Cascorbi *et al.* 2001
exon 13	C1474T		Arg492Cys	linker region	1.4	Kim *et al.* 2001
exon 21	C2650T		Leu884Leu	4 intracellular loop	2.7	Kim *et al.* 2001
	G2677T G2677A	2032582	Ala893Ser Ala893Thr	4 intracellular loop	41.6 1.9	Cascorbi *et al.* 2001
exon 26	C3435T	1045642	Ile1145Ile	C-terminus	53.9	Cascorbi *et al.* 2001

* The positions of the SNPs correspond to GenBank accession number M14758 with the first base of the ATG start codon set to 1.

Subsequently, several studies were performed to determine the allele and genotype distribution of the C3435T in different ethnic groups (Schwab *et al.* 2003). The lowest frequency for the T3435 allele has been observed in Africans, ranging from 10% (Ghanaian) to 27% (Sudanese). Higher frequency for the T3435 allele was found in Caucasians, ranging from 46% (Northern Italian) to 57% (Portuguese). In Southwest Asians, the frequency of the T3435 allele was 66%. Also a marked variation in the allele frequency for other SNPs in the *MDR1* has been found between different ethnic groups.

The present study aimed to investigate the functional significance of the C3435T SNP in the *MDR1* on Pgp expression and activity, based on the data from Hoffmeyer *et al.* (2000). A second SNP, G2677T, was included in the study, since it was found to be in a strong linkage disequlibrium with the C3435T SNP (Tanabe *et al.* 2001) and unlike the C3435T results in amino acid change. Also, both of these SNPs occur with the highest frequency in Caucasians (Cascorbi *et al.* 2001).

2. General properties of MRP1 and MRP2

The human multidrug resistance-associated protein 1 (MRP1/ABCC1) and 2 (MRP2/ABCC2) were first identified in 1992 by Cole *et al.* in doxorubicin selected human small cell lung cancer cells, and in 1996 by Taniguchi *et al.* in cisplatin resistant human head and neck cancer cell line, respectively. Subsequent analysis showed that these transporters conferred MDR against a wide range of anticancer drugs. The resistance profiles of MRP1, MRP2, and Pgp transfected cells are similar, and typically include crossresistance to the anthracyclines, vinca alkaloids, taxanes, and epipodophyllotoxins.

2.1. Tissue distribution of the MRP1 and MRP2

Unlike Pgp, MRP1 is localized to the basolateral membrane of epithelial cells and therefore pumps its substrates into the interstitial space rather than into bile, urine, or gut (Leslie *et al.* 2001). Studies on *mrp1*(-/-) knockout mice have revealed that MRP1 may have an important protective role in some tissues.

In normal liver, mrp1 expression in the basolateral membrane of hepatocytes is very low, but it is induced during liver regeneration, endotoxemia, and obstructive cholestasis (Roelofsen *et al.* 1997; Vos *et al.* 1998; Pei *et al.* 2002). In the small intestine, the highest levels of mrp1 were found in the Paneth cells, located in the crypts (Peng *et al.* 1999). Mrp1 expression in villi enterocytes was not detectable. In colon, all of the cells lining the crypt-villous axis of the colon wall contained mrp1. In *mrp1*(-/-) mice, an increased accumulation of

etoposide in colon and concomitantly an increased crypt degeneration and frequency of colitis has been observed during etoposide treatment (Wijnholds *et al.* 2000). No differences in AUC of etoposide after intravenous administration were observed in knockout and control mice, indicating that mrp1 does not contribute to renal, liver, or intestinal clearance, but protects the proliferative cell compartment in the crypt. In mice, mrp1 is present in the basal epithelial layer of the tongue and mucosal layer of the cheek (Wijnholds *et al.* 1998). The lack of mrp1 protein in knockout mice resulted in increased oropharyngeal toxicity during etoposide administration (Wijnholds *et al.* 1998). These cells have the basolateral membrane facing the blood circulation and mrp1 protects the basal epithelium in the oropharyngeal cavity of wild type mice.

In human brain, MRP1 expression in capillary endothelial cells has not been detected (Seetharaman *et al.* 1998), indicating that MRP1 probably does not form blood-brain barrier. In rats and mice, mrp1 has been found in epithelium of choroid plexus (Wijnholds *et al.* 2000; Rao *et al.* 1999). The latter is responsible for the production of cerebrospinal fluid. In rats, after intracerebroventricular administration, estradiol 17-β-D-glucuronide, a substrate for mrp1, was eliminated rapidly from the cerebrospinal fluid to blood (Nishino *et al.* 1999). Simultaneous administration of probenecid, an inhibitor for mrp1, reduced the elimination of estradiol 17-β-D-glucuronide. The *mrp1*(-/-) mice accumulated 10 fold more etoposide in the cerebrospinal fluid than did control animals whereas levels in brain and plasma concentrations did not differ. These findings indicate that mrp1 is a part of the blood-cerebrospinal fluid barrier.

In kidney, MRP1 was expressed in the basolateral membrane of the epithelial cells of the loop of Henle and the urinary collecting ducts in the marrow and cortex, as well as in glomeruli and proximal tubules (Flens *et al.* 1996; Peng *et al.* 1999; Schaub *et al.* 1999). *Mrp1*(-/-) knockout mice acquired hypotonic polyuria when treated with etoposide (Wijnholds *et al.* 1998). It was supposed that etoposide treatment caused damage in the basolateral membrane of the collecting ducts, leading to reduced tubular responsiveness to vasopressin.

Relatively high levels of MRP1 have been found in the sex hormone producing Leydig cells in the interstitial tissue and in the basal membrane of Sertoli cells in the seminiferous tubules of the testis (Flens *et al.* 1996; Wijnholds *et al.* 1998). No MRP1 expression in the endothelial layer of the blood vessels in the testis, a location for Pgp, was observed. Estrogen is synthesized in the testis and is required for normal testicular function. Recently, using MRP1 expressing cell lines, Qian *et al.* (2001) showed that MRP1 transports estrogen conjugates. MRP1 expression in Leydig cells may serve to protect the testis from the potential feminizing effects of endogenously produced estrogen conjugates. Treatment with etoposide resulted in increased impairment of spermatogenesis in *mrp*1 knockout mice (Wijnholds *et al.* 1998), indicating that mrp1 plays an important role in the protection of the Sertoli cells.

Regardless of the increased sensitivity toward vincristine and etoposide, in terms of lethality (Wijnholds *et al.* 1998; Johnson *et al.* 2001) and increased bone marrow toxicity (Lorico *et al.* 1997; Johnson *et al.* 2001) in *mrp1*(-/-) mice, there is no direct evidence that MRP1 contributes to the systemic drug absorption or excretion, consistent with its low expression in gut and liver. However, the basolateral location of MRP1 seems to protect basal cell layers from destruction. At the cellular level, MRP1 contributes to the resistance of cells to a range of anticancer drugs, as demonstrated *in vitro* in knockout and transfected cell lines (Allen *et al.* 2000; Lorico *et al.* 1996).

Unlike MRP1, but similar to Pgp, MRP2 localizes to the apical membrane of epithelial cells and is predominantly expressed in the liver and intestine (Schinkel and Jonker 2003). *In vivo* MRP2 has been found in the bile canalicular membrane of hepatocytes, in the luminal membrane of the small intestinal epithelium, in the luminal membrane of the proximal tubules of the kidney, and in the syncytiotrophoblasts of term placenta. In human jejunum, MRP2 mRNA levels, as measured by RT-PCR, were amongst the highest of all tested ABC transporters (Taipalensuu *et al.* 2001). In brain, mrp2 is expressed in capillary endothelial cells (Miller *et al.* 2000), possibly forming a functional component of the blood-brain barrier. Similar location with Pgp, in a range of tissues important for the pharmacokinetics of drugs, indicates that MRP2 may contribute to the systemic drug absorption and disposition. As shown by Dietrich *et al.* (2001a,b), the mrp2 deficiency in rats increased bioavailability and impaired intestinal and hepatobiliary excretion of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

Within haematopoetic cells, Pgp has been found to be expressed in all subsets of peripheral blood cells, except in granulocytes (Drach *et al.* 1992), and Pgp contribution to immunological functions has been supposed. Limited number of studies with variable results has investigated MRP1 and MRP2 in human blood cells. Therefore, the present study aimed to assess the expression of MRP1 and MRP2 in different subsets of peripheral blood mononuclear cells (PBMC).

2.2. Substrates, inhibitors, and inducers of MRP1 and MRP2

There are many similarities between the spectrum of substrates transported by MRP1 and MRP2, with both extruding various exogenous and endogenous compounds conjugated with sulfate, glucuronide, or glutathione (Table 3). Many good substrates of these transporters are highly charged or hydrophilic conjugates which enter intact cells very poorly. Therefore, for the characterization of substrate specificity, vesicular uptake in inside-out vesicles derived from MRP1 or MRP2 transfected cells is determined. *In vivo* studies, the toxicological relevance of MRP1 and MRP2 has been investigated using *mrp1* (-/-) knockout mice and mrp2 deficient rats.

Table 3. Some substrates, inhibitors, and inducers of MRP1 and MRP2.

Substrates	MRP1	MRP2
Anticancer agents		
daunorubicin	+	+
doxorubicin	+	+
etoposide	+	+
vinblastine	+	+
vincristine	+	+
methotrexate	+	+
cisplatin		+
Miscellaneous		
leukotriene C_4	+	+
glutathione	+	+
glutathione disulfide	+	+
estradiol-17β-D-glucuronide	+	+
estrone 3-sulfate	+	
monoglucuronosyl bilirubin	+	+
bisglucuronosyl bilirubin	+	+
saquinavir	+	+

Inhibitors	MRP1	MRP2
probenecid	+	+
sulfinpyrazone	+	+
MK571	+	+
indomethacin	+	
buthionine sulfoximine	+	+
valspodar	+	+
cyclosporine A	+	+
Inducers		
cisplatin		+
vinblastine	+	+
dexamethasone		+
phenobarbital		+
rifampicin		+
sulindac	+	

Modified from Faber *et al.* 2003; Sun *et al.* 2003; Schinkel and Jonker 2003; Gerk and Vore 2002; Leslie *et al.* 2001; Borst *et al.* 1999.

The first substrate, shown to be actively transported by MRP1 using inside-out vesicles, was the glutathione conjugated leukotriene C_4 (Leier *et al.* 1994). *Mrp1*(-/-) knockout mice are viable, fertile, and have no physical or histological abnormalities. However, *mrp1*(-/-) mice have an impaired response to inflammatory stimulus and decreased secretion of leukotriene C_4 from leukotriene synthesizing cells (Wijnholds *et al.* 1997; Verbon *et al.* 2002; Schultz *et al.* 2001). There is no *mrp2* knockout mouse, but there are natural mutant GY/TR$^-$ and EHBR rats, lacking mrp2. Studies of mrp2 deficient rats indicated that this protein was required for the excretion of a wide range of conjugated organic anions, including bilirubin glucuronides into the bile (Borst *et al.* 1999; Leslie *et al.* 2001).

The involvement of glutathione is an important feature of MRP-mediated drug resistance. Transport of vincristine and daunorubicin in membrane vesicles from MRP1 expressing cell lines has been found to be dependent on the concentration of ATP and glutathione (Renes *et al.* 1999). Zaman *et al.* (1995)

demonstrated that the depletion of intracellular glutathione resulted in a complete reversal of resistance to vinca alkaloids and anthracyclines in lung carcinoma cells transfected with MRP1. They also showed that the cells transfected with MRP1 secreted more glutathione into the medium than parental cells. Since stable glutathione conjugates of vincristine and daunorubicin could not be detected in media, it was supposed that MRP1 mediates co-transport of glutathione and hydrophobic drugs. Also in MRP2 transfected cells, increased apical glutathione secretion has been demonstrated (Paulusma *et al.* 1999), vinblastine transport occurred stoichiometrically with glutathione transport (Evers *et al.* 2000), and depletion of cellular glutathione resulted in decreased drug resistance (Cui *et al.* 1999).

In primary cultures of rat hepatocytes, an induction of mrp2 mRNA and protein expression after treatment with cisplatin and vinblastine has been found (Schrenk *et al.* 2001), with possible impact on the acquisition of MDR during chemotherapy of tumors. In rats, *mrp2* was also inducible by dexamethasone and phenobarbital. MRP2 has been implicated in drug and estrogen induced cholestasis. Pregnancy and treatment of rats with ethinylestradiol markedly decreased mrp2 protein expression (Gerk and Vore 2002). In clinical studies, expression of MRP2 mRNA and protein was decreased in patients with obstructive cholestasis (Shoda *et al.* 2001). In another clinical study, MRP2 mRNA and protein expression in the apical membrane of duodenal enterocytes in healthy volunteers was significantly induced by rifampin treatment (600mg/day 9 days) (Fromm *et al.* 2000). No increase in MRP1 expression was observed in the same study. Upregulation of MRP1 by vinblastine, but not by cisplatin, doxorubicin, phenobarbital, rifampicin, and tamoxifen, has been found in mammary carcinoma cells (Schrenk *et al.* 2001).

To reverse MRP-mediated resistance, to improve the efficacy of anticancer therapy, the number of small molecular inhibitors that can be used in intact cells is quite limited. Several non-steroidal anti-inflammatory drugs, as indomethacin and mefenamic acid, have been demonstrated to significantly enhance the cytotoxicity of anticancer drugs *in vitro* when coadministered in MRP1 overexpressing cell lines (Duffy *et al.* 1998).

2.3. Biochemical and genetical aspects of MRP1 and MRP2

MRP1 and MRP2 have the same basic structure as Pgp, but in addition they have an N-terminal extension consisting of 5 putative transmembrane segments (Fig. 1) (Borst *et al.* 1999). The N-terminus of MRP1 and MRP2 is located extracellularly. These proteins are N-glycosylated at the N-terminus and at the sixth extracellular loop, and share 49% amino acid identity.

The MRP1 protein comprises 1531 amino acids and is encoded by the *MRP1* gene, which is located in chromosome 16p13.1 (Grant *et al.* 1997). Recently,

identification of several SNPs in the human *MRP1* has been reported (Perdu *et al.* 2001; Ito *et al.* 2001; Conrad *et al.* 2001; Saito *et al.* 2002b).

The MRP2 protein comprises 1545 amino acids and *MRP2* gene is located to chromosome 10q24 (Toh *et al.* 1999). Mutations in the *MRP2* gene were reported to cause Dubin-Johnson syndrome (Kartenbeck *et al.* 1996; Paulusma *et al.* 1997; Toh *et al.* 1999), an autosomal recessive disease characterized by conjugated hyperbilirubinemia. As shown in several studies, hepatobiliary excretion of conjugated bilirubin is mediated by MRP2 (Gerk and Vore 2002).

The present study aimed to determine the frequencies of genetic polymorphisms in the human *MRP1* in healthy Caucasians. Three of the SNPs T825C, T1684C, and G4002A, located in exons 8, 13, and 28, respectively, were selected for the study. Other SNPs identified previously in exonic regions of *MRP1* occurred with a low frequency, and were observed by a single investigator, but not confirmed by the others. A novel silent mutation G816A in exon 8 was identified in this study and the frequency of the G816A in Caucasians was also determined.

AIMS OF THE STUDY

The specific aims of this study were:

1. to assess the impact of the genetic polymorphisms G2677T and C3435T in the *MDR1* gene, we determined
1.1. Rh123 efflux in peripheral blood $CD56^+$ and $CD4^+$ cells from healthy individuals with different *MDR1* genotypes;
1.2. the absorption of a single oral dose of digoxin in healthy subjects with different *MDR1* genotypes;
1.3. MDR1 mRNA expression in peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells from healthy individuals with different *MDR1* genotypes;
2. to determine the MRP1 and MRP2 mRNA expression in human peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells;
3. to investigate the prevalence of the genetic polymorphisms G816A, T825C, T1684C, and G4002A in the *MRP1* gene in healthy Caucasians, and to assess the functional importance of these polymorphisms by determining MRP1 mRNA levels in peripheral blood $CD4^+$ cells from healthy individuals with different *MRP1* genotypes.

METHODS AND MATERIALS

1. Study subjects

The study protocols were approved by the Ethics Committee of the Humboldt University. All subjects gave their written informed consent before entry into the study. The subjects took no medication and were ascertained to be healthy by a clinical examination and routine laboratory tests.

46 male healthy volunteers (age range 19 to 45 years, median 25 years) were recruited for the Rh123 efflux study.

A total of 50 healthy male Caucasians (nonsmokers, age range 19 to 45 years, median 26 years) were enrolled in the digoxin study. Their weight ranged from 45.1 kg to 74.6 kg. Electrocardiogram, urine analysis, and tests for hepatitis B and C, and HIV were performed before the study. All volunteers refrained from alcohol, coffee, tea, and cola beverages consumption.

45 healthy Caucasians (39 males and 6 females, age range 21 to 33 years, median 25 years) were enrolled in the MDR1 mRNA expression study.

20 healthy volunteers (17 males and 3 females, age range 21 to 33 years, median 25 years) were enrolled in the MRP1/MRP2 mRNA expression study.

The prevalence of the *MRP1* genetic polymorphisms was studied in DNA samples from 230 healthy unrelated Caucasians of German origin (194 males and 36 females, age range 20 to 59 years, median 28 years).

To assess the functional importance of the *MRP1* polymorphisms, MRP1 mRNA levels in $CD4^+$ cells were determined in 61 healthy volunteers (47 males and 14 females, age range 21 to 32 years, median 26 years).

2. Materials

Biocoll lymphocyte separating solution (density 1.077 g/ml) was from Biochrom KG (Berlin, Germany). FITC- and PE-conjugated antibodies were obtained from Immunotech Beckman Coulter (Munich, Germany); APC- and PerCP-Cy5.5-conjugated antibodies were from Becton Dickinson (San Jose, CA, USA). Rh123, verapamil hydrochloride, BSA fraction V powder, and DMSO were purchased from Sigma Chemicals (Munich, Germany). DMEM, FBS, and PBS were obtained from Gibco-BRL (Karlsruhe, Germany). All oligonucleotide primers and hybridisation probes for PCR and RT-PCR were from Tib Molbiol (Berlin, Germany). 10 × PCR buffer, $MgCl_2$, and dNTPs were obtained from Rapidozym (Berlin, Germany). Recombinant RNasin® ribonuclease inhibitor, T7 RNA polymerase and RQ1-DNase were purchased from Promega (Madison, WI, USA); 5 × First Strand Buffer, 10 mM DTT, M-MLV reverse transcriptase and *Taq* DNA Polymerase from Invitrogen (Karlsruhe, Germany). BioTherm™ DNA polymerase was from GeneCraft (Munster, Germany).

3. Rh123 efflux study

PBMC were isolated from 20 ml whole blood by gradient centrifugation over Biocoll lymphocyte separating solution. To allow Rh123 accumulation, cell suspension ($1x10^6$ cells/ml in DMEM/10%FBS) was incubated for 30 min at 37°C in 5% CO_2 with Rh123 in a final concentration of 150 ng/ml (0.39 μM). After accumulation, the control sample was put on ice for a measurement of the baseline dye uptake. The efflux samples were performed for 30 min and 90 min efflux at 37°C in 5% CO_2 in a Rh123 free medium for the measurement of Rh123 efflux in $CD56^+$ and $CD4^+$ cells, respectively. In parallel, one sample was incubated with verapamil hydrochloride in a concentration of 1 μg/ml (2 μM). After efflux, samples were washed with cold PBS and stained with PE-anti-CD56 or PE-anti-CD4 antibody. PE-mouse IgG_1 antibody was used as an isotype control.

After staining, cells were analysed using a FACScan™ flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm argon laser. The viability of isolated cells was determined using a trypan-blue exclusion and was always greater than 97%. The samples were gated on forward scatter *versus* side scatter to include lymphocytes. A second gate was set around the PE-$CD56^+$ or PE-$CD4^+$ cells. A minimum of 2000 of $CD56^+$ and $CD4^+$ events were collected per sample. Data was expressed as median Rh123 fluorescence in $CD56^+$ and $CD4^+$ cells. Median Rh123 fluorescence in control sample, after baseline dye uptake, was set as 100% (Fig. 2). Rh123 fluorescence in efflux sample was used to calculate % of Rh123 retained in the cells in comparison to control. Final data was presented as the mean±SD of Rh123 efflux for each genotype group. The coefficient of variation for interassay variability of the Rh123 efflux was less than 2%.

4. Digoxin study

4.1. Study protocol

After an overnight fast, each subject received a single oral dose of 1 mg digoxin (Dilanacin™, Arzneimittelwerk Dresden GmbH, Radebeul, Germany) along with 200 ml water. Venous blood samples (5 ml) for drug analysis were collected before and 10, 20, 30, 35, 40, 45, 50, 60, 75, 90, 120, 180, and 240 min after digoxin intake. The plasma was separated by centrifugation and stored at –22°C until analysis.

4.2. Digoxin concentration measurements and pharmacokinetic analysis

Digoxin plasma concentrations were determined by an immunoassay (IMx® Digoxin Assay, Abbott Laboratories, USA). The lower limit of quantification was 0.3 μg/l. The plasma samples of each volunteer were analysed in duplicate together with calibration and quality control samples. Further calculations were done with the respective mean values. The interassay coefficient of variation at plasma concentrations of 0.9 μg/l, 1.9 μg/l, and 3.2 μg/l were 8.37%, 5.75%, and 4.68%, respectively.

C_{max} and t_{max} of digoxin were derived directly from the measured values. The t_{max} for digoxin ranged from 0.5 to 3 h in our samples. Thus, an AUC from 0 to 4 h (AUC_{0-4h}) was considered a suitable measure of digoxin absorption. AUC_{0-4h} was calculated by use of the trapezoidal rule, using WinNonlin™ (version 1.5, Pharsight Corporation, Mountain View, CA, USA).

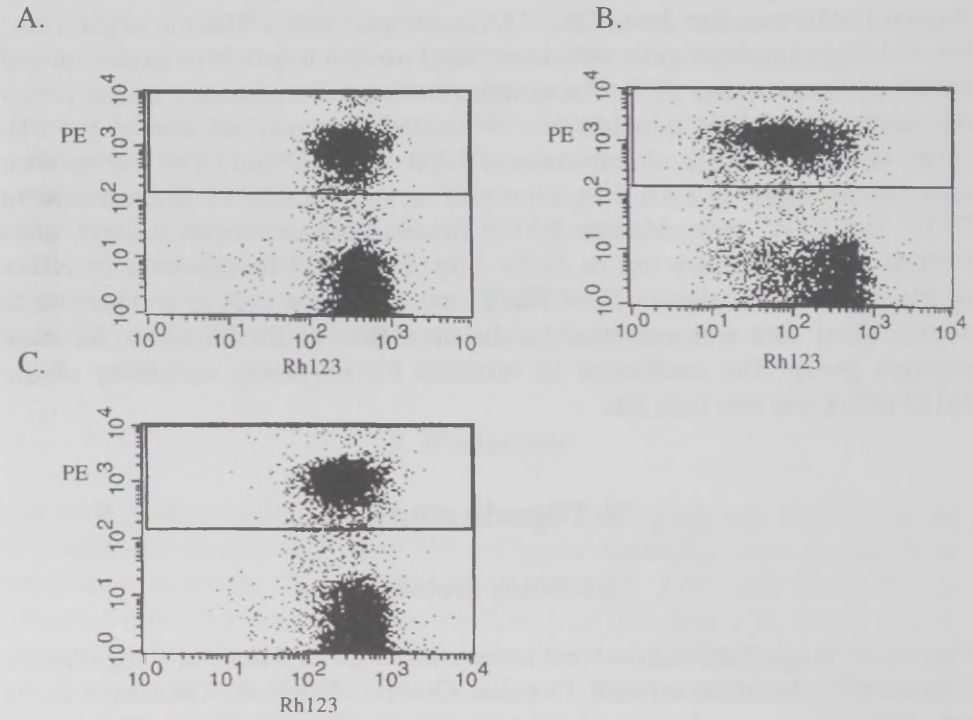


Figure 2. Two-dimensional flow cytometric dot plots of a two-colour flow cytometric assay of $CD56^+$ natural killer cells. PE-CD56 *versus* Rh123 fluorescence is shown. Rh123 fluorescence was determined in cells gated for lymphocytes and PE-CD56 profile. Median Rh123 fluorescence in control sample (A) was set as 100%. Rh123 fluorescence in efflux samples incubated for 30 min in a Rh123 free medium without verapamil (B) and with 2 μM verapamil (C) was used to calculate % of Rh123 retained in the cells in comparison to control.

5. Principles of the LightCycler™ technology

The LightCycler technology comprises fluorescence based method for the detection of PCR amplification in real-time (Meuer *et al.* 2001). PCR reaction mixture contains, in addition to the reaction components used for conventional PCR, two sequence-specific oligonucleotides (hybridisation probes) labelled with fluorescent dyes. The hybridisation probes hybridize during the annealing step of each PCR cycle adjacently to target DNA within a primer set (Fig. 3). One of the probes (donor) has a fluorescein label at its 3´end and another probe (acceptor) has LC Red640 or LC Red705 label at its 5´end. The donor's fluorescein dye is excited by the LightCycler Light Emitting Diode and thereupon emits fluorescent light at 530 nm. The emitted energy excites the acceptor LC Red640 or LC Red705 dye that subsequently emits fluorescent light at 640 nm or 705 nm, respectively. This energy transfer, referred to as fluorescence resonance energy transfer, occurs only when both of the hybridisation probes are bound to their target and locate in close proximity (1-5 nucleotides apart). The intensity of the light emitted by the LC Red640 or LC Red705 is measured by the LightCycler optics.

LightCycler technology allows cDNA quantification and detection of genetic polymorphisms.

During quantification, the fluorescence is monitored at each cycle and the second derivative maximum method automatically calculates the cycle numbers, referred to as crossing point, where the second derivative is at its maximum. A calibration curve is generated by plotting the crossing point *versus* the logarithm of copies in respective standard sample (Fig. 4). The calibration curve, in turn, is used to estimate the number of copies in unknown samples.

For the detection of genetic polymorphisms, melting curve analysis of the PCR products is performed subsequently to an amplification run. The cycle program for melting curve analysis comprises a slow heating (temperature elevation of 0.1–0.2°C/sec) to monitor the melting of the hybridisation probes from double stranded DNA. When hybridisation probes melt, the fluorescence decreases. Melting temperature has been defined as a temperature at which 50% of the probe has separated from the template. One of the hybridisation probes, referred to as sensor, covers the predicted site of the mutation and is complementary to the wild type allele (or to the mutated sequence). If the amplified gene carries a point mutation within this specific region, a single mismatch significantly reduces the melting temperature of the sensor. Therefore, the wild type sample (if sensor is complementary to the wild type sequence) has a higher melting temperature than the mutant sample. For visualisation of melting temperatures, melting peaks are displayed by plotting the negative derivative of fluorescence *versus* temperature (Fig. 5).

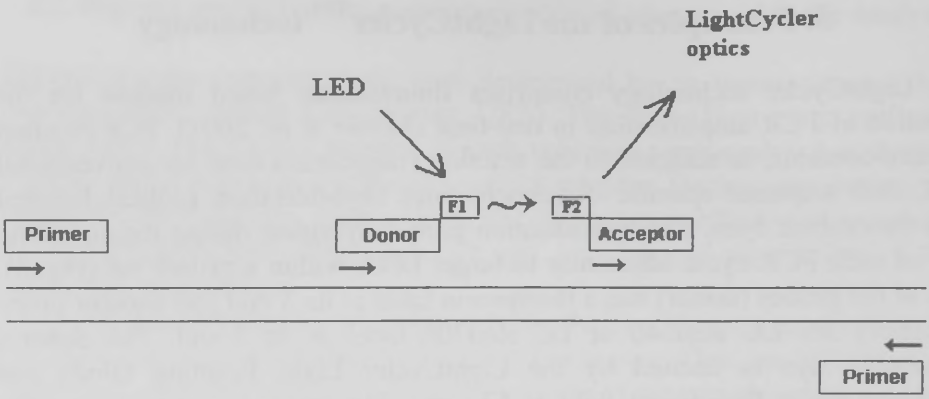


Figure 3. Schematic illustration of the principles of the LightCycler technology. Donor excitation by a Light Emitting Diode (LED) leads to the fluorescence resonance energy transfer and excitation of acceptor dye. The light emitted by the acceptor dye is measured by the LightCycler optics. F1 – fluorescein label, F2 – LC Red640 or LC Red705 label.

6. Quantitative determination of the MDR1, MRP1, and MRP2 mRNA expression in a LightCycler

6.1. Cell sorting and isolation of total RNA

PBMC were isolated from 20 ml whole blood by gradient centrifugation over Biocoll lymphocyte separating solution. Cells were stained with FITC-anti-CD4, PE-anti-CD56, APC-anti-CD8, and PerCP-Cy5.5-anti-CD19 antibody, and sorted using FACSDiva™ Turbosorter (Becton Dickinson). Purity of sorted cells was analysed with FACSCalibur™ and was ≥ 98% (n=10). Total RNA from FACS-sorted cells was isolated with the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA concentration was measured spectrophotometrically and samples were maintained at –80°C until analysis.

Table 4. Oligonucleotide primers and hybridisation probes used for real-time quantitative RT-PCR.

Gene	Sequence*	Position (bp)
MDR1		
Forward primer	5'-CCATCATTGCAATAGCAGG-3'	(3018–3036)
Reverse primer	5'-GAGCATACATATGTTCAAACTTC-3'	(3163–3185)
LightCycler probes	5'-TGGGAAGATCGCTACTGAAGCAAT-3'-fluorescein	(3100–3123)
	5'-LC Red640-AACTTCCGAACCGTTGTTTCTTTGA-3'-phosphate	(3128–3152)
MRP1		
Forward primer	5'-TTCCGGAACTACTGCCTGCGCTA-3'	(4079–4101)
Reverse primer	5'-GGGTCCTGGGGGATGATGGTGA-3'	(4143–4164)
LightCycler probes	5'-CTCGTTGATCCGAAATAAGCCCAGGGTCA-3'-fluorescein	(4231–4203)
	5'-LC Red640-ACGACTTCCCAGCTCCCGTCCGCC-3'-phosphate	(4199–4176)
MRP2		
Forward primer	5'-GGCTGAGATTGGAGAGAAGGGTATA-3'	(2292–2314)
Reverse primer	5'-ACTGCAGACAGGGGGTCAT-3'	(2409–2391)
LightCycler probes	5'-TTTGGTAGGTAGCTCTGGCCAGGCT-3'-fluorescein	(2369–2345)
	5'-LC Red640-ATCCGCTGCTTCTGACCCCCAC-3'-phosphate	(2343–2322)
T7 primer**	5'-AGAGCGTAATACGACTCACTATAGGGTATCTGCAGA-	
RT primer***	5'-TTTTTTTTTTTTTTT-	

* Area amplified refers to sequences with accession numbers as follows: NM_000927 (MDR1), NM_004996 (MRP1), NM_000392 (MRP2).

** T7 promoter sequence was incorporated at the 5'-end of the forward primers to obtain templates for *in vitro* external standard RNA synthesis.

*** dT(15) sequence was incorporated at the 5'end of the reverse primers to obtain templates for *in vitro* external standard RNA synthesis.

6.2. Real-time quantitative RT-PCR assays

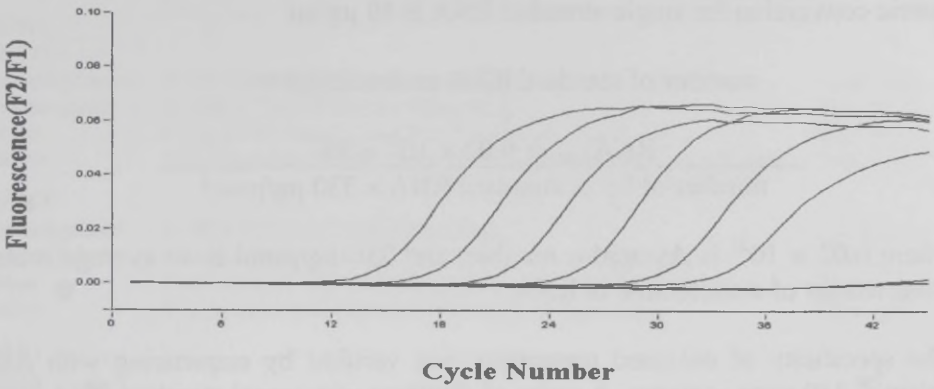
cDNA was synthesized from total RNA in a 25µl final reaction volume containing 50 ng of sample RNA or dilutions of standard RNA (run in the same plate but separate tubes), 1 × First Strand Buffer, 10 mM DTT, 0.4 mM of each dNTP, 8 U ribonuclease inhibitor, 120 U M-MLV reverse transcriptase and 0.8 µM oligo dT(15) primer. The mixture was pre-incubated for 10 min at 26°C, then incubated at 42°C for 60 min followed by heating at 95°C for 2 min, and cooled.

Quantitative PCR was performed with gene-specific primers and hybridisation probes shown in Table 4 in a LightCycler. PCR amplification was carried out in a 16 µl reaction volume containing 5 µl of cDNA (of 25 µl of total cDNA), 1.25 U *Taq* DNA Polymerase, 1.6 µl of 10 × PCR buffer, and $MgCl_2$, dNTPs, reverse and forward primer, hybridisation probes, BSA and DMSO optimised for each reaction. The PCR reactions were initiated with a denaturation at 94°C for 60 sec, followed by the amplification with 45 cycles at 95°C 0 sec, annealing (depending on the primer pair) for 10 sec, and 72°C for 10 sec. Calibration curves were generated from serial dilutions of the external standard cDNAs and used to estimate the number of mRNA copies in samples (Fig. 4). Final data was expressed as the mean±SD of the MDR1 (or MRP1 or MRP2) mRNA copies/ng of total RNA. The coefficient of variation for MDR1, MRP1, and MRP2 mRNA levels were 8.9%, 26.1%, 21.5%, respectively. PCR products migrated as a single fragment with an expected size on an agarose gel. The specificity of PCR products was verified by sequencing (ABI Prism™ 310, Applied Biosystems, Foster City, CA, USA).

6.3. Synthesis of external standards for quantitative real-time RT-PCR

In order to establish the calibration standard curve, external standard RNA was prepared. RT-PCR was carried out in a two-step reaction for each gene as described above, using primers listed in Table 4. The subsequent PCR with T7 primer (T7 promoter sequence was incorporated at the 5'-end of the forward primer) and RT primer (dT(15) sequence was incorporated at the 5'-end of the respective reverse primer) was carried out to obtain a template for *in vitro* transcription. After purification with Ultrafree MC 30.000 filters, the template was used for *in vitro* transcription at 37°C for 90 min using the DNA-dependent T7 RNA polymerase. After digestion with RNase-free RQ1-DNase, RNA transcripts were purified using RNeasy Mini Kit and RNA Cleanup Protocol from QIAGEN. All of the RNA standards were quantified spectrophotometrically and maintained at –80°C. The number of standard RNA molecules was determined as follows:

A.



B.

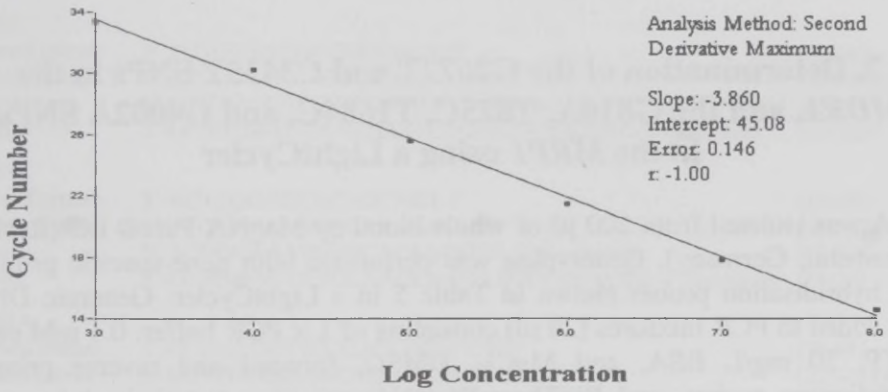


Figure 4. Representative amplification plot for MDR1 mRNA expression. (A) Amplification curves of serial dilutions of standard RNA, ranging from $1x10^8$ to $1x10^3$ starting copies, were displayed by plotting the fluorescence data *versus* cycle number. No-template controls for RT and PCR were also performed. (B) Calibration curve was generated by plotting the crossing point *versus* the logarithm of the number of copies of standard RNA. Crossing point values for unknown samples were interpolated on the calibration curve and used to calculate the number of copies.

$$\text{RNA concentration } (RNA_{conc}) = A_{260} \times 40\ \mu g/ml$$

where A_{260} is an absorbance at 260 nm, and when $A_{260}=1$, then spectrophotometric conversion for single stranded RNA is 40 μg/ml

$$\text{number of standard RNA molecules/}\mu l = \frac{RNA_{conc} \times 6.02 \times 10^{23} \times 10^{-9}}{\text{number of bp of standard RNA} \times 330\ \mu g/\mu mol}$$

where 6.02×10^{23} is Avogadro number, and 330 μg/μmol is an average molecular weight of a nucleotide in RNA.

The specificity of obtained transcripts was verified by sequencing with ABI Prism™ 310 gene analyser. A series of dilutions of external standard RNA were performed.

7. Determination of the G2677T and C3435T SNPs in the *MDR1*, and the G816A, T825C, T1684C, and G4002A SNPs in the *MRP1* using a LightCycler

DNA was isolated from 200 μl of whole blood by MagNA Pure® LC (Roche, Mannheim, Germany). Genotyping was performed with gene-specific primers and hybridisation probes shown in Table 5 in a LightCycler. Genomic DNA was added to PCR mixtures (20 μl) consisting of 1 × PCR buffer, 0.1 mM each dNTP, 30 mg/L BSA, and $MgCl_2$, DMSO, forward and reverse primer, hybridisation probes, and BioTherm™ DNA polymerase optimised for each reaction. After an initial denaturation at 95°C for 30 sec, the amplification was performed by means of 45 cycles of denaturation at 95°C for 0 sec, annealing (depending on the primer pair) for 15 sec, and extension at 72°C for 15 sec. Melting curve acquisition was from 35°C to 95°C at 0.1–0.2°C/sec, optimised for each reaction. Confirmation of the LightCycler analysis was performed by sequencing on an ABI Prism™ 310 automatic sequencer and by PCR-RFLP. Typical results for melting curve analysis, as determined in a LightCycler, are shown in Fig. 5. At position 2677 in the *MDR1* three nucleotide variants (G, T, or A) have been identified. The frequency of the A2677 allele has been observed to be very low in Caucasians (Cascorbi *et al.* 2001). In our study, individuals carrying the A allele (genotype GA with melting temperature 58°C and TA with melting temperature 52/59°C) were excluded from the analysis.

Table 5. Oligonucleotide primers and hybridisation probes used for PCR.

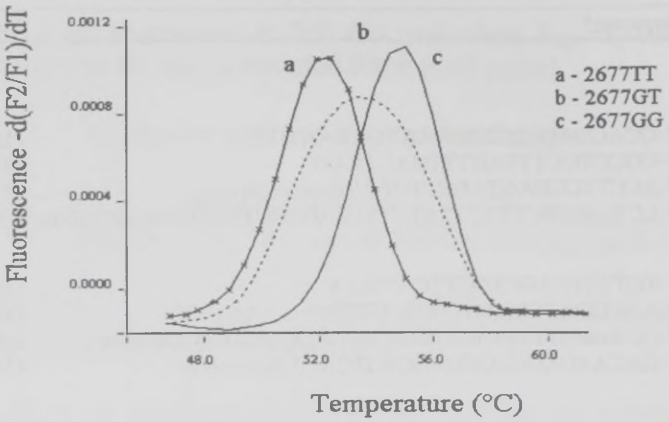
Gene	Sequence*	Position (bp)
MDR1		
G2677T		
Forward primer	5'-GCAGGAGTTGTTGAAATGAAAATG-3'	(156-179)
Reverse primer	5'-CGCCTGCTTTAGTTTGACTCA-3'	(232-252)
Sensor	5'-ACCTTCCCAGTACCTTCT-3'-fluorescein	(215-232)
Anchor	5'-LC Red640-CTTTCTTATCTTTCAGTGCTTGTCC-3'-phosphate	(186-210)
C3435T		
Forward primer	5'-TGTTTTCAGCTGCTTGATGG-3'	(15-34)
Reverse primer	5'-AAGGCATGTATGTTGGCCTC-3'	(192-211)
Sensor	5'-LC Red640-GGAAGAGATCGTGAGGGCAG-3'-phosphate	(167-186)
Anchor	5'-GACAACAGCCGGGTGGTGTCA-3'-fluorescein	(144-164)
MRP1		
G816A		
Forward primer	5'-TGTGGTAGGGGGCTGCA-3'	(13-29)
Reverse primer	5'-TTCGCATCCACCTTGGAACT-3'	(125-144)
Sensor	5'-LC-Red705-GTTCCAGGCAGCCAGTGAA-3'-phosphate	(66-84)
Anchor	5'-CCCACAACGGCTTCACCTCCTT-3-fluorescein	(42-63)
T825C		
Forward primer	5'-TGTGGTAGGGGGCTGCA-3'	(13-29)
Reverse primer	5'-TTCGCATCCACCTTGGAACT-3'	(125-144)
Sensor	5'-GTGAAGGTCGTGTACTCC-3'-fluorescein	(80-97)
Anchor	5'-LC-Red640-CCAAGGATCCTGCCCAGCCGAA-3'-phosphate	(99-120)
T1684C		
Forward primer	5'-ACTCGGGGCACAGCAGT-3'	(33-49)
Reverse primer	5'-TTGAACAAGGCCAAAGACA-3'	(149-167)
Sensor	5'-ACGGCAAATGTGCACAAGGCC-3'-fluorescein	(78-98)
Anchor	5'-LC-Red640-CCTGCAAGCAAGAACGCCCAGTG-3'-phosphate	(54-76)
G4002A		
Forward primer	5'-GCCAGCATTCCCACCACACCT-3'	(28-48)
Reverse primer	5'-GCTTGCCAGCTCTGGCTCACC-3'**	(251-271)
Sensor	5'-GGTCAGGGACGACTTCCC-3'-fluorescein	(95-112)
Anchor	5'-LC-Red640-CTCCCGTCCGCCCCACG-3'-phosphate	(76-92)

* Area amplified refers to sequences with accession numbers as follows: M29440 (G2677T), M29445 (C3435T), AF022830 (G816A, T825C), AF022835 (T1684C), AF022850 (G4002A).

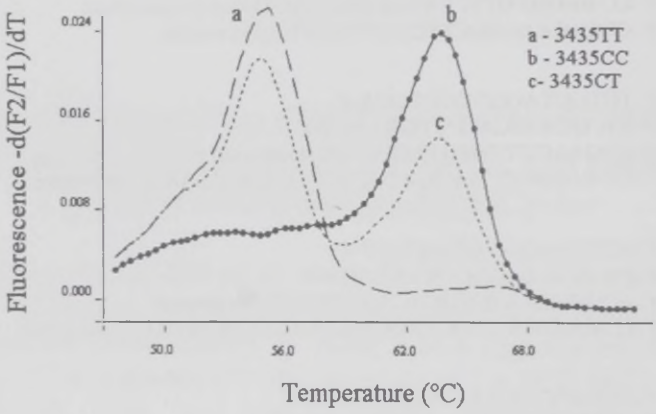
** PCR primer as reported by Ito *et al.* (2001).

The position of the variable base in sensor is underlined.

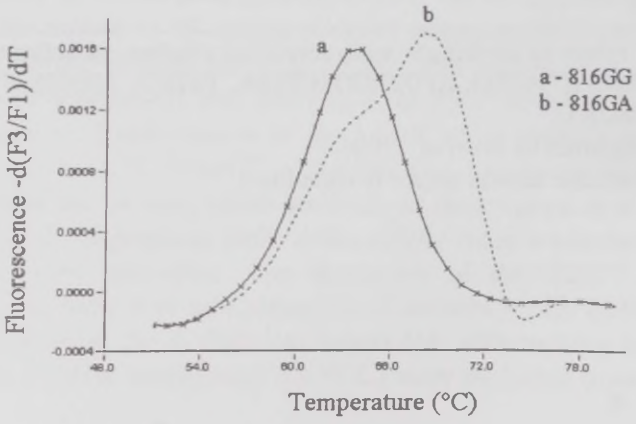
A.


Fluorescence -d(F2/F1)/dT
0.0012
0.0008
0.0004
0.0000
a
b
c
a - 2677TT
b - 2677GT
c - 2677GG
48.0
52.0
56.0
60.0
Temperature (°C)

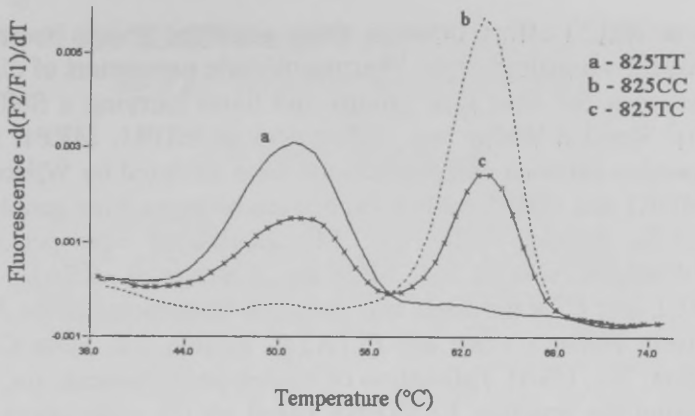
B.


Fluorescence -d(F2/F1)/dT
0.024
0.016
0.008
0.000
a
b
c
a - 3435TT
b - 3435CC
c- 3435CT
50.0
56.0
62.0
68.0
Temperature (°C)

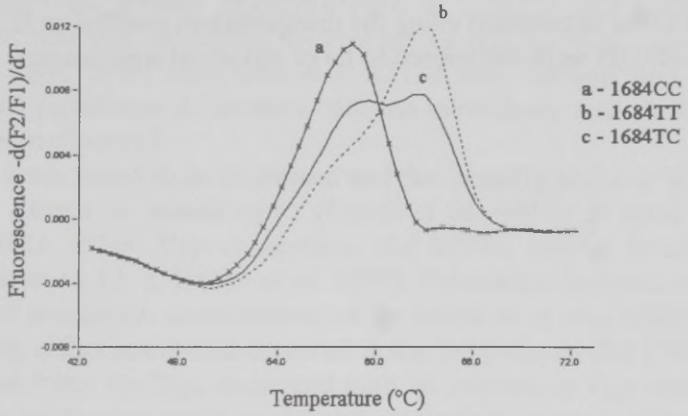
C.


Fluorescence -d(F3/F1)/dT
0.0016
0.0012
0.0008
0.0004
0.0000
-0.0004
a
b
a - 816GG
b - 816GA
48.0
54.0
60.0
66.0
72.0
78.0
Temperature (°C)

D.



E.



F.

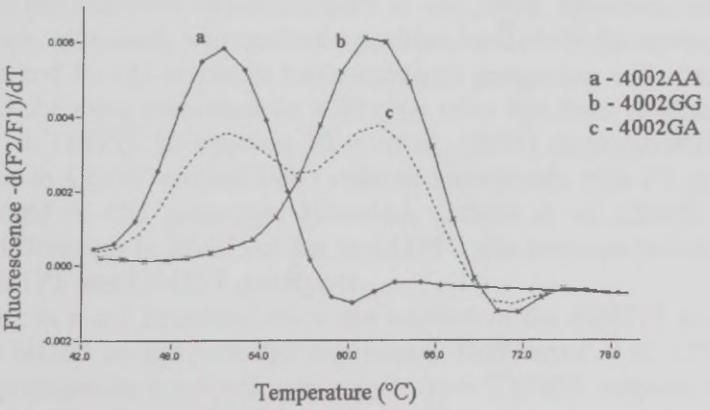


Figure 5. Melting curve analysis of the G2677T (A) and C3435T (B) SNPs in the *MDR1*, and the G816A (C), T825C (D), T1684C (E) and G4002A (G) SNPs in the *MRP1* using a LightCycler.

8. Statistical analysis

Differences in Rh123 efflux between three genotype groups were compared using the Student's unpaired *t*-test. Pharmacokinetic parameters of digoxin were compared between the wild type groups and those carrying a SNP using the nonparametric Kruskal-Wallis test. Differences in MDR1, MRP1, and MRP2 mRNA expression between cell populations were analysed by Wilcoxon signed ranks test. MDR1 and MRP1 mRNA expression between three genotype groups was analysed by Kruskal-Wallis test. All calculations were performed with SPSS™ for Windows (version 10.0, SPSS Inc., Chicago, IL, USA).

OR, 95%CI, and *P* for the allele and genotype frequencies of the *MRP1* were calculated using Fisher's exact test (STATA, version 7.0, Stata Corporation, College Station, TX, USA). Estimation of haplotype frequencies for *MRP1* was performed using the program FASTEH+ based on the expectation-maximization algorithm (Zhao and Sham 2002). Linkage disequilibrium between the pairs of SNPs was determined using the disequlibrium coefficient D'.

P values of 0.05 were considered to be of statistical significance.

RESULTS AND DISCUSSION

1. Impact of the G2677T and C3435T SNPs in the *MDR1* gene on Pgp function and expression

1.1. Rh123 efflux study

A clear difference concerning the Rh123 efflux pattern was observed between the $CD56^+$ and $CD4^+$ cells (Table 6), which is in accordance with studies published previously (Drach *et al.* 1992). Statistical analysis revealed no significant differences in Rh123 efflux in $CD56^+$ and $CD4^+$ cells between individuals with different *MDR1* genotypes. The verapamil effect on Pgp activity was analysed after 30 min of Rh123 efflux in $CD56^+$ cells and after 90 min efflux in $CD4^+$ cells. Verapamil was a strong inhibitor of Rh123 efflux, but this effect was not associated with the genetic polymorphisms at G2677T and C3435T. When Rh123 efflux rate was calculated by comparing fluorescence measurement in $CD56^+$ and $CD4^+$ cells, incubated in a Rh123 free medium with or without verapamil, no significant differences between individuals with different *MDR1* genotypes were observed.

Pgp has been found to be expressed and functionally active in all peripheral blood cells, except in granulocytes (Drach *et al.* 1992). A good correlation between Rh123 efflux, Pgp expression, and MDR1 mRNA levels has been found (Drach *et al.* 1992; Robey *et al.* 1999). For studies designed to overcome Pgp mediated resistance, measurement of the extent of *in vivo* inhibition of Pgp mediated drug efflux is deemed essential. It has been shown that PSC833 effect, a selective inhibitor for Pgp, decreased with an increase in Pgp expression and the high Pgp expression could explain an incomplete reversal of Pgp-mediated Rh123 efflux by PSC833 in some patients (Robey *et al.* 1999). No Pgp inhibitor was used for pretreatment of control cells in our study. However, one sample, incubated with verapamil, was used as a positive control for the method.

An impaired Rh123 efflux in haematopoietic progenitor cells from *mdr1a/1b*(-/-) knockout mice compared to wild type mice has been demonstrated by Schinkel *et al.* (1997). In humans, Hitzl *et al.* (2001) demonstrated a slower Rh123 efflux in $CD56^+$ natural killer cells in individuals with TT genotype at 3435 compared to CC genotype. However, Calado *et al.* (2002) found no significant differences in Rh123 efflux in $CD34^+$ cells between individuals with different G2677T and C3435T genotypes.

In contrast to *mdr1* knockout mice, the carriers of the G2677T and C3435T SNPs in the *MDR1* do not lack Pgp expression. Hoffmeyer *et al.* (2000) found that Pgp expression in duodenal enterocytes from TT3435 subjects was significantly lower than from CC3435 subjects. There is a considerable overlap between the substrate specificity of Pgp and other ABC transporters. Daoud *et al.* (2000) reported that Rh123 also binds to multiple sites on the MRP1. There-

fore, it is not possible to exclude a confounding effect of other transporters on Rh123 efflux.

Table 6. Rh123 efflux in $CD56^+$ and $CD4^+$ cells from individuals with different G2677T and C3435T genotypes of the *MDR1* gene.

	G2677T			C3435T		
	GG(n=22)	GT(n=14)	TT(n=9)	CC(n=17)	CT(n=14)	TT(n=15)
$CD56^+$						
30 min	27.5±4.7	26.9±5.0	24.6±3.7	28.0±5.3	26.7±4.4	25.5±3.9
verpamil*	88.7±6.0	86.0±7.2	89.5±5.3	89.7±6.3	84.6±6.7	88.8±5.0
$CD4^+$						
90 min	62.6±7.8	60.5±7.5	60.5±8.7	64.4±7.0	60.1±7.8	59.3±8.1
verpamil*	91.8±3.9	93.8±4.1	90.3±5.7	92.8±3.3	91.6±5.0	91.7±5.5

Data are presented mean±SD in %. After Rh123 accumulation, cells were incubated for 30 min and 90 min in a Rh123 free medium for the measurement of Rh123 efflux in $CD56^+$ and $CD4^+$ cells, respectively, with (*) or without 2 μM verapamil. Numbers in parenthesis indicate the number of individuals. One individual was determined to carry the A allele at 2677 and was excluded from the G2677T analysis. *P* values according to unpaired t-test exceeded values 0.05.

1.2. Digoxin study

Pharmacokinetic parameters of a single oral dose of 1 mg digoxin, characterized by $AUC_{0\text{-}4h}$, C_{max}, and t_{max}, were not significantly different between individuals with different *MDR1* genotypes (Table 7, Fig. 6). No significant differences were observed after normalization to ideal body weight (data not shown). Digoxin is considered a good probe for Pgp, since it's metabolized in a minor extent in humans. The experimental design was chosen to assess the impact of the *MDR1* SNPs on the absorption of digoxin, since Pgp acts primarily as an efflux pump across apical membranes of enterocytes.

Several studies have determined the potential effects of the G2677T and C3435T SNPs in the *MDR1* on disposition of drugs (Table 8). Comparison of the C_{max} values for 0.25 mg digoxin after oral administration on steady state conditions revealed that subjects homozygous for TT had significantly higher C_{max} values than those with CC genotype (Hoffmeyer *et al.* 2000). Comparison of the $AUC_{0\text{-}\infty}$ values for a single oral dose of 180 mg fexofenadine revealed no significant differences between healthy individuals with different G2677T and C3435T genotypes (Drescher *et al.* 2002). No association between the C3435T mutation and the doses needed to maintain similar cyclosporine A trough concentrations was observed in renal transplant recipients (von Ahsen *et al.* 2001).

The conflicting results obtained by different authors could partially be explained by differences in study design. Various transporters, other than ABC transporters, have been reported to contribute to the disposition of drugs. For instance, in humans, digoxin has been shown to be a substrate for the organic anion transporting polypeptides, also expressed in intestine (Hagenbuch and Meier 2003). A large variation in the distribution of the kinetic data within the genotype groups in our study indicates that further transporters and hitherto unknown SNPs may influence the absorption of digoxin.

Table 7. Pharmacokinetic parameters of a single oral dose of 1 mg digoxin in individuals with different G2677T and C3435T genotypes in the *MDR1* gene.

Genotype	C_{max} (µg/l)	T_{max} (h)
2677GG (n=12)	4.22±1.5	1.00±0.4
2677GT (n=21)	3.91±1.0	1.00±0.6
2677TT (n=7)	3.69±0.8	1.10±0.5
3435CC (n=12)	4.12±1.2	0.92±0.2
3435CT (n=25)	3.87±1.3	1.05±0.6
3435TT (n=13)	4.14±1.1	0.95±0.4

Data are presented as mean±SD. *P* values according to Kruskal-Wallis test exceeded values 0.05. Individuals carrying the A allele at 2677 were excluded from the analysis of the G2677T SNP. *n* = number of individuals.

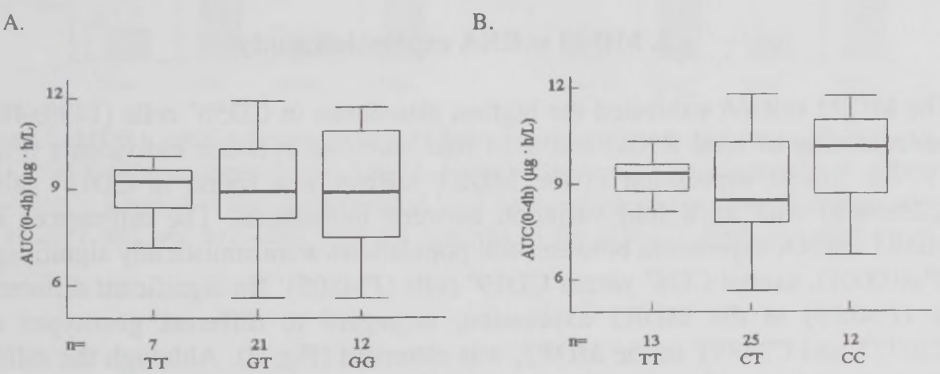


Figure 6. AUC_{0-4h} data for a single oral dose of 1 mg digoxin in healthy individuals in relation to the G2677T (A) and C3435T (B) *MDR1* polymorphisms. The boxes represent the distribution of the 25th to 75th percentiles, the bars represent the 5th to 95th percentiles. Median values for each genotype group are given within the boxes. *P* values according to Kruskal-Wallis test exceeded values 0.05. *n* = number of subjects.

Table 8. Some of the studies, where the impact of the G2677T and C3435T SNPs in the *MDR1* on the disposition of drugs that are substrates for Pgp has been investigated.

Drug	SNP	Result	Reference
Digoxin	C3435T	C_{max}: TT>CC	Hoffmeyer *et al.* 2000
	C3435T	AUC_{0-4h}: CC>CT=TT	Sakaeda *et al.* 2001
Fexofenadine	C3435T	$AUC_{0-\infty}$: CC=TT	Drescher *et al.* 2002
	G2677T	GG=GT=TT	
	C3435T	AUC_{0-4h}: CT>CC>TT	Kim *et al.* 2001
	G2677T	GT=GG>TT	
Talinolol	C3435T	$AUC_{0-\infty}$: CC=CT=TT	Siegmund *et al.* 2002
	G2677T	GG=GT=TT	
Nelfinavir	C3435T	Plasma concentration: CC>CT>TT	Fellay *et al.* 2002
Cyclosporine A	C3435T	Dose adjusted through concentration: CC=CT=TT	von Ahsen *et al.* 2001
	C3435T	AUC_{0-24h}: CC=CT=TT	Min *et al.* 2002
Docetaxel	C3435T	Clearance: CC=CT=TT	Goh *et al.* 2002
Tacrolimus	C3435T	Concentration:dose ratio CC=CT=TT	Goto *et al.* 2002

Modified from Fromm 2003.

1.3. MDR1 mRNA expression study

The MDR1 mRNA exhibited the highest abundance in $CD56^+$ cells (1430±485 molecules/ng of total RNA) and a 14 fold variation between individuals (Fig. 7). The lowest expression of the MDR1 mRNA was found in $CD19^+$ cells (223±98.8) with an 8 fold variation between individuals. The differences in MDR1 mRNA expression between cell populations were statistically significant ($P<0.0001$), except $CD4^+$ *versus* $CD19^+$ cells ($P>0.05$). No significant difference ($P>0.05$) in the MDR1 expression, in regard to different genotypes at G2677T and C3435T in the *MDR1*, was observed (Fig. 7). Although the difference was not significant, both polymorphisms were associated with the lowest level of mRNA in homozygous mutant individuals in $CD8^+$ and $CD56^+$ cells, and heterozygous individuals displayed an intermediate phenotype.

The association between the *MDR1* genetic polymorphisms and MDR1 mRNA and/or Pgp expression has been investigated in several studies. Higher

mRNA levels, in duodenal enterocytes in healthy subjects homozygous for TT at 3435 than in CT or CC individuals, were observed by Nakamura *et al.* (2002). In contrast, quantification of MDR1 transcripts and Pgp expression in PBMC from HIV-infected individuals showed significantly lower mRNA and protein levels in 3435 TT than in CC subjects (Fellay *et al.* 2002). A good correlation between Pgp expression and SNP in the promoter region T-129C of the *MDR1* was found in placental samples from Japanese women (Tanabe *et al.* 2001). However, the G2677T and C3435T SNPs did not show statistically significant effect. Markedly lower renal Pgp expression and an increased risk for development of renal epithelial tumors was observed in individuals homozygous for TT than those homozygous for CC at 3435 (Siegsmund *et al.* 2002). In contrast, in previously untreated acute myeloid leukaemia patients the CC3435 genotype demonstrated lower bone marrow MDR1 mRNA expression and significantly decreased overall survival in comparison with TT3435 genotype when treated according to protocol (Illmer *et al.* 2002).

The conflicting results obtained by different authors indicate the complex regulation of the *MDR1* gene expression. These results also raise the possibility of differential gene regulation in various body tissues.

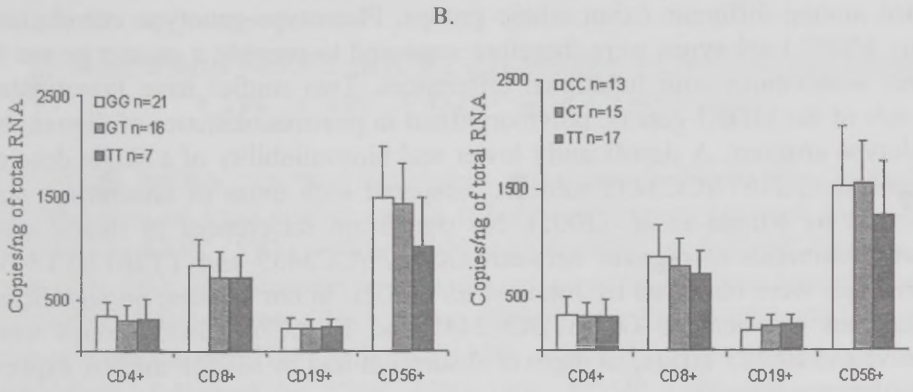


Figure 7. MDR1 mRNA levels in PBMC from individuals with different G2677T (A) and C3435T (B) genotypes in the *MDR1*. Each bar represents the mean±SD. *P* values according to Kruskal-Wallis test exceeded 0.05. One individual was determined to carry TA at 2677 and was excluded from the analysis of the G2677T SNP. *n* = number of subjects.

1.4. General discussion concerning the impact of the G2677T and C3435T SNPs in the *MDR1* on Pgp activity and expression

It's known that the expression of the *MDR1* in gastrointestinal tract and kidney significantly alters the extent of drug absorption and excretion. Numerous

SNPs, identified within the *MDR1* gene, support that wide interindividual variation in disposition of drugs, which are substrates of Pgp, may have a genetic basis. In several studies, the potential role of the SNPs in the *MDR1* in Pgp expression and activity has been investigated, but the results published so far are conflicting.

In the first published studies solely the impact of the C3435T polymorphism in the *MDR1* was investigated, on a basis of the study from Hoffmeyer *et al.* (2000). Later, a strong association between the SNPs G2677T and C3435T was observed (Tanabe *et al.* 2001). The G to T transversion at position 2677 results in amino acid Ala 893 change by Ser. The C3435T SNP is a silent polymorphism that does not result in its encoded Ile amino acid change. Therefore, it was supposed that the linked polymorphism G2677T rather than C3435T is causative for individual differences in Pgp expression and function. When the studies were extended to investigate the impact of the G2677T on Pgp, the results remained conflicting. More recently, strong linkage disequilibrium between the SNPs T1236C in exon 12, G2677T in exon 21, and C3435T in exon 26 was observed in a population of European Americans and African Americans (Kim *et al.* 2001), and in Asian subjects (Tang *et al.* 2002). The linkage disequilibrium, spanning the 40 kb between exon 12 and exon 26, varied among different Asian ethnic groups. Phenotype-genotype correlations using *MDR1* haplotypes were therefore supposed to provide a greater power to detect associations with functional differences. Two studies have investigated the role of the MDR1 genetic polymorphisms in pharmacokinetics of digoxin by haplotype analysis. A significantly lower oral bioavailability of a single dose of digoxin in GG2677/CC3435 subjects compared with those of noncarriers was observed by Kurata *et al.* (2002). No significant differences in steady state pharmacokinetics of digoxin between GG2677/CC3435 and TT2677/TT3435 individuals were observed by Johne *et al.* (2002). In our studies, no significant differences between the GG2677/CC3435 and TT2677/TT3435 groups were observed in Rh123 efflux, in digoxin absorption and in MDR1 mRNA expression (data not shown).

Synonymous coding SNPs do not lead to amino acid changes, but the variant mRNA transcripts may exhibit altered stability and/or splicing, these changes may alter protein expression. Nonsynonymous coding SNPs result in amino acid differences that may affect overall protein structure, cellular localization, and affinity with individual substrates. Introduction of different amino acid substitutions into various positions along the Pgp affected drug resistance in varying degrees. For instance, cells carrying the Val to Ala change at position 338 exhibited increased resistance to colchicine and doxorubicin, and reduced resistance to vinblastine (Loo and Clarke 1994). While cells carrying the Gly to Val change at position 341 have decreased resistance to colchicine and doxorubicin, but resistance to vinblastine and actinomycin D was unchanged. Using a vaccinia virus-based expression system, no modification of Pgp cell surface localization and expression was observed by the G2677T *MDR1* SNP (Kimchi-

Sarfaty *et al.* 2002). Transport studies indicated that the substrate specificity of the pump to several compounds was not substantially affected by the tested polymorphism. There is a possibility that the G2677T SNP could affect disposition of some drugs, other than investigated so far. However, this does not explain the conflicting results as obtained from different authors using the same probe drug.

In summary, no significant difference in MDR1 mRNA expression between individuals with different G2677T and C3435T genotypes, and accordingly the lack of association between the *MDR1* SNPs and Rh123 efflux in PBMC and digoxin pharmacokinetics was observed in the present studies. These findings, together with evidence from studies published previously, indicate that *MDR1* genetic polymorphisms G2677T and C3435T have probably no clinically important effect on Pgp function. It has been hypothesized that the linkage between the silent SNP C3435T and different unobserved causal SNPs may explain contradictory results associating the T3435 allele with different functional changes of *MDR1* (Tang *et al.* 2002). Further studies are required to elucidate the significance of this explanation.

2. MRP1 and MRP2 mRNA expression in peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells

The MRP1 mRNA exhibited the highest abundance in $CD4^+$ cells (7400±3200 molecules/ng of total RNA) with a 6.7 fold difference between individuals, followed by $CD8^+$ (5700±2500), $CD19^+$ (4000±1800), and $CD56^+$ (3600±1300) cells (Fig. 8A). The differences between cell populations were statistically significant ($P<0.01$), except $CD19^+$ *versus* $CD56^+$ cells. The MRP2 mRNA expression was highest in $CD4^+$ cells (670±280) with a 5.2 fold difference between individuals, followed by $CD8^+$ (570±190), $CD56^+$ (490±220), and $CD19^+$ (420±160) cells (Fig. 8B). The differences observed were statistically significant ($P<0.05$). No correlation between the MRP1 and MRP2 mRNA expression was observed.

Within haematopoietic cells it has been found that $CD3^+$ cells exhibited the highest and $CD19^+$ cells the lowest MRP1 mRNA levels (Legrand *et al.* 1996). Protein analysis determined that $CD4^+$ cells exhibited higher MRP1 expression than other cells. In another study, Abbaszadegan *et al.* (1994) showed that regardless of the cell lineage, normal peripheral blood and bone marrow haematopoietic cells express similar basal levels of the MRP1 mRNA. Recently, Laupèze *et al.* (2001) investigated MRP activity in normal mature leucocytes using the carboxy-2',7'-dichlorofluorescein efflux assay. Similar cellular export was evidenced in $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, $CD15^+$, $CD20^+$, and $CD56^+$ cells, whereas $CD34^+$ cells showed higher efflux rate. Investigation

of the mRNA expression revealed that leucocytes were found to mostly express MRP1, whereas MRP2 transcripts were not present or only at very low levels.

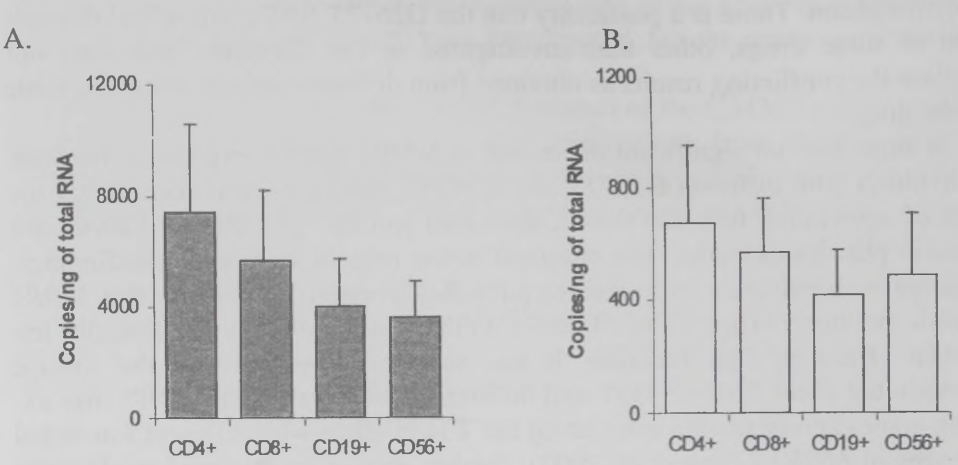


Figure 8. Quantitative determination of the MRP1 (A) and MRP2 (B) mRNA levels in PBMC. Each bar represents the mean±SD, as determined in 20 individuals. The differences in MRP1 expression between cell populations were statistically significant ($P<0.01$), except $CD19^+$ *versus* $CD56^+$ cells. All differences in MRP2 expression were statistically significant ($P<0.05$).

MRP1 and MRP2 have similar substrate specificity. There are no drugs yet that specifically inhibit a single MRP species without effect on other transporters. Therefore, a sensitive technique RT-PCR, instead of efflux assays, was used in the present study. Similar to the results reported previously (Legrand *et al.* 1996), the MRP1 mRNA expression was found to be highest in $CD4^+$ cells. However, larger interindividual variation (20 fold) than in the present study was observed by Legrand *et al.* (1996). In contrast to Laupèze *et al.* (2001), the MRP2 mRNA was evidenced in all cell populations investigated.

The impact of the MRP1 and MRP2 transcription in PBMC remains to ascertain. Pgp has been found to be highly expressed in $CD8^+$ and $CD56^+$ cytotoxic cells (Drach *et al.* 1992). In the present study, $CD4^+$ cells exhibited the highest abundance of the MRP1 and MRP2 mRNA, with more than 7 fold higher MRP1 expression. This indicates that the expression of different ABC transporter genes in haematopoietic cells may be variously regulated during differentiation and miscellaneous ABC transporters may have different function. For instance, Pgp has been reported to be involved in the natural killer and T cell mediated cytotoxicity (Klimecki *et al.* 1995; Gupta *et al.* 1992), and in the transport of cytokines (Drach *et al.* 1996). MRP1, unlike Pgp, facilitates the secretion of leukotrienes (Leier *et al.* 1994), the major mediators of inflamma-

tion. Further studies are required to specify the physiological function of the MRP1 and MRP2 transporters in PBMC cells.

Several HIV protease inhibitors and reverse transcriptase inhibitors are substrates of MRP1 and MRP2 (Williams *et al.* 2002; Huisman *et al.* 2002). The highest level of the MRP1 and MRP2 mRNA was found in $CD4^+$ cells, at major site of virus replication. Hence, MRPs may have an important role in limiting drug exposure within these cells and therefore the efficacy of HIV therapy. Recently, Jones *et al.* (2001) demonstrated that the accumulation of ritonavir, indinavir, saquinavir, and nelfinavir in MRP1 expressing cells was significantly reduced in comparison with control cells. MRP1 inhibitor MK571 partially reversed these effects. An inverse relationship between the MRP1 expression and intracellular accumulation of ritonavir and saquinavir in PBMC from HIV-infected individuals has been shown by Meaden *et al.* (2002). Both MRP1 and MRP2 expression in PBMC indicates that not only MRP1, but also MRP2 and probably other MRPs account for the resistance to anti-HIV-drugs. Recently, several SNPs in the human *MRP1* and *MRP2* gene were identified (Saito *et al.* 2002b). Conrad *et al.* (2002) found that G1299T polymorphism in the *MRP1* increased resistance to doxorubicin. Genetic polymorphisms in the *MRP2* have been found to result in a conjugated hyperbilirubinemia named Dubin-Johnson syndrome (Toh *et al.* 1999). Further studies should confirm if these mutations also affect MRP1 and MRP2 expression in PBMC and therefore contribute to therapeutic outcome of HIV therapy.

3. Frequencies of the *MRP1* genetic polymorphisms and their functional significance in Caucasians

3.1. Detection of a novel G816A SNP in the human *MRP1*

Using a LightCycler technique, we established real-time PCR assays for the SNPs located in exon 8 (T825C), in exon 13 (T1684C), and in exon 28 (G4002A) in the *MRP1*. Unexpectedly, a novel silent mutation in exon 8, a G to A transversion at position 816 (Pro to Pro at codon 272) was identified (Fig. 9). The derivative melting curves on the LightCycler are highly reproducible under constant conditions (Meuer *et al.* 2001). Shifts greater than 1°C from the characteristic melting curve suggests the presence of two mismatches, as occurred also in our study. First, the long sensor probe (position at 813–834 bp) used for T825C genotyping, determined 4 individuals with atypical melting curves. Subsequent sequencing showed that all these individuals, but not subjects with typical melting curves for T825C, were heterozygous GA at position 816. Hence, the G816A substitution destabilized the binding of the long T825C sensor. A new, shorter sensor probe (position at 817-834 bp, see methods) for

T825C detection, and another pair of hybridisation probes (position at 779-800 bp anchor, and 803-821 bp sensor) for G816A detection were designed. When designing hybridisation probes to recognize the G816A and T825C polymorphisms, oligonucleotides that cover the both mutation sites should be avoided. As reported previously, aberrant derivative melting curves have effectively identified unexpected variants in factor V, hereditary haemochromatosis, and cystic fibrosis (Meuer *et al.* 2001).

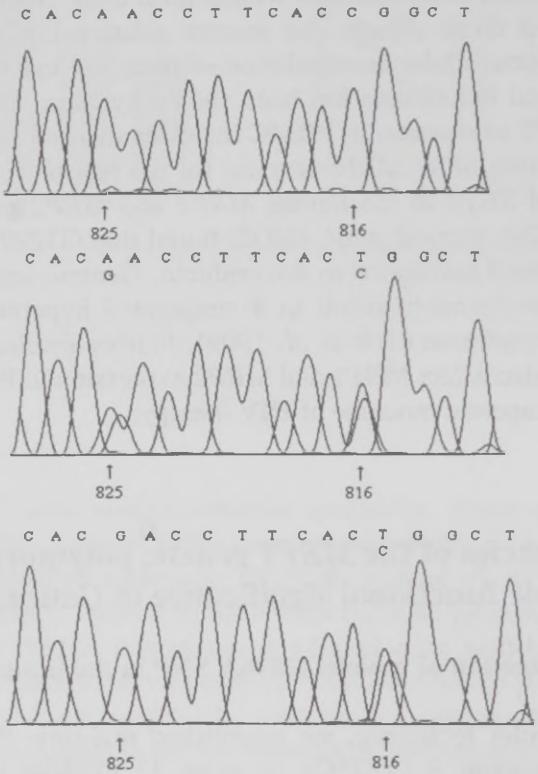


Figure 9. Identification of G816A and T825C polymorphisms in the human *MRP1* gene by sequence analysis (reverse primer).

3.2. Frequencies of the G816A, T825C, T1684C, and G4002A SNPs in the *MRP1*

The results of *MRP1* genotyping are given in Table 9.

Table 9. Observed frequencies of *MRP1* genetic variants in 230 healthy Caucasians. Expected genotype frequencies were calculated according to the Hardy-Weinberg equilibrium from the allele frequencies.

Position	Allele	Allele frequency %	Geno-type	Number of subjects	Genotype frequency		
					% Observed	95% CI	% Expected
816	G	95.9	GG	211	91.7	87.40–94.95	91.9
	A	4.1	GA	19	8.3	5.05–12.60	7.9
			AA	0	0.0	0.0–0.02	0.0
825	T	70.0	TT	115	50.0	43.36 – 56.64	49.0
	C	30.0	TC	92	40.0	33.62 – 46.64	42.0
			CC	23	10.0	6.45 – 14.63	9.0
1684	T	20.0	TT	15	6.5	3.70 – 10.53	4.0
	C	80.0	TC	62	27.0	21.34 – 33.18	32.0
			CC	153	66.5	60.02 – 72.59	64.0
4002	G	71.7	GG	118	51.3	44.65 – 57.93	51.4
	A	28.3	GA	94	40.9	34.45 – 47.52	40.6
			AA	18	7.8	4.70 – 12.09	8.0

The novel mutation G816A was found heterozygously in 19 of 230 individuals with an allele frequency of 4.1%. The allele frequency of the T allele at position 825 was found to be 70.0% in Caucasians, as determined in the present study, and 62.5% in Japanese (n=48), as reported by Ito *et al.* (2001) (OR 1.4, 95% CI 0.86–2.26, P=0.18). The frequency of the G allele at position 4002 was 71.7% in Caucasians and 84.4% in Japanese (OR 0.47, 95% CI 0.24–0.86, P=0.01). When genotyping results of the T1684C polymorphism were compared between Caucasians and Japanese, it was found that the frequency of T allele was 20.0% in Caucasians, and 80.2% in Japanese (OR 0.06, 95% CI 0.03–0.11, P<0.0001). In the first published *MRP1* sequence (AF022835), derived from a lung cancer cell line, a T nucleotide at position 1684 was reported and defined as a wild type allele. We found that a C allele at position 1684 was the most common variant for Caucasians.

A marked difference in the frequencies of the T1684C and G4002A SNPs in the *MRP1* was observed between Caucasians and Japanese. Three additional mutations T1062C, C2007T, and G2168A have been identified in Japanese by Ito *et al.* (2001) and Saito *et al.* (2002b). None of these mutations was found by Conrad *et al.* (2001) by screening 36 healthy Caucasians. Three of the SNPs C1299T, C1704T, and G2021T, identified by Conrad *et al.* (2001), and the

G816A mutation, identified in the present study, were not found in Japanese. These findings suggest an ethnic variation of the polymorphisms in the *MRP1*.

Estimation of the haplotypes based on the expectation-maximization algorithm showed that of 16 possible haplotypes only 11 were observed, and four haplotypes GTCG, GTCA, GCCG, and GTTG together accounted for 80% of the haplotypes (Table 10). Linkage disequilibrium between the different pairs of SNPs was determined using the statistic D'. A D' value of 1 indicates complete linkage disequlibrium, while a value of 0 corresponds to complete linkage equilibrium. In our population, a strong linkage disequilibrium between the G816A and T825C SNPs (D'=1.000, $P<0.001$), and between the G186A and T1684C SNPs (D'=0.996, $P<0.001$) was observed (Table 11).

Table 10. Distribution of haplotype frequencies (%) for the four SNP loci of the *MRP1* gene in Caucasians.

Haplotype	Estimated
GTCG	38.96
GTCA	18.12
GCCG	14.28
GTTG	9.49
GCTG	6.30
GCCA	4.55
GTTA	3.44
ACCG	2.68
ACCA	1.42
GCTA	0.74
ACTG	0.03

Table 11. Linkage analysis of the *MRP1* SNPs in Caucasians.

	G816A	T825C	T1684C	G4002A
G816A		D'=1.000 $P<0.001$	D'=0.996 $P<0.001$	D'=0.075 $P=0.706$
T825C			D'=0.074 $P=0.393$	D'=0.194 $P=0.226$
T1684C				D'=0.237 $P=0.258$
G4002A				

3.3. MRP1 mRNA expression in individuals with different *MRP1* genotypes

Functional significance of the SNPs in the *MRP1* was investigated in peripheral blood $CD4^+$ cells. MRP1 mRNA expression was 10300±3800 molecules/ng of total RNA with an 8 fold variation between individuals. Statistical analysis revealed no significant (P>0.05) difference in MRP1 expression with regard to different genotypes in the *MRP1* (Fig. 10). Also the novel mutation G816A was not found to determine MRP1 mRNA expression, since homozygous wild type GG individuals displayed 10400±3400 and heterozygous GA individuals 9830±5500 MRP1 mRNA molecules/ng of total RNA.

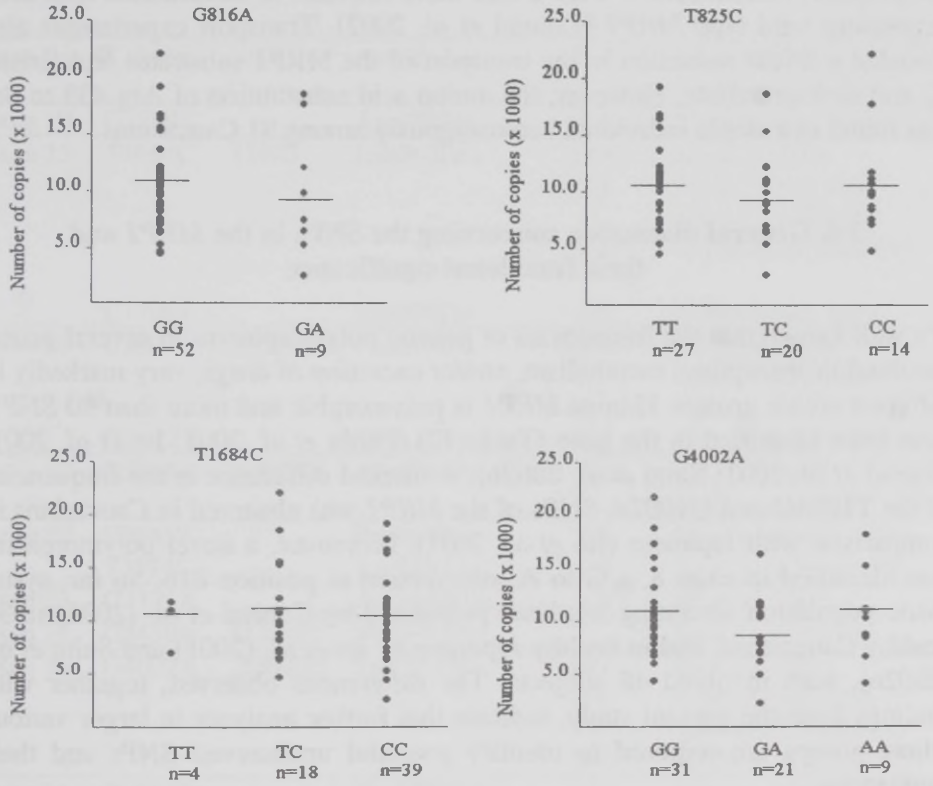


Figure 10. Quantitative determination of the MRP1 mRNA expression in peripheral blood $CD4^+$ cells from individuals with different *MRP1* genotypes. Data are presented as MRP1 mRNA copies/ng of total RNA. Bars represent the mean values for each group. No difference in MRP1 mRNA expression was observed between subjects with the different *MRP1* genotypes ($P > 0.05$).

All mutations investigated in this study were silent mutations, which do not alter the amino acid sequence. However, synonymous SNPs may affect RNA folding and stability (Shen *et al.* 1999). As shown by Conrad *et al.* (2001), sense mutations T825C and C1704T seemed to correlate with a lower MRP1 mRNA expression in peripheral blood lymphocytes, but the numbers of samples for each genotype were too low for statistical analysis. In the present study, no correlation between the MRP1 mRNA levels in $CD4^+$ cells and G816A, T825C, T1684C, and G4002A SNPs in the *MRP1* was found.

Two of the mutations identified by Conrad *et al.* (2001), G1299T and G2012T, cause the exchange of amino acids. In subsequent transfection studies, the mutant G2012T (Gly671Val) showed a phenotype similar to wild-type MRP1 (Conrad *et al.* 2001). Transfected HeLa cells expressing G1299T (Arg433Ser) mutant *MRP1* were 2 fold more resistant to doxorubicin than cells expressing wild type *MRP1* (Conrad *et al.* 2002). Transport experiments also revealed a 2 fold reduction in the transport of the MRP1 substrates leukotriene C_4 and estrone sulfate. However, the amino acid substitution of Arg 433 to Ser was found in a single individual heterozygously among 91 Caucasians.

3.4. General discussion concerning the SNPs in the *MRP1* and their functional significance

It's well known that the frequencies of genetic polymorphisms in several genes, involved in absorption, metabolism, and/or excretion of drugs, vary markedly in different ethnic groups. Human *MRP1* is polymorphic and more than 80 SNP's have been identified in the gene (Table 12) (Perdu *et al.* 2001; Ito *et al.* 2001; Conrad *et al.* 2001; Saito *et al.* 2002b). A marked difference in the frequencies of the T1684C and G4002A SNPs of the *MRP1* was observed in Caucasians in comparison with Japanese (Ito *et al.* 2001). Moreover, a novel polymorphism was identified in exon 8, a G to A transversion at position 816. So far, systematic population screening has been performed by Conrad *et al.* (2001) in 36 healthy Caucasians, and in healthy Japanese by Ito *et al.* (2001) and Saito *et al.* (2002b), both involved 48 subjects. The differences observed, together with findings from the present study, indicate that further analyses in larger various ethnic groups are required to identify potential unobserved SNPs and their frequencies.

Table 12. Summary of genetic polymorphisms identified in exonic sequences of the human *MRP1* (GenBank accession numbers AF022824-AF022853).

Location	SNP*	NCBI SNP rs	Amino acid substitution	Mutant allele frequency (%)	Reference
Exon 2	G128C		Cys43Ser	1.0	Ito *et al.* 2001
	C218T		Thr73Ile	1.0	Ito *et al.* 2001
Exon 3	T350C		Thr117Ala	100.0	Perdu *et al.* 2000
Exon 8	G816A		Pro272Pro	4.1	Oselin *et al.* 2003 (V)
	T825C	246221	Val275Val	37.5	Ito *et al.* 2001
				2.8	Conrad *et al.* 2001
					Saito *et al.* 2002b
				30.0	Oselin *et al.* 2003 (V)
Exon 9	T1062C	35587	Asn354Asn	35.4	Ito *et al.* 2001
					Saito *et al.* 2002b
Exon 10	G1299T		Arg433Ser	1.4	Conrad *et al.* 2001
Exon 13	T1684C	35605	Leu562Leu	19.8	Ito *et al.* 2001
				8.3	Conrad *et al.* 2001
					Saito *et al.* 2002b
				80.0	Oselin *et al.* 2003 (V)
	C1704T		Tyr568Tyr	2.8	Conrad *et al.* 2001
Exon 16	C2007T	2301666	Pro669Pro	8.3	Ito *et al.* 2001
					Saito *et al.* 2002b
	G2012T		Gly671Val	2.8	Conrad *et al.* 2001
Exon 17	G2168A	4148356	Arg723Gln	7.3	Ito *et al.* 2001
					Saito *et al.* 2002b
Exon 20	C2665T		Pro889Pro	1.0	Ito *et al.* 2001
	T2694C		Asn898Asn	1.0	Ito *et al.* 2001
Exon 23	G3173A		Arg1058Gln	1.0	Ito *et al.* 2001
Exon 24	G3450A	4148377	Pro1150Pro		Saito *et al.* 2002b
Exon 28	G4002A	2239330	Ser1334Ser	15.6	Ito *et al.* 2001
				2.8	Conrad *et al.* 2001
					Saito *et al.* 2002b
				28.3	Oselin *et al.* 2003 (V)
Exon 31	C4524T		Tyr1508Tyr	1.0	Ito *et al.* 2001

Allele frequencies for genetic variants observed by Saito *et al.* (2002b) were not given in the publication.

* The positions of the SNPs correspond to GenBank accession number L05628 with the first base of the ATG start codon set to 1.

Individuals carrying polymorphisms in the *MRP1* might be subject to a variety of effects. Altered MRP1 expression and/or activity could influence the pharmacokinetic properties of drugs that are substrates for MRP1. Transport of glutathione disulfide and leukotriene C_4 (Leier *et al.* 1996; Leier *et al.* 1994), suggests MRP1 involvement in oxidative stress and anti-inflammatory responses. In the present study, we observed that MRP1 mRNA expression in PBMC was the highest among the ABC transporters studied. In $CD56^+$ cells, which have been considered to express high levels of Pgp and MDR, MRP1 mRNA expression was more than 2 fold higher than that of the MDR1 expression. Also in $CD4^+$ cells, target for HIV therapy, the MRP1 mRNA expression was found to be the highest. Accordingly, interindividual variation in the expression of MRP1 has the highest potential to modify therapeutic outcome of HIV therapy and other therapies related to PBMC.

Limited number of studies has investigated the effects of genetic variants in the *MRP1*. No significant differences in MRP1 mRNA expression in peripheral blood $CD4^+$ cells between individuals with different *MRP1* genotypes were observed in the present study. However, further studies are required to determine the potential contribution of *MRP1* SNPs to pharmacokinetic properties of drugs and therapeutic response as well as to anti-inflammatory and antioxidative defence system.

CONCLUSIONS

In the past decade, several ABC transporters have been identified. The contribution of various SNPs in the ABC transporter genes to the pharmacokinetics of drugs as well as to the aetiology of several genetic disorders has been reported.

Main purpose of the present work was to assess the functional importance of the SNPs in the human ABC transporters, *MDR1* and *MRP1*, genes. The studies gave the following conclusions:

1. Using a Rh123 efflux assay, we could not find significant differences in Pgp activity in peripheral blood $CD56^+$ and $CD4^+$ cells from healthy individuals with different MDR1 genotypes at G2677T and C3435T.

2. The G2677T and C3435T SNPs in the *MDR1* did not contribute to the absorptive pharmacokinetics of a single oral dose of 1 mg digoxin in healthy volunteers.

3. The genetic polymorphisms G2677T and C3435T in the *MDR1* gene did not have any significant effect on the MDR1 mRNA expression in PBMC from healthy subjects.

4. On a mRNA level MRP1 and MRP2 were found to be expressed in human peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells, with more than 7 fold higher MRP1 expression in all cell populations.

5. A novel polymorphism G816A (Pro to Pro at codon 272) was identified in the human *MRP1* gene and marked ethnic variations in the frequencies of the *MRP1* G816A, T825C, T1684C, and G4002A SNPs were observed.

6. In healthy individuals, MRP1 mRNA expression in $CD4^+$ cells was not found to correlate with genetic polymorphisms G816A, T825C, T1684C, and G4002A in the *MRP1*.

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SUMMARY IN ESTONIAN

MDR1, MRP1 JA MRP2 ABC TRANSPORTERID: GENEETILISE POLÜMORFISMI TÄHTSUS MDR1 JA MRP1 GEENIDES

Rakkudel on võime transportida teatud aineid rakust läbi rakumembraani rakuvälisesse keskkonda. Valke, mis seda funktsiooni teostavad, nimetatakse transporteriteks. Käesolev uurimistöö käsitleb ATP-ga seonduvaid (inglise k. ATP-binding cassette — ABC) transportereid.

Ravimite imendumisel, jaotumisel ja eritumisel osalevad järgmised ABC transporterid: MDR1 ehk P-glükoproteiin, MRP1 ja MRP2. P-glükoproteiin oli esimene ABC transporter, mis isoleeriti esmalt kemoterapeutikumide suhtes resistentsetest kasvajarakkudest. Praeguseks on teada, et lisaks kasvajarakkudele ekspresseerub P-glükoproteiin ka normaalsetel rakkudel, eeskätt soole enterotsüütide apikaalsel membraanil, maksarakkude kanalikulaarsel pinnal ja proksimaalsete neerutorukeste epiteelirakkudes. P-glükoproteiin viies neist rakkudest välja paljusid ravimeid, vähendab nii nende imendumist gastrointestinaaltraktist ja kiirendab eliminatsiooni maksa ning neerude kaudu. Sarnaselt P-glükoproteiiniga transpordivad ka MRP1 ja MRP2 mitmeid ravimeid rakust rakuvälisesse keskkonda, kuid võrreldes P-glükoproteiiniga on nende substraatide spekter erinev.

Mitmes hiljuti avaldatud uuringus on leitud, et P-glükoproteiini ja MRP1 kodeerivad geenid on polümorfsed. Käesoleva uurimistöö peamine eesmärk oli hinnata, kas geneetilised mutatsioonid nimetatud geenides omavad funktsionaalset tähtsust ning kas individuaalsed erinevused ravimite imendumises ja eritumises on seletatavad geneetilise polümorfsusega nimetatud geenides.

Töö eesmärgid ja uurimismeetodid

1. Selgitada, kas MDR1 geeni polümorfsed variandid G2677T ja C3435T mõjutavad P-glükoproteiini ekspressiooni ja funktsiooni tervetel isikutel. Selle eesmärgi täitmiseks viisime läbi järgmised uuringud:

1.1. voolutsütomeetriat kasutades mõõtsime erineva genotüübiga isikute perifeerse vere $CD56^+$ ja $CD4^+$ rakkudes P-glükoproteiini substraadi, rodamiin 123, väljavoolu määra;

1.2. erineva genotüübiga isikutel määrasime P-glükoproteiini substraadi, digoksiini, farmakokineetilised parameetrid 1 mg ühekordse suukaudse annuse manustamise järgselt, digoksiini kontsentratsiooni määramiseks seerumis kasutasime immunoloogilist meetodit;

1.3. kvantitatiivset RT-PCR kasutades määrasime erineva genotüübiga isikute perifeerse vere $CD4^+$, $CD8^+$, $CD19^+$ ja $CD56^+$ rakkudes MDR1 geeni mRNA ekspressiooni. Erinevate rakutüüpide eraldamiseks perifeerse vere mononukleaarsetest rakkudest kasutasime voolutsütomeerit.

2. Selgitada, kas ABC transporterid MRP1 ja MRP2 on ekspresseeritud tervete isikute perifeerse vere $CD4^+$, $CD8^+$, $CD19^+$ ja $CD56^+$ rakkudes, selleks määrasime voolutsütomeetriga sorteeritud rakupopulatsioonides MRP1 ja MRP2 geeni mRNA ekspressiooni, kasutades kvantitatiivset RT-PCR.
3. Uurida MRP1 geeni erinevate alleelide T825C, T1684C ja G4002A esinemissagedust eurooplastel ja hinnata, kas nimetatud mutatsioonid omavad funktsionaalset tähtsust. Selleks määrasime kvantitatiivset RT-PCR kasutades erineva genotüübiga isikute perifeerse vere $CD4^+$ rakkudes MRP1 geeni mRNA ekspressiooni.

Töö tulemused ja järeldused

1. Rodamiin 123 väljavoolu määr, digoksiini farmakokineetilised parameetrid ja MDR1 geeni mRNA ekspressioon ei erinenud oluliselt erineva G2677T ja C3435T genotüübiga isikutel. Seega ei määra uuritud alleelide kandlus MDR1 geenis individuaalseid erinevusi P-glükoproteiini ekspressioonis ja aktiivsuses. Samuti pole isikutevaheline varieeruvus P-glükoproteiini substraatide farmakokineetikas seletatav uuritud polümorfismide kandlusega MDR1 geenis.
2. MRP1 ja MRP2 geenid olid ekspresseeritud kõigis uuritud rakutüüpides, kusjuures MRP1 geeni ekspressioon oli umbes 7 korda kõrgem. MRP1 ja MRP2 transportides rakust rakuvälisesse keskkonda mitmeid ravimeid, vähendavad nende rakusisest kontsentratsiooni. Seega võivad individuaalsed erinevused MRP1 ja MRP2 ekspressioonis perifeerse vere rakkudel mõjutada nendele rakkudele suunatud ravi, näiteks HIV ravi, efektiivsust.
3. Leidsime uue geneetilise mutatsiooni inimese MRP1 geeni lookuses G816A ja märkimisväärseid erinevusi teiste uuritud mutatsioonide esinemissagedustes eurooplastel võrreldes jaapanlastega. Erinevate MPR1 genotüüpidega (G816A, T825C, T1684C ja G4002A) isikutel ei esinenud perifeerse vere $CD4^+$ rakkudes olulist erinevust MRP1 geeni mRNA ekspressioonis. Kas nimetatud geneetiliste mutatsioonide kandlus mõjutab ravimite farmakokineetikat, vajab edasisi täiendavaid uuringuid.

PUBLICATIONS

I

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ORIGINAL ARTICLE

MDR1 polymorphisms G2677T in exon 21 and C3435T in exon 26 fail to affect rhodamine 123 efflux in peripheral blood lymphocytes

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ABSTRACT

P-glycoprotein (Pgp) is a member of the ABC-transporter family, and in humans, is encoded by the MDR1 gene. Recently, several single-nucleotide polymorphisms in the MDR1 gene were identified. The aim of the present study was to evaluate the effect of the MDR1 genetic polymorphisms G2677T and C3435T on Pgp activity in $CD56^+$ and $CD4^+$ peripheral blood cells. Using flow cytometry, rhodamine 123 (Rh123) efflux was determined in 46 male healthy volunteers. Median Rh123 fluorescence in control sample, after baseline dye uptake, was set as 100%. Rh123 fluorescence in efflux samples, exposed to different efflux periods, was used to calculate the percentage of Rh123 retained in the cells in comparison with control. There was no significant difference in Rh123 efflux in $CD56^+$ cells after 5, 10, 15, and 30 min efflux between individuals with different MDR1 genotypes. Also, in $CD4^+$ cells after 15, 30, 60, and 90 min, Rh123 efflux did not reveal statistically different results for the three genotypes at 2677 and 3435. Rh123 efflux was not enhanced by a 10-day rifampin administration, as determined in 15 individuals before and after rifampin treatment. In conclusion, we found no impact of the MDR1 G2677T and C3435T polymorphisms on Pgp activity in $CD56^+$ and $CD4^+$ peripheral blood lymphocytes.

INTRODUCTION

The human MDR1 gene encodes a 170-kDa plasma membrane protein, P-glycoprotein (Pgp), a member of the ATP-binding cassette transporters [1]. In several cell lines, overexpression of Pgp has been found to correlate with decreased intracellular accumulation of drugs that are substrates for Pgp. In contrast, mice with a targeted disruption of the MDR1a gene display a two-fold to 50-fold higher concentration of Pgp substrates in many tissues [2–4].

P-glycoprotein substrates comprise a wide spectrum of therapeutic agents, such as anticancer drugs paclitaxel, etoposide; human immunodeficiency virus-protease inhibitors saquinavir, indinavir; immunosuppressive drugs dexamethsone, cyclosporin A, etc. [2–7]. Digoxin has been identified *in vitro* and in animal experiments as a substrate of renal and intestinal Pgp [2,8]. Pgp expression in enterocytes from healthy volunteers has been found to correlate significantly with plasma area under the concentration–time curve values for digoxin after its oral administration [9]. Recently, several single-nucleotide polymorphisms in the MDR1 gene were identified [10,11]. The C3435T polymorphism in exon 26 was found to determine intestinal Pgp expression and digoxin absorption [11]. Nevertheless, comparison of the area under the concentration–time curve values for fexofenadine, another substrate for Pgp, revealed no significant differences between individuals with different C3435T and G2677T genotypes [12].

The aim of the present study was to assess the effect of the genetic polymorphisms G2677T and C3435T in the MDR1 gene on Pgp activity in peripheral blood lymphocytes. We measured rhodamine 123 (Rh123) efflux in peripheral blood $CD56^+$ natural killer (NK) and $CD4^+$ T-helper cells from healthy individuals with different MDR1 genotypes. To evaluate whether the rifampin treatment modulates Pgp expression in peripheral blood lymphocytes, Rh123 efflux was determined before and after a 10-day rifampin administration.

METHODS

Clinical study

The study protocol was approved by the Ethics Committee of the Humboldt University. Based on the genotype in exon 26 of the MDR1, 46 male healthy volunteers (age range 19–45 years) were recruited for Rh123 efflux study after an informed consent. The subjects took no medication and were ascertained to be healthy by a clinical examination and routine laboratory tests. In 15 individuals Rh123 efflux was determined before and after a 10-day rifampin treatment (600 mg/day, given in the morning; Grünenthal GmbH, Aachen, Germany).

Materials

Oligonucleotide primers and hybridization probes for polymerase chain reaction (PCR) were purchased from Tib Molbiol (Berlin, Germany); Rh123, verapamil hydrochloride, and BSA fraction V powder from Sigma Chemicals (Munich, Germany). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and Phosphate Buffered Saline (PBS) were obtained from Gibco-BRL (Karlsruhe, Germany); Biocoll lymphocyte separating solution (density 1.077) was from Biochrom KG (Berlin, Germany). Phycoerythrin (PE)-conjugated antibodies (CD56, CD4, and IgG_1) were obtained from Immunotech Beckman Coulter GmbH (Munich, Germany). Rh123 and verapamil stock solutions were prepared in ethanol in a concentration of 0.1 and 1 mg/mL, respectively, and maintained at 4 °C.

DNA isolation and genotyping of the MDR1 exon 26 C3435T and exon 21 G2677T polymorphisms

The DNA was isolated from 200 µL of whole blood by MagNA Pure LC (Roche, Mannheim, Germany). Genotyping was performed on real-time PCR assays in a LightCycler™ (Roche). G2677T mutation in exon 21 (GenBank Accession No. M29440), was detected with primers 5′-GCA GGA GTT GTT GAA ATG AAA ATG (forward) and 5′-CGC CTG CTT TAG TTT GAC TCA (reverse), and hybridization probes 5′-ACC TTC CCA GTA CCT TCT (sensor) and 5′-LC Red640-CTT TCT TAT CTT TCA GTG CTT GTC C (anchor). To detect C3435T mutation in exon 26 (GenBank Accession No. M29445) primers 5′-TGT TTT CAG CTG CTT GAT GG (forward) and 5′-AAG GCA TGT ATG TTG GCC TC (reverse) were used. Fluorescent detection was performed with hybridization probes 5′-GAC AAC AGC CGG GTG GTG TCA (anchor) and 5′-LC Red640-GGA AGA GAT CGT GAG GGC AG (sensor).

Rhodamine 123 efflux assay

Peripheral blood mononuclear cells were isolated from 20 mL whole blood by centrifugation over Biocoll lymphocyte separating solution (density 1.077). Cells were washed twice with PBS and diluted to 1×10^6 cells/mL in DMEM/10% FBS. To allow Rh123 accumulation, cell suspension was incubated for 30 min at 37 °C in 5% CO_2 with Rh123 in a final concentration of 150 ng/mL. After accumulation, cells were aliquoted into 1.5 mL Eppendorf tubes, centrifuged for 5 min at 400 *g* and washed twice with PBS to remove extracellular Rh123. After washing, the control sample was put on ice for measurement of the baseline dye uptake. All other samples were resuspended in 1 mL DMEM/10% FBS and performed for 5, 10, 15, and 30 min efflux in $CD56^+$; and for 15, 30, 60, and 90 min efflux in $CD4^+$ cells at 37 °C in 5% CO_2. In preliminary experiments, the Rh123 efflux time was varied from 5 to 120 min. Four different timepoints were chosen to evaluate the early and late phase of the efflux. One sample was incubated with verapamil in a concentration of 1 µg/mL for 30 min in $CD56^+$ and for 90 min in $CD4^+$ cells. After efflux, samples were washed once with cold PBS, stained with PE-labeled anti-CD56 antibody, PE-labeled anti-CD4 antibody or PE-labeled mouse IgG_1 as a negative control, for 30 min on ice in the dark. After staining, cells were washed twice with cold PBS/1% BSA and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) with a 488 nm argon laser.

The viability of isolated cells was determined using trypan-blue exclusion and was always >97%. Rh123 fluorescence was collected after 530-nm bandpass filter and PE fluorescence was collected after 585-nm bandpass filter. The appropriate threshold value was set to exclude clumps and debris. Fluorescence compensation was determined using samples stained with either Rh123 or PE alone. The samples were gated on forward scatter vs. side scatter to include lymphocytes. A second

gate was set around the PE-CD56$^+$ or PE-CD4$^+$ cells. A minimum of 2000 of CD56$^+$ and CD4$^+$ events were collected per sample. Data was expressed as median Rh123 fluorescence in CD56$^+$ and CD4$^+$ cells. Median Rh123 fluorescence in control sample, after baseline dye uptake, was set as 100%. Rh123 fluorescence in efflux samples, exposed to different efflux periods, was used to calculate the percentage of Rh123 retained in the cells in comparison with control.

Statistical analysis

Data were presented as mean ± SD. The Student's unpaired *t*-test was used to compare the mean Rh123 efflux between individuals with different genotypes. The paired *t*-test was used to compare the mean Rh123 fluorescence before and after rifampin treatment. All calculations were performed with SPSS™ for Windows (version 10.0; SPSS Inc., Chicago, IL, USA). *P*-values ≤ 0.05 were considered to be of statistical significance.

RESULTS

MDR1 genetic polymorphisms analyzed with real-time PCR assays in a LightCycler™

For the G2677T polymorphism, the melting temperature of the fluorescent hybridization probes was 56 °C to the homozygous wild-type GG, 52 °C to the mutant TT sample and 54 °C to heterozygous GT (*Figure 1*). DNA samples with known genotypes was let run in parallel. At position 2677 in exon 21, three nucleotide variants (G, T, or A) have been identified. The frequency of the

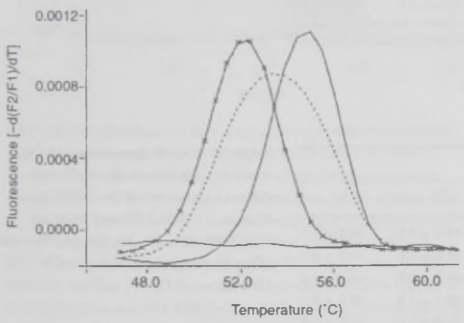

Figure 1 Genotyping of the G2677T polymorphism in the MDR1 gene with hybridization probes on the LightCycler™. Typical results for a homozygous 2677T allele (—x—), heterozygous GT (- - -), and a sample homozygous for 2677G allele (——).

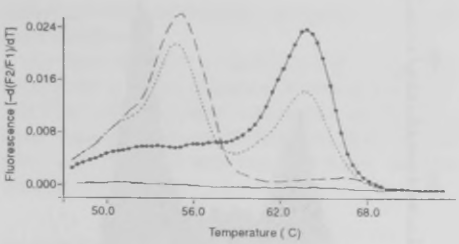

Figure 2 Genotyping of the C3435T exon 26 polymorphism of the MDR1 gene on the LightCycler™. The homozygous TT sample (— —), heterozygous CT (- - -), and the homozygous wild-type CC sample (-•-•-).

Table I Distribution of G2677(T.A) and C3435T polymorphisms of the MDR1 gene in 46 individuals.

	C3435T		
G2677T	CC	CT	TT
GG	15	7	0
GT	2	6	6
TT	0	0	9
TA	0	1	0

2677A allele has been observed to be very low in Caucasians. In our study, one individual was determined to carry the A allele (genotype GA with melting temperature 58 °C) and was excluded from the analysis. For the C3455T polymorphism, the melting point was 64 °C when hybridized to the wild-type allele and 55 °C when hybridized to the mutant allele (*Figure 2*). The heterozygous sample generated both peaks. A strong association between the G2677T and C3435T polymorphisms was observed (*Table I*).

Influence of the G2677T and C3435T genetic polymorphisms in the MDR1 on Rh123 efflux in CD56$^+$ NK and CD4$^+$ T-helper cells

Based on Rh123 fluorescence measurement, two clearly separated populations of lymphocytes were determined after 90 min efflux, indicating cells with higher and lower Pgp activity (*Figure 3*). Therefore, two subpopulations of peripheral blood mononuclear cells, CD56$^+$ cells with the highest and CD4$^+$ cells with intermediate expression of Pgp [13], were selected for the analysis.

In CD56$^+$ cells we could detect very quick Rh123 efflux. After 5 min incubation in Rh123-free medium

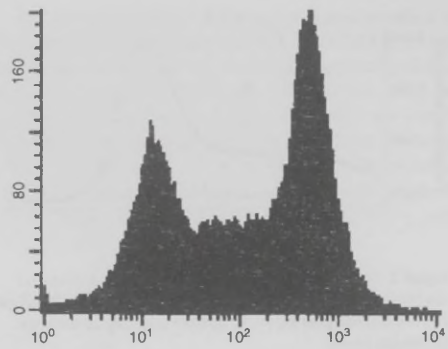

Figure 3 Rh123 fluorescence in lymphocytes after 90 min efflux. Peripheral blood mononuclear cells were incubated with Rh123 in a final concentration of 150 ng/mL and were exposed to 90 min efflux in Rh123-free medium. The histogram presents Rh123 fluoresence in gated lymphocytes indicating two large subpopulations of cells with higher and lower Pgp activity.

more than 10% of Rh123 had been effluxed. However, there was no statistical significance in Rh123 efflux in $CD56^+$ cells between individuals with different MDR1 genotypes (*Table II*). A clear difference concerning the Rh123 efflux pattern was observed between the $CD56^+$ and $CD4^+$ cells. The data shows more than 20% difference in efflux rate in $CD56^+$ and $CD4^+$ cells already after 15 min efflux. Similar to the results above, there was no statistical significance in Rh123 efflux in $CD4^+$ cells between individuals with different genotypes in the MDR1 gene (*Table III*). Slower efflux in peripheral blood and haematopoietic cells may render them more vulnerable to cytotoxic compounds, but it seems to be independent of the genetic polymorphisms in MDR1 gene.

Verapamil effect *in vitro* and rifampin effect *in vivo* on Pgp-mediated Rh123 efflux

The verapamil effect on Pgp activity was analyzed after 30 min of Rh123 efflux in $CD56^+$ cells (*Figure 4*) and after 90 min efflux in $CD4^+$ cells. Verapamil was a strong inhibitor of Rh123 efflux, but this effect was not associated with the genetic polymorphisms at G2677T and C3435T (*Tables II and III*). In addition, when Rh123 efflux rate was calculated by comparing fluorescence measurement in $CD56^+$ and $CD4^+$, incubated for 30 and 90 min in Rh123-free medium, respectively, with or without verapamil, no significant difference between

	G2677T			C3435T		
Efflux	GG (n = 22)	GT (n = 14)	TT (n = 9)	CC (n = 17)	CT (n = 14)	TT (n = 15)
5 min	80.2 ± 4.7	80.7 ± 6.0	77.8 ± 3.4	80.9 ± 5.0	79.9 ± 6.3	78.8 ± 3.6
10 min	67.2 ± 6.1	67.8 ± 5.1	63.6 ± 5.7	68.9 ± 5.7	65.6 ± 5.6	65.2 ± 5.6
15 min	55.6 ± 6.7	57.0 ± 7.4	52.5 ± 4.6	56.8 ± 7.6	54.4 ± 6.5	54.7 ± 5.6
30 min	27.5 ± 4.7	26.9 ± 5.0	24.6 ± 3.7	28.0 ± 5.3	26.7 ± 4.4	25.5 ± 3.9
Verapamil*	88.7 ± 6.0	86.0 ± 7.2	89.5 ± 5.3	89.7 ± 6.3	84.6 ± 6.7	88.8 ± 5.0

Data are presented in percentage as mean ± SD. *P*-values according to unpaired *t*-test exceeded values 0.05. Numbers in parenthesis indicate the number of individuals.

*Cells were incubated 30 min in Rh123-free medium with verapamil (1 μg/mL).

Table II Rh123 fluorescence in $CD56^+$ natural killer cells from individuals with different genotypes of the MDR1 gene in exon 21 (G2677T) and exon 26 (C3435T) after 5, 10, 15, and 30 min efflux in comparison with control (100%).

	G2677T			C3435T		
Efflux	GG (n = 22)	GT (n = 14)	TT (n = 9)	CC (n = 17)	CT (n = 14)	TT (n = 15)
15 min	83.6 ± 5.9	84.8 ± 5.0	82.1 ± 5.2	84.2 ± 5.3	84.2 ± 6.5	82.6 ± 5.0
30 min	76.1 ± 7.3	75.9 ± 5.5	76.1 ± 5.7	77.2 ± 5.5	75.0 ± 7.6	75.7 ± 6.3
60 min	67.0 ± 7.8	67.5 ± 6.2	66.4 ± 7.7	68.6 ± 7.5	66.7 ± 6.6	65.6 ± 7.4
90 min	62.6 ± 7.8	60.5 ± 7.5	60.5 ± 8.7	64.4 ± 7.0	60.1 ± 7.8	59.3 ± 8.1
Verapamil*	91.8 ± 3.9	93.8 ± 4.1	90.3 ± 5.7	92.8 ± 3.3	91.6 ± 5.0	91.7 ± 5.5

Data are presented in percentage as mean ± SD. *P*-values according to unpaired *t*-test exceeded values 0.05. Numbers in parenthesis indicate the number of individuals.

*Cells were incubated 90 min in Rh123-free medium with verapamil (1 μg/mL).

Table III Rh123 fluorescence in $CD4^+$ T-helper cells from individuals with different genotypes of the MDR1 gene in exon 21 (G2677T) and exon 26 (C3435T) after 15, 30, 60, and 90 min efflux in comparison with control (100%).

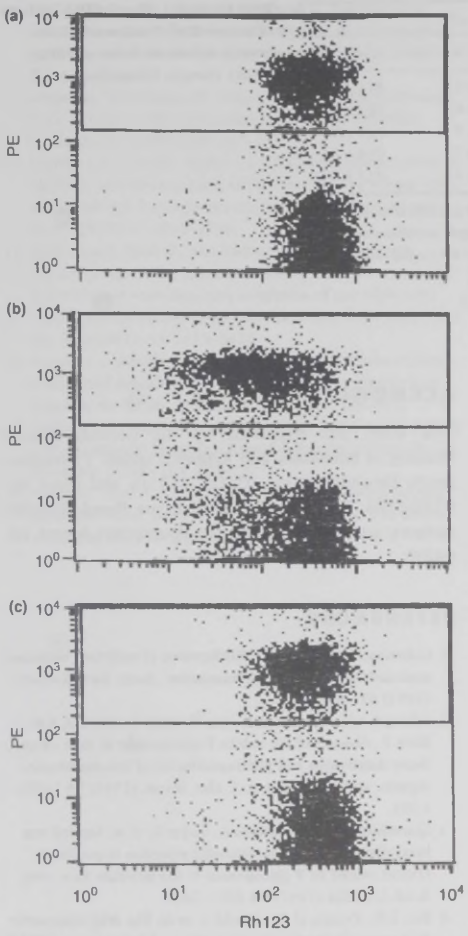

Figure 4 Two-dimensional flow-cytometric dot plots of a two-color flow cytometric assay of natural killer cells. PE-CD56 vs. Rh123 fluorescence is shown. Peripheral blood mononuclear cells were incubated with Rh123 in a final concentration of 150 ng/mL. After accumulation, control sample was put on ice for measurement of the baseline dye uptake. All other samples were exposed to efflux in Rh123-free medium. After staining with PE-conjugated anti-CD56 antibody, median Rh123 fluorescence was determined in cells gated for lymphocytes and PE-CD56 profile. Median Rh123 fluorescence in control sample (a) was set as 100%. Rh123 fluorescence in efflux samples incubated for 30 min in a Rh123-free medium without verapamil (b) and with verapamil (c) was used to calculate the percentage of Rh123 retained in the cells in comparison with control.

individuals with different MDR1 genotypes was observed (data not shown).

We could not find a significant induction of Pgp activity after a 10-day rifampin administration in $CD56^+$ and $CD4^+$ cells in healthy individuals (*Table IV*). Also, there was no difference in Rh123 efflux in $CD56^+$ and $CD4^+$ cells incubated with verapamil before and after rifampin treatment.

DISCUSSION

In the present study, we determined Rh123 efflux in peripheral blood $CD56^+$ NK and $CD4^+$ T-helper cells from healthy individuals with different MDR1 genotypes at G2677T and C3435T. Statistical analysis revealed no significant difference in Pgp activity between individuals with different MDR1 genotypes.

P-gp has been found to be expressed and functionally active in all peripheral blood cells, except in granulocytes [14]. The expression and function of Pgp appeared to be the highest in $CD56^+$ NK and $CD8^+$ T cells. A good correlation between Rh123 efflux, Pgp expression and MDR1 mRNA level has been found, indicating that Rh123 efflux provides a surrogate marker for Pgp expression [14,15]. For studies designed to overcome Pgp-mediated resistance, measurement of the extent of *in vivo* inhibition of Pgp-mediated drug efflux is deemed essential. Also, it has been shown that the effect of PSC833, a selective inhibitor for Pgp, decreases with an increase in Pgp expression and the high Pgp expression could explain an incomplete reversal of Pgp-mediated Rh123 efflux by PSC833 in some patients [15]. Therefore, no Pgp inhibitor was used for pretreatment of control cells in our study. However, one sample, incubated with verapamil in a concentration of 1 μg/mL, was used as a positive control for the method.

A strong association between the two polymorphisms G2677T in exon 21 and C3435T in exon 26 has been found [16]. The G → T transversion at position 2677 results in amino acid Ala893 change by Ser. MDR1 mutation C3435T in exon 26 does not change amino acid sequence and is not located at a promoter region. Therefore, it was supposed that the linked mutation G2677T rather than C3435T is causative for individual differences in Pgp expression. No association between the Rh123 efflux and the G2677T mutation was found in the present study. Hitzl et al. demonstrated a slower Rh123 efflux in $CD56^+$ NK cells in individuals with TT genotype at 3435 compared with CC genotype [17]. However, Calado et al. found no significant differences in

Efflux	$CD56^+$ cells Before (n = 15)	After (n = 15)	Efflux	$CD4^+$ cells Before (n = 15)	After (n = 15)
5 min	79.0 ± 3.2	80.2 ± 3.3	15 min	84.9 ± 5.8	86.1 ± 5.7
10 min	63.9 ± 6.1	67.1 ± 6.5	30 min	76.9 ± 6.4	77.7 ± 6.0
15 min	53.6 ± 5.5	55.7 ± 6.4	60 min	67.7 ± 7.1	68.1 ± 7.2
30 min	25.5 ± 3.7	27.3 ± 6.4	90 min	62.8 ± 7.3	63.9 ± 9.8
Verapamil*	88.2 ± 6.2	88.4 ± 5.4	Verapamil**	90.5 ± 4.3	92.7 ± 5.5

Data are presented in percentage as mean ± SD in comparison with control, set as 100% after baseline dye uptake. *P*-values according to paired *t*-test exceeded values 0.05 indicating no significant difference in Rh123 efflux before and after rifampin administration. Numbers in parenthesis indicate the number of individuals.
*Cells were incubated 30 min in Rh123-free medium with verapamil (1 μg/mL).
**Cells were incubated 90 min in Rh123-free medium with verapamil (1 μg/mL).

Table IV Rh123 efflux in $CD56^+$ natural killer and $CD4^+$ T-helper cells from healthy individuals before and after 10-day rifampin administration.

Rh123 efflux in $CD34^+$ cells between different G2677T and C3435T genotypes [18]. There is a considerable overlap between the substrate specificity of Pgp and other ABC family transporters. Recently, Daoud et al. reported that Rh123 also binds to multiple sites on the multidrug-resistant protein 1 [19]. The contribution of ABC transporters other than Pgp to the transport of known Pgp substrates might explain the lack of association with MDR1 genetic polymorphisms as found in several studies. We also consider that the differences between our study and Hitzl et al. may result from different experimental design.

Experimental evidence obtained from human colon carcinoma cell lines indicated that Pgp expression was upregulated by rifampin [20]. Pgp induction by rifampin treatment in enterocytes from healthy volunteers has been found by Hoffmeyer et al. [11], with the highest induction in individuals with CC genotype at 3435 of the MDR1. In our study, Rh123 efflux in $CD56^+$ NK and $CD4^+$ T-helper cells was not enhanced after a 10-day rifampin treatment. Greiner et al. detected increased Pgp levels in enterocytes from healthy volunteers during rifampin administration [9]. Concomitantly, the oral bioavailability of digoxin decreased 30.1%. Renal clearance was not altered and only a limited effect was found after intravenous digoxin administration. These findings indicate the possibility of tissue-specific regulation of Pgp expression.

In conclusion, using a Rh123 efflux assay we could not find significant differences in Pgp activity in peripheral blood lymphocytes from healthy individuals with different MDR1 genotypes at G2677T and C3435T. However, other mutations in the MDR1 gene could determine the genetically determined effect on Pgp activity.

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II

Gerloff T, Schaefer M, Johne A, **Oselin K**, Meisel C, Cascorbi I, Roots I. MDR1 genotype do not discriminate between absorptive pharmacokinetic parameters of a single oral dose of 1 mg digoxin in healthy white males. British Journal of Clinical Pharmacology 2002;54:610–616.

MDR1 genotypes do not influence the absorption of a single oral dose of 1 mg digoxin in healthy white males

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Aims A noncoding single nucleotide polymorphism (SNP) in exon 26 3435C > T of the highly polymorphic MDR1 gene has been demonstrated to alter digoxin absorption after induction of the MDR1 gene product P-glycoprotein by rifampicin or after multiple oral dosing. The aim of the study was to investigate the effects of the major known MDR1 SNPs on the absorption of digoxin after a single oral dose in a large sample without drug pretreatment.

Methods Fifty healthy white male subjects between the age of 18 and 40 years were enrolled. Following an overnight fast, all subjects received a single oral dose of 1 mg digoxin. Venous blood samples were taken at intervals up to 4 h post dose to obtain a pharmacokinetic profile.

Results AUC(0,4 h), C_{max} and t_{max}, used as indices of digoxin absorption, were not significantly different in any of the genotype groups tested. In particular, there was no significant difference between homozygous carriers of the C and T allele in exon 26 3435 (AUC(0,4 h) 9.24 and 9.38 μg l^{-1} h, C_{max} 4.73 and 3.81 μg l^{-1}, t_{max} 0,83 and 01.14 h).

Conclusions This lack of effect of the major MDR1 SNPs on digoxin absorption might be explained by saturation of the maximum transport capacity of intestinal Pgp at the dose used.

Keywords: drug absorption, P-glycoprotein, pharmacogenetic effects

Introduction

The intestinal epithelium is not only involved in the absorption of nutrients, water, xenobiotics and drugs, but also has barrier and excretory functions. The latter are mainly provided by P-glycoprotein (Pgp), the product of the MDR1 gene, which is a major determinant of drug export from enterocytes into the gut lumen. Pgp is a member of the ATP-binding cassette (ABC) superfamily of membrane transporters [1] and was originally discovered through its ability to confer resistance to antineoplastic agents in tumour cells [2]. A broad range of diverse, structurally unrelated compounds, including the cardiac glycoside digoxin [3], anthracycline antibiotics, vinca alkaloids, epipodophyllotoxins [4, 5], daunomycin, and the immunosuppressant cyclosporin A [6] are transported from cells by Pgp in an ATP-dependent manner. Pgp is located as a 170 kDa integral membrane protein in the apical pole of luminal epithelial cells of the stomach, the small intestine, the colon [7], the biliary canaliculi of the liver, and the brush border of the renal proximal tubules [8]. The expression of Pgp in these tissues is related to its role in the excretion of substances into the gut, the bile, and the urine. Furthermore Pgp can be found in the endothelium of capillary blood vessels in brain, testis, and other blood–tissue barrier sites [9–11]. The extensive tissue distribution and the wide variety of therapeutically relevant compounds that are transported by Pgp, clearly indicate its important role in drug absorption, distribution, and elimination. Therefore, alterations of the transport function or the degree of expression of Pgp are likely to affect the pharmacokinetics of many drugs.

Recently a number of single nucleotide polymorphisms (SNPs) of the MDR1 gene have been identified [12–14]. Most of the SNPs are intronic or silent and therefore do not change the amino acid composition of Pgp. However, the silent polymorphism in exon

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26 3435C > T was found to correlate significantly with the amount of intestinal Pgp protein and the extent of absorption of digoxin in a small number of subjects ($n = 8$) pretreated with rifampicin or given multiple doses (0.25 mg day^{-1}) of digoxin [12]. However, it is unclear whether this pharmacogenetic effect requires Pgp induction or steady state conditions. Therefore, in the present study we investigated the effects of the major MDR1 SNPs, including that in exon 26 3435C > T on the absorption of a single 1 mg oral dose digoxin in a large sample of 50 subjects without pretreatment with rifampicin.

Methods

Subjects

A total of 50 healthy unrelated Caucasian male volunteers (nonsmokers) with a median age of 26 years were enrolled from the Berlin area. They were considered to be healthy as determined by their medical histories, physical examination, electrocardiogram, urine analysis, and routine tests of biochemistry, haematology, hepatitis B and C, and HIV. Their weight ranged from 45.1 kg to 74.6 kg. All volunteers refrained from alcohol, coffee, tea or cola beverages consumption and did not take medications during the study. All subjects gave their written informed consent before entry into the study. The investigation was approved by the local ethics committee of the Charité Medical Center, Humboldt University of Berlin. MDR1 genotype was determined using polymerase chain reaction (PCR)–based restriction fragment length polymorphism (RFLP) assays [13].

Study protocol

After an overnight fast, each subject received a single oral dose of 1 mg digoxin (Dilanacin™, Arzneimittelwerk Dresden GmbH, Radebeul) along with 200 ml water. Venous blood samples (5 ml) for drug analysis were collected into syringes containing ethylene diaminetetraacetic acid (EDTA) before and 10, 20, 30, 35, 40, 45, 50, 60, 75, 90, 120, 180, and 240 min after digoxin intake. The plasma was separated by centrifugation and immediately stored in polypropylene tubes at −22 °C.

Digoxin concentration measurements

Digoxin plasma concentrations were determined by a microparticle enzyme immunoassay (IMx® Digoxin Assay, Abbott Laboratories; USA). The lower limit of quantification was 0.3 μg l^{-1}. The plasma samples of each volunteer were analyzed in duplicate together with calibration and quality control samples. Further calculations were done with the respective mean values. The inter-assay coefficients of variation at plasma concentrations of 0.9 μg l^{-1}, 1.9 μg l^{-1} and 3.2 μg l^{-1} were 8.37%, 5.75% and 4.68%, respectively.

Pharmacokinetic analysis

Previous investigations demonstrated that pharmacokinetic differences between the genotypic groups occur primarily during the initial hours of absorption and are best reflected by area under the plasma concentration time curves (AUCs) covering the early hours after oral drug intake [15–17]. The t_{max} for digoxin is known to be a variable parameter [18] ranging from 0.5 to 3 h in our sample. Thus, an AUC from 0 to 4 h AUC(0,4 h) was considered a suitable measure of digoxin absorption. AUC(0,4 h) was calculated by use of the trapezoidal rule, using WinNonlin™ (professional edition; version 1.5, Pharsight Corporation, Mountain View, CA, USA). C_{max} and t_{max} of digoxin were derived directly from the measured values.

Statistical analysis

The primary aim of this exploratory study was to evaluate the functional relevance of major known MDR1 SNPs on digoxin absorption represented by AUC(0,4 h). For a two groups comparison the Mann–Whitney-U two sample test was used. When more than two groups were being compared the Kruskal–Wallis test was used. A P value of <0.05 was considered significant. Calculations were done using SPSS™ software (version 9.0, SPSS Inc., Chicago, USA).

Results

To study the influence of MDR1 variant genes on digoxin pharmacokinetics we included SNPs occurring at a frequency of at least 10% in the Caucasian population (exon 6 + 139C > T, exon 17–76T > A, 3435C > T), SNPs changing the amino acid composition (61 A > G, 1199G > A, 2677G > T > A), and a SNP that directly precedes the translation start codon of the MDR1 gene (exon 2–1G > A). The allelic frequency distribution of the investigated genetic variants in our sample (Table 1) is in good agreement with previous studies [12, 13] and did not show significant deviation from Hardy–Weinberg equilibrium.

After administration of a single oral dose of 1 mg digoxin the pharmacokinetics of the absorptive phase characterized by AUC(0,4 h), C_{max} and t_{max} was not significantly different between the genotypic groups. Comparison of the linked MDR1 polymorphisms in exon 21 2677 and exon 26 3435 also did not discrimi-

Table 1 Location, position, effects of protein expression, allelic frequencies and genotype prevalence of the MDR1 variants studied.

Location	*Position*	*Allele*	*Effect*	*Allelic frequency (%)*	*Allelic frequency (14) (%)*	*Genotype*	*Genotype prevalence (observed) (%)*	*Genotype prevalence (95% confidence interval)*
Intron 1	Exon 2–1	G	initiation of translation?	93.0	91.0	G/G	86.0	73.3, 94.2
		A		7.0	9.0	G/A	14.0	5.8, 26.7
						A/A	0.0	0.0, 0.1
Exon 2	cDNA 61	A	21Asn	87.0	88.8	A/A	74.0	59.7, 85.4
		G	21Asp	13.0	11.2	A/G	26.0	14.6, 40.3
						G/G	0.0	0.0, 0.1
Intron 6	Exon 6 + 139	C	?	61.0	62.8	C/C	36.0	22.9, 50.8
		T		39.0	37.2	C/T	50.0	35.5, 64.7
						T/T	14.0	5.8, 26.7
Exon 11	cDNA 1199	G	400Ser	98.0	94.5	G/G	96.0	86.3, 99.5
		A	400Asn	2.0	5.5	G/A	4.0	0.5, 13.7
						A/A	0.0	0.0, 0.1
Intron 16	Exon 17–76	T	?	53.0	53.8	T/T	28.0	16.2, 42.5
		A		47.0	46.2	T/A	50.0	35.5, 64.5
						A/A	22.0	11.5, 35.7
Exon 21	cDNA 2677	G	893Ala	52.0	56.5	G/G	24.0	13.1, 38.2
		T	893Ser	38.0	41.6	G/T	42.0	28.2, 57.0
		A	893Thr	10.0	1.9	T/T	14.0	5.8, 26.7
						G/A	14.0	5.8, 26.7
						T/A	6.0	1.3, 16.6
						A/A	0.0	0.0, 0.1
Exon 26	cDNA 3435	C	Wobble	49.0	46.1	C/C	24.0	13.1, 38.2
		T		51.0	53.9	C/T	50.0	35.5, 64.5
						T/T	26.0	14.6, 40.3

The positons of the MDR1 polymorphisms correspond to the MDR1 cDNA (GenBank accession no. AC002457/AC005068). The first base of the ATG start codon is set to no. 1. Intronic SNPs are described as (exon ± n), with n bases upstream (–) or downstream (+) of the exons. Eight polymorphisms were analysed.

nate between subjects carrying the genotype exon 21 2677GG/exon 26 3435CC and exon 21 2677TT/exon 26 3435TT. Additional homozygous allelic combinations were not investigated due to low frequencies. Pharmacokinetic data and statistical analysis are summarized in Table 2. There were also no significant differences after normalization to ideal body weight to eliminate interindividual differences in weight and height of the subjects. There was considerable variation in the kinetic parameters within each genotypic group (Figure 1).

Discussion

Very recently a number of MDR1 SNPs were detected and tested preliminarily for their ability to alter the pharmacokinetics of orally administered digoxin [12]. The main findings were that a polymorphism in exon 26 (3435C > T), although noncoding, resulted in a more than 2-fold decrease in duodenal epithelial Pgp protein content in homozygous T/T subjects compared with carriers of the C/C variant. This was accompanied by a significantly higher digoxin bioavailability in the T/T genotype. The finding was supported by a lowered efflux of rhodamine-123 from $CD56^+$ natural killer cells, mediated by Pgp, in 3435TT compared with CC carriers [19]. These studies were limited because of the small number of subjects investigated. Accordingly the aim of the present study was to extend the sample number to seek for additional pharmacokinetic effects of the major MDR1 SNPs and to evaluate the impact of the known functional active exon 26 3435C>T polymorphism.

In contrast to the previously reported results [12] no significant differences in AUC(0,4 h), C_{max} and t_{max} were observed between MDR1 genotype combinations in this study.

A major finding of one of the previous studies was that the observed difference in the bioavailability of digoxin between genotypes with respect to exon 26 3435C > T SNP was observed only after pretreatment with rifampicin [12]. This antibiotic is reported to be a strong inducer of intestinal Pgp expression [16], increasing protein content 3.5 fold above control levels. The ability of rifampicin to increase epithelial Pgp content was significantly higher in individuals carrying the C/C

Table 2 Pharmacokinetic parameters and statistical analysis.

			AUC(0,4 h) ($\mu g\ l^{-1}\ h$)			C_{max} ($\mu g\ l^{-1}$)			t_{max} (h)		
Location	*Position*	*Genotype*	P *value*	*Mean*	*Difference between mean of SNP and wild type groups (95% CI)*	P *value*	*Mean*	*Difference between mean of SNP and wild type groups (95% CI)*	P *value*	*Mean*	*Difference between mean of SNP and wild type groups (95% CI)*
Intron 1	Exon 2–1	G/G	0.426	9.17		0.780	4.00		0.232	1.02	
		G/A		9.73	0.563 (−0.81, 1.94)		3.98	0.00 (−1.01, 0.97)		0.80	−0.22 (−0.59, 0.15)
Exon 2	cDNA 61	A/A	0.091	9.51		0.382	4.11		0.815	0.96	
		A/G		8.51	−1.00 (−2.05, 0.06)		3.68	−0.43 (−1.20, 0.34)		1.09	0.13 (−0.17, 0.42)
Intron 6	Exon 6 + 139	C/C	0.785	9.22		1.000	4.10		0.907	0.96	
		C/T		9.17	0.05 (−1.05, 1.15)		3.90	0.19 (−0.58, 0.97)		1.00	−0.04 (−0.33, 0.25)
		T/T		9.60	−0.38 (−1.84, 1.08)		4.08	0.01 (−1.21, 1.24)		1.06	−0.10 (−0.46, 0.25)
Exon 11	cDNA 1199	G/G	–	9.21		–	4.03		–	0.98	
		G/A		10.21	–		3.26	–		1.18	–
Intron 16	Exon 17–76	T/T	0.718	9.01		0.294	3.88		0.228	1.07	
		T/A		9.46	−0.45 (−1.58, 0.68)		4.21	−0.33 (−1.14, 0.47)		0.92	0.15 (−0.17, 0.47)
		A/A		9.07	−0.05 (−1.39, 1.28)		3.67	0.21 (−0.76, 1.17)		1.05	0.03 (−0.33, 0.38)
Exon 21	cDNA 2677	G/G	0.875	9.06		0.648	4.22		0.736	1.00	
		G/T		9.10	−0.03 (−1.28, 1.21)		3.91	0.30 (−0.57, 1.17)		1.00	−0.06 (−0.36, 0.35)
		T/T		8.95	0.11 (−1.51, 1.74)		3.69	0.53 (−0.77, 1.83)		1.10	−0.10 (−0.50, 0.30)
		G/A		9.91	−0.84 (−2.65, 0.96)		4.00	0.21 (−1.30, 1.73)		0.85	0.15 (−0.20, 0.50)
		T/A		10.50	–		4.39	–		0.96	–
Exon 26	cDNA 3435	C/C	0.295	9.72		0.799	4.12		0.871	0.92	
		C/T		8.89	0.83 (−0.39, 2.05)		3.87	0.26 (−0.64, 1.15)		1.05	−0.12 (−0.46, 0.22)
		T/T		9.51	0.20 (−1.10, 1.51)		4.14	−0.02 (−0.98, 0.94)		0.95	−0.03 (−0.30, 0.24)
Exon 21/	cDNA	2677 3435									
Exon 26	2677/	G/G C/C	0.393	9.06		0.712	3.84		0.478	0.90	
	3435	T/T T/T		9.06	0.00 (−1.78, 1.78)		3.69	0.15 (−1.07, 1.36)		1.11	−0.21 (−0.68, 0.26)

Pharmacokinetic parameters were compared between the wildtype groups and those carrying a SNP using the nonparametric Mann–Whitney-U two sample test or Kruskal–Wallis test. A P value of < 0.05 was considered significant. P values and 95% CI were not given for subject groups with a lower number than 4.

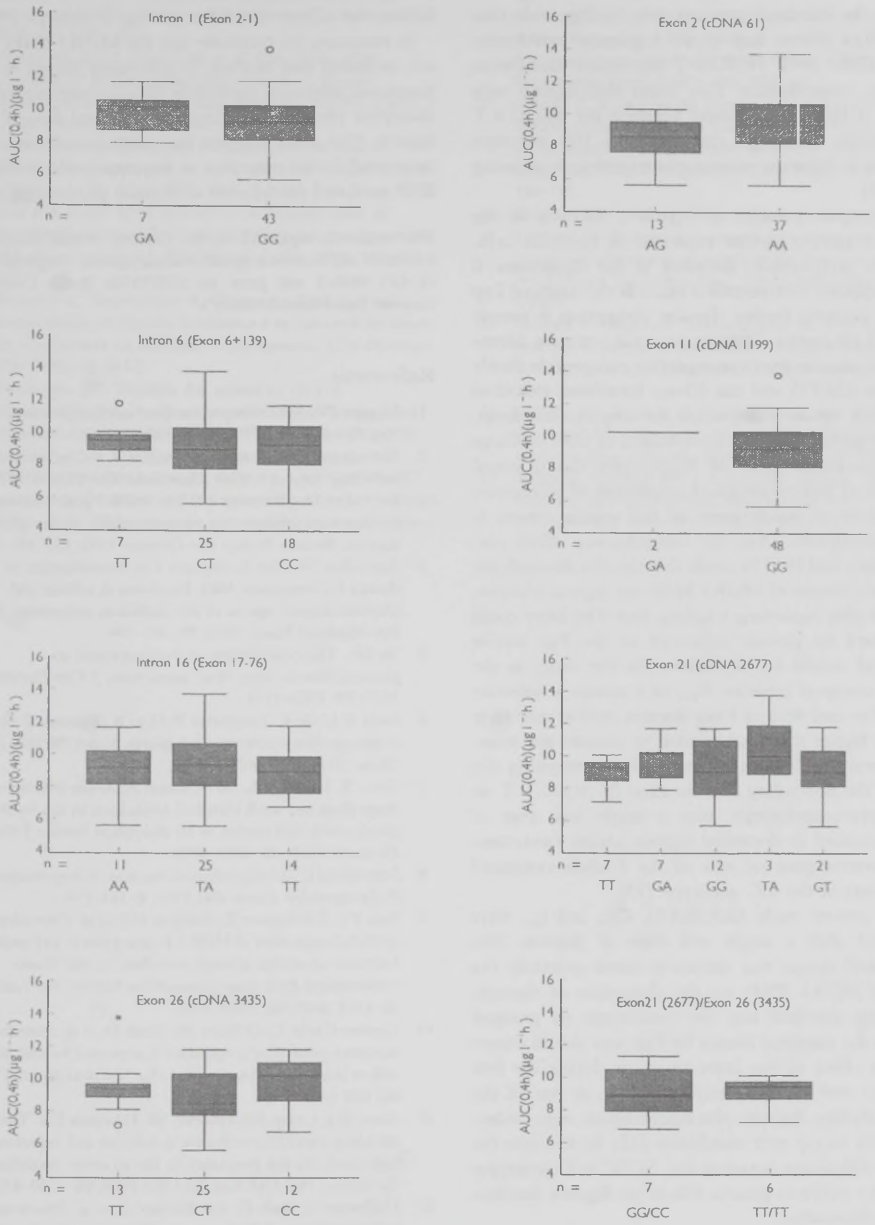

Figure 1 AUC(0,4 h) data for digoxin after a 1 mg oral dose in relation to the MDR1 polymorphisms. *n* = number of subjects in each group.

genotype in MDR1 exon 26 3435C > T than in subjects homozygous for the T allele. Since digoxin is transported back into the intestinal lumen mainly by Pgp, induction of this efflux system may be an important prerequisite for the MDR1 SNP 3435C > T dependent modulation of digoxin bioavailability. This could also explain why there was a lack of association between the 3435C > T polymorphism and Pgp expression in 100 placentas obtained from Japanese women not treated with inducing agents [14].

The transport capacity of Pgp is a function of the amount of carrier protein expressed in epithelial cells. Digoxin is preferentially absorbed in the duodenum. If the local digoxin concentration exceeds the maximal Pgp secretory capacity, further digoxin absorption is merely dependent on passive diffusion or uptake carriers. Members of the organic anion-transporting polypeptide family in humans (OATP) and rats (Oatp) have been identified as candidate uptake transporters for amphipathic drugs, including digoxin [20, 21]. Localization of OATPs/Oatps to the basolateral pole of hepatocytes facing portal venous blood and to the apical membrane of enterocytes [22], suggests an involvement of this uptake system in digoxin absorption. Thus, the counteracting effects passive diffusion and OATPs could dramatically diminish the modulatory actions of MDR1 SNPs on digoxin pharmacokinetics after exceeding a ceiling dose. The latter could be increased by protein induction of the Pgp carrier system and would be dependent on the drug, as the maximal transport capacity V_{max} of a carrier is substrate specific. The oral dose of 1 mg digoxin used in our study might be higher than that needed to saturate the transport capacity of Pgp. A recent study investigating the effects of the noncoding SNP in exon 26 3435C > T on digoxin pharmacokinetics after a single low dose of 0.25 mg resulted in decreased digoxin plasma concentration in homozygous subjects of the T allele compared with carriers of the CC genotype [23].

In the present study AUC(0,4 h), C_{max} and t_{max} were determined after a single oral dose of digoxin. This experimental design was chosen to assess primarily the impact of MDR1 SNPs on the absorption of digoxin. Since drugs absorbed into the enterocytes are pumped back into the intestinal lumen by Pgp, one would expect the major effect of the latter to occur during the first hours after oral administration. However, in one of the previous studies, digoxin pharmacokinetics were monitored under steady state conditions [12]. In this case the observed differences between the 3435C > T genotypes may be due partly to genetic effects on digoxin distribution and elimination.

In our study there was a large variation in digoxin pharmacokinetics within each genotypic group. This high variability indicates that additional factors may influence the absorption of digoxin. These may include other as yet undetected SNPs or dietary and other environmental factors, that induce or inhibit the Pgp activity [5, 24, 25].

In summary, we conclude that the MDR1 SNPs studied, including that in exon 26 previously shown to have functional relevance, apparently do not contribute to the absorptive pharmacokinetics of a single oral dose of 1 mg digoxin. Our study suggests that other genetic and environmental factors may play an important role in MDR1 SNP mediated modulation of digoxin pharmacokinetics.

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III

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Quantitative determination of MDR1 mRNA expression in peripheral blood lymphocytes: a possible role of genetic polymorphisms in the MDR1 gene

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Abstract

Background P-glycoprotein is a transmembrane efflux pump that extrudes a wide variety of drugs, thereby reducing their intracellular access. In humans, P-glycoprotein is encoded by the MDR1 gene. Recently, several single nucleotide polymorphisms in the MDR1 gene were identified. Moreover, it was postulated that, in addition to the full-length P-glycoprotein, a 'mini' P-glycoprotein was also present in lymphocytes.

Materials and methods We investigated the effect of the genetic polymorphisms G2677T and C3435T in the MDR1 gene on MDR1 mRNA expression in FACS-sorted peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells. MDR1 mRNA expression was determined in 45 healthy individuals using a real-time quantitative RT-PCR.

Results We detected the highest expression of MDR1 mRNA in $CD56^+$ cells, followed by $CD8^+ > CD4^+ > CD19^+$ cells. However, genetic polymorphisms of the MDR1 gene failed to affect ($P > 0{\cdot}05$) MDR1 mRNA levels in the peripheral blood lymphocytes. Furthermore, the transcript levels for the MDR1 N-terminal half were almost two-fold lower than that of the MDR1 C-terminal half in all cell populations investigated ($P < 0{\cdot}0001$).

Conclusions An almost two-fold difference in MDR1 C- and N-terminal half expressions supports the presence of mini-P-glycoprotein, an alternatively spliced form of the full-length molecule, in peripheral blood lymphocytes.

Keywords P-glycoprotein, peripheral blood lymphocytes, MDR1 mRNA expression, MDR1 genetic polymorphism.
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Introduction

P-glycoprotein (Pgp)-related transport of chemotherapeutics is a well-characterized mechanism of chemoresistance in cancer therapy. Overexpression of Pgp has been found in different cancers, correlating with lack of response to chemotherapy and poor survival in some malignancies [1,2]. Within haematopoietic cells, Pgp has been detected in peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells, with the highest level in cells with cytotoxic activity, $CD56^+$ natural killer (NK) and $CD8^+$ T cells [3,4]. In several studies, NK and T cell-mediated cytotoxicity was inhibited by Pgp pharmacological inhibitors and anti-Pgp monoclonal antibodies [5,6]. In human T lymphocytes, Pgp has been reported to participate in the transport of cytokines, including IL-2, IL-4 and IFN-γ [7,8]. These findings indicate that Pgp may play a role in specific immunological functions.

In humans, Pgp is encoded by the MDR1 gene, a member of the ATP-binding cassette transporter family. Recently, several single nucleotide polymorphisms in the MDR1 gene were identified [9]. C3435T in exon 26 was found to determine intestinal Pgp expression. Comparison of area-under-the-curve (AUC) values for digoxin after oral administration revealed that subjects homozygous for TT had significantly higher AUC values than those with CC genotype. Fellay *et al.* [10] demonstrated that the C3435T polymorphism

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predicted immune recover after initiation of antiretroviral treatment in HIV-1-infected individuals, as estimated by a greater rise in $CD4^+$ cell count in TT than in CC or CT patients. However, no association between the C3435T mutation and the doses needed to maintain similar cyclosporine A trough concentrations was observed in renal transplant recipients [11].

The aim of the present study was to evaluate Pgp expression, by the means of the mRNA level, in peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells from healthy individuals with different MDR1 genotypes. The influence of two partially linked polymorphisms, G2677T in exon 21 and C3435T in exon 26 of the MDR1 gene, was investigated in 45 healthy individuals. A previous study has shown that NK cells did not express detectable levels of full-length 170 kDa Pgp [12]. The presence of a small-molecular-weight mini-Pgp in peripheral blood NK cells was postulated. Hence, two specific primer pairs, designed to distinguish the full-length and the suggested mini-Pgp were used in RT-PCR.

Materials and methods

Clinical study

The study protocol was approved by the Ethics Committee of Humboldt University. After an informed consent, 45 healthy Caucasians (six female and 39 male, age range 21–33 years) were enrolled. The subjects took no medication and were ascertained to be healthy by a clinical examination.

Materials

All oligonucleotide primers and hybridization probes for PCR and RT-PCR were purchased from Tib Molbiol (Berlin, Germany). Biocoll lymphocyte separating solution (density 1·077) was from Biochrom KG (Berlin, Germany). Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies were obtained from Immunotech Beckman Coulter (Munich, Germany); allophycocyanin (APC)- and periclinin chlorophyll protein cyanine 5 conjugate (PerCP-Cy5·5)-conjugated antibodies were obtained from Becton Dickinson (San Jose, CA USA). Recombinant Rnasin® ribonuclease inhibitor, T7 RNA polymerase and RQ1-DNase were purchased from Promega (Madison, WI, USA); and M-MLV reverse transcriptase and *Taq* DNA polymerase from Invitrogen (Karlsruhe, Germany).

DNA isolation and genotyping of MDR1 exon 21 G2677T and exon 26 C3435T polymorphisms

DNA was isolated from 200 µL of whole blood by MagNA Pure LC (Roche, Mannheim, Germany). Genotyping was performed on real-time PCR assays in a LightCycler™ (Roche), as described previously [13].

Cell sorting assay and isolation of total RNA

Peripheral blood mononuclear cells (PBMC) were isolated from 20 mL of whole blood by gradient centrifugation over Biocoll lymphocyte separating solution (density 1·077) according to the manufacturer's protocol. The lymphocyte layer was washed twice with PBS/0·1%BSA and stained with FITC-anti-CD4, APC-anti-CD8, PerCP-Cy5·5-anti-CD19 and PE-anti-CD56 antibody. Appropriate isotype controls were used. Cells were sorted using FACSDiva 8-color Turbosorter (Becton Dickinson). The samples were gated on forward-scatter vs. side-scatter to include lymphocytes. A second gate was set around the FITC-, APC-, PerCP-Cy5·5- or PE-positive cells. Purity of sorted cells was analyzed with FACSCalibur and was = 98% (n = 10). Total RNA from FACS-sorted cells was isolated with the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA concentration was measured spectrophotometrically and samples were maintained at −80 °C until analysis.

Real-time quantitative RT-PCR (LightCycler) analysis

cDNA was synthesized from total RNA in a 25-µL final reaction volume containing 50 ng of sample RNA or dilutions of standard RNA (run in the same plate but separate tubes), 1 × First Strand Buffer, 10 mM of dithiothreitol, 0·4 mM of each dNTP, 8 U of ribonuclease inhibitor, 120 U of M-MLV reverse transcriptase and 0·8 µM of oligo dT(15) primer. The mixture was preincubated for 10 min at 26 °C, then incubated at 42 °C for 60 min followed by heating at 95 °C for 2 min, and cooled. Quantitative PCR was performed with gene-specific primers in the LightCycler™. The principles of real-time quantitative PCR in a LightCycler has been described elsewhere [14]. To avoid amplification of possible contaminating genomic DNA, the selected forward or reverse primers and/or hybridization probes were designed to span intron/exon junctions. The sequences of primers and hybridization probes are shown in Tables 1 and 2. Also, control reactions without reverse transcriptase were run to verify the absence of contaminating DNA.

PCR amplification was carried out in a 16-µL reaction volume containing 5 µL of cDNA (of 25 µL of total cDNA), 1·25 U of *Taq* DNA polymerase, 1·6 µL of 10× PCR buffer, and $MgCl_2$, dNTPs, reverse and forward primer, hybridization probes, BSA and DMSO optimized for each reaction. The PCR reactions were initiated with denaturation at 94 °C for 60 s, followed by amplification with 45 cycles at 95 °C 0 s, annealing (depending on the primer pair) for 10 s, and 72 °C for 10 s. Annealing temperatures were 55 °C for MDR1 C-terminal half, 60 °C for MDR1 N-terminal half and 60 °C for the housekeeping gene β2-microglobulin. The PCR products migrated as a single fragment with an expected size on an agarose gel. The specificity was verified by direct-sequencing with each one-side primer (ABI Prism™ 310, Applied Biosystems, Foster City, CA).

Table 1 Oligonucleotide primers used for real-time quantitative RT-PCR

Gene	Forward primers* (position)	Reverse primers* (position)	Product (bp)
MDR1 C-half	5′-CCATCATTGCAATAGCAGG-3′ (3018–3036)	5′-GAGCATACATATGTTCAAACTTC-3′ (3163–3185)	168
MDR1 N-half	5′-AAAGTCGGAGTATCTTCTTCCAAG-3′ (359–382)	5′-CCAATTTGAATAGCGAAACATTGA-3′ (536–559)	201
β2-micro globulin	5′-CCAGCAGAGAATGGAAAGTC-3′ (113–132)	5′-CATGTCTCGATCCCACTTAAC-3′ (350–370)	258
T7 primer**	5′-AGAGCGTAATACGACTCACTATAG-GGTATCTGCAGA-		
RT primer***	5′-TTTTTTTTTTTTTTT-		

*Area amplified refers to sequences with accession numbers as follows: NM_000927 (MDR1), NM_004048 (β2-microglobulin).
**T7 promoter sequence was incorporated at the 5′-end of the forward primers to obtain templates for *in vitro* external standard RNA synthesis.
***dT(15) sequence was incorporated at the 5′-end of the reverse primers to obtain templates for *in vitro* external standard RNA synthesis.

Table 2 Sequences of hybridization probes used for real-time quantitative RT-PCR

Gene	Sensor* (position)	Anchor* (position)
MDR1 C-half	5′-TGGGAAGATCGCTACTGAAGCAAT-3′-fluorescein (3100–3123)	5′-LC Red640 – AACTTCCGAACCGTTGTTTC-TTTGA-3′- phosphate (3128–3152)
MDR1 N-half	5′-TTCTTCTTTGCTCCTCCATTGCGG-3′-fluorescein (442–465)	5′-LC Red640 – CCCCTTCAAGATCCATCCC-GACC-3′- phosphate (418–440)
β2-micro globulin	5′-TTCTTCAGTAAGTCAACTTCAATG-TCGGA-3′- fluorescein (170–198)	5′-LC Red705 – ATGAAACCCAGACACATAG-CAATTCAG-3′- phosphate (140–166)

*Area amplified refers to sequences with accession numbers as follows: NM_000927 (MDR1), NM_004048 (β2-microglobulin).

Synthesis of external standards for quantitative real-time RT-PCR analysis

In order to establish the calibration standard curve, external standard RNA was prepared. RT-PCR was carried out in a two-step reaction for each gene, as above-described using the primers listed in Table 1. The subsequent PCR with T7 primer (T7 promoter sequence was incorporated at the 5′-end of the forward primer) and RT primer [dT(15) sequence was incorporated at the 5′-end of the respective reverse primer] was carried out to obtain a template for *in vitro* transcription. After purification with Ultrafree MC 30·000 filters, the template was used for *in vitro* transcription at 37 °C for 90 min using the DNA-dependent T7 RNA polymerase. After digestion with RNase-free RQ1-DNase, RNA transcripts were purified using RNeasy Mini Kit and RNA Cleanup Protocol from QIAGEN. All of the RNA standards were quantified spectrophotometrically and maintained at – 80 °C. The number of standard RNA molecules was determined as described elsewhere [15]. A series of dilutions of external standard RNA, ranging from 10^{10} to 10^{6} copies for β2-microglobulin, and from 10^{8} to 10^{3} copies for MDR1 C- and N-terminal half, were performed. The specificity of obtained transcripts was verified by sequencing with the ABI Prism™ 310 gene analyser.

Quantification of mRNA in the LightCycler™

The data was evaluated with the LightCycler™-run software, version 3·5. The Ct (threshold cycle) values were calculated using the second derivative maximum method. A calibration curve was generated from serial dilutions of the standard RNA by plotting Ct vs. the logarithm of the copies of standard RNA, and was used to calculate the number of RNA copies in the samples. The housekeeping gene β2-microglobulin was used for normalization of MDR1 mRNA expression. Final results were expressed as MDR1 cDNA copies/ng of total RNA and as the normalized ratio of MDR1 cDNA $\text{copies}_{\text{sample}} \times$ β2-microglobulin cDNA $\text{copies}_{\text{mean}}/$β2-microglobulin cDNA $\text{copies}_{\text{sample}}$.

Statistical analysis

Data were presented as the mean ± SD. MDR1 mRNA levels between cell populations and differences in the MDR1 C- and N-terminal half expressions were analyzed by the Wilcoxon signed ranks test. MDR1 mRNA expression between the three genotypes was analyzed by the Kruskal–Wallis test. All calculations were performed with SPSS™ for Windows (version 10·0, SPSS Inc., Chicago, IL.). A *P*-value of 0·05 was considered to be of statistical significance.

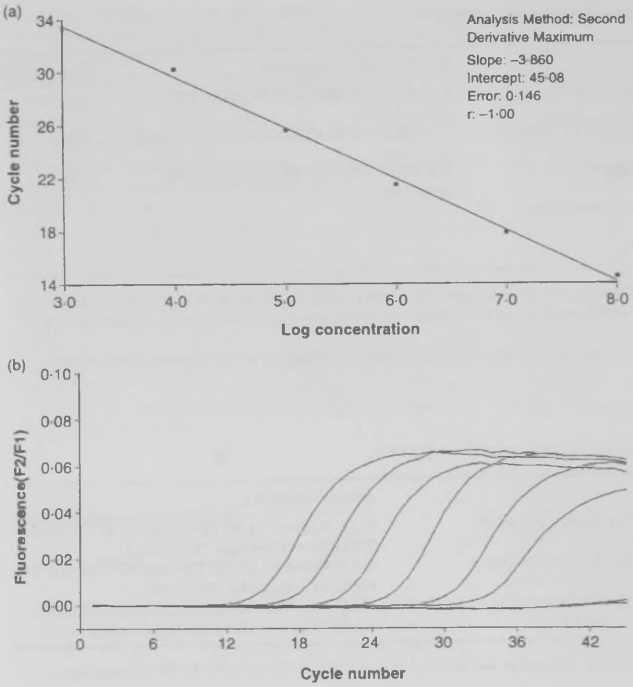

Figure 1 Amplification plot for MDR1 C-terminal half mRNA expression. (a) Calibration curve was generated by plotting the Ct value vs. the log of starting copies of standard RNA. Ct values for samples were interpolated on the calibration curve and used to calculate the number of copies in samples. (b) Amplification curves of serial dilutions of standard RNA, ranging from 1×10^8 to 1×10^3 starting copies, were displayed by plotting the fluorescence data vs. cycle number. No template controls for RT and PCR were also performed.

Results

Quantification of the MDR1 mRNA in CD4$^+$, CD8$^+$, CD19$^+$, and CD56$^+$ cells

Calibration curves were generated from serial dilutions of the external standard cDNAs. The calibration curves were linear as shown by a correlation coefficient (R^2 value) of ≥ 0·98 (Fig. 1a). The slopes (≥ −3·860) were close to the theoretical optimum (−3·322), which indicates a very high PCR efficiency. The coefficients of variation for MDR1 C-terminal half, MDR1 N-terminal half, and β2-microglobulin mRNA levels were 8·9%, 35·9% and 9·7%, respectively, as determined with data obtained from six separate RT-PCR experiments with the same RNA sample ($n = 5$). Figure 1(b) shows a representative amplification plot of MDR1 C-terminal half; similar standard curves were obtained for β2-microglobulin and MDR1 N-terminal half.

Using 50 ng of total RNA, MDR1 mRNA was detected and quantified by RT-PCR in FACS-sorted CD4$^+$, CD8$^+$, CD19$^+$, and CD56$^+$ cells. The MDR1 mRNA C-terminal half exhibited the highest abundance in the CD56$^+$ cells (mean $1{\cdot}43 \times 10^3$ molecules/ng of total RNA ± $4{\cdot}85 \times 10^2$) and a 14-fold variation between individuals (Fig. 2).

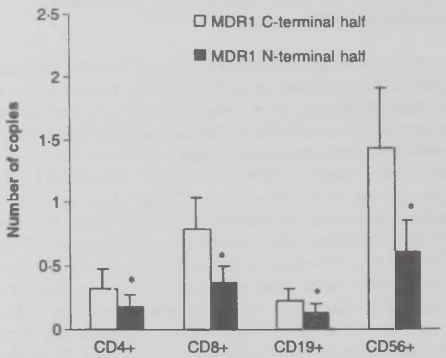

Figure 2 Quantitative determination of the MDR1 C- and N-terminal half mRNA levels in human peripheral blood lymphocytes. Each bar represents the mean ± SD number of copies ($\times 10^3$) ng^{-1} of total RNA, as determined in 45 individuals. *Differences between the C- and N-terminal half expression were significant ($P < 0{\cdot}0001$) in all cell populations, as determined with Wilcoxon's signed ranks test.

The lowest expression of the MDR1 mRNA (c-terminal half) was found in the $CD19^+$ cells (mean $2{\cdot}23 \times 10^2$ molecules/ng of total RNA ± 98·8) with an 8-fold variation between individuals. The differences in MDR1 mRNA expression between cell populations were statistically significant ($P < 0{\cdot}0001$), except $CD4^+$ vs. $CD19^+$ ($P > 0{\cdot}09$). All pairwise differences remained statistically significant at 5% level after applying Bonferroni's correction for multiple comparison.

Effect of MDR1 genetic polymorphisms on MDR1 mRNA expression

We compared the mRNA levels of MDR1 C-terminal half in $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells between individuals with different genotypes in the MDR1 gene. Statistical analysis revealed no significant ($P > 0{\cdot}05$) difference in the MDR1 expression in regard to different genotypes in exon 21 G2677T and in exon 26 C3435T (Fig. 3a–b). Although the difference was not significant, both mutations were associated with the lowest level of mRNA in homozygous mutant individuals in $CD8^+$ and $CD56^+$ cells, and heterozygous individuals displayed an intermediate phenotype. However, these differences in MDR1 expression were not found when the transcript levels were normalized (Table 3). The mean β2-microglobulin values used for normalization were $5{\cdot}8 \times 10^5$ copies for $CD4^+$, $6{\cdot}0 \times 10^5$ copies for $CD8^+$, $3{\cdot}22 \times 10^5$ copies for $CD19^+$, and $6{\cdot}64 \times 10^5$ copies for $CD56^+$ cells, as determined in 45 individuals.

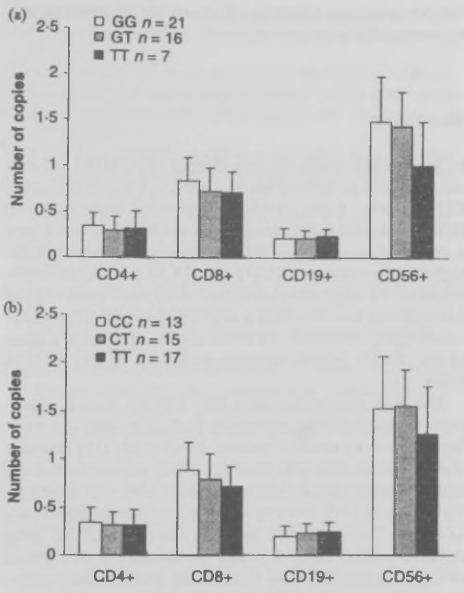

Figure 3 The mRNA levels of the MDR1 C-terminal half in FACS-sorted peripheral blood lymphocytes from individuals with different genotypes in (a) exon 21 G2677T and (b) exon 26 C3435T of the MDR1 gene. One individual was determined to carry TA in exon 21 and was excluded from the analysis of the G2677T polymorphism. Data are presented as mean ± SD number of copies ($\times 10^3$) ng^{-1} of total RNA. *P*-values according to the Kruskal–Wallis test exceeded 0·05.

Mini-Pgp expression in $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells

Pgp is composed of two homologous halves, C- and N-terminal half, each containing six transmembrane domains and an ATP-binding domain [16]. To elucidate the presence of mini- and full-length Pgp in PBMC, primers amplifying the C- and N-terminal half of Pgp were designed. Previous studies have shown that mini-Pgp contains the C-terminal half of the molecule and is not glycosylated [12,17,18]. Hence, the primers amplifying the C-terminal half determined both the mini- and the full-length molecule, but the primers amplifying the N-terminal half determined only the full-length Pgp. Using these primer pairs, we detected the expression of both the N- and C-terminal half of Pgp in all cell types investigated. Indeed, the transcript levels for the MDR1 N-terminal half were almost two-fold lower than that of the MDR1 C-terminal half ($P < 0{\cdot}0001$) in all cell populations (Fig. 2). A comparison of β2-microglobulin-normalized data displayed the same pattern (data not shown). As found for the MDR1 C-terminal half, the genotype

Table 3 Normalized values of MDR1 mRNA (C-terminal half) levels in peripheral blood lymphocytes from individuals with different MDR1 genotypes

	G2677T in exon 21			C3435T in exon 26		
	GG ($n = 21$)	GT ($n = 16$)	TT ($n = 7$)	CC ($n = 13$)	CT ($n = 15$)	TT ($n = 17$)
$CD4^+$	3·47 ± 1·69	3·73 ± 2·18	3·69 ± 1·43	3·23 ± 1·39	4·18 ± 2·33	3·24 ± 1·52
$CD8^+$	8·62 ± 3·68	8·67 ± 2·27	6·90 ± 2·00	8·34 ± 3·53	9·02 ± 2·61	7·97 ± 2·99
$CD19^+$	2·62 ± 1·49	2·30 ± 0·70	2·60 ± 1·45	1·87 ± 0·90	2·94 ± 1·17	2·59 ± 1·34
$CD56^+$	15·5 ± 6·13	15·3 ± 4·49	13·0 ± 6·04	12·6 ± 3·69	18·2 ± 6·07	14·8 ± 6·03

Mean ± SD ($\times 10^2$) are presented. The data was expressed as a ratio of MDR1 cDNA $copies_{sample} \times$ β2-microglobulin $cDNAcopies_{mean}$/β2-microglobulin cDNA $copies_{sample}$. Numbers in parenthesis indicate the number of individuals. One individual was determined to carry TA in exon 21 and was excluded from analysis of the G2677T polymorphism. *P*-values according to the Kruskal–Wallis test exceeded 0·05.

did not have any apparent effect on the N-terminal half expression (data not shown).

Discussion

In the present study, we investigated the MDR1 mRNA expression in peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells from healthy individuals with different G2677T and C3435T genotypes in the MDR1 gene. Using a real-time quantitative RT-PCR assay, we detected the highest expression of MDR1 mRNA in the $CD56^+$ cells, followed by the $CD8^+ > CD4^+ > CD19^+$ cells. These findings are in accordance with studies published previously [3,4]. However, we could find no significant impact of the MDR1 genetic polymorphisms on MDR1 mRNA levels.

The association between the MDR1 genetic polymorphisms and Pgp expression and/or activity has been investigated in several studies. Hitzl *et al.* [19] demonstrated a slower Rh123 efflux in $CD56^+$ cells in individuals with TT genotype at 3435 compared with CC genotype. Calado *et al.* [20] investigated the functional importance of G2677T and C3435T polymorphisms in $CD34^+$ cells. A Rh123 efflux assay revealed no significant differences in Pgp function between individuals with different genotypes. Higher mRNA levels, in duodenal enterocytes in subjects homozygous for TT at 3435 than in CT or CC individuals, were observed by Nakamura *et al.* [21]. In contrast, quantification of MDR1 transcripts and Pgp expression in PBMC from HIV-infected individuals showed significantly lower levels of MDR1 mRNA and Pgp in 3435 TT subjects [10]. The conflicting results obtained by different authors indicate the complex regulation of the MDR1 expression. Therefore, it is unlikely that variable responsiveness of patients to drugs, which are substrates for Pgp, could be attributable solely to single nucleotide polymorphisms in MDR1 gene. Also, other genetic and non-genetic determinants might have an impact on Pgp function.

Several reports have identified the presence of a small-molecular-weight Pgp in a drug-resistant variety of cell lines from different species [18]. Recently, Trambas *et al.* [12] reported that the mini-Pgp is constitutively expressed in human NK cells. The classic 170-kDa Pgp protein was not detected by Western blot analysis, instead smaller Pgp products of approximately 70 and 80 kDa were featured. It was suggested that both the 70 and 80 kDa proteins contain the C-terminal half of full-length Pgp. Moreover, when a cDNA containing full-length mRNA of the hamster pgp1 gene was transfected into cells, both the full-length 4·3 kb mRNA and a 2·3-kb transcript were produced [17]. Later experiments showed that the 2·3-kb transcript encoded a 57-kDa miniprotein. Full-length Pgp contains N-linked glycosylation sites. All three sites are located within the first extracellular loop at amino acid residues 91, 94 and 99 [22]. Nevertheless, previous studies have shown that mini-Pgp is not glycosylated [17,18]. In the present study, an attempt to elucidate the presence of both mini- and full-length Pgps in human peripheral blood lymphocytes was performed. Two primer pairs, amplifying specifically the C-terminal half and the N-terminal half, upstream from N-glycosylation sites of the Pgp molecule, were used.

Based on the results obtained, we made the following conclusions: (i) on the mRNA level, both the C- and N-terminal half of Pgp are expressed in PBMC; (ii) we suggest that the full-length molecule is present in PBMC on the mRNA level. It is not very probable that only mini-Pgp, containing the C-terminal half of the molecule, is expressed in NK cells, as demonstrated by Trambas *et al.* [12]. It has been suggested that alternative splicing of the MDR1 gene could result in production of mini-Pgp. However, the molecule cleavage of full-length Pgp may also occur post-transcriptionally, and both C- and N-terminal half could be expressed as a form of small molecular weight Pgp. And (iii) almost two-fold lower MDR1 N-terminal half than C-terminal half expression in PBMC could indicate the presence of mini-Pgp, an alternatively spliced form of the full-length molecule. We also consider that the differences observed might result from the different efficacy of RT-PCR, especially the lower RT efficacy for long N-terminal transcripts compared with short C-terminal products.

In conclusion, using a quantitative real-time RT-PCR assay, we determined MDR1 mRNA expression in human FACS-sorted peripheral blood lymphocytes. The MDR1 mRNA level was highest for the $CD56^+$ cells, followed by the $CD8^+ > CD4^+ > CD19^+$ cells. However, the genetic polymorphisms, G2677T in exon 21 and C3435T in exon 26 of the MDR1 gene, did not have any apparent effect on MDR1 mRNA expression. Furthermore, we demonstrated that human PBMC express both the C- and N-terminal half of MDR1 on a mRNA level. An almost two-fold difference in the C- and N-terminal half expression supports the presence of a small molecular weight Pgp, an alternatively spliced form of MDR1, in human peripheral blood lymphocytes. Further studies are required to verify the presence and role of mini-Pgp in these cells.

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IV

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Quantitative determination of the human MRP1 and MRP2 mRNA expression in FACS-sorted peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells

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Abstract: *Objectives:* ATP-binding cassette (ABC) transporters extrude a wide variety of endogenous and exogenous compounds. In cancer cells, they are known to confer multidrug resistance. The aim of the present study was to determine the expression of the multidrug resistance-associated protein 1 (MRP1) and 2 (MRP2), which are members of the subfamily C of the ABC transporters family, in human hematopoietic cells. *Methods:* $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells were isolated from whole blood by FACS-sort in 20 healthy volunteers. MRP1 and MRP2 mRNA levels were quantified using real-time reverse transcriptase–polymerase chain reaction (RT–PCR) assays on a LightCycler™ (Roche, Mannheim, Germany). *Results:* The MRP1 mRNA exhibited the highest abundance in $CD4^+$ cells ($7.4 \times 10^3 \pm 3.19 \times 10^3$ molecules/ng of total RNA), followed by $CD8^+ > CD19^+ > CD56^+$ cells. The MRP2 mRNA expression was highest in $CD4^+$ cells ($6.7 \times 10^2 \pm 2.84 \times 10^2$), followed by $CD8^+ > CD56^+ > CD19^+$ cells. No correlation between the MRP1 and MRP2 mRNA expression was observed. Interestingly, β2-microglobulin mRNA expression in $CD19^+$ cells was found to be twofold lower in comparison with other cells. *Conclusions:* On an mRNA level both MRP1 and MRP2 were expressed in peripheral blood cells, with more than sevenfold higher MRP1 expression in all cell populations investigated. The impact of the MRP1 and MRP2 transcription in these cells remains to study. The use of β2-microglobulin as a housekeeping gene could have a critical impact on the interpretation of RT–PCR data.

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Key words: ATP-binding cassette transporters; multidrug resistance-associated protein 1 and 2; human PBMC

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ATP-binding cassette (ABC) transporters play an important role in the transport of various endogenous and exogenous compounds across biologic membranes (1). In cancer cells, they are known to confer multidrug resistance through enhanced efflux of chemotherapeutics. The human multidrug resistance-associated protein 1 (MRP1) and 2 (MRP2) belong to subfamily C of the ABC transporters, and were first identified by Cole *et al.* (1992) (2) in human small cell lung cancer cells, and by Taniguchi *et al.* (1996) (3) in cisplatin-resistant human head and neck cancer cell line, respectively. Within hematopoietic cells it has been found that $CD3^+$ cells exhibited the highest and $CD19^+$ cells the lowest MRP1 mRNA levels (4). Protein analysis determined that $CD4^+$ cells exhibited higher MRP1 expression than other cells. In another study, Abbaszadegan *et al.* (5) showed that regardless of the cell lineage, normal peripheral blood and bone marrow hematopoietic cells express similar basal levels of the MRP1 mRNA. Laupèze *et al.* (6) investigated MRP activity in normal mature leukocytes using the

carboxy-2′,7′-dichlorofluorescein efflux assay. Similar cellular export was evidenced in $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, $CD15^+$, $CD20^+$, and $CD56^+$ cells, whereas $CD34^+$ cells showed higher efflux rate. Investigation of the mRNA expression revealed that leukocytes were found to mostly express MRP1, whereas MRP2 transcripts were not present or only at very low levels.

The variable and limited results available in the literature, characterizing MRP1 and MRP2 expression in human peripheral blood cells, encouraged us to determine the MRP1 and MRP2 mRNA expression in $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells. MRP1 and MRP2 have similar substrate specificity (7). There are no drugs yet that specifically inhibit a single MRP species without effect on other transporters. Therefore, we used a sensitive technique, reverse transcriptase–polymerase chain reaction (RT–PCR), instead of efflux assays, to discriminate between the MRP1 and MRP2.

Materials and methods

Study protocol was approved by the ethics committee of the Humboldt University. After an informed consent, 20 healthy volunteers (17 males and three females, age range 21–33 yr) were enrolled in the study.

FITC

Peripheral blood mononuclear cells (PBMC) were isolated from 20 mL whole blood by gradient centrifugation over Biocoll lymphocyte separating solution (Biochrom KG, Berlin, Germany). Cells were stained with fluorescein isothiocyanate-anti-CD4, PE-anti-CD56 (Immunotech Beckman Coulter, Munich, Germany), APC-anti-CD8, and PerCP-Cy5.5-anti-CD19 antibody (Becton Dickinson, San Jose, CA, USA) and sorted using FACSDiva Turbosorter (Becton Dickinson). Appropriate isotype controls were used. Purity of sorted cells was analyzed with FACSCalibur and was ≥98%. Total RNA from FACS-sorted cells was isolated with the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA concentration was measured spectrophotometrically and samples were maintained at −80°C until analysis.

RT–PCR

cDNA was synthesized from 50 ng of sample RNA and a series of dilutions of external standard RNA using a 120 U M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany) and 0.8 μM oligo dT(15) primer. The mixture was preincubated for 10 min at 26°C, then incubated at 42°C for 60 min followed by heating at 95°C for 2 min, and cooled. Quantitative PCR was performed on the LightCycler™ (Roche, Mannheim, Germany) with gene specific primers and hybridization probes listed in Table 1. All oligonucleotide primers and hybridization probes were purchased from Tib Molbiol (Berlin, Germany). To avoid amplification of possible contaminating genomic DNA, the selected forward or reverse primers were designed to span intron/exon junctions. Also, control reactions without reverse transcriptase were run to verify the absence of contaminating DNA.

The PCR amplification was carried out in a 16-μL reaction volume containing 5 μL of cDNA (of 25 μL of total cDNA) and 1.25 U of *Taq* DNA polymerase. The PCR reactions were initiated with denaturation at 94°C for 60 s, followed by amplification with 45 cycles at 95°C 0 s, annealing (64°C for MRP1, 63°C for MRP2, and 60°C for β2-microglobulin) for 10 s, and 72°C for 10 s. The data was evaluated with the LightCycler™ run software, version 3.5. The threshold cycle (Ct) was calculated using the second derivative maximum method. A calibration curve was generated from serial dilutions of the standard RNA by plotting Ct vs. the log of the copies of standard RNA, and used to calculate the number of copies in the samples. The housekeeping gene β2-microglobulin was used for normalization of the MRP1 and MRP2 mRNA expression. Final results were expressed as MRP1 (or MRP2) cDNA copies per ng of total RNA and as the normalized ratio of MRP1 (or MRP2) cDNA copies per 10^3 copies of β2-microglobulin cDNA.

In order to establish the calibration standard curve, external standard RNA was prepared as described previously (8). Briefly, RT–PCR was carried out in a two-step reaction for each gene, as described above using the primers listed in Table 1. To obtain a template for *in vitro* transcription, subsequent PCR with T7 primer (T7 promoter sequence was incorporated at the 5′-end of the forward primer) and RT primer [dT(15) sequence was incorporated at the 5′-end of the reverse primer] was carried out. *In vitro* transcription was performed at 37°C for 90 min using the DNA-dependent T7 RNA polymerase. After purification, RNA standards were quantified spectrophotometrically. A series of dilutions of standard RNA was performed and maintained at −80°C.

Statistical analysis

Data were presented as mean ± SD. mRNA expression between the different cells was analyzed by Wilcoxon signed ranks test. All calculations were performed with SPSS™ for Windows (version

Table 1. Oligonucleotide primers and hybridization probes used for real-time quantitative RT–PCR

Gene	Sequence*	Position (bp)
MRP1		
Forward primer	5′-TTCCGGAACTACTGCCTGCGCTA-3′	4079–4101
Reverse primer	5′-GGGTCCTGGGGGATGATGGTGA-3′	4305–4326
LightCycler probes	5′-CTCGTTGATCCGAAATAAGCCCAGGGTCA-3′-fluorescein	4231–4203
	5′-LC Red640-ACGACTTCCCAGCTCCCGTCCGCC-3′-phosphate	4199–4176
MRP2		
Forward primer	5′-GGCTGAGATTGGAGAGAAGGGTATA-3′	2292–2314
Reverse primer	5′-ACTGCAGACAGGGGGTCAT-3′	2409–2391
LightCycler probes	5′-TTTGGTAGGTAGCTCTGGCCAGGCT-3′-fluorescein	2369–2345
	5′-LC Red640-ATCCGCTGCTTCTGACCCCCAC-3′-phosphate	2343–2322
β2-microglobulin		
Forward primer	5′-CCAGCAGAGAATGGAAAGTC-3′	113–132
Reverse primer	5′-CATGTCTCGATCCCACTTAAC-3′	350–370
LightCycler probes	5′-TTCTTCAGTAAGTCAACTTCAATGTCGGA-3′-fluorescein	170–198
	5′-LC Red705-ATGAAACCCAGACACATAG CAATTCAG-3′-phosphate	140–166

*Area amplified refers to sequences with accession numbers as follows: NM_004996 (MRP1), NM_000392 (MRP2), NM_004048 (β2-microglobulin).

10.0; SPSS, Inc., Chicago, IL, USA). *P*-values of 0.05 were considered to be of statistical significance.

Results

The coefficients of variation for MRP1, MRP2, and β2-microglobulin mRNA levels were 26.1%, 21.5%, and 13.6%, respectively. The MRP1 mRNA exhibited the highest abundance in $CD4^+$ cells ($7.4 \times 10^3 \pm 3.2 \times 10^3$ molecules/ng of total RNA) with a 6.7-fold difference between individuals, followed by $CD8^+$ ($5.7 \times 10^3 \pm 2.5 \times 10^3$), $CD19^+$ ($4 \times 10^3 \pm 1.8 \times 10^3$), and $CD56^+$ ($3.6 \times 10^3 \pm 1.3 \times 10^3$) cells (Fig. 1A). The differences between cell populations were statistically significant ($P < 0.01$), except $CD19^+$ vs. $CD56^+$ cells. Also the MRP2 mRNA expression was highest in $CD4^+$ cells ($6.7 \times 10^2 \pm 2.8 \times 10^2$) with a 5.2-fold difference between individuals, followed by $CD8^+$ ($5.7 \times 10^2 \pm 1.9 \times 10^2$), $CD56^+$ ($4.9 \times 10^2 \pm 2.2 \times 10^2$), and $CD19^+$ ($4.2 \times 10^2 \pm 1.6 \times 10^2$) cells (Fig. 1B). The differences observed were statistically significant ($P < 0.05$). No correlation between the MRP1 and MRP2 mRNA expression was observed (data not shown).

The MRP1 and MRP2 mRNA levels normalized to β2-microglobulin are shown in Table 2. However, we found that the mean β2-microglobulin expression in $CD19^+$ cells was significantly lower ($2.6 \times 10^5 \pm 7.8 \times 10^4$; $P < 0.001$) in comparison with other cells ($5.5 \times 10^5 \pm 1.8 \times 10^5$ in $CD4^+$, $5 \times 10^5 \pm 1.2 \times 10^5$ in $CD8^+$, and $5.5 \times 10^5 \pm 1.9 \times 10^5$ in $CD56^+$ cells). This indicates that the use of β2-microglobulin to normalize mRNA levels may have a critical impact on the interpretation of RT–PCR data.

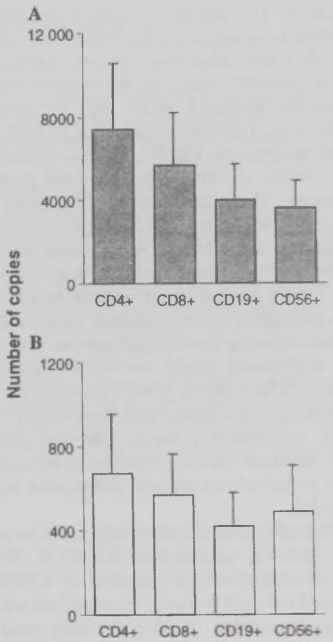

Fig. 1. Quantitative determination of the MRP1 (A) and MRP2 (B) mRNA levels in human peripheral blood lymphocytes. Each bar represents the mean ± SD number of copies per ng of total RNA, as determined in 20 individuals. The differences in MRP1 expression between cell populations were statistically significant ($P < 0.01$), except $CD19^+$ vs. $CD56^+$ cells. All differences in MRP2 expression were statistically significant ($P < 0.05$).

Table 2. Normalized MRP1 and MRP2 mRNA expression in human peripheral blood lymphocytes ($n = 20$)

Cell	MRP1	MRP2
$CD4^+$	12.6 ± 4.6^1	1.3 ± 0.3^3
$CD8^+$	10.0 ± 3.0	1.2 ± 0.4^3
$CD19^+$	14.4 ± 6.0^1	$1.7 \pm 0.5^{1,2}$
$CD56^+$	$5.9 \pm 2.2^{1,2,3}$	$1.0 \pm 0.5^{1,2,3}$

Mean ± SD of MRP1 or MRP2 cDNA copies per 10^3 copies of β2-microglobulin are presented.
Values are significantly different from $^1CD8^+$, $^2CD4^+$, and $^3CD19^+$ cells.
$P < 0.05$ according to Wilcoxon signed ranks test.

Discussion

The MRP1 is highly expressed throughout the body, but poorly in the liver and intestine (7). In contrast, MRP2 is predominantly expressed in the liver. In the present study, we found that both MRP1 and MRP2 were expressed in peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells, with more than sevenfold higher MRP1 expression in all cell populations.

Similar to the results published previously (4), the highest expression of the MRP1 was observed in $CD4^+$ cells. However, larger interindividual variation (20-fold) than in the present study was observed by Legrand *et al.* (4). In contrast to Laupèze *et al.* (6) who found that MRP2 mRNA was not present in PBMC, in the present study MRP2 mRNA was evidenced in all cell populations investigated. The main goal of this study was to investigate MRP1 and MRP2 expression in different subtypes of PBMC. A sensitive method as quantitative real-time RT–PCR was used. However, it would have a great impact to confirm the protein expression in cell surface. Our study group consisted of young healthy volunteers and any age-related differences could not be determined. No significant differences in MRP1 and MRP2 mRNA expression between males and females (17 vs. three subjects, respectively) were observed (data not shown). Because of the small number of female subjects potential sex-related differences could not be excluded.

β2-Microglobulin is commonly used as a housekeeping gene for quantitative RT–PCR. We found that β2-microglobulin expression in $CD19^+$ cells was almost twofold lower in comparison with other cells. The expression of 10 commonly used housekeeping genes in 13 different human tissues was found to vary considerably (9). β2-Microglobulin expression level was 112-fold higher in leukocytes compared with fetal brain, indicating large tissue specific variation. Although it was concluded that β2-microglobulin was a good marker for normalization of leukocyte mRNA expression, its expression in different subtypes of PBMC was not studied. Our data indicates that the use of β2-microglobulin for normalization of the target mRNA expression in different subsets of PBMC could lead to misinterpretation of the data. There is no universal control gene for normalization and the validity of the conclusions is highly dependent on the applied control. Therefore, the use of an absolute number of copies of target gene mRNA per total RNA seems to be appropriate, at least for homogenous samples as PBMC.

The impact of the MRP1 and MRP2 transcription in PBMC remains unknown. P-glycoprotein, a member of the subfamily B of the ABC transporters encoded by the MDR1 gene, has been found to be highly expressed in $CD8^+$ and $CD56^+$ cytotoxic cells (10). In the present study, $CD4^+$ cells exhibited the highest abundance of the MRP1 and MRP2 mRNA. This indicates that the expression of different ABC transporter genes in hematopoietic cells may be variously regulated during differentiation. Miscellaneous ABC transporters may have different function. For instance, P-glycoprotein has been reported to be involved in the natural killer and T cell mediated cytotoxicity (11, 12) and in the transport of cytokines (13). MRP1, unlike P-glycoprotein, facilitates the secretion of leukotrienes, the major mediators of inflammation (14).

Several HIV protease inhibitors and reverse transcriptase inhibitors are substrates for MRP1 and MRP2 (15, 16). We found that both MRP1 and MRP2 were expressed in PBMC with the highest level in $CD4^+$ cells, at major site of virus replication. MRPs may have an important role in limiting drug exposure within these cells and therefore the efficacy of HIV therapy. Jones *et al.* (17) demonstrated that the accumulation of ritonavir, indinavir, saquinavir, and nelfinavir in MRP1 expressing cells was significantly reduced in comparison with control cells. MRP1 inhibitor MK571 partially reversed these effects. Meaden *et al.* showed an inverse relationship between the MRP1 expression and intracellular accumulation of ritonavir and saquinavir in PBMC from HIV-infected individuals (18). Both MRP1 and MRP2 expression in PBMC indicates that not only MRP1, but also MRP2 and probably other MRPs account for the resistance to anti-HIV-drugs.

Clinical relevance of the expression of the MRP1 and MRP2 in PBMC is not limited to HIV therapy. These ABC transporters also extrude a number of anticancer drugs (7). Lower expression of MRP1 and MRP2 in $CD19^+$ and $CD56^+$ cells could predispose these cells to cytotoxicity during chemotherapy. Also, individual differences in the expression of MRP1 and MRP2 could determine

individual sensitivity toward hematologic side effects of various drugs.

In conclusion, on an mRNA level, both MRP1 and MRP2 were expressed in human peripheral blood $CD4^+$, $CD8^+$, $CD19^+$, and $CD56^+$ cells. Further studies are required to specify the physiologic function and clinical importance of the MRP1 and MRP2 transporters in these cells.

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SHORT COMMUNICATION

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Frequency of *MRP1* genetic polymorphisms and their functional significance in Caucasians: detection of a novel mutation G816A in the human *MRP1* gene

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Abstract *Objective*: The aim of the present study was to determine the frequency of the G816A, T825C, T1684C, and G4002A genetic polymorphisms of the human *MRP1* gene in 230 healthy Caucasians. The functional assessment of these mutations was performed in fluorescence-activated cell sorting (FACS)-sorted peripheral blood $CD4^+$ cells in a further 61 healthy volunteers by determining MRP1 mRNA expression.
Methods: Genotyping of the *MRP1* was carried out using real-time polymerase chain reaction (PCR) assays. Quantitative determination of the MRP1 mRNA expression was performed with real-time reverse-transcription-PCR.
Results: A novel silent mutation G816A in exon 8 was found in this study. Allele frequencies of the 816A, 825C, 1684C, and 4002A were 4.1, 30.0, 80.0, and 28.3%, respectively. The frequency of the T825C polymorphism was comparable with that found in a previous Japanese study. In contrast, the frequency of the T1684C (OR 0.06, 95% CI 0.03–0.11, $P<0.0001$, vs Japanese) and the G4002A (OR 0.47, 95% CI 0.24–0.86, $P=0.01$, vs Japanese) was significantly rarer. The mean MRP1 mRNA expression in peripheral blood $CD4^+$ cells was $1.03\times10^4\pm3.8\times10^3$ molecules/ng of total RNA with an eightfold variation among individuals. However, MRP1 mRNA expression in $CD4^+$ cells was not found to correlate with genetic polymorphisms investigated in this study.
Conclusions: The genotypic results observed show an ethnic difference in the frequencies of the *MRP1* genetic polymorphisms between Japanese and Caucasians. Further studies are required to better understand the clinical consequences of the *MRP1* genetic variants.

Keywords MRP1 · Genetic polymorphisms · mRNA expression

Introduction

The ATP-binding cassette (ABC) transporters play an important role in the transport of drugs as well as endogenous substances across biological membranes [1]. The human multi-drug resistance-associated protein 1 (MRP1) belongs to subfamily C of the ABC transporters (ABCC1), and was first identified in 1992 by Cole et al. [2] in doxorubicin-selected human small cell lung cancer cells. Recently, identification of several single nucleotide polymorphisms (SNPs) in the human *MRP1* has been reported [3, 4, 5, 6].

In the present study, the prevalence of the genetic polymorphisms G816A, T825C, T1684C, and G4002A in the *MRP1* was investigated in 230 healthy Caucasians. All these mutations were silent mutations, which do not alter the amino acid sequence. However, wobble mutations might affect RNA folding and stability [7]. Therefore, to assess the functional importance of these polymorphisms, peripheral blood $CD4^+$ cells were analyzed for MRP1 mRNA levels using a quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) assay.

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Materials and methods

Study population

We studied DNA samples from 230 healthy Caucasians of German origin (194 males and 36 females, age range 20–59 years, mean 30 years). MRP1 mRNA levels in fluorescence-activated cell sorting (FACS)-sorted peripheral blood $CD4^+$ cells were deter-

mined in a further 61 healthy volunteers (47 males and 14 females, age range 21–32 years, mean 26 years). The study protocol was approved by the ethics committee of the Humboldt University, Berlin, Germany.

Genotyping of the *MRP1* using LightCycler assays

The reaction mixture (20 µl) for PCR amplification consisted of 1×PCR buffer, 0.1 mM each dNTP, 30 mg/l bovine serum albumin (BSA), and 1 U BioTherm DNA polymerase. The concentration of $MgCl_2$, dimethyl sulfoxide, primers and hybridization probes (Table 1) was optimized for each reaction. After an initial denaturation at 95°C for 30 s, the amplification was performed by means of 45 cycles of denaturation at 95°C for 0 s, annealing (Table 1) for 15 s, and extension at 72°C for 15 s. Melting curves were recorded subsequently to an amplification run from 35°C to 95°C at 0.2°C/s, 35°C to 85°C at 0.15°C/s, 40°C to 85°C at 0.1°C/s, and 40°C to 95°C at 0.2°C/s for the detection of the G816A, T825C, T1684C, and G4002A SNPs, respectively. Confirmation of the LightCycler analysis was performed by sequencing and PCR-restriction fragment length polymorphism (RFLP) analysis.

Quantitative determination of the MRP1 mRNA expression

The principles of the methods, used for cell sorting, total RNA isolation and real-time quantitative RT-PCR on the LightCycler, have been described previously [8]. The human MRP1 mRNA (NM_004996) was amplified with gene specific primers 5′-TTC CGG AAC TAC TGC CTG CGC TA-3′ (forward, 0.3 µM) and 5′-GGG TCC TGG GGG ATG ATG GTG A-3′ (reverse, 0.3 µM, annealing temperature 64°C). The reaction mixture also included hybridization probes 5′-CTC GTT GAT CCG AAA TAA GCC CAG GGT CA-3′-flourescein (0.13 µM) and 5′-LC-Red640-ACG ACT TCC CAG CTC CCG TCC GCC-3′-phosphate (0.13 µM). PCR amplification for the housekeeping gene β2-microglobulin (NM_004048) was carried out, as described previously [8].

Statistical analysis

Odds ratios (OR) with their 95% confidence interval (CI) were calculated using Fisher's exact test (STATA, version 7.0, Stata Corporation, College Station, TX, USA). MRP1 mRNA levels were presented as the mean ± SD. Differences in MRP1 mRNA expression between the genotypes were analyzed by Kruskal-Wallis test. Calculations were performed with SPSS for Windows (version 10.0, SPSS Inc., Chicago, IL, USA). *P* values of 0.05 were considered to be of statistical significance.

Results

Genotyping of human *MRP1*

A novel G816A substitution in exon 8 of the *MRP1* was identified heterozygously in 19 of 230 subjects. First, the long sensor probe (position at 813–834 bp) used for T825C genotyping, determined four individuals with atypical melting curves. Subsequent sequencing showed that all these individuals, but not subjects with typical melting curves for T825C, were heterozygous GA at position 816. The G816A substitution destabilized the binding of the long T825C sensor. Therefore a new, shorter sensor probe (position at 817–834 bp, see Methods) for T825C detection, and another pair of hybridization probes (position at 779–800 bp anchor, and 803–821 bp sensor) for G816A detection was designed.

The results of *MRP1* genotyping in 230 healthy Caucasians are given in Table 2. A linkage dysequilibrium between the G816A and T825C polymorphisms was observed, since the [816GA, 825TT] was not observed

Table 1 Oligonucleotide primers and hybridization probes used for polymerase chain reaction (PCR)

SNP	Sequence*	Position (bp)	Annealing (°C)
G816A			60
Forward	5′-TGTGGTAGGGGGCTGCA-3′	(13–29)	
Reverse	5′-TTCGCATCCACCTTGGAACT-3′	(125–144)	
Sensor	5′-LC-Red705-GTTCCAGGCAGCCAGTGAA-3′-phosphate	(66–84)	
Anchor	5′-CCCACAACGGCTTCACCTCCTT-3-fluorescein	(42–63)	
T825C			60
Forward	5′-TGTGGTAGGGGGCTGCA-3′	(13–29)	
Reverse	5′-TTCGCATCCACCTTGGAACT-3′	(125–144)	
Sensor	5′-GTGAAGGTCGTGTACTCC-3′-fluorescein	(80–97)	
Anchor	5′-LC-Red640-CCAAGGATCCTGCCCAGCCGAA-3′-phosphate	(99–120)	
T1684C			56
Forward	5′-ACTCGGGGCACAGCAGT-3′	(33–49)	
Reverse	5′-TTGAACAAGGCCAAAGACA-3′	(149–167)	
Sensor	5′-ACGGCAAATGTGCACAAGGCC-3′-fluorescein	(78–98)	
Anchor	5′-LC-Red640-CCTGCAAGCAAGAACGCCCAGTG-3′-phosphate	(54–76)	
G4002A			71
Forward	5′-GCCAGCATTCCCACCACACCT-3′	(28–48)	
Reverse	5′-GCTTGCCAGCTCTGGCTCACC-3′**	(251–271)	
Sensor	5′-GGTCAGGGACGACTTCCC-3′-fluorescein	(95–112)	
Anchor	5′-LC-Red640-CTCCCGTCCGCCCCACG-3′-phosphate	(76–92)	

*Area amplified refers to sequences with accession numbers as follows: AF022830 (G816A, T825C), AF022835 (T1684C), AF022850 (G4002A)

**PCR primer as reported by Ito et al. [4]
The position of the variable base in sensor is underlined

Table 2 Observed frequencies of *MRP1* genetic variants in 230 healthy Caucasians. Expected genotype frequencies were calculated according to the Hardy-Weinberg equilibrium from the allele frequencies

Position	Allele	Frequency %	Genotype	Number of subjects	Frequency		
					% Observed	95% CI	% Expected
816	G	95.9	GG	211	91.7	87.40–94.95	91.9
	A	4.1	GA	19	8.3	5.05–12.60	7.9
			AA	0	0.0	0.0–0.02	0.0
825	T	70.0	TT	115	50.0	43.36–56.64	49.0
	C	30.0	TC	92	40.0	33.62–46.64	42.0
			CC	23	10.0	6.45–14.63	9.0
1684	T	20.0	TT	15	6.5	3.70–10.53	4.0
	C	80.0	TC	62	27.0	21.34–33.18	32.0
			CC	153	66.5	60.02–72.59	64.0
4002	G	71.7	GG	118	51.3	44.65–57.93	51.4
	A	28.3	GA	94	40.9	34.45–47.52	40.6
			AA	18	7.8	4.70–12.09	8.0

(4% expected vs 0% observed; $P < 0.01$) and the [816GA, 825CC] was more frequently observed than expected (0.007% expected vs 0.04% observed; $P < 0.001$).

MRP1 mRNA expression in peripheral blood $CD4^+$ cells from individuals with different *MRP1* genotypes

The mean MRP1 mRNA expression in peripheral blood $CD4^+$ cells was $1.03 \times 10^4 \pm 3.8 \times 10^3$ molecules/ng of total RNA with an eightfold variation among individuals. However, statistical analysis revealed no significant ($P > 0.05$) difference in MRP1 expression with regard to different genotypes in the *MRP1* (Fig. 1). A comparison of β2-microglobulin-normalized data displayed the same pattern (data not shown).

Discussion

In the present study, genotype frequencies of the four genetic polymoprhisms in the human *MRP1* were determined in 230 healthy Caucasians. T825C, T1684C, and G4002A polymorphisms have been described previously [4, 5, 6]. A novel mutation G816A was identified in this study. No functional importance of these mutations was observed, as determined by MRP1 mRNA expression in peripheral blood $CD4^+$ cells.

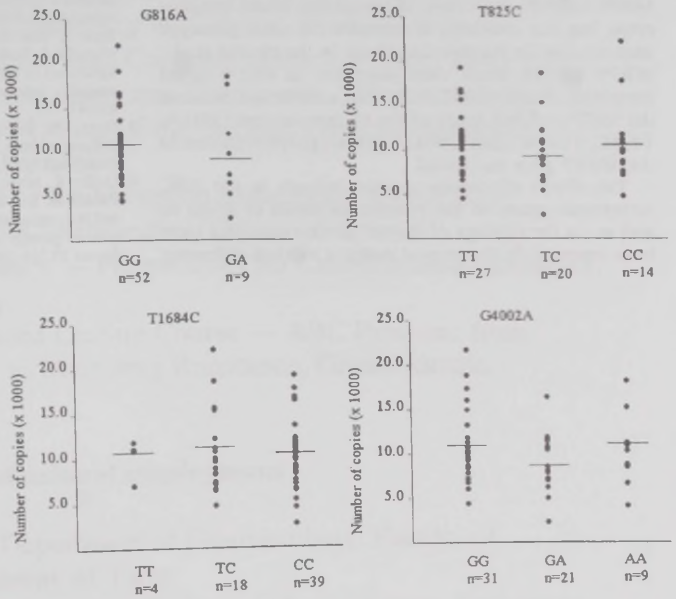

Fig. 1 Quantitative determination of the MRP1 mRNA expression in peripheral blood $CD4^+$ cells from individuals with different *MRP1* genotypes. Data are presented as number of copies of MRP1 mRNA/ng of total RNA. *Bars* represent the mean values for each genotype. No difference in MRP1 mRNA expression was observed between subjects with the different *MRP1* genotypes ($P > 0.05$)

MRP1 is polymorphic and more than 80 SNPs have been identified in the gene [3, 4, 5, 6]. Most of the SNPs are located in intronic sequences. Three of the SNPs—T825C, T1684C, and G4002A, located in exons 8, 13, and 28, respectively—were selected for the present study. Other SNPs identified previously in exonic regions occurred with a low frequency (1%), and were observed only by a single investigator, but not confirmed by the others.

A novel silent mutation in exon 8, a G to A transversion at position 816 (Pro to Pro at codon 272) was identified in this study with an allele frequency of 4.1%. The allele frequency of the T allele at position 825 was found to be 70.0% in Caucasians, as determined in the present study, and 62.5% in Japanese ($n=48$), as reported by Ito et al. [4] (OR 1.4, 95% CI 0.86–2.26, $P=0.18$). The frequency of the G allele at position 4002 was 71.7% in Caucasians and 84.4% in Japanese (OR 0.47, 95% CI 0.24–0.86, $P=0.01$). The results obtained indicate an ethnic difference in the frequency of *MRP1* genetic polymorphisms. When genotype results of the T1684C polymorphism were compared between Caucasians and Japanese, it was found that the frequency of T allele was 20.0% in Caucasians, and 80.2% in Japanese (OR 0.06, 95% CI 0.03–0.11, $P<0.0001$). In the first published *MRP1* sequence (AF022835), derived from a lung cancer cell line, a T nucleotide at position 1684 was reported and defined as a wild-type allele. We found that a C allele at position 1684 was the most common variant for Caucasians.

As shown by Conrad et al. [5], sense mutations T825C and C1704T seemed to correlate with a lower MRP1 mRNA expression in peripheral blood lymphocytes, but the numbers of samples for each genotype were too low for statistical analysis. In the present study, MRP1 mRNA levels were measured in FACS-sorted peripheral blood $CD4^+$ cells. No correlation between the MRP1 mRNA levels of the 61 samples and G816A, T825C, T1684C, and G4002A genetic polymorphisms in the *MRP1* gene was found.

The effects of various genetic variants in the ABC transporter genes on the pharmacokinetics of drugs as well as on the etiology of several genetic disorders have been reported. In the present study, a marked difference in the frequencies of the *MRP1* genetic polymorphisms was observed between Caucasians and Japanese. Further studies are required to determine the prevalence of the *MRP1* genetic variants in different ethnic groups and their functional importance in vivo.

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