Published in final edited form as:

J Pharmacol Exp Ther. 2008 June ; 325(3): 859–868. doi:10.1124/jpet.108.136523.

THE HUMAN MULTIDRUG RESISTANCE PROTEIN 4 (MRP4, ABCC4): FUNCTIONAL ANALYSIS OF A HIGHLY POLYMORPHIC GENE

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Abstract

ABCC4 encodes MRP4, a member of the ATP-binding cassette family of membrane transporters involved in the efflux of endogenous and xenobiotic molecules. The aims of this study were to identify single nucleotide polymorphisms of *ABCC4*, and to functionally characterize selected non-synonymous variants. Resequencing was performed in a large ethnically diverse population. Ten non-synonymous variants were selected for analysis of transport function based on allele frequencies and evolutionary conservation. The reference and variant MRP4 cDNAs were constructed by site-directed mutagenesis and transiently transfected into human embryonic kidney cells (HEK 293T). The function of MRP4 variants was compared by measuring the intracellular accumulation of two antiviral agents, azidothymidine (AZT) and adefovir (PMEA). A total of 98 variants were identified in the coding and flanking intronic regions of *ABCC4*. Of these, 43 variants are in the coding region and 22 are non-synonymous. In a functional screen of ten variants, there was no evidence for a complete loss of function allele. However, two variants (G187W and G487E) showed a significantly reduced function compared to reference with both substrates, as evidenced by higher intracellular accumulation of AZT and PMEA compared to the reference MRP4 (43% and 69% increase in accumulation for G187W compared with the reference MRP4, with AZT and PMEA, respectively). The G187W variant also showed decreased expression following transient transfection of HEK 293T cells. Further studies are required in order to assess the clinical significance of this altered function and expression and to evaluate substrate specificity of this functional change.

Introduction

The multidrug resistance protein 4 (MRP4), encoded by *ABCC4*, belongs to the superfamily of ATP-binding cassette (ABC) transporters. It has been implicated in the transport of antiviral agents, such as the nucleoside/nucleotide analogs azidothymidine (AZT), adefovir (9-(2-phosphonylmethoxyethyl)adenine or PMEA), tenofovir, lamivudine, and ganciclovir (Schuetz et al., 1999; Adachi et al., 2002; Anderson et al., 2006; Imaoka et al., 2007), anticancer drugs (methotrexate, 6-mercaptopurine, 6-thioguanine, camptothecins (Lee et al., 2000; Chen et al., 2002; Wielinga et al., 2002; Leggas et al., 2004; Tian et al., 2005)), as well as endogenous

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Recommended section assignment: Metabolism, transport, and pharmacogenomics

molecules such as prostaglandins, steroids, bile acids, cyclic nucleotides, and folate (Chen et al., 2002; van Aubel et al., 2002; Reid et al., 2003; Zelcer et al., 2003; Denk et al., 2004).

MRP4 is ubiquitously expressed, with a high expression in the prostate, as well as in hematopoietic stem cells and blood cells (Su et al., 2004) (<http://symatlas.gnf.org/SymAtlas/>). It is also present in the kidney proximal tubules (van Aubel et al., 2002), in the liver (Rius et al., 2003) and in the brain (Leggas et al., 2004). Interestingly, its localization in most tissues is apical but basolateral localization has been demonstrated in brain choroid plexus epithelial cells, in prostate and in hepatocytes (Lee et al., 2000; van Aubel et al., 2002; Rius et al., 2003; Leggas et al., 2004). Like most efflux transporters, no disease has been directly linked to altered MRP4 activity. Recently, a study with *Abcc4*^{-/-} mice showed that the absence of this transporter did not induce obvious abnormalities. However, it resulted in acute PMEA toxicity, suggesting a protective role of MRP4 in the bone marrow, spleen, thymus and gastrointestinal tract. Moreover, these data suggested that MRP4 may reduce the passage of PMEA and probably other nucleotide analogs into the brain (Belinsky et al., 2007). This is in agreement with a previous report stating that *Abcc4*^{-/-} mice accumulated more topotecan in the brain and cerebrospinal fluid than wildtype mice (Leggas et al., 2004). Therefore, its physiological role could include detoxification of drugs, and also of endogenous molecules. With respect to endogenous substrates, up-regulation of MRP4 in the liver of rats and humans with obstructive cholestasis provides a mechanism to eliminate excess bile salts (Denk et al., 2004; Gradhand et al., 2008).

Although *ABCC4* is a highly polymorphic gene (Saito et al., 2002), few data are available concerning the function of its variants. Recent studies have investigated the functional effects of several *ABCC4* single-nucleotide polymorphisms (SNPs) on drug disposition. Anderson *et al.* showed a 20% increase in lamivudine-triphosphate intracellular concentrations in patients carrying the 4131T>G variant, while the 3724G>A variant was associated with a trend for elevated AZT-triphosphate, suggesting a reduced MRP4 efflux function (Anderson et al., 2006). Interestingly, the 4131T>G variant is in the 3'-untranslated region (UTR) of the gene, while the 3724G>A variant is synonymous and there is no clear mechanism explaining these effects. In another study, no association was observed between two non-synonymous and seven synonymous *ABCC4* variants and tenofovir disoproxil fumarate-induced renal proximal tubulopathy (Izzedine et al., 2006). Most recently, 74 genetic variants in *ABCC4* were shown to have no effect on MRP4 mRNA and protein expression in Caucasian cholestatic and non-cholestatic patients (Gradhand et al., 2008).

The aims of this study were to identify genetic variants of *ABCC4* in a cohort of healthy individuals of different ethnic groups, and to functionally characterize selected non-synonymous variants *in vitro*. AZT and PMEA, the first nucleoside/nucleotide analogs reported to be substrates of MRP4 (Schuetz et al., 1999), have been selected for this study. AZT and PMEA are used in the treatment of HIV and Hepatitis B infections, respectively, and altered function of MRP4 might contribute to interindividual variability in the response to these antivirals.

Methods

Identification of *ABCC4* variants

Genomic DNA was obtained from an ethnically diverse population of 270 healthy individuals (African Americans, Caucasians, Asian Americans and Mexican Americans) in the San Francisco Bay area, as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project. DNA collection and genotyping was approved by the University of California San Francisco Institutional Review Board. Resequencing led to the identification of variants in the exonic and adjoining intronic regions. The primer sequences

used for resequencing are available at <http://www.pharmgkb.org>. The neutral parameter θ (the proportion of nucleotide sites that are expected to be polymorphic in a sample of sequences drawn at random from a population), nucleotide diversity π , (average proportion of nucleotide differences between all possible pairs of sequences in a sample), and Tajima's D statistic (used to detect departures from the standard neutral model), were calculated in each ethnic group for synonymous, non-synonymous and non-coding sites according to Tajima (Tajima, 1989; Tajima, 1993).

Haplotype analysis

Haplotypes were statistically inferred in each ethnic group using the PHASE method (Stephens et al., 2001) for variants with a minor allele frequency > 5%. The analysis was run ten times, and haplotypes estimated in at least seven runs were reported. Analyses were carried out using all variable sites of *ABCC4* above the mean allele frequency cut-off. A seaperate analysis excluded the intronic sites.

Construction of *ABCC4* variants and non-functional mutant

The *ABCC4* cDNA was cloned from human kidney using the following primers: forward AGCATCCCTGCTTGAGGTCCA, and reverse ACGGACTTGACATTTTGGTTGG. The cDNA was originally inserted into the pCR®2.1-TOPO® vector (Invitrogen Corporation, Carlsbad, CA), and subsequently into the pcDNA5/FRT vector (Invitrogen). This plasmid contained two synonymous *ABCC4* SNPs (rs1678339 and rs1189466) which were reversed by site-directed mutagenesis to obtain the reference *ABCC4*. The previously described reference sequence (GenBank accession number NM_005845) contains two SNPs (rs11568681/Leu18Ile and rs1557070) with low frequencies (<10%), which are not found in our reference sequence. This reference plasmid was used as a template for constructing the ten non-synonymous variants selected for this study, as well as a non-functional mutant. Variant constructs were obtained by site-directed mutagenesis, using the *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's protocol and the primers reported in Table 1. The mutations were confirmed by sequencing, and complete sequencing of each plasmid was also performed in order to check that no new mutations were introduced during the mutagenesis procedure.

Functional assays of MRP4 variants

Human embryonic kidney epithelial cells transformed with SV40 T antigen (HEK 293T) were obtained from the Gladstone Institute of Virology and Immunology (San Francisco, CA, USA). They were maintained in MEM Eagle's medium with Earle's Balanced Salt Solution, supplemented with sodium pyruvate, penicillin, streptomycin, and non-essential amino acids (Cell Culture Facility, University of California, San Francisco, CA), as well as fetal bovine serum (Invitrogen). HEK 293T cells were seeded onto poly-D-lysine coated 24-well plates (BD Biosciences Discovery Labware, Bedford, MA). The following day, the cells were transfected with 0.8 μ g DNA (*ABCC4* reference, variant or non-functional mutant) and 1 μ l Lipofectamine 2000 (Invitrogen) in each well, according to the manufacturer's protocol. Accumulation studies were performed 24 hours after transfection. Cells were washed with phosphate buffered saline and incubated with 100 nM [3 H] bis(pivaloyloxymethyl) (9-(2-phosphonylmethoxyethyl)adenine ([2-(6-aminopurin-9-yl)ethoxymethyl-(2,2-dimethylpropanoyloxymethoxy)phosphoryl]oxymethyl 2,2-dimethylpropionate, bis-POM-PMEA) or [3 H] azidothymidine (1-[4-azido-5-(hydroxymethyl)oxolan-2-yl]-5-methylpyrimidine-2,4-dione, AZT) (Moravek Biochemicals, Inc., Brea, CA) at 37°C. The effect of endogenous MRP4 was minimized by running the assay for 30 min for AZT and 60 min for PMEA. After 30 min of accumulation, the ratio of accumulation of AZT between transfected and untransfected cells reaches its peak, while the assay with PMEA needs to be

longer to allow the intracellular release of PMEA from bis-POM-PMEA by esterases. For studies of accumulation as a function of concentration, higher concentrations of substrates were obtained by adding radiolabeled substrates to different concentrations of unlabeled AZT (Toronto Research Chemicals Inc., North York, ON, Canada) and bis-POM-PMEA (Moravek). Accumulation was stopped by removing the substrate and washing the cells three times with ice-cold phosphate buffered saline. The cells were lysed with 800 µl/well of an aqueous solution containing 0.1 N NaOH and 0.1% sodium dodecyl sulfate. An aliquot of the lysate (775 µl) was added to 3.25 ml of Ecolite scintillation fluid (MP Biomedicals, Irvine, CA), and intracellular amounts of the radiolabeled substrates were determined by scintillation counting. These data were then normalized to protein concentration in each well, measured using a BCA™ protein assay kit (Pierce Biotechnology Inc., Rockford, IL).

RNA and protein expression

Cells were seeded onto 12-well plates and transfected. Twenty-four hours after transfection, RNA or proteins were extracted from the cells. For RNA extraction, TRIZOL™ reagent was added to the wells (Invitrogen), and RNA was extracted according to the manufacturer's protocol. ABCC4 mRNA was quantified in each sample by real-time RT-PCR (TaqMan®) and compared to its basal expression in cells transfected with the empty vector (endogenous ABCC4). For protein extraction, the cells were lysed, and proteins were extracted using centrifugation. Protein concentrations were quantified by the BCA™ assay, and these samples were used for Western blot analysis. A monoclonal antibody to MRP4 (M4-I10) and a polyclonal antibody to rat IgG (Axxora, LLC, San Diego, CA) were used to visualize MRP4 using the Immobilon™ Western detection reagent (Millipore corporation, Billerica, CA). Lysates from three separate transfections were analyzed by Western blotting and the density of the MRP4 signal was quantified using image analysis and expressed relative to that of GAPDH.

Subcellular localization (immunocytochemistry)

Cells were grown on poly-D-lysine coated coverslips (BD Biosciences Discovery Labware) in a 24-well plate. They were transfected the next day, and stained 24 hours later. The same antibody to MRP4 that was used for Western blotting was used for immunocytochemistry. A fluorescent goat anti-rat secondary antibody (Invitrogen) was used for the detection of MRP4. Staining was visualized under a Zeiss Axioskop epifluorescence microscope.

Design of a non-functional mutant

In addition to the naturally occurring variants studied, a non-functional mutant was designed as a functional negative control. This mutant, G538D, has an aspartate instead of a glycine at the fourth position in the ABC signature of the first nucleotide binding domain (NBD; Fig. 1). A corresponding mutation was previously reported to lead to non-functional, though stable, P-gp (Bakos et al., 1997) and MRP1 (Ren et al., 2004).

Statistics

The accumulation of AZT and PMEA in cells transfected with the reference, variants or mutant MRP4, were compared using an unpaired Student's *t*-test with Bonferroni correction for multiple testing.

Results

ABCC4 variants

A total of 98 variants were identified in 8660 bp in the coding and flanking intronic regions of the *ABCC4* gene encoding MRP4, with a frequency of approximately 11 variants per kb of

sequence. Of these, 43 variants are in the coding region and 22 are non-synonymous. For simplicity, only the coding variants, the variants within 10 base pairs from the exons and UTR variants are listed in Table 2. All the variants have been deposited in dbSNP and can be found at <http://www.pharmgkb.com> (Nguyen et al., 2006). Non-synonymous SNPs are well distributed throughout the protein, with the majority of them located in the intracellular regions (17 out of 22) (Fig. 1). Most high frequency SNPs (>5% in at least one population) are either synonymous (9 SNPs) or non-coding (25 SNPs). Only three non-synonymous variants (G187W, K304N and M744V) have frequencies higher than 5% in a given ethnic group. A relatively high number of singletons was found among the non-synonymous variants (14 out of 22) compared to synonymous variants (6 out of 21).

Values for θ and π were calculated for coding and non-coding sites in the different populations studied (Fig. 2). The same pattern of genetic variation is observed with each ethnic group, with slightly higher values for African Americans. Specifically, synonymous sites are highly variable, while non-synonymous sites have very low θ and π values. On average, the values are slightly higher for non-coding compared to coding regions. The θ values for the entire sequenced region in each population are in the same range as the previously reported average value of 10.4×10^4 for 24 human membrane transporters (not including *ABCC4*), while π values are higher than the reported value of 5.09×10^4 (Leabman et al., 2003). The ratio between non-synonymous and synonymous π values ($\pi_{NS}/\pi_S = 0.07$) is lower than the average value for membrane transporters (0.23) indicating a high degree of negative selection (Leabman et al., 2003). The Tajima's D values associated with these parameters are negative with the exception of non-coding and synonymous sites in Asian Americans and synonymous sites in African Americans (data not shown).

***ABCC4* haplotypes**

Initially, *ABCC4* haplotypes were inferred from all variable sites with a frequency higher than 5% in at least one population (corresponding to 37 sites in coding and non-coding regions of the gene). However, as a consequence of the high polymorphism observed within the *ABCC4* gene, as many as 257 haplotypes were identified (data not shown). Most haplotypes had a low frequency, with only 111 predicted for more than one chromosome. The most frequent haplotype was present in 8.6% of Asians Americans and 3.2% of Caucasians, but was absent in African Americans and Mexicans. Only two of the 257 haplotypes were shared by all four populations, and 229 haplotypes were specific to one population. Therefore, the information given by this haplotype analysis was limited and was consistent with minimal linkage disequilibrium across most of the gene. A separate analysis was performed only on coding and UTR sites that are present in at least 5% in one or more populations. This reduced the number of haplotypes to 71. Of these, only 45 were present in more than one chromosome (Fig. 3), and 34 were seen in 3 chromosomes or more. Fourteen haplotypes had a frequency of 5% or greater in at least one population. Significant interethnic variability was observed in haplotype distribution. Considering the most common 45 haplotypes represented in Figure 3, African Americans had the greatest number (32), while polymorphisms in Caucasians were represented by only 12 haplotypes, of which ten were shared with African Americans. It should be noted that because of the 5% minor allele frequency cut-off, only three non-synonymous variants were included in this haplotype analysis, consistent with no haplotype containing more than one non-synonymous variant.

Variant selection for functional studies

Ten non-synonymous variants were chosen for this study based on a frequency of $\geq 5\%$ in the populations studied (G187W, K304N and M744V), high evolutionary conservation (all variants except M744V) or a high Grantham value (G187W > C956S > P403L = G487E > K304N). Evolutionary conservation was determined by alignment of orthologous amino acid

sequences from seven mammalian species. Variants referred to as 'evolutionarily conserved' show a complete conservation across the seven species, with the exception of an arginine to lysine change for variant K304N in two species. The majority of these variants are localized in the intracellular region of the protein (Fig. 1).

Transport of AZT and PMEA by MRP4

Accumulation data confirmed previous reports indicating that AZT and PMEA are substrates of MRP4 (Schuetz et al., 1999; Imaoka et al., 2007). Like other nucleoside/nucleotide analogs, these molecules require phosphorylation for antiviral activity. AZT undergoes three sequential phosphorylations inside the cell and its monophosphate form is effluxed by MRP4. PMEA is a monophosphate and only two phosphorylation steps are required for its activity. PMEA is effluxed before being phosphorylated (Schuetz et al., 1999). The intracellular accumulation of both drugs was decreased when the cells were transfected with the reference ABCC4 cDNA compared to the cells transfected with the empty pcDNA5/FRT vector (~ 55% and 84% decrease for AZT and PMEA, respectively; Fig. 4A), due to MRP4-mediated efflux. As expected a mutation in a highly conserved glycine of NBD1 leads to a non-functional MRP4. This is illustrated by the fact that the accumulation of both substrates in the cells transfected with the G538D construct is not statistically different from the vector-transfected cells (Fig. 4A). Therefore, this mutant can be used as a negative control to validate the data obtained in the functional studies and confirms that MRP4 is responsible for the decrease observed in intracellular concentrations of AZT and PMEA.

Functional analysis of ABCC4 variants

The accumulation data showed that none of the SNPs is completely deleterious, and all MRP4 variants tested are functional. However, the function of two variants (G187W and G487E) was significantly reduced compared to the reference, and this was observed both with AZT and PMEA ($p < 0.001$ and $p < 0.005$, Student's t-test with Bonferroni correction for ten comparisons) (Fig. 4B). The P78A and P403L variants also showed a significantly lower function when transporting AZT ($p < 0.005$), but not PMEA (although a trend for reduced function was observed). Interestingly, the C956S variant showed a slight increase in function with PMEA ($p < 0.001$). The G187W variant had the greatest decrease in function, with a 43% and 69% increase in accumulation of AZT and PMEA, respectively, compared to reference MRP4.

In order to characterize the functional difference observed with some of the variants, we measured the intracellular accumulation of AZT and PMEA as a function of the extracellular concentration for the reference and the variants that showed a reduced function with one or both substrates (P78A, G187W, and G487E), as well as the C956S variant which was more functional with respect to PMEA transport (Fig. 5). For each MRP4 variant or the reference protein, the efflux transport velocity was estimated at each concentration by subtracting the concentration measured in the transfected cells from the intracellular accumulation in empty vector transfected cells. The transport velocity curves (Fig. 5) suggest that the differences reported with some of the variants (G187W and G487E) are real and concentration-independent, and this effect seems more pronounced with AZT.

Expression of the variants

To determine whether the functional variations observed were related to differences in expression, the mRNA and protein expression of ABCC4/MRP4 were investigated by TaqMan (data not shown) and Western Blot (Fig. 6). Endogenous expression of MRP4 was evident in HEK 293T cells, and this was greatly increased by transfection of ABCC4 plasmids. Taqman data showed that the transfection led to a significant increase in ABCC4 mRNA with all the

variants, with an average increase of ~100-fold. Negligible differences were observed between the RNA levels of the ABCC4 variants and reference.

MRP4 protein was also highly expressed in the cells, and variability in expression was observed among the variants and reference protein. The MRP4 and GAPDH signals were quantified using image analysis and the G187W variant was expressed at a significantly lower level than that of the reference MRP4 (data not shown). None of the other differences were significant when lysates from three separate transfections were blotted and quantified. It should be noted that the MRP4 protein was detected in the empty vector control cells with a longer exposure of the blot.

To check that the functional differences were not due to differences in localization of the variants at the cell membrane, immunocytochemistry studies were carried out with the less functional variants (G187W, G487E and P78A), the non-functional mutant (G538D) and the more functional variant (C956S). The variant MRP4 proteins are all expressed at the membrane and no significant difference in cell membrane localization can be seen between MRP4 reference and these variants (Fig. 7). A negative control with empty vector shows almost no signal with the same exposure time.

Discussion

Resequencing of *ABCC4* indicated a high degree of polymorphism. Among nine other ABC transporters that were resequenced in similar populations (*ABCC1*, *ABCC2*, *ABCC3*, *ABCC5*, *ABCC6*, *ABCB1*, *ABCB4*, *ABCB11* and *ABCG2*), only *ABCC6* has more variants. Considering only haplotypes inferred from coding and UTR variants, several blocks of variable sites are evident, in particular within exon 8, and across exons 22 to 23 and 26 to 31. The linkage disequilibrium patterns for the 14 variable sites in these 45 haplotypes is similar across all ethnic groups (data not shown), suggesting that this diversity happened in the ancestral population.

Genetic variation at synonymous sites is high while it is relatively low and similar to other ABC transporters at non-synonymous sites. The low π_{NS}/π_S ratio suggests that this gene has a low tolerance for non-synonymous compared to synonymous variations. This could explain why most non-synonymous variants are rare. However, one may infer from this low ratio that a mutation affecting the protein structure would be deleterious, but this has not been confirmed clinically. Similar to most ABC efflux transporters, no disease has been associated with a non-functional MRP4.

This study confirms the role of MRP4 in transporting AZT and PMEA by a direct measurement of transport in cells transfected with a known *ABCC4* sequence. The lipophilic ester prodrug of PMEA, bis(POM)-PMEA, has been used to increase passive intracellular uptake (Srinivas et al., 1993; Hatse et al., 1998). PMEA is then rapidly released intracellularly from the prodrug by esterases (Srinivas et al., 1993) and is effluxed unchanged by MRP4 (Schuetz et al., 1999). Endogenous MRP4 protein expressed in HEK 293T could contribute to the cellular efflux. This contribution seems greater in the case of AZT which shows a less dramatic difference between empty vector and MRP4 transfected cells than PMEA. Other transporters are likely to be involved in AZT and PMEA uptake and efflux (OAT1 and MRP5 for PMEA (Cihlar et al., 1999; Wijnholds et al., 2000), OAT1–4 and MXR for AZT (Takeda et al., 2002; Wang et al., 2004)) and passive diffusion of these antivirals will be determined by their lipophilicity. Differences in these alternate pathways for AZT and PMEA transport may explain the differences observed between these two substrates. MRP4 expression was also high in other cell lines, including MDCKII, CHO-K1, HepG2, and CV-1 cells. Moreover, because

MRP4 is highly expressed in the kidney (van Aabel et al., 2002), HEK 293T cells provide a realistic model for studying the function of this transporter.

MRP4-mediated transport of AZT and PMEA was observed with concentrations as low as 20 nM. Reported $C_{\max}$ values are 3.7 μ M following a 200 mg dose of AZT (Singlas et al., 1989) and 67 nM for a 10 mg dose of PMEA (<http://www.hepsera.com>). Therefore, an intermediate donor concentration of 100 nM was used in the first screen of the variants. All ten non-synonymous variants of MRP4 that were tested are functional. Four variants have significantly lower function, with the G187W variant displaying the greatest reduction in function. In contrast, the C956S variant had a significantly higher transport of PMEA. In all cases, a similar trend was observed with both substrates; this strongly suggests that the functional differences observed are real and substrate-independent within this same chemical group. The velocity curves can in theory be used to estimate and compare kinetic parameters ($V_{\max}$ and K_m) related to the transport of AZT and PMEA by MRP4 variant and reference proteins. However, the assay described in this study is an indirect measurement of the efflux, and is based on a difference in accumulation between transfected and untransfected cells. The sensitivity of the analytical method did not allow direct measurement of the amount of substrate being effluxed following accumulation. Therefore, while the $V_{\max}$ values could in theory reflect efflux by MRP4 (because the transport velocities related to MRP4 variants and reference have been normalized to empty vector transfected cells), the K_m values would only be apparent and correspond to the extracellular concentration to which the cells are exposed (instead of the intracellular concentration to which the transporter is really exposed).

A mutant designed to be non-functional by replacing a highly conserved glycine in NBD1 with an aspartate showed no difference in intracellular accumulation compared to the empty vector-transfected cells, confirming that the reduction of substrate accumulation seen with the reference and the variants is due to efflux by MRP4. This validates the MRP4 efflux data for AZT and PMEA and is consistent with earlier results with P-gp and MRP1, where a mutation in a specific conserved amino acid in one of the two NBDs is sufficient to lead to a total loss of function (Bakos et al., 1997; Ren et al., 2004). The glycine at the fourth position of the ABC signature is almost fully conserved in the ABC transporter family (it is present in 99% of 1000 transporters analyzed in different species).

The functional differences observed with most of the variants cannot be attributed to differences in expression of the proteins at the membrane or total MRP4 expression levels. One exception to this is the G187W variant, which had lower levels of antiretroviral transport and showed decreased expression by Western blotting. A similar observation has been made for several MRP2, OATP1B1 and OATP1A2 variants (Tirona et al., 2001; Hirouchi et al., 2004; Lee et al., 2005). Interestingly, the non-functional mutant is also correctly expressed at the membrane, showing that its loss of function is not due to a decreased stability or impaired trafficking to the membrane. What are other possible mechanisms to explain the functional differences? One hypothesis implicates the modifications in the chemical structure of the protein when replacing one amino acid with another. The Grantham value for G187W is the greatest among the non-synonymous variants of MRP4 ($D=184$), indicating that this variant has the greatest structural change, with respect to composition, polarity, and molecular volume (Grantham, 1974). This could contribute to a certain extent to the reduction in function. It is supported by the fact that the G487E and C956S variants also have high Grantham values ($D=98$ and 102 , respectively) and altered functions. However, this estimator can probably not account completely for these data, since another variant with a high Grantham value (K304N) does not show any functional difference.

The ~ 50% reduction in function observed with the G187W variant could be clinically relevant, while the small differences observed with the other variants (G487E, P78A, P403L and C956S)

are less likely to be significant. The G187W variant is present at a frequency of 2.5–13% in various ethnic groups. A decrease in MRP4 transport of antivirals could be beneficial as a result of higher target cell concentrations but could also result in a higher incidence of toxicity related to increased systemic and/or tissue levels of MRP4 substrates. The G187W MRP4 variant could also play a role at the organism level, since MRP4 is expressed in the kidney and the liver where it may be involved in antiviral elimination (Zamek-Gliszczynski et al., 2006; Imaoka et al., 2007). The expression of MRP4 variants in the liver has been evaluated in a recent clinical study, which showed no association between MRP4 polymorphism and RNA or protein expression in human liver (Gradhand et al., 2008). Interestingly, there was a trend toward decreased protein levels of 187W MRP4 in normal livers, consistent with our Western blot data. AZT is primarily eliminated by hepatic metabolism before being excreted in the urine, while PMEA is mainly renally excreted (http://us.gsk.com/products/assets/us_retrovir.pdf, <http://www.hepsera.com>). Therefore, the consequences of altered MRP4 transport on AZT and PMEA pharmacokinetics should be investigated.

Previous reports have shown a certain degree of substrate specificity associated with variants in membrane transporters (Erdman et al., 2006; Jeong et al., 2007), including a different specificity between endogenous substrates and drugs (Urban et al., 2006). This specificity should be further investigated for MRP4 which has both physiological and xenobiotic substrates. Inhibitory interactions of xenobiotics with MRP4 non-synonymous variants should also be considered. This is particularly relevant for nucleoside/nucleotide analogs used for treating HIV infection that are always administered as combination therapy.

In summary, *ABCC4* is a highly polymorphic gene, as illustrated by the large number of variants and haplotypes identified in this study. Convincing evidence of AZT and PMEA transport by MRP4 is provided in this first functional characterization of MRP4 non-synonymous variants. None of the ten non-synonymous variants of MRP4 which were studied are completely deleterious. The G187W variant shows the greatest decrease in function and lower expression in this in vitro system. The clinical consequences of this altered function (e.g. increased response or higher incidence of side effects) require further investigation.

Acknowledgements

We thank Jason Gow for excellent technical advice and helpful discussions and Libusha Kelly for the determination of evolutionary conservation of the variants studied.

This work was supported by NIH grant GM61390 (DLK), Swiss National Science Foundation postdoctoral fellowship PBGEB-111224 (NA), and Health and Labour Sciences Research Grant H16-iyaku-026 (TN).

Abbreviations

ABC, ATP-binding cassette
 AZT, azidothymidine
 cDNA, complementary DNA
 HEK 293T, Human embryonic kidney epithelial cells transformed with SV40 T antigen
 HIV, human immunodeficiency virus
 MRP, Multidrug resistance-associated protein
 NBD, nucleotide binding domain
 PMEA, 9-(2-phosphonylmethoxyethyl)adenine
 bis-POM-PMEA, bis(pivaloyloxymethyl)PMEA
 SNP, single nucleotide polymorphism
 SOPHIE, Studies of Pharmacogenetics in Ethnically Diverse Populations
 UTR, untranslated region

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Figure 1.

Transmembrane prediction for MRP4 and localization of the non-synonymous variants and non-functional mutant constructed for this study (NBDs: nucleotide binding domains, EC: evolutionarily conserved, EU: evolutionarily unconserved, red circles: non-synonymous SNPs, green circles: synonymous SNPs, black circle: non-functional mutant)

Figure 2.

Population genetics statistics for *ABCC4*. Shown are the π (A) and θ (B) parameters for *ABCC4* in the different populations studied (mean $\pm$ SD). Values were calculated for the total (solid), coding (diagonal), non-coding (open), synonymous (checkered), and non-synonymous (gray) variant sites.

	Exon												Ethnicity					
	1	5	6	8	11	18	22	23	26	28	31	CA	AA	AS	ME			
*1	C	G	C	G	G	G	C	A	G	C	T	A	G	T	0.244	0.032	0.025	0.110
*12					A	A								G	0.138	0.026	0.067	0.170
*10	T				A	A								G	0.000	0.000	0.058	0.000
*35	T				A	A									0.000	0.000	0.025	0.000
*29					A	A									0.031	0.032	0.108	0.050
*33					A	A					G				0.019	0.000	0.017	0.000
*34				T	A	A					G				0.019	0.000	0.000	0.000
*30			T		A	A									0.106	0.071	0.050	0.030
*32			T		A	A	T								0.000	0.032	0.000	0.000
*13			T		A	A								G	0.031	0.013	0.017	0.050
*31			T		A	A					G				0.062	0.019	0.000	0.010
*2														G	0.212	0.077	0.150	0.100
*9	T													G	0.000	0.000	0.017	0.000
*3				T										G	0.006	0.006	0.075	0.050
*5				T					A					G	0.000	0.013	0.000	0.000
*4				T							G			G	0.000	0.032	0.000	0.000
*17				T							G				0.000	0.000	0.000	0.030
*19				T						C			A		0.000	0.019	0.000	0.000
*18				T									A		0.000	0.013	0.000	0.000
*16				T											0.062	0.077	0.067	0.080
*42		T													0.000	0.000	0.042	0.020
*27		T									G				0.000	0.000	0.008	0.030
*6		T												G	0.000	0.000	0.058	0.070
*7								A						G	0.000	0.026	0.000	0.000
*20											G				0.069	0.006	0.000	0.120
*8											G			G	0.000	0.013	0.000	0.000
*40					A		T					G			0.000	0.026	0.000	0.000
*15					A		T					G		G	0.000	0.013	0.000	0.000
*14					A		T							G	0.000	0.019	0.000	0.000
*37					A		T								0.000	0.045	0.000	0.000
*38					A		T							A	0.000	0.038	0.000	0.000
*43														A	0.000	0.026	0.000	0.000
*44					A					C			A		0.000	0.032	0.000	0.000
*39					A		T			C			A		0.000	0.013	0.000	0.000
*45					A	A				C			A		0.000	0.019	0.000	0.000
*28					A										0.000	0.038	0.000	0.040
*11					A									G	0.000	0.000	0.000	0.020
*41					A			G				G			0.000	0.013	0.000	0.000
*36					A	A			A						0.000	0.013	0.000	0.000
*24			T		A	A			A	T		G			0.000	0.032	0.058	0.000
*23					A	A			A	T		G			0.000	0.000	0.033	0.000
*26					A	A	T		A	T		G			0.000	0.013	0.000	0.000
*25					A	A			A	T		G		G	0.000	0.000	0.017	0.000
*22				T					A	T		G			0.000	0.000	0.075	0.000
*21									A	T		G			0.000	0.013	0.017	0.000

Figure 3.

Sequence alignments and frequencies of *ABCC4* haplotypes. For simplification, only variants in the coding and UTR regions were used for haplotype estimation. A total of 71 haplotypes were constructed using the PHASE method with the sites present in more than 5% in at least one population. Only the 45 haplotypes that were found more than once are shown. Haplotypes have been aligned according to sequence similarity. The color code is as follows: a black box means reference sequence; red and green boxes correspond to non-synonymous and synonymous changes, respectively; the variants in the 5' and 3' UTR regions are colored in yellow. Haplotypes were numbered according to evolutionary considerations and population

frequency (*2 being the most common), but *1, which has no variant sites, has been set as the reference.

A.

B.

Figure 4.

Intracellular accumulation of AZT (gray bars) and PMEA (open bars) for reference and variant MRP4. Accumulation is expressed relative to reference MRP4 for negative controls (A) and the selected variants (B). Cells were incubated at 37°C with 100 nM AZT for 30 min, or 100 nM PMEA for one h. The values shown are the mean $\pm$ standard deviation, for $n = 6-12$ replicates. Significant differences between reference and variants were assessed by a Student's *t*-test with Bonferroni correction for multiple testing (* $p < 0.005$, ** $p < 0.001$)

Figure 5. Concentration-dependence of (A) AZT and (B) PMEA accumulation in cells expressing either the reference MRP4 or one of the variants. The values represent the mean $\pm$ SD of $n=3$ (AZT) or $n=4$ (PMEA) determinations (■ reference, ◆ P78A, ▲ G187W, △ G487E, and ○ C956S).

Figure 6. Expression of MRP4 in transfected HEK 293T cells. Protein was isolated 24 hours after transfection with pcDNA5/FRT, MRP4 reference and MRP4 variants and immunoreactive MRP4 was detected by Western blot. GAPDH (lower panel) was measured as a loading control.

Figure 7.

Localization of MRP4 proteins by immunocytochemistry. Cells were grown on cover slips and transfected with the different variants. Twenty four h after transfection, cells were incubated with a monoclonal antibody to MRP4 (M4-I10) and a fluorescent goat anti-rat secondary antibody. A: Empty vector, B: MRP4 reference, C: P78A, D: G187W, E: P403L, F: G487E, G: C956S, H: G538D. All images are shown at 200 $\times$ magnification.

Table 1

Primers used for constructing the variants and the non-functional mutant by site-directed mutagenesis.

Variant	Sequence ^a
P78A	F: GAATGACGCACAGAAGGCTTCTTTAACAAGAGC R: GCTCTTGTTAAAGAAGCCTTCTGTGCGTCATTTC
G187W	F: GTAACATGGCCATGTGGAAGACAACCACAG R: CTGTGGTTGTCTTCCACATGGCCATGTTAC
K293E	F: GTACGCCTGGGAAGAGTCATTTTCAAATC R: GATTTGAAAATGACTCTTCCCAGGCGTAC
K304N	F: CCAATTTGAGAAATAAGGAGATTTCCAAG R: CTTGGAAATCTCCTTATTTCTCAAATTGG
P403L	F: GCGCAACCGTCAGCTGCTGTCAGATGGTAAAAAG R: CTTTTTACCATCTGACAGCAGCTGACGGTTGCGC
G487E	F: CCCTGGGTGTCTCGGAAACTCTGAGGAG R: CTCCTCAGAGTTTCCGAGAACACCCAGGG
K498E	F: GTAATATTTTATTTGGGAAGGAATACGAAAAGG R: CCTTTTCGTATTCTTCCCAAATAAAATATTAC
M744V	F: GGGCAAACAAACAAAGTGTGCTAAATGTCACTG R: CAGTGACATTTAGCACACTTTGTTTGTGTTGCC
C956S	F: CGTCTGGATGCCATCTCTGCCATGTTTGTGTCATC R: GATGACAAACATGGCAGAGATGGCATCCAGACG
V1071I	F: CACAAGAAAAGATTGGCATTGTGGGAAG R: CTTCCCACAATGCCAATCTTTTCTTGTG
G538D	F: GGAACCACGCTGAGTGGAGACCAGAAAGCACGGGTAAACC R: GGTTTACCCGTGCTTTCTGGTCTCCACTCAGCGTGGTTCC

^aThe mutations introduced in the sequence are underlined.

Table 2

Selected genetic variants in *ABCC4*^a.

Exon	Nucleotide Position	Golden Path Position ^b	Variant Flag ^b	Nucleotide change	Amino acid or intronic position	Amino acid change	Grantham score	AA (n=160)	CA (n=160)	Allele frequency ^c AS (n=120)	ME (n=100)
1	–49	chr13:94751618	rs3751333	C > T	5'-UTR	-	-	0.013	0.019	0.102	0.03
1	52	chr13:94751518	rs11568681	C > A	18	Leu to Ile	5	0.006	0.044	0.034	0.01
1	IVS1+10	chr13:94751486	rs11568682	C > T	intronic	-	-	0.006	0.019	0	0
2	IVS 2+7	chr13:94697891	rs11568700	C > T	intronic	-	-	0.031	0	0	0
3	IVS 3–5	chr13:94697355	rs4148437	T > C	intronic	-	-	0.087	0.331	0.183	0.25
3 ^d	232	chr13:94697304	rs11568689	C > G	78	Pro to Ala	27	0	0	0.008	0
4	IVS 4–10	chr13:94685099	rs11568638	C > T	intronic	-	-	0.025	0	0	0
4	IVS 4+10	chr13:94684855	rs11568637	A > G	intronic	-	-	0.031	0	0.117	0
5	551	chr13:94661017	rs11568657	T > C	184	Met to Thr	81	0.013	0	0	0
5	559	chr13:94661009	rs11568658	G > T	187	Gly to Trp	184	0	0.025	0.108	0.130
6	669	chr13:94659805	rs899494	C > T	223	syn	-	0.219	0.200	0.125	0.090
6	717	chr13:94659757	rs11568674	T > C	239	syn	-	0	0	0.008	0
7	877	chr13:94658089	rs11568684	A > G	293	Lys to Glu	56	0.006	0	0	0
8	912	chr13:94657036	rs2274407	G > T	304	Lys to Asn	94	0.181	0.087	0.225	0.160
8	951	chr13:94656997	rs2274406	G > A	317	syn	-	0.619	0.406	0.458	0.390
8	969	chr13:94656979	rs2274405	G > A	323	syn	-	0.312	0.406	0.458	0.320
8	1035	chr13:94656913	rs11568703	G > A	345	syn	-	0	0.013	0	0
8	1067	chr13:94656881	rs11568701	C > T	356	Thr to Met	81	0	0	0	0.010
8	IVS 8+8	chr13:94656779	rs11568702	T > A	intronic	-	-	0	0.013	0	0
9	1208	chr13:94645146	rs11568705	C > T	403	Pro to Leu	98	0.006	0	0	0
11	1458	chr13:94637043	rs11568670	G > A	486	syn	-	0	0.006	0	0
11	1460	chr13:94637041	rs11568668	G > A	487	Gly to Glu	98	0	0	0.008	0
11	1492	chr13:94637009	rs11568669	A > G	498	Lys to Glu	56	0.025	0	0	0
11	1497	chr13:94637004	rs1557070	C > T	499	syn	-	0.238	0	0	0
14	1737	chr13:94620874	rs11568664	T > C	579	syn	-	0.006	0	0	0
15	IVS 15–7	chr13:94616629	rs11568696	A > G	intronic	-	-	0.031	0	0	0
15	1875	chr13:94616572	rs11568699	A > G	625	Ile to Met	10	0.006	0.006	0	0
15	2000	chr13:94616447	rs11568697	C > T	667	Pro to Leu	98	0	0	0	0
15	2001	chr13:94616446	rs11568698	C > T	667	syn	-	0.013	0	0	0
16	2100	chr13:94614708	rs11568666	C > T	700	syn	-	0	0.013	0	0
18	2230	chr13:94613455	rs9282570	A > G	744	Met to Val	21	0.050	0	0	0
18	2269	chr13:94613416	rs3765534	G > A	757	Glu to Lys	56	0.025	0.013	0.033	0.030
19	2364	chr13:94611535	rs11568709	C > T	788	syn	-	0.006	0	0	0
20	2459	chr13:94566253	rs11568659	G > T	820	Arg to Ile	97	0.006	0	0	0
21	2560	chr13:94533521	rs11568694	G > T	854	Val to Phe	50	0.006	0	0	0
21	2577	chr13:94533504	rs11568691	C > T	859	syn	-	0	0.006	0	0
22	2698	chr13:94525795	rs11568673	G > T	900	Val to Leu	32	0	0.006	0	0.010
22	2712	chr13:94525781	rs1678339	G > A	904	syn	-	0.156	0.031	0.217	0.020
23	2844	chr13:94524542	rs1189466	C > T	948	syn	-	0.075	0.031	0.208	0.020
23	2847	chr13:94524539	rs11568708	C > T	949	syn	-	0.019	0	0	0
23	2867	chr13:94524519	rs11568707	G > C	956	Cys to Ser	112	0.006	0	0	0
26	3211	chr13:94513114	rs11568653	G > A	1071	Val to Ile	29	0.006	0	0	0
26	3255	chr13:94513070	rs11568652	C > A	1085	syn	-	0.013	0	0	0.010
26	3310	chr13:94513015	rs11568655	T > C	1104	syn	-	0.100	0	0	0.010
26	3348	chr13:94512977	rs1751034	A > G	1116	syn	-	0.231	0.169	0.242	0.200
27	3425	chr13:94503381	rs11568644	C > T	1142	Thr to Met	81	0	0.006	0	0
28	3609	chr13:94494541	rs11568695	G > A	1203	syn	-	0.206	0	0	0.010
29	3659	chr13:94494013	rs11568639	G > A	1220	Arg to Gln	43	0.006	0	0	0
29	3723	chr13:94493949	rs11568640	C > T	1241	syn	-	0.006	0	0	0
30	3774	chr13:94484956	rs11568704	G > A	1258	syn	-	0.037	0	0	0

Exon	Nucleotide Position	Golden Path Position ^b	Variant Flag ^b	Nucleotide change	Amino acid or intronic position	Amino acid change	Grantham score	AA (n=160)	Allele frequency ^c AS (n=120)	ME (n=100)
31	IVS 31–3	chr13:94471940	rs9524765	C > T	intronic	-		0.225	0	0.02
31	3941	chr13:94471867	rs11568688	A > G	1314	Gln to Arg	43	0.006	0	0
31	4016	chr13:94471792	rs3742106	T > G	3' UTR	-		0.287	0.388	0.470

AA: African Americans, CA: Caucasians, AS: Asian Americans, ME: Mexican Americans

^a Only coding region, UTR and intronic variants within 10 bp of the splice site are shown.

^b Golden path positions are from March 2006 assembly and variant flags are from dbSNP build 126.

^c The number of chromosomes sequenced in each ethnic group is shown in parentheses.

^d Non-synonymous variants selected for this study are in bold.