The Human Multidrug Resistance Protein 4 (MRP4, ABCC4): Functional Analysis of a Highly Polymorphic Gene

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ABSTRACT

ABCC4 encodes multidrug resistance protein 4 (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 nonsynonymous variants. Resequencing was performed in a large ethnically diverse population. Ten nonsynonymous 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 nonsynonymous. 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 to assess the clinical significance of this altered function and expression and to evaluate substrate specificity of this functional change.

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), antineoplastic drugs [methotrexate, 6-mercaptopurine, 6-thioguanine, camptothecins (Lee et al., 2000; Chen et al., 2002; van Aubel et al., 2002), anticancer drugs [methotrexate, 6-mercaptopurine, 6-thioguanine, camptothecins (Lee et al., 2000; Chen et al., 2002; Wiolninga et al., 2002; Leggas et al., 2004; Tian et al., 2005)], as well as endogenous 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 and 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). It is interesting that 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). Similar to 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 wild-type mice (Leggas et al., 2004). Therefore, its physiological role could include detoxification of drugs, as well as that 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. (2006) showed a 20% increase in lamivudine-triphosphate intracellular concentrations in patients carrying the 4131T>G variant, whereas the 3724G>A variant was associated with a trend for elevated AZT-triphosphate, suggesting a reduced MRP4 efflux function (Anderson et al., 2006). It is interesting that the 4131T>G variant is in the 3′-untranslated region (UTR) of the gene, whereas the 3724G>A variant is synonymous and there is no clear mechanism explaining these effects. In another study, no association was observed between two nonsynonymous 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 noncholestatic 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 nonsynonymous 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 human immunodeficiency virus and hepatitis B infections, respectively, and altered function of MRP4 might contribute to interindividual variability in the response to these antivirals.

Materials and 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, nonsynonymous, and noncoding sites according to Tajima (Tajima, 1989, 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 10 times, and haplotypes estimated in at least seven runs were reported. Analyses were carried out using all available sites of ABCC4 above the mean allele frequency cut-off. A separate analysis excluded the intronic sites.

Construction of ABCC4 Variants and Nonfunctional Mutant. The ABCC4 cDNA was cloned from human kidney using the following primers: forward 5′-AGCATCCTGTTGAGGTCAA-3′ and reverse 5′-ACGGACTTGACATTTGTTG-3′. The cDNA was originally inserted into the pCR2.1-TOPO vector (Invitrogen, 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/Leu181Ile and rs1557070) with low frequencies (<10%) that are not found in our reference sequence. This reference plasmid was used as a template for constructing the 10 nonsynonymous variants selected for this study, as well as a nonfunctional 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 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). They were maintained in minimal essential Eagle’s medium with Earle’s balanced salt solution, supplemented with sodium pyruvate, penicillin, streptomycin, and nonessential 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-L-lysine-coated 24-well plates (BD Biosciences Discovery Labware, Bedford, MA). The next day, the cells were transfected with 0.8 μg of DNA (ABCC4 reference, variant or nonfunctional mutant) and 1 μl of Lipofectamine 2000 (Invitrogen) in each well, according to the manufacturer’s protocol. Accumulation studies were performed 24 h after transfection. Cells were washed with phosphate-buffered saline and incubated with 100 nM [3H]bis(pivaloyloxymethyl)[9-(2-phosphonylmethoxyethyl)]adenosine(2′,2′-dideoxy-L-2′-aminopurine-9-yl)ethoxymethyl-2,2′-dimethylpropanoyloxyxymethoxyphosphoryl)oxyethyl] 2,2′-dimethylpropanoate (bis-POM-PMEA) or [3H]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, whereas 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 Biochemicals, Inc.). 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 liquid scintillation fluid (MP Biomedical, 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).
TABLE 1

<table>
<thead>
<tr>
<th>Variant</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt; (5′–3′)</th>
</tr>
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<tbody>
<tr>
<td>P78A</td>
<td>F: GAATGACCGCAGAAGGCTCTTTTAAACAAGAGC</td>
</tr>
<tr>
<td></td>
<td>R: GCTCTTGTTAAAGAAGCCTCTTGCTGCTATTTC</td>
</tr>
<tr>
<td>G187W</td>
<td>F: GATACTTTGGCATTTGGAAGACACCCAGAC</td>
</tr>
<tr>
<td></td>
<td>R: CTGTTGTTGCTTCACATGGGCCATGTTGATTA</td>
</tr>
<tr>
<td>K293E</td>
<td>F: CTGAGGCTGGAGAAAGTAGATTTCTTTAAC</td>
</tr>
<tr>
<td></td>
<td>R: GATTTGAATGAGCTCCTCCAGGAGC</td>
</tr>
<tr>
<td>K304N</td>
<td>F: CCAATTAGAAGAATTAAAGGAGATTTCC</td>
</tr>
<tr>
<td></td>
<td>R: CTTCCTAGAGTTTGGACAGAGGAG</td>
</tr>
<tr>
<td>P403L</td>
<td>F: GCCCAACCGCTAGCTGCTAGTGAAGTAAAAAG</td>
</tr>
<tr>
<td></td>
<td>R: CTTTTTACCATCTAGCAGCTGTTGCTGGCC</td>
</tr>
<tr>
<td>G487E</td>
<td>F: CCGTGGGTGTCTCAGAACACTGAGGAGG</td>
</tr>
<tr>
<td></td>
<td>R: CTCTCTAGAGTTTGGAGGAG</td>
</tr>
<tr>
<td>K498E</td>
<td>F: GTAATATTTTATTTGAGAAGAATAGCAGAGG</td>
</tr>
<tr>
<td></td>
<td>R: CTTTTCTGTATTCTCTCCAAATAATATTAC</td>
</tr>
<tr>
<td>M744V</td>
<td>F: CGGCAACACAAAGAAGTAGTGCAATTGTAAG</td>
</tr>
<tr>
<td></td>
<td>R: TAGTGACATTGACACAGAGATTCTTGGCC</td>
</tr>
<tr>
<td>C956S</td>
<td>F: CTGCTGGTGCATCTGCTGCAATGTCGATTC</td>
</tr>
<tr>
<td></td>
<td>R: CATGAAATGATGGAGATGACAGATCACAGC</td>
</tr>
<tr>
<td>V1071I</td>
<td>F: CACAAGAAAGATGGAATGGTGAGAAG</td>
</tr>
<tr>
<td></td>
<td>R: CTCCCCAAGATGCCATCTTTCTGTG</td>
</tr>
<tr>
<td>G538D</td>
<td>F: GGAACCCGCTGAGTGAGAGAAGGGACAGCACGGGTAAACC</td>
</tr>
<tr>
<td></td>
<td>R: GGTTTACCCTGCTTTCTGCTGCTCCACTCAGG</td>
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<sup>a</sup>The mutations introduced in the sequence are underlined.

**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 reverse transcription-polymerase chain reaction (TaqMan) and compared to its basal expression in human membrane transporters (not including ABCC4). ABCC4 mRNA levels were normalized to GAPDH. 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. In addition to the naturally occurring variants studied, a nonfunctional 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 protein. A functional negative control. This mutant, G538D, has an aspartate instead of a glycine at the fourth position in the ABC signature of the protein.

**Subcellular Localization (Immunocytochemistry).** Cells were grown on poly-D-lysine-coated coverslips (BD Biosciences) and then grown on poly-D-lysine-coated coverslips (BD Biosciences, Billerica, MA). 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.

**Design of a Nonfunctional Mutant.** In addition to the naturally occurring variants studied, a nonfunctional 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 mutant, G538D, was 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.

**Results**

**ABCC4 Variants.** A total of 98 variants were identified in 8660 base pairs in the coding and flanking intronic regions of the ABCC4 gene encoding MRP4, with a frequency of approximately 11 variants per kilobase of sequence. Of these, 43 variants are in the coding region, and 22 are nonsynonymous. For simplicity, only the coding variants, the variants within 10 base pairs from the exons and UTR variants are listed in Table 2. All of the variants have been deposited in dbSNP and can be found at http://www.pharmgkb.com (Nguyen et al., 2006). Nonsynonymous SNPs are well distributed throughout the protein, with the majority of them located in the intracellular regions (17 of 22) (Fig. 1). Most high-frequency SNPs (>5% in at least one population) are either synonymous (9 SNPs) or noncoding (25 SNPs). Only three nonsynonymous 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 nonsynonymous variants (14 of 22) compared to synonymous variants (6 of 21).

Values for θ and π were calculated for coding and noncoding 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, whereas nonsynonymous sites have very low values. The frequencies of nonsynonymous sites in Asian Americans and synonymous sites in African Americans are in the same range as the previously reported average value of 10.4 × 10<sup>4</sup> for 24 human membrane transporters (not including ABCC4), whereas π values are higher than the reported value of 5.09 × 10<sup>4</sup> (Leabman et al., 2003). The ratio between nonsynonymous and synonymous π values (π<sub>NS</sub>/π<sub>S</sub> = 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 noncoding 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 noncoding 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 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 three 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 Fig. 3, African Americans had the greatest number (32), whereas polymorphisms in Caucasians were represented by only 12 haplotypes, of which 10 were shared with African Americans. It should be noted that because of the 5% minor allele frequency cut-off, only three nonsynonymous variants were included in this haplotype analysis, consistent with no haplotype containing more than one nonsynonymous variant.

**Variant Selection for Functional Studies.** Ten nonsynonymous variants were chosen for this study based on a frequency of ≥5% in the populations studied (G187W, K304N, and M744V), high evolutionary conservation (all variants with the exception of 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). Similar to 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 nonfunctional 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 that all of the 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’s correction for 10 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). It is interesting that 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.

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 of 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 was 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 nonfunctional 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 had more variants. With consideration to only haplotypes inferred from coding and UTR variants, several blocks of variable sites were 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 were similar across all ethnic groups (data not shown), suggesting that this diversity happened in the ancestral population.

Genetic variation at synonymous sites is high, although it is relatively low and similar to other ABC transporters at nonsynonymous sites. The low $\pi_{NS}/\pi_S$ ratio suggests that this gene has a low tolerance for nonsynonymous compared to synonymous variations. This could explain why most nonsynonymous 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 nonfunctional 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 intracel-
lularly 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 probably involved in AZT and PMEA uptake and efflux (OAT1 and MRPI 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 (Madin-Darby canine kidney II), Chinese hamster ovary-K1, HepG2, and CV-1 cells. Moreover, because MRP4 is highly expressed in the kidney (van Aubel 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 \( C_{\text{\text{max}}} \) values are 3.7 \( \mu \text{M} \) 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 10 nonsynonymous 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 \( (V_{\text{\text{max}}} \text{ and } K_{\text{m}}) \) 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, whereas the \( V_{\text{\text{max}}} \) 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 \( K_{\text{m}} \) 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 nonfunctional 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-glycoprotein 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 MRPI, OATP1B1, and OATP1A2 variants (Tirona et al., 2001; Hirouchi et al., 2004; Lee et al., 2005). It is noteworthy that the nonfunctional 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 nonsynonymous 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 probably cannot account completely for these data, because 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, whereas 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 to 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, because 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). It is interesting that there was a trend toward decreased protein levels of 187W MRP4 in various ethnic groups. A decrease in MRP4 transport of nucleoside-based antiviral drugs could also result in a higher incidence of toxicity related to increased systemic and/or tissue levels of MRP4 substrates.

In summary, ABC4 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 nonsynonymous variants. None of the 10 nonsynonymous variants of MRP4, which were studied, is 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.

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References


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