Analysis of Pseudoxanthoma Elasticum–Causing Missense Mutants of ABCC6 *In Vivo;* Pharmacological Correction of the Mislocalized Proteins

Viola Pomozi[1], Christopher Brampton[2], Krisztina Fülöp[1], Li-Hsieh Chen[2], Ailea Apana[2], Qiaoli Li[3], Jouni Uitto[3], Olivier Le Saux[2,4] and András Váradi[1,4]

Mutations in the *ABCC6* gene cause soft-tissue calcification in pseudoxanthoma elasticum (PXE) and, in some patients, generalized arterial calcification of infancy (GACI). PXE is characterized by late-onset and progressive mineralization of elastic fibers in dermal, ocular, and cardiovascular tissues. GACI patients present a more severe, often prenatal arterial calcification. We have tested 10 frequent disease-causing *ABCC6* missense mutants for the transport activity by using Sf9 (Spodoptera frugiperda) cells, characterized the subcellular localization in MDCKII (Madin–Darby canine kidney (cell line)) cells and in mouse liver, and tested the phenotypic rescue in zebrafish. We aimed at identifying mutants with preserved transport activity but with improper plasma membrane localization for rescue by the chemical chaperone 4-phenylbutyrate (4-PBA). Seven of the mutants were transport-competent but mislocalized in mouse liver. The observed divergence in cellular localization of mutants in MDCKII cells versus mouse liver underlined the limitations of this 2D *in vitro* cell system. The functionality of ABCC6 mutants was tested in zebrafish, and minimal rescue of the morpholino-induced phenotype was found. However, 4-PBA, a drug approved for clinical use, restored the plasma membrane localization of four ABCC6 mutants (R1114P, S1121W, Q1347H, and R1314W), suggesting that allele-specific therapy may be useful for selected patients with PXE and GACI.

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INTRODUCTION

Ectopic calcification occurs in various common conditions, such as diabetes, hypercholesterolemia, and chronic renal insufficiency, as well as in certain genetic conditions. Pseudoxanthoma elasticum (PXE, OMIM 26480) is one of these heritable disorders characterized by late-onset and progressive mineralization of elastic fibers in dermal, ocular, and cardiovascular tissues. Generalized arterial calcification of infancy (GACI, OMIM 614473) also presents a calcification phenotype with characteristics similar to the arterial phenotype of PXE, although it is more severe in some instances with prenatal mineralization of the arterial blood vessels. Loss-of-function mutations in *ABCC6* cause both PXE and a subset of GACI cases (most GACI patients carry mutations in the *Ectonucleotide Pyrophosphatase/Phosphodiesterase 1* (*ENPP1*) gene) (Nitschke *et al.*, 2012; Li *et al.*, 2013). ENPP1 is a cell surface enzyme generating inorganic pyrophosphate, a solute that regulates cell differentiation and serves as an essential physiologic inhibitor of calcification. Interestingly, heterozygous *ABCC6* mutations confer an increased susceptibility to cardiovascular diseases (Trip *et al.*, 2002; Köblös *et al.*, 2010; Martin *et al.*, 2011), although this finding is somewhat controversial (Hornstrup *et al.*, 2011). *ABCC6* is primarily expressed in the liver and at a lower level in kidneys. The corresponding protein is localized in the basolateral plasma membrane (Pomozi *et al.*, 2013), and it facilitates the sinusoidal efflux of an unknown metabolite(s) toward the bloodstream. Because the ectopic calcification occurs in peripheral tissues, PXE is considered as metabolic disease (Jiang *et al.*, 2009; Uitto *et al.*, 2010). On the same line of reasoning, this might be true for ABCC6-connected GACI as well.

The number of disease-causing ABCC6 variants identified thus far largely exceeds 300 (Pfendner *et al.*, 2007), and the mutation spectra of ABCC6 in PXE and in GACI overlap. In spite of this large number of mutations, no clear genotype–phenotype correlation was established for PXE (Le Saux *et al.*, 2001; Chassaing *et al.*, 2005; Pfendner *et al.*, 2007). In the light of the phenotype divergence between the PXE- and

[1]*Institute of Enzymology, RCNS, Hungarian Academy of Sciences, Budapest, Hungary;* [2]*Department of Cell and Molecular Biology, John A Burns School of Medicine, University of Hawaii, Honolulu, Hawaii, USA and* [3]*Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, Pennsylvania, USA*

[4]*These authors contributed equally to this work.*

Correspondence: András Váradi, Institute of Enzymology, RCNS, Hungarian Academy of Sciences, Karolina ut 29, 1113 Budapest, Hungary. E-mail: varadi@enzim.hu

Abbreviations: 4-PBA, 4-phenylbutyrate; GACI, generalized arterial calcification of infancy; LTC4, leukotriene C4; MDCKII, Madin–Darby canine kidney (cell line); PXE, pseudoxanthoma elasticum; wt, wild type

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ABCC6-related GACI patients, notably the severity and the affected tissues, the lack of clear correlation is not surprising.

Most of the disease-causing mutations in ABCC6 are missense, and a significant fraction of these clusters in areas critical to the physiological function of the protein (Fülöp *et al.*, 2009; Le Saux *et al.*, 2011). In fact, amino-acid substitutions in ABC transporter proteins, such as ABCC6, tend to result in the loss of function either through changes in the catalytic/transport activity, in the intracellular trafficking, the conformational stability, or any combination thereof.

We have characterized several ABCC6 mutants with substantial residual transport activity but abnormal intracellular processing, that is, with no or only partial plasma membrane localization (Le Saux *et al.*, 2011). Deciphering consequences of disease-associated missense mutations may assist in the development of individualized, mutation-based therapeutic applications. Specifically, these mutant proteins appear as excellent candidates for ''folding-correction'' in order to adjust their intracellular trafficking. Indeed, we have identified one such mutant, R1314W, whose cellular localization was normalized using the chemical chaperone sodium 4-phenylbutyrate (4-PBA) in both *in vitro* and *in vivo* experiments. In contrast to pharmacoperones, such chemical chaperones have the notable advantage of promoting folding with no direct interaction with the proteins, thus not interfering with their function (Ulloa-Aguirre and Conn, 2011). In support of this approach, several studies have shown that 4-PBA can partially rescue the intracellular trafficking of the frequent DeltaF508 variant of ABCC7/CFTR (cystic fibrosis transmebrane regulator) and maybe some of its channel function (Rubenstein and Zeitlin, 2000). Of particular interest is that 4-PBA is also approved by US Food and Drug Administration for clinical use in urea cycle disorders and thalassemia (Dover *et al.*, 1992; Perrine *et al.*, 1993; Maestri *et al.*, 1996). Many membrane proteins with disease-causing mutations have also been successfully subjected *in vitro* to 4-PBA treatment, resulting in marked improvement of their folding/trafficking: ABCA1 (Sorrenson *et al.*, 2012), ABCA3 (Cheong *et al.*, 2006), LDL-receptor (Tveten *et al.*, 2007), BSEP11/ABCB11 (Hayashi and Sugiyama, 2007, 2009; Lam *et al.*, 2007), ATP7B (van den Berghe *et al.*, 2009), ATP8B1 (van der Velden *et al.*, 2010), and ATP-sensitive potassium channel/ABCC8 (Powell *et al.*, 2011).

In the present work, we have fully characterized 10 ABCC6 missense mutants associated with PXE and GACI with respect to their transport activity, stability, and conformation/folding both *in vitro* and *in vivo* for the purpose of evaluating their rescue potential by 4-PBA. The *in vitro* testing was performed in cell-free assays, as well as in cultures of polarized and nonpolarized MDCKII (Madin–Darby canine kidney (cell line)) cells. The *in vivo* analysis relied on two complementary animal models. First, the transient expression of the mutant proteins in mouse liver was carried out to determine their subcellular tissue localization, and second in zebrafish embryo system (Li *et al.*, 2010) to evaluate the rescue of the morpholino-induced developmental phenotype provided by the human ABCC6 variants.

RESULTS

We have generated 12 ABCC6 variants using PCR mutagenesis (Table 1), 11 of which are associated with PXE, 1 of which is also a causative mutation in GACI. In addition to 10 missense mutants, R1141X was also used as a negative control in certain experiments. Six of the mutants were partially characterized in our previous studies (Iliás *et al.*, 2002; Le Saux *et al.*, 2011). The disease-associated mutations were selected for their frequency in PXE patients (S Terry of PXE International, personal communication), by that in GACI (Nitschke *et al.*, 2012; Li *et al.*, 2013), and by their predicted localization in the homology model (Fülöp *et al.*, 2009). Their positions in the membrane topology model are indicated in Figure 1a. We also included an N-terminally truncated version (delABCC6), lacking amino acids 2–275, that is, domains TMD0 and L0. The mutated cDNAs were cloned into baculovirus vector for Sf9 (Spodoptera frugiperda) insect cell expression, into retroviral vector for expression in MDCKII cells (Sinkó *et al.*, 2003), into the pLIVE vector for liver-specific *in vivo* expression in mice, and into Bluescript II SK+ vector for *in vitro* transcription of human ABCC6 cDNA to mRNA for subsequent injection into zebrafish embryos.

Biochemical characterization of transport activity

Inside-out vesicles of Sf9 cells expressing the human ABCC6 variants were used in the rapid filtration transport assay in the presence of two model substrates: LTC4 (leukotriene C4) (50 nM) and *N*-ethylmaleimide-glutathione (4 μM, not shown). As shown in Figure 1b, the wild-type (wt) ABCC6 is fully functional in the biochemical transport assay, as shown earlier (Iliás *et al.*, 2002), and 7 of the 10 missense mutant variants included in this study were also active as transporters. Two missense variants (V1298F and G1321S) showed very little, if any, transport activity (Iliás *et al.*, 2002). The R1339C was found to be unstable in Sf9 cells, similar to that shown earlier (Le Saux *et al.*, 2011), and its transport activity could not be assayed. The delABCC6 mutant showed low transport activity. The nonsense R1141X mutant was not expressed in Sf9 cells.

Subcellular localization *in vitro*

Each mutant (with the exception of R1141X) was individually expressed in MDCKII cells, which were then grown either on plastic wells as nonpolarized cells or on Transwell filters, which ensures development of monolayers of polarized cells. The wtABCC6 was present predominantly in the plasma membrane in both types of cell cultures, and specifically in the basolateral compartment of the polarized cells (Figure 2, columns 1 and 3). The delABCC6 was found to be entirely intracellular in both cell culture types, thus serving as an intracellular (''incorrect'') localization control. Eight of the ten missense mutants when expressed in polarized and nonpolarized MDCKII cultures did not show wt–like subcellular localization (Figure 2, columns 1 and 3). In nonpolarized cells, only S1121W and the transport-deficient V1298F were targeted to the plasma membrane. In addition to these, three other mutants, R1114P, R1138Q, and T1301I, were also

found in the basolateral plasma membrane when expressed in polarized MDCKII cells (Figure 2, columns 1 and 3).

Investigation of subcellular localization *in vivo* in mouse liver

ABC transporters are frequently studied in cultures of kidney-derived MDCKII cells, but this cell type does not represent the physiology of the liver where ABCC6 is primarily present. Therefore, to determine subcellular localization of mutant ABCC6 variants, we used hydrodynamic tail vein injection of pLIVE vectors into normal C57BL/6J mice. The vectors contained the appropriate cDNA constructs under the control of a liver-specific mouse albumin promoter. As we have demonstrated previously, we could achieve a high level of liver-specific expression, whereas no human protein was detected in other organs (Le Saux *et al.*, 2011). Each mutant was injected into at least three mice, and the livers were harvested at 24 hours after injection. To detect possible intracellular trafficking defects of the mutant ABCC6 proteins, expression and the subcellular distribution of ABCC6 proteins in the liver were determined by immunohistochemistry and confocal microscopy, and 50–100 cells expressing the human protein were individually imaged to assess subcellular localization. As we have shown previously, the wtABCC6 is targeted to the plasma membrane, and it shows colocalization with the endogenous mouse Abcc6 (Figure 3, column 1), whereas delABCC6 was intracellular. The R1459C and the transport-inactive V1298F mutants were found in subcellular localization identical to the wt, whereas all the other mutants showed various degrees of intracellular localization, as illustrated in Figure 3, column 1. Interestingly, S1121W, which showed wt–like plasma membrane localization in both MDCKII expression systems, was mostly intracellular in mouse liver, similar to delABCC6. It is worth noting that R1114P and R1138Q mutants were found both intracellularly and in the plasma membrane. The R1141X mutant was not expressed in the mouse liver.

Rescue of the developmental phenotype of morpholino-silenced zebrafish

Our earlier work indicated that both the mouse *Abcc6* and human *ABCC6* mRNA provided a nearly complete rescue of the developmental phenotype triggered by morpholino-mediated silencing of the *Abcc6a* gene in zebrafish embryos (Li *et al.*, 2010; Zhou *et al.*, 2013). In the present study, wt and mutant human *ABCC6* mRNAs were transcribed *in vitro* and injected together with a morpholino into zebrafish embryos. At least 100 embryos were injected for each mRNA variant. As shown in Figure 4, the wt human *ABCC6* rescues the zebrafish phenotype, and statistical analysis shows that the extent of rescue was 90.6% (Table 1). Next, the injection of the nonsense R1141X mRNA showed that this mutant was ineffective in rescuing the morpholino-mediated phenotype (4.8%). Eight of the ten disease-causing missense mutants were not able to counteract the effect of gene silencing by the Abcc6a-specific morpholino (0–5.1% rescue). The efficacy of S1121W was somewhat better (7.9%), whereas that of the V1298F was significantly higher (32.0%). These data are detailed in Table 1.

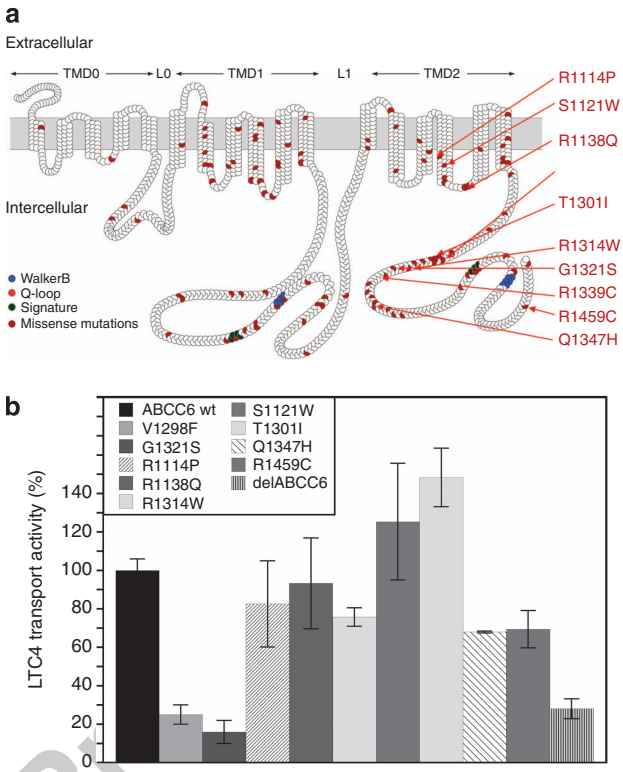


Figure 1. Positions of the investigated mutants in the membrane topology model of ABCC6 protein and their transport activity. (**a**) The model is based on our earlier publication (Tusnády *et al.*, 2006); the positions of the various domains are indicated by horizontal arrows. Conserved sequence motifs, ABC signatures, Q-loops, and Waker B motifs are colored, and the positions of known disease-causing missense mutations are indicated with red color. Arrows point to the positions of mutants investigated in the present study. (**b**) Protein variants were expressed in Sf9 insect cell system, and their ATP-dependent transport activity was assayed in the presence of 50 nM [^{3}H]LTC4 as substrate. Transport activity was compared with that of the wild-type (wt) protein (100%). Each assay has been performed on two independent membrane preparations in triplicate. LTC, leukotriene C4.

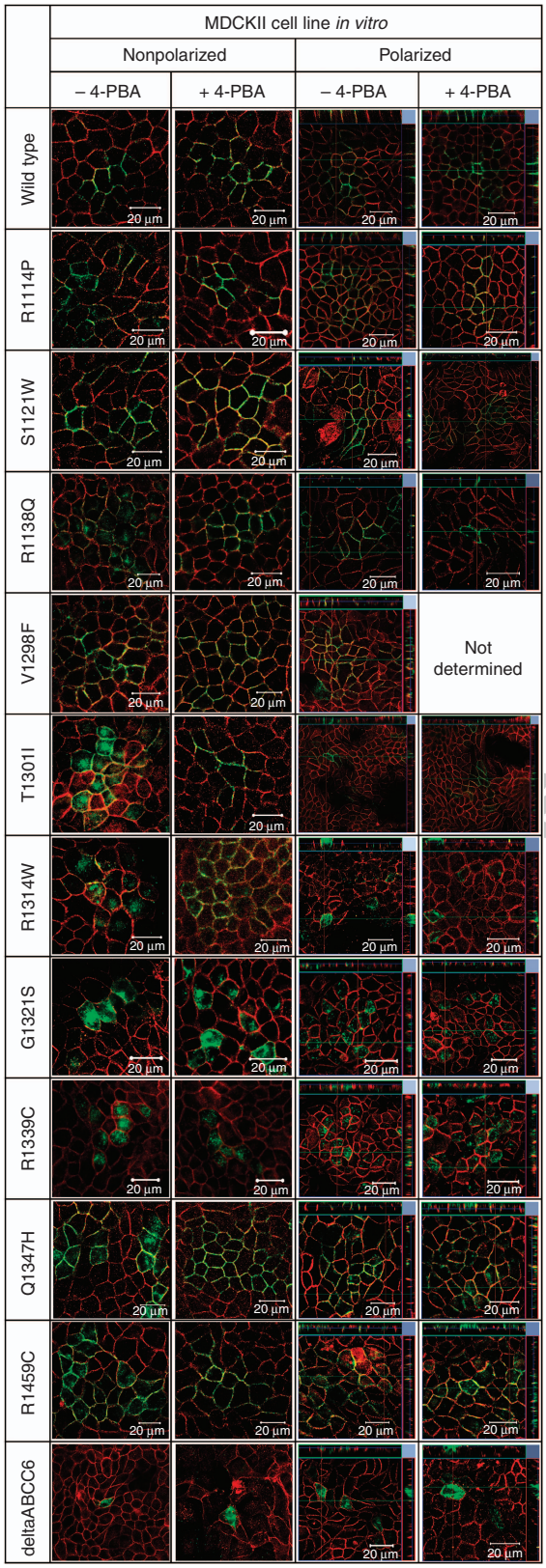
Pharmacological correction of "mistargeted" ABCC6 mutants

The main aim of this study was to evaluate the potential of the chemical chaperone/corrector 4-PBA to restore the normal intracellular trafficking of each mutant that retained substantial transport activity. We performed these experiments both *in vitro* in cell cultures and *in vivo* in mouse liver. Note that this type of experiment was not performed *in vivo* with the two inactive mutants, V1298F and G1321S, or with R1459C, as this mutant was found in the plasma membrane without 4-PBA treatment. The effect of 4-PBA treatment on the mutants expressed in nonpolarized or in polarized MDCKII cells showed different results (see Figure 2, columns 2 and 4). For instance, mutants Q1347H and R1459C were found mostly intracellularly in nonpolarized and in polarized MDCKII cells, yet the 4-PBA treatment resulted in plasma membrane localization only when these two variants were expressed in nonpolarized cells. More importantly, we found additional discrepancies when the effect of 4-PBA was investigated in mouse liver. No 4-PBA-induced plasma membrane rescue was observed for R1138Q and T1301I in mouse liver, whereas

the same compound was effective in nonpolarized MDCKII cells (compare Figure 2 and Figure 3). As for the R1314W mutant, 4-PBA treatment resulted in plasma membrane targeting both in polarized and nonpolarized MDCKII cells, as well as in mouse liver. 4-PBA treatment was also effective in the case of Q1347H *in vivo*, while it facilitated plasma membrane targeting only in nonpolarized MDCKII cells (but not in polarized cell cultures). No plasma membrane targeting was achieved in the case of mutant R1339C irrespective of which experimental system was used.

We found that the same concentration of 4-PBA used in MDCKII cell cultures was toxic to zebrafish, and further experiments with this compound were not pursued.

The *in vitro* and *in vivo* localization data, along with those obtained by the 4-PBA treatment, are summarized in Table 1.



DISCUSSION

In the present study, we characterized the transport activity, stability, and conformation/trafficking of ten disease-causing *ABCC6* missense mutations associated with PXE and GACI using *in vitro* and *in vivo* models. The results significantly expand our understanding of the structural and functional consequences of disease-causing mutations in an ABC transporter, specifically ABCC6, as well as the model systems, which were used to study this protein. We also demonstrated the feasibility of pharmacological correction of certain disease-causing missense mutants.

Interestingly, we found that the majority of the missense ABCC6 variants (7 of 10) retained a high level of LTC4 and *N*-ethylmaleimide-glutathione transport efficiency as determined by cell-free inside-out vesicles derived from Sf9 cells. Of the three remaining mutants, only two displayed decreased transport function (Figure 1b), whereas the other mutant could not be stably expressed in Sf9 cells (R1339C). These results suggested that the disease-causing *ABCC6* missense mutations that are not directly affecting transport and/or ATP catalytic activity resulted primarily in lower stability and/or cytoplasmic retention of the mutant proteins. To confirm this possibility, we expressed this series of ABCC6 mutants and controls in MDCKII cells, an *in vitro* model traditionally used in studies of other ABC transporters. The MDCKII cells were cultured under both nonpolarized and polarized conditions. We achieved a good level of expression of the R1339C mutant in this culture model. The results from these experiments revealed that the cellular localization of the mutants was variable under the polarized and nonpolarized condition, in the majority of the mutants tested (7 out of 10), which highlighted the influence of the polarization status of this *in vitro* model. Additional divergence of the results was observed after the treatment of the cultures with 1 mM 4-PBA, which was

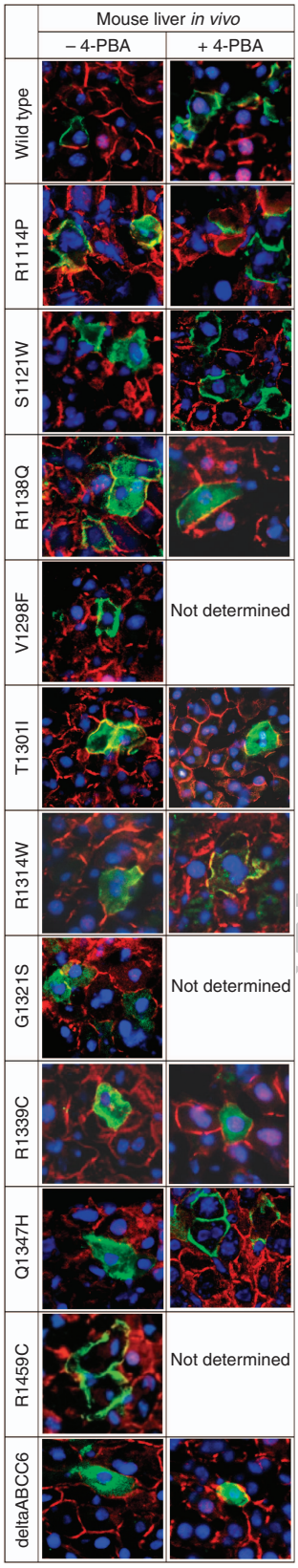
Figure 2. Subcellular localization of ABCC6 variants expressed in MDCKII (Madin–Darby canine kidney) cells and the effect of 4-phenylbutyrate (4-PBA) treatment on their localization. ABCC6 was detected by immunofluorescence using mAb M6-II7 (green color); anti-Na,K-ATPase pAb was used to detect Na,K-ATPase as a plasma membrane localization marker (red color). Cells overexpressing ABCC6 variants were grown with or without 1 mM 4-PBA either on plastic (nonpolarized) or on Transwell membrane (polarized). Confocal laser microscopy images were collected together with Z-stack images in the case of polarized cell cultures. Bar = 20 μm.

intended to correct their cellular localization, as described previously (Le Saux *et al.*, 2011). For both mutants Q1347H and R1459C, the chemical chaperone was effective in correcting its cellular trafficking only in nonpolarized cultures.

As ABCC6 has a specialized efflux function in the liver, we extended our studies to *in vivo* models whereby we transiently expressed the ABCC6 mutants in C57BL/6J mouse liver along with positive and negative controls. As we previously showed (Le Saux *et al.*, 2011), a high degree of liver-specific transient expression can be achieved in this fully differentiated organ in adult mice. When we compared the cellular localization of the mutants in polarized MDCKII cells with the liver of C57BL/6J mice, divergence was observed in localization of six ABCC6 variants (Table 1). Such discrepancy was further observed when 4-PBA was used for treatment (Table 1). The observed differences in localization of the same mutant after treatment in polarized MDCKII cells versus mouse liver underline the limitations of a 2D MDCKII cell culture system.

Therefore, we propose the use of hydrodynamic tail vein injection of plasmids for localization studies of liver-specific ABC proteins, such as ABCC6.

The second *in vivo* model, the zebrafish (*Danio rerio*), was used to investigate the functionality of the ABCC6 disease-causing mutants. The biological basis of this experimental



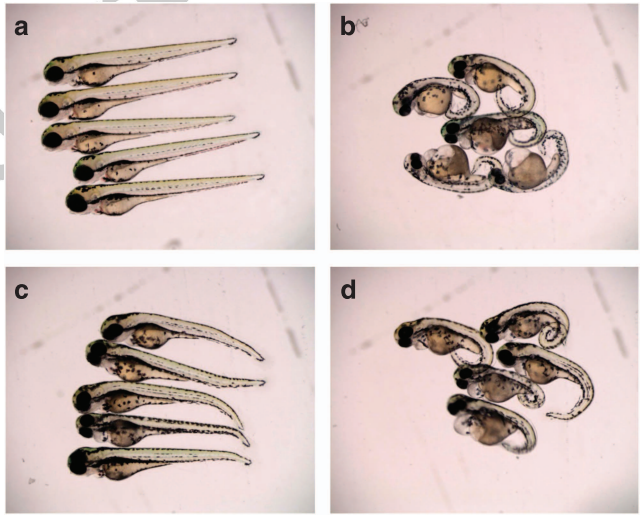


Figure 4. Rescue of the morpholino-induced phenotype by human ABCC6 mRNA in zebrafish embryos. Human ABCC6 cDNA variants were cloned into Bluescript II SK+ vector, mRNA was generated by *in vitro* transcription, and 2–4 ng of mRNA was injected into ~100 embryos together with 12–18 ng of MO1 morpholino (MO1; for sequence, see Li *et al.*, 2010). Animals were photographed 3 days after injection. (**a**) Control animals injected with standard control morpholino. (**b**) Animals injected with morpholino. (**c**) Animals injected with morpholino and with wtABCC6 mRNA. (**d**) Animals injected with morpholino and with ABCC6 R1141X mRNA. Wt, wild type.

Figure 3. Subcellular localization of ABCC6 variants expressed in mouse liver and the effect of 4-phenylbutyrate (4-PBA) treatment on their localization. The human and mouse ABCC6/Abcc6 were detected on frozen sections by immunofluorescence using mAb M6-II7 (green color) and the S-20 polyclonal antibody (red), respectively. Mice received three intraperitoneal injections of 4-PBA (100 mg/kg^{-1} per day) before performing hydrodynamic tail-vein injections and received 6.25 mg ml^{-1} 4-PBA in drinking water during the entire experiment.

Table 1. Summary of the characterization and rescue of disease-causing ABCC6 mutants

		Localization in mouse liver		Localization in MDCKII cell line				
				Nonpolarized		Polarized		
ABCC6 variant	Sf9 transport activity	Without treatment	After 4-PBA treatment	Without treatment	After 4-PBA treatment	Without treatment	After 4-PBA treatment	Zebrafish + mRNA rescue (%)
Wild type	Active	PM[1]	PM	PM	PM	PM	PM	90.6
R1114P	Active	IC > PM	PM (rescue)	IC < PM	PM (rescue)	PM	PM	0.0
S1121W	Active	IC > PM	PM (rescue)	PM	PM	PM	PM	7.9
R1138Q	Active	IC > PM	IC > PM (no effect)	IC	PM (rescue)	PM	PM	1.8
V1298F	<20%	PM	ND	PM	PM	PM	ND	32.0
T1301I	Active	IC > PM	IC > PM (no effect)	IC > PM	PM (rescue)	PM	PM	5.1
R1314W[1]	Active	IC > PM	PM (rescue)	IC	PM (rescue)	IC > PM	PM (rescue)	0.0
G1321S	<20%	IC	ND	IC > PM	IC > PM (no effect)	IC	IC (no effect)	0.0
R1339C	Not stable	IC	IC (no effect)	IC	IC (no effect)	IC	IC (no effect)	0.0
Q1347H	Active	IC > PM	PM (rescue)	IC > PM	PM (rescue)	IC = PM	IC = PM (no effect)	0.8
R1459C	Active	PM	ND	IC = PM	PM (rescue)	IC < PM	IC < PM (no effect)	0.0
delABCC6	<20%	IC	IC	IC	IC	IC	IC	ND
R1141X	Stop	ND	ND	ND	ND	ND	ND	4.8

Abbreviations: IC, intracellular; ND, not determined; PM, plasma membrane.
[1]This mutation is associated with both PXE and GACI phenotypes (Nitschke *et al.*, 2012; Li *et al.*, 2013).

system was that both mouse *Abcc6* mRNA (Li *et al.*, 2010) and human *ABCC6* mRNA (Zhou *et al.*, 2013) provided nearly complete rescue of the morpholino-induced developmental phenotype (see Figure 4). These observations indicated that, in spite of the different phenotypes in zebrafish and in patients with PXE or GACI, the fish and the human proteins fulfill a similar transport function in the two organisms. Minimal level of rescue was provided by the disease-causing mutants (0–7.9%) irrespective of their transport activity or their cellular localization, with the exception of V1298F (32.0%; Table 1). This mutant showed correct plasma membrane localization in mouse liver, but was otherwise transport deficient with very low, if any, activity. We hypothesized that V1298F probably possesses some residual transport activity of the endogenous zebrafish substrate.

In addition, it is noteworthy that the PXE-associated mutant R1459C (Chassaing *et al.*, 2005) was found to be an active transporter, and it localized in the plasma membrane in mouse hepatocytes, suggesting that R1459C could be a neutral polymorphism rather than a disease-causing mutant. However, as this variant could not rescue the zebrafish phenotype, it probably is a genuine disease-causing mutant. The case of R1459C illustrates the necessity of using multiple model systems in parallel to study the functional consequences of ABCC6 mutations.

The major finding of our study was that 4-PBA treatment restored the plasma membrane localization of three transport-competent missense mutants, R1114P, S1121W, and Q1347H in mouse liver, in addition to R1314W, which served as a positive control in the present study. Our data indicate that the *in vivo* mouse model system provides the best approach to answer the question of subcellular location of ABCC6 and bodes well for the next stage of research, that is, verifying whether the pharmacological correction of the plasma membrane localization of candidate mutants results in physiological rescue of ectopic calcification.

Over 50% of PXE patients harbor missense mutations, and our results show that the majority of such mutations likely cause mislocalization of the protein. Our data are promising and with translational potential, as they suggest that allele-specific therapy can be useful for PXE patients, as well as for those GACI patients who harbor mutations in *ABCC6*. In this context, it should be noted that 4-PBA is a drug that has already been approved by Food and Drug Administration for clinical use, and its efficacy in PXE and GACI patients could be studied in upcoming clinical trials.

MATERIALS AND METHODS

Primary and secondary antibodies

The following primary antibodies were used: anti-human ABCC6: M6II-7 (rat, 1:100; generous gift from George Scheffer, University Medical Center, Amsterdam, The Netherlands); anti-mouse Abcc6: S-20 (rabbit, 1:200); and anti-Na, K-ATPase (chicken, 1:200; Santa Cruz Biotechnology, Dallas TX). The secondary antibodies used were

as follows (Life Technologies, Eugene, OR): anti-rat Alexa Fluor 488 (1:250); anti-rabbit Alexa Fluor 594 (1:250); and anti-chicken Alexa Fluor 594 (1:250).

Mice

All mice were kept under standard laboratory conditions, and the study was approved by the Institutional Animal Care and Use Committee of the University of Hawaii and by the Institutional Animal Care and Use Committee of the RCNS of the Hungarian Academy of Sciences.

Expression of ABCC6 variants in Sf9 insect cells, vesicular transport

Expression of ABCC6 variants in Sf9 insect cells and vesicular transport was performed as described (Sarkadi *et al.*, 1992; Bakos *et al.*, 1998; Szakács *et al.*, 2001; Iliás *et al.*, 2002). Briefly, Sf9 cells were cultured at 27 °C in TNM-FH insect medium supplied with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin. For transfection of Sf9 cells, BaculoGold kit (BD Biosciences, Durham, NC) was used. We isolated individual virus clones using the end-point dilution method. Sf9 membrane vesicles from cells overexpressing wt or mutant ABCC6 were prepared and incubated with [^{3}H]LTC4 (130 Ci mmol^{-1}; Perkin-Elmer, Waltham, MA) in the presence or absence of 4 mM MgATP at 37 °C. The transport reaction was terminated and the vesicles were transferred onto a nitrocellulose filter. Radioactivity was determined by liquid scintillation counter.

The ABCC6 model (Fülöp *et al.*, 2009) was analyzed with the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.

Expression of ABCC6 variants in MDCKII cells

MDCKII cells were cultured in a humidified 37 °C, 5% CO_2 incubator, in DMEM culture medium with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin either on standard 24-well plastic plates (for nonpolarized cultures) or on BD Falcon cell culture inserts (BD Biosciences, Durham, NC; for polarized cultures). Expression of ABCC6 variants was achieved by retroviral transfection: Phoenix-Ampho packaging cells were transfected with the recombinant retrovirus vectors containing wt or mutant *ABCC6*. The transfection was performed using the calcium phosphate method. Cell clones overexpressing ABCC6 were selected by end-point dilution (Sinkó *et al.*, 2003). For immunocytochemistry, MDCKII cells were first washed and fixed with 4% paraformaldehyde and precooled methanol, and they were then incubated with blocking buffer for 1 hour at room temperature. After removal of the blocking buffer, samples were incubated with the appropriate primary antibodies for 2 hours at room temperature. After washing, the cells were incubated with the secondary antibodies for 1 hour. Nuclei were stained with 4′,6-diamidino-2-phenylindole for 5 minutes. Samples were kept at 4 °C until microscopic analysis.

Liver-specific expression of ABCC6 variants in mice and immunohistochemical staining of mouse liver samples

Liver-specific expression of ABCC6 variants in mice and immunohistochemical staining of mouse liver samples were performed as described in our previous papers (Le Saux *et al*, 2011; Pomozi *et al.*, 2013). Briefly, pLIVE plasmid (Mirus Bio, Madison, WI) containing the wt or mutant *ABCC6* was delivered into the mice by hydrodynamic tail-vein injection (70 μg of plasmid DNA in 1.8 ml). At least four mice were injected with each form of the human ABCC6 cDNA. Mice were killed by standard CO_2 procedures 2 days after hydrodynamic tail vein injections. For immunohistochemistry, 8-μm-thick frozen sections were prepared from frozen liver tissue, slices were fixed in methanol, and then washed in Dulbecco's phosphate-buffered saline. After incubation in blocking buffer for 1 hour, the appropriate primary antibodies were added for 90 minutes, followed by incubation with secondary antibodies for 1 hour. Nuclei were stained with 4′,6-diamidino-2-phenylindole for 5 minutes. Samples were kept at 4 °C until microscopic analysis.

4-PBA treatment of MDCKII cells and mice

MDCKII cells were cultured in the presence of 1 mM 4-PBA (Tocris Biosciences, Ellisville, MO). Mice received three intraperitoneal injections of 4-PBA (100 mg/kg^{-1} per day) before performing hydrodynamic tail-vein injections and additionally received approximate dosage of 1000 mg/kg^{-1} per day for 3 days in the drinking water.

Rescue experiments in zebrafish

Rescue experiments in zebrafish were performed as described in our recent publications (Li *et al.*, 2010; Zhou *et al.*, 2013). Briefly, human *ABCC6* variants were cloned in Bluescript II SK+ vector, and mRNA was generated by *in vitro* transcription using the mMessage mMachine kit (Ambion, Austin, TX). mRNA (2–4 ng per embryo) was injected into ~100 one- to four-cell-stage embryos, together with 12–18 ng of MO1 morpholino, and the embryonic phenotype and survival were determined at 3 days after injection.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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