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Premature Termination Codon Read-Through in the *ABCC6* Gene: Potential Treatment for Pseudoxanthoma Elasticum

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Abstract

Pseudoxanthoma elasticum (PXE) is an autosomal recessive disorder manifesting with ectopic connective tissue mineralization, caused by mutations in the *ABCC6* gene, ~35% of all mutations being premature termination mutations. In this study, we investigated the therapeutic potential of the nonsense codon read-through-inducing drug, PTC124, in treating PXE. The ability of this drug to facilitate read-through of nonsense mutations was examined in HEK293 cells transfected with human *ABCC6* expression constructs harboring seven different PXE associated nonsense mutations, and evaluated by immunofluorescence and In-Cell ELISA. Our data demonstrated that PTC124 did not exhibit cell toxicity in concentrations up to 40 μg/ml, and the facilitated read-through was not only dose dependent but also sequence context dependent. Considering the redundancy of the genetic code, it was postulated that in case of the most common recurrent nonsense mutation, p.R1141X, the read-through may result in substitution of the arginine 1141 by either glycine, tryptophan or cysteine. Their potential pathogenicity was tested in a recently developed zebrafish mRNA rescue assay, and demonstrated that all three mRNA transcripts were able to rescue *abcc6a* morpholino-induced phenotype of zebrafish. Thus, our results suggest that read-through of nonsense mutations in *ABCC6* by PTC124 may have potential for pharmacologic treatment of PXE.

INTRODUCTION

Pseudoxanthoma elasticum (PXE) is a multi-system heritable disorder characterized by ectopic mineralization in the skin, eyes, and the vascular systems (Neldner, 1988; Uitto *et al.*, 2010). The early cutaneous findings consist of yellowish papules on the predilection sites, *i.e.*, sides of the neck and antecubital fossae, coalescing into inelastic plaques of

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leathery skin which can extend to involve the entire integument. While the skin findings are primarily of cosmetic concern, they signify the possibility of development of serious, debilitating, and occasionally life-threatening complications in the eyes and the cardiovascular system. The characteristic ophthalmologic finding in patients with PXE is the presence of angioid streaks, and after the age of 30 years their prevalence approaches 100%. Angioid streaks derive from breaks upon aberrant mineralization of the elastic lamina of Bruch's membrane, which separates the pigmented layer of the retina from the choroid of the eye. These fractures lead to neovascularization from choriocapillaris, and subsequent leakage of newly formed vessels leads to hemorrhage and scarring. These progressive pathologic changes cause loss of visual acuity and eventually lead to central blindness if left untreated (Finger *et al.*, 2009; Georgalas *et al.*, 2011). The cardiovascular involvement, primarily reflecting mineralization of medium-sized arterial blood vessels, manifests with hypertension, intermittent claudication, bleeding from the gastrointestinal vessels, and, occasionally, myocardial infarcts and strokes.

PXE is caused by mutations in the *ABCC6* gene which encodes a transmembrane efflux transporter protein, ABCC6, expressed primarily in the liver and the kidneys (Belinsky and Kruh, 1999). The precise function of this protein remains to be disclosed, however, the critical role of the *ABCC6* gene in the pathogenesis of PXE has been confirmed by development of transgenic mice through targeted ablation of the corresponding mouse gene (Gorgels *et al.*, 2005; Klement *et al.*, 2005). Close to 600 distinct mutations representing well over 1000 mutant alleles have so far been encountered in the *ABCC6* gene (http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6). While the repertoire of these distinct mutations is varied, approximately 35% of all mutations are nonsense mutations which result in synthesis of truncated, non-functional ABCC6 protein. The most common recurrent nonsense mutation is p.R1141X which accounts for ~30% of all pathogenic PXE mutations in Caucasian patient populations.

Recently, pharmaceutical compounds have been developed to read through premature termination codons which allow synthesis of full-length, potentially functional protein. These include 1,2,4-oxadiazole, known as PTC124, a non-aminoglycoside nonsense mutation suppressor (Du *et al.*, 2008; Peltz *et al.*, 2013; Welch *et al.*, 2007). The pharmacologic safety of this molecule has already been established, and clinical trials have been initiated to examine its effectiveness in treatment of cystic fibrosis and muscular dystrophy in cases caused by nonsense mutations (Finkel, 2010; Hirawat *et al.*, 2007; Wilschanski *et al.*, 2011). In this study, we have examined the efficacy of PTC124 in suppressing a number of nonsense mutations in the *ABCC6* gene.

RESULTS

In vitro assay system for ABCC6 expression

A total of 25 distinct nonsense mutations have been encountered in the *ABCC6* gene in patients with PXE. In this study, we focused on seven of them, including the most common stop codon mutation, p.R1141X, in which the arginine at position 1141 (codon CGA) has been replaced by a stop codon (TGA) (Table 1). We first constructed an ABCC6 expression vector by cloning full-length human *ABCC6* cDNA with a 3'-end DDK tag into the

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pCMV6-Entry vector. The expression of the full-length ABCC6 protein from this vector was examined by transfecting a number of different cell lines, including HEK293, NIH3T3, HeLa and MLE-10 cells, followed by immuno detection of the DDK tag at the carboxy terminal end of the newly synthesized protein using a specific antibody. While this vector was readily expressed in a number of cell lines tested, the best transfection and translation efficiency was noted in HEK293 cells (a human embryonic kidney epithelial cell line) (Figure 1a). We subsequently tested an expression vector containing the mutant *ABCC6* cDNA into which different premature stop codon mutations were introduced by site-directed mutagenesis (Table 1). Transfection of the mutant constructs into HEK293 cells did not show any evidence of synthesis of full-length protein, although the transfection efficiency, as determined by parallel transfection with a CMV-EGFP construct, was comparable to that noted with the wild-type construct (Figure 1b).

PTC124 induces read-though of nonsense mutations in *ABCC6*

PTC124, a non-aminoglycoside premature termination codon suppressor, was then added to the incubation medium of cells transfected with mutant constructs. First, testing of PTC124 in different concentrations up to 72 hours of incubation indicated facilitation of the synthesis of full-length ABCC6 polypeptide, as determined by immunostaining with an anti-DDK antibody (Figure 1c,d). Testing of two mutant constructs, p.R1164X and p.R1275X, in the presence of varying concentrations of PTC124 indicated that the highest level of expression was noted with 5 μg/ml, as quantitated by In-Cell ELISA (Figure 2). Subsequently, all mutant ABCC6 expression constructs were tested for the read-through efficacy of PTC124 at 5 μg/ml at 48 or 72 hours of incubation. The results indicated varying degrees of enhancement with different mutant constructs at 48 hours, but in each case, the amount of full-length protein was increased when the incubation was continued up to 72 hours (Figure 3). Thus, PTC124 is able to induce the read-through of a number of termination codon mutations in *ABCC6* in a time-dependent manner, but the degree of enhancement appears to be context dependent, influenced by the precise nucleotide sequence of the stop codon and the immediate 3' nucleotide present in the sequence (Howard *et al.*, 2000; Rowe and Clancy, 2009).

Since PTC124 was more efficient in inducing the read-through at 5 μg/ml than at 10 or 20 μg/ml concentration, subsequent experiments were performed to examine the toxicity of PTC124 in cell culture at different concentrations (0–100 μg/ml) up to 72 hours of incubation. The viability of HEK293 cells was determined by MTT colorimetric assay. The results indicated that PTC124 at the concentrations 0–20 μg/ml did not affect the viability of the cells in comparison to cells incubated with the vehicle (DMSO). At concentrations over 40 μg/ml, PTC124 elicited a statistically significant reduction of cell viability ($p < 0.05$) (Figure 4).

A zebrafish system to test the functionality of ABCC6 polypeptides

While the precise mechanisms of PTC124 in facilitating read-through of stop codon mutations in the *ABCC6* gene are currently unknown, it apparently replaces the stop codon with another amino acid through the mispairing of a near-cognate aminoacyl tRNA (Nilsson and Ryden-Aulin, 2003). In case of the p.R1141X mutation, considering the codon

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redundancy and possible combinations of the corrected mutations from the stop codon (TGA) to an amino acid, in addition to arginine (CGA) this position could be occupied either by cysteine (TGC), tryptophan (TGG) or glycine (GGA). Since a number of missense mutations have also been encountered in *ABCC6* in patients with PXE, there is a possibility, at least theoretical, that the full-length polypeptide synthesized in the presence of PTC124 is nonfunctional.

In order to test the functionality of the full-length ABCC6 protein synthesized in the presence of PTC124, which instead of an arginine in position 1141 could contain cysteine, tryptophan or glycine, we utilized a zebrafish mRNA rescue system recently developed by us (Li *et al.*, 2011). In this system, injection of 1 to 4-cell zebrafish embryos with a morpholino corresponding to the exon 7/intron 7 border of the *abcc6a* gene decreases the gene expression by up to 80% and induces a phenotype of pericardial edema and curled tail associated with early demise. Microinjecting the embryos with full-length human *ABCC6* mRNA together with the morpholino rescues this phenotype (Li *et al.*, 2010) (Figure 5). In contrast, injection of human *ABCC6* mRNA harboring the p.R1141X stop codon mutation failed to reverse the phenotype (Figure 5, Table 2), suggesting that this mRNA rescue system can be utilized to check the pathogenicity of mutant human ABCC6 proteins, including those harboring the missense amino acids as a result of PTC124 read-through. We, therefore, engineered mutant human *ABCC6* constructs which encode either cysteine, tryptophan or glycine in position 1141 using site-directed mutagenesis kit, and these mutant mRNAs were injected into zebrafish embryos in parallel to wild-type mRNA containing arginine in the corresponding position. Quantitation of embryonic lethality allowed us to determine whether the read-through ABCC6 protein is functional or not in rescuing the zebrafish phenotype. The results indicated that the lethality of the embryos injected with the abcc6a morpholino at day 1 post-fertilization was around 70% but this was reversed to the control level (~10–12%) by the human wild-type mRNA harboring arginine at position 1141 or as noted in embryos injected with universal standard control morpholino (Table 2). Similarly, the constructs harboring either cysteine, tryptophan or glycine in place of arginine reversed the morpholino induced lethality (Figure 5 and Table 2).

DISCUSSION

The overall goal of this study is to develop a pharmacologic treatment for a subset of patients with PXE, *viz.*, those harboring nonsense mutations in one or both *ABCC6* alleles. Our data, which demonstrated read-through of a stop codon in the presence of PTC124, attest to the feasibility of this approach. These data demonstrated significant expression of the full-length polypeptide under the *in vitro* culture conditions tested by PTC124, with the maximum effect at 5 μg/ml concentration. Increasing the concentration of PTC124 did not increase the read-through, consistent with findings by others (Welch *et al.*, 2007). It should be noted that PTC124 at this concentration resulted only in partial correction of the ABCC6 expression. While the precise level of expression of ABCC6 needed in individual patients to prevent ectopic mineralization is currently unknown, at least 50% level, and possibly much lower, is sufficient as attested to by the fact that heterozygous carriers of a mutation do not develop mineralization phenotypes (Uitto *et al.*, 2011). In further studies, the *in vivo* efficacy

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of PTC124 can be tested in a preclinical animal model of knock-in mice harboring human *ABCC6* with p.R1141X mutations in both alleles on *Abcc6*$^{-/-}$ background.

While PTC124 has been shown to facilitate read-through of premature termination codons in a number of genes, the information on the amino acid that substitutes the stop codon is not known in case of *ABCC6*. It is conceivable that the presence of such an amino acid carried by a near-cognate tRNA (instead of the parent wild-type arginine in case of p.R1141X) could be pathogenic, essentially representing a missense mutation. To test the potential pathogenicity of such amino acid substitutions, we have developed an innovative zebrafish mRNA rescue system that will provide information as to whether the amino acid substitution in ABCC6 is pathogenic or not (Li *et al.*, 2011). Our results showed that substitution of arginine at position 1141 by cysteine, tryptophan or glycine did not alter the functionality of the protein. It should be noted that this innovative zebrafish mRNA rescue system should be applicable to other proteins as well, an issue that has not been addressed in development of similar pharmacologic approaches for other heritable diseases.

PTC124 is an orally bioavailable compound that promotes read-through of nonsense mutations demonstrated in a number of cell culture and mouse model systems (Kayali *et al.*, 2012; Peltz *et al.*, 2013; Welch *et al.*, 2007). The pharmacokinetics and safety of this compound in healthy adult volunteers have been documented in single dose and multi-dose studies with doses ranging from 10–50 mg per kg per dose twice per day for 14 days (Hirawat *et al.*, 2007). PTC124 administered orally was well tolerated as a single dose up to 100 mg per kg. At higher doses, 150 and 200 mg per kg, PTC124 induced mild headache, dizziness, and gastrointestinal events. With multiple doses of 50 mg per kg per dose twice a day, no significant side effects were detected and there was no evidence of protein elongation due to non-specific ribosomal read-through of normal stop codons (Hirawat *et al.*, 2007). Collectively, these studies suggested that PTC124 is safe for clinical trials.

Concomitant to these initial safety studies, the efficacy of PTC124 was tested in mouse models, including a model for cystic fibrosis harboring the CFTR-G542X stop codon mutation containing transgene (Du *et al.*, 2008). Feeding these mice with PTC124 resulted in the appearance of human CFTR and restored 24–29% of its activity, *i.e.*, cAMP-stimulated *trans*-epithelial chloride transport, in comparison to wild-type mice. These authors suggested that in light of its oral bioavailability, safety, toxicology profile in animal studies, and efficacy with other nonsense alleles, PTC124 has the potential to be an important therapeutic agent for the treatment of inherited diseases caused by nonsense mutations. Similar studies have demonstrated the efficacy of PTC124 in promoting dystrophin production in primary muscle cells from patients with muscular dystrophy and from *mdx* mice expressing dystrophin nonsense alleles (Finkel, 2010; Kayali *et al.*, 2012). Based on these considerations, clinical trials have been initiated to test the efficacy of PTC124 for treatment of cystic fibrosis, a disease in which approximately 10% of the subjects harbor a premature stop codon mutation (Wilschanski *et al.*, 2011). While the study on cystic fibrosis is ongoing, another study is currently recruiting patients with Duchenne's muscular dystrophy caused by premature stop codon mutations in the dystrophin gene (see ClinicalTrials.gov). Finally, the feasibility of PTC124-mediated read-through of nonsense mutation in genes associated with a number of metabolic disorders (see *e.g.*, (Ho *et al.*,

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2013; Sanchez-Alcudia *et al.*, 2012; Tan *et al.*, 2011) as well as in hereditary cancer syndromes (Bordeira-Carrico *et al.*, 2012) has been tested.

It should be noted that while PXE is considered to be a rare disease (<200,000 affected individuals in the U.S.A.), its estimated prevalence, ~1:50,000, suggests that there are at least 7,000 affected individuals in the United States, and if the same prevalence is extrapolated to the global level, as many as 150,000 individuals are affected with PXE in the world (Uitto, 2012). Considering the fact that 100% of patients with PXE develop angioid streaks, and the majority of them encounter loss of visual acuity eventually leading to blindness if left untreated, successful therapy of these patients with PTC124 would potentially prevent blindness in thousands of individuals worldwide. Furthermore, prevention or amelioration of vascular complications would significantly reduce the morbidity and mortality associated with this, currently intractable, disorder.

MATERIALS AND METHODS

Reagents, plasmid and site-directed mutagenesis

PTC124 (Ataluren) was purchased from Selleckchem (Houston, TX) and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Deisenhofen, Germany) just prior to use. A full-length wild-type human *ABCC6* cDNA linked to a nucleotide sequence encoding DDK peptide tag at the 3' end was cloned into pCMV6-Entry expression vector purchased from OriGene (RC211938, Rockville, MD). Using this construct as a template, seven different *ABCC6* nonsense DNA-constructs were obtained by site-directed mutagenesis following the manufacturer's instructions (Agilent, Santa Clara, CA).

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY), and incubated at 37°C under 5% CO2. Cells were transfected with different *ABCC6* cDNA-constructs using Lipofectamine® 2000, as suggested by the manufacturer (Invitrogen, Grand Island, NY). After four hours of incubation the medium was replaced with fresh culture medium without or with PTC124 at different concentrations. At different time points the cells were evaluated for synthesis of ABCC6-DDK fusion protein either by immunofluorescence or in-cell ELISA. For each construct, at least three independent transfection experiments were performed.

Immunofluorescence

Immunofluorescence was performed on 4% paraformaldehyde-fixed cells (15 min at room temperature). Non-specific sites were blocked for 60 min in 2% bovine serum albumin, and cells were then incubated overnight at 4°C with mouse anti-DDK antibody (1:2000, OriGene). Following three washes in PBS, cells were incubated for 1 hour with Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody. After triple washing with PBS, the cells were mounted with antifade reagent and stained with DAPI (Molecular Probes, Eugene, OR, USA). The stained cells were analyzed using a fluorescent microscope (Zeiss, Göttingen, Germany).

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In-Cell ELISA

To quantify the efficiency of read-through, the treated cells in 96-well plate were evaluated by Colorimetric In-Cell ELISA Kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. The absorbance was measured at 460 nm within 30 minutes of stopping the reaction. The value was normalized to corresponding untreated controls and converted to percentage using the expression of the wild-type ABCC6-DDK construct as 100%.

Cell proliferation assay

For PTC124 cytotoxicity assay, HEK293 cells were seeded in 96-well plates in triplicate at a density of 2000 cells/well and incubated with PTC124 at concentrations from 0 to 100 μg/ml. The medium was changed daily. At 72 hours, cell proliferation was determined by a colorimetric assay using CellTitCellTiter 96® Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol. The spectrophotometric absorbance of each sample was measured at 495 nm using Biorad microplate reader (Hercules, CA)

Zebrafish mRNA rescue assay

To test the potential pathogenicity of missense substitutions after read-through of *ABCC6* induced by PTC124, a zebrafish mRNA rescue assay were performed. Capped full-length human *ABCC6* mRNA corresponding to wild-type, R1141X or putative read-through substitutions of argine1141 by cysteine, tryptophan or glycine was transcribed from an expression vector pCMV-Tag4B using T3 mMessage mMachine kit (Ambion, Austin, TX). The morpholino was injected into one- to four-cell-stage embryos either alone or in combination with the mRNA as above (2.4 mmol). The injected zebrafish embryos were followed for their phenotype and survival rate.

Statistical analysis

The data were expressed as mean ± SEM. Statistical analyses were performed using a two-sided Student's *t*-test, and statistical significance was accepted at $p < 0.05$.

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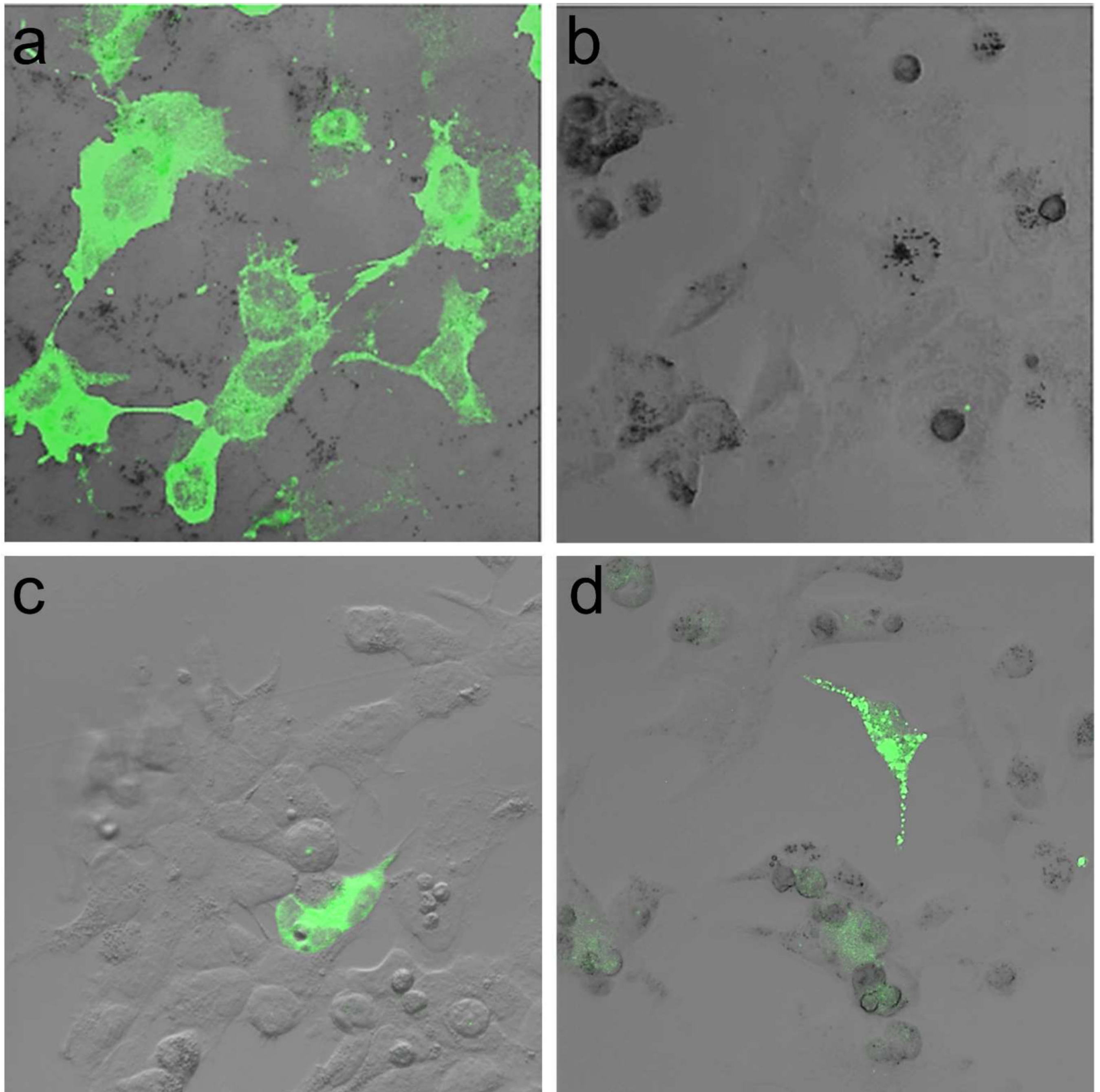

Figure 1. Expression of wild-type and mutant human *ABCC6* cDNA, and demonstration that PTC124 induces read-through of a stop codon

An expression vector (p.CMV6-Entry) containing full-length wild-type human *ABCC6* cDNA (a) or a corresponding mutant cDNA harboring p.R1141X premature termination codon (b,c) containing a 3'-end sequence which encodes a DDK reporter peptide, or p.R1275X (d) were transfected into HEK293 cells in culture without (a,b) or with (c,b) PTC124 (10 μg/ml for 72 hours). The cell cultures were then stained with an anti-DDK

antibody for the expression of full-length polypeptide. Note relatively low level of expression in some cells (asterisks).

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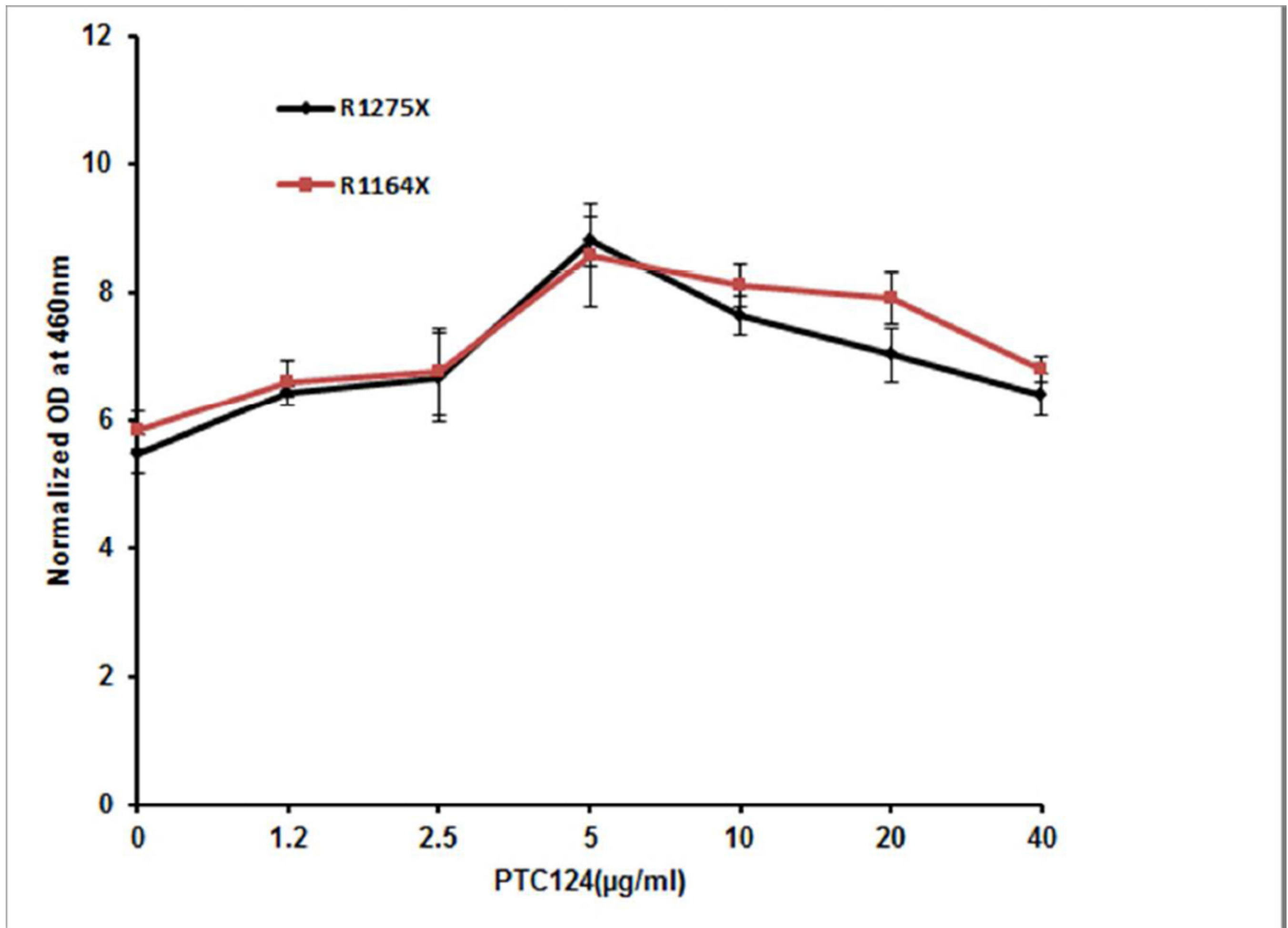

Figure 2. Read-through efficiency of stop codon mutations p.R1275X and p.R1164X by PTC124 in different concentrations

HEK293 cells were transfected with expression vectors containing full-length human *ABCC6* cDNA harboring one of the two nonsense mutations. The cells were placed on medium containing PTC124 in concentrations indicated, and the read-through of full-length polypeptide was quantitated by an In-Cell ELISA with an anti-DDK antibody. The values represent determinations from three independent cultures (mean ± SEM).

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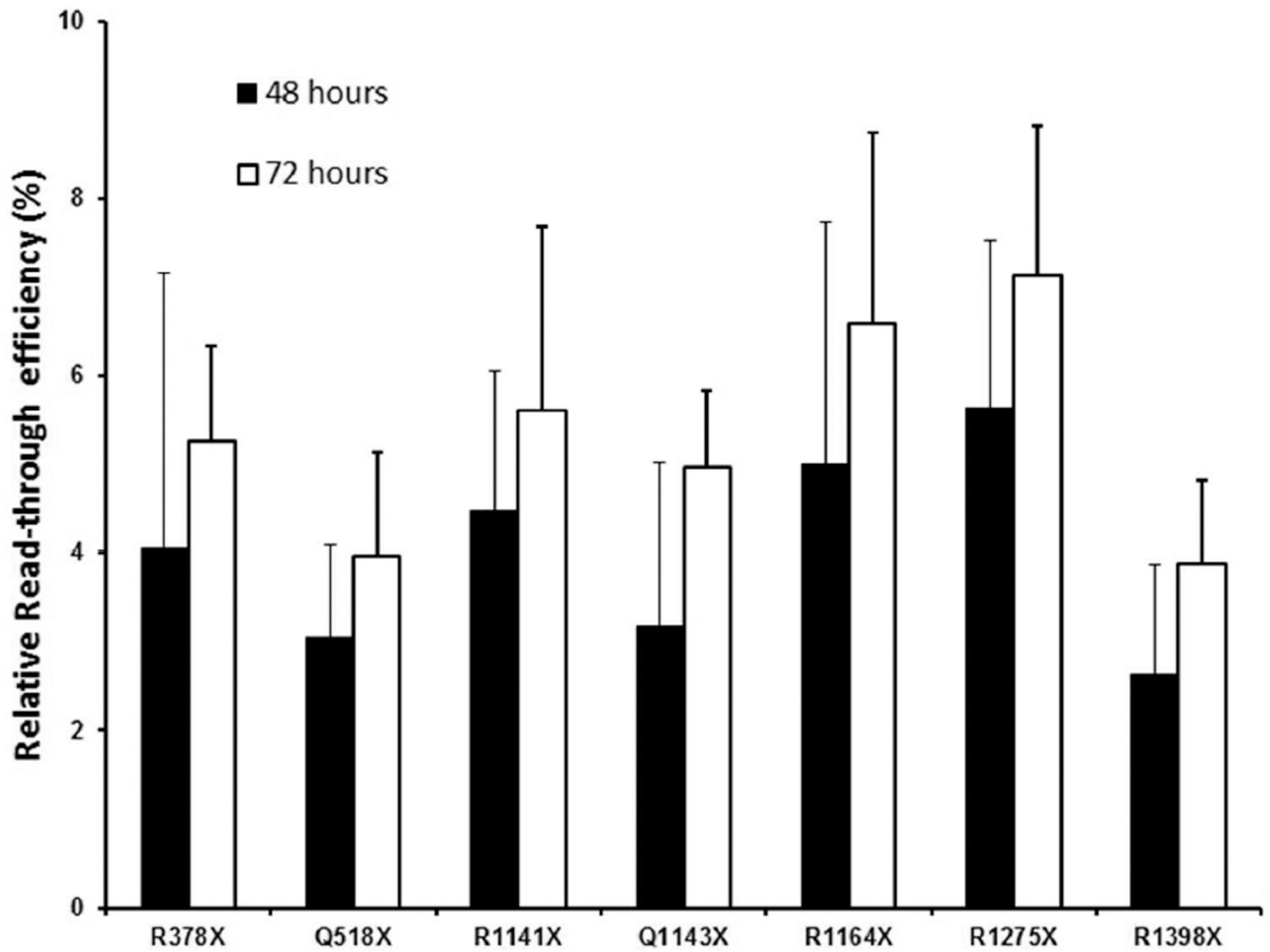

Figure 3. Read-through efficiency of different nonsense mutations by PTC124 (5 μg/ml) at 48 and 72 hrs of incubation

HEK293 cells were transfected with the mutant expression vectors as indicated in the legend to Figure 2. The nucleotide sequences of the stop codon mutations are shown in Table 1. The expression of full-length polypeptide was quantitated by an In-Cell ELISA.

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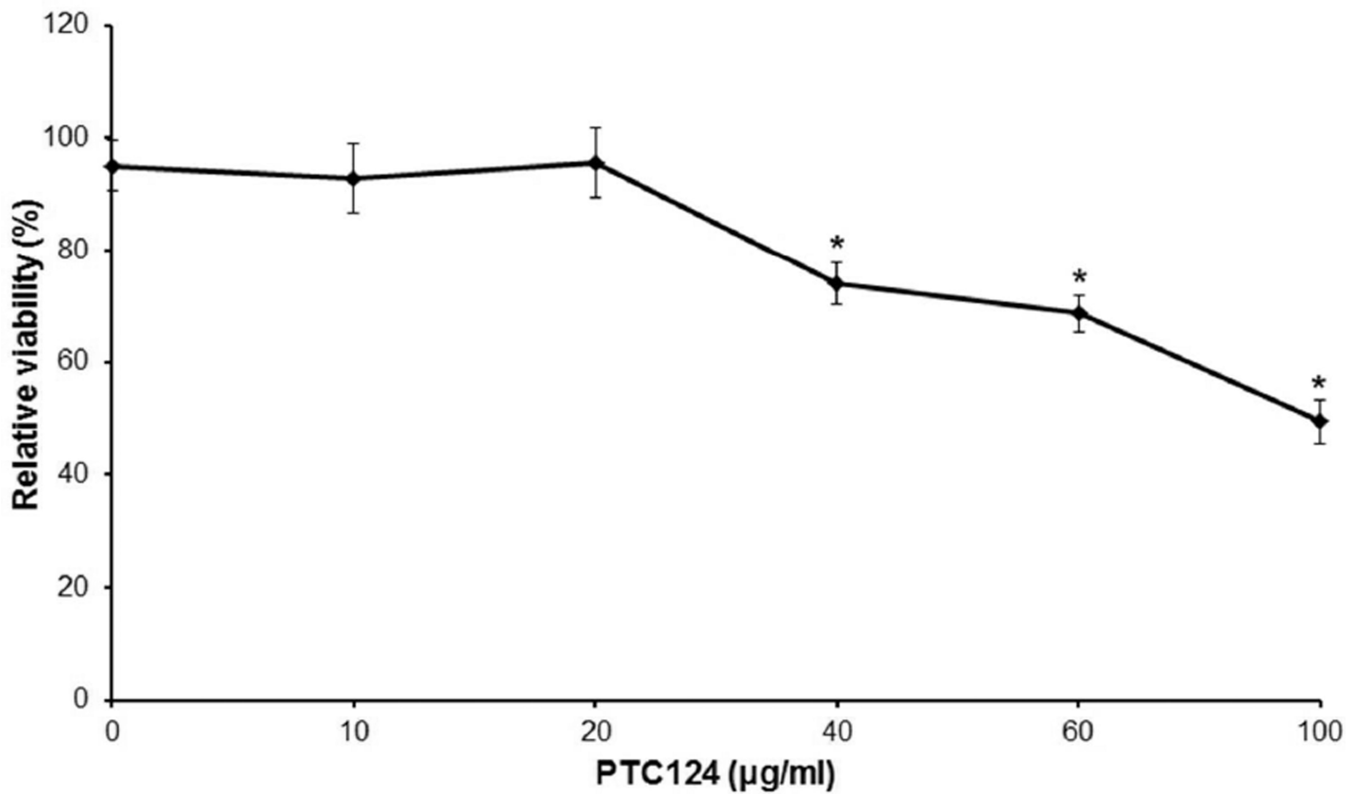

Figure 4. Demonstration that PTC124 is not toxic to the cells in culture up to 40 μg/ml

HEK293 cells were incubated for 72 hrs with varying concentrations of PTC124, and the viability of cells was determined by MTT colorimetric assay. PTC124 in the concentration range of 0–20 μg/ml did not affect the viability of the cells after normalization to the viability of cells incubated with the corresponding concentration of vehicle (DMSO) in parallel. At concentrations 40, 60 and 100 μg/ml, PTC124 elicited a statistically significant reduction of cell viability (*$P < 0.05$; Student's t-test) (mean ± SEM; n = 3).

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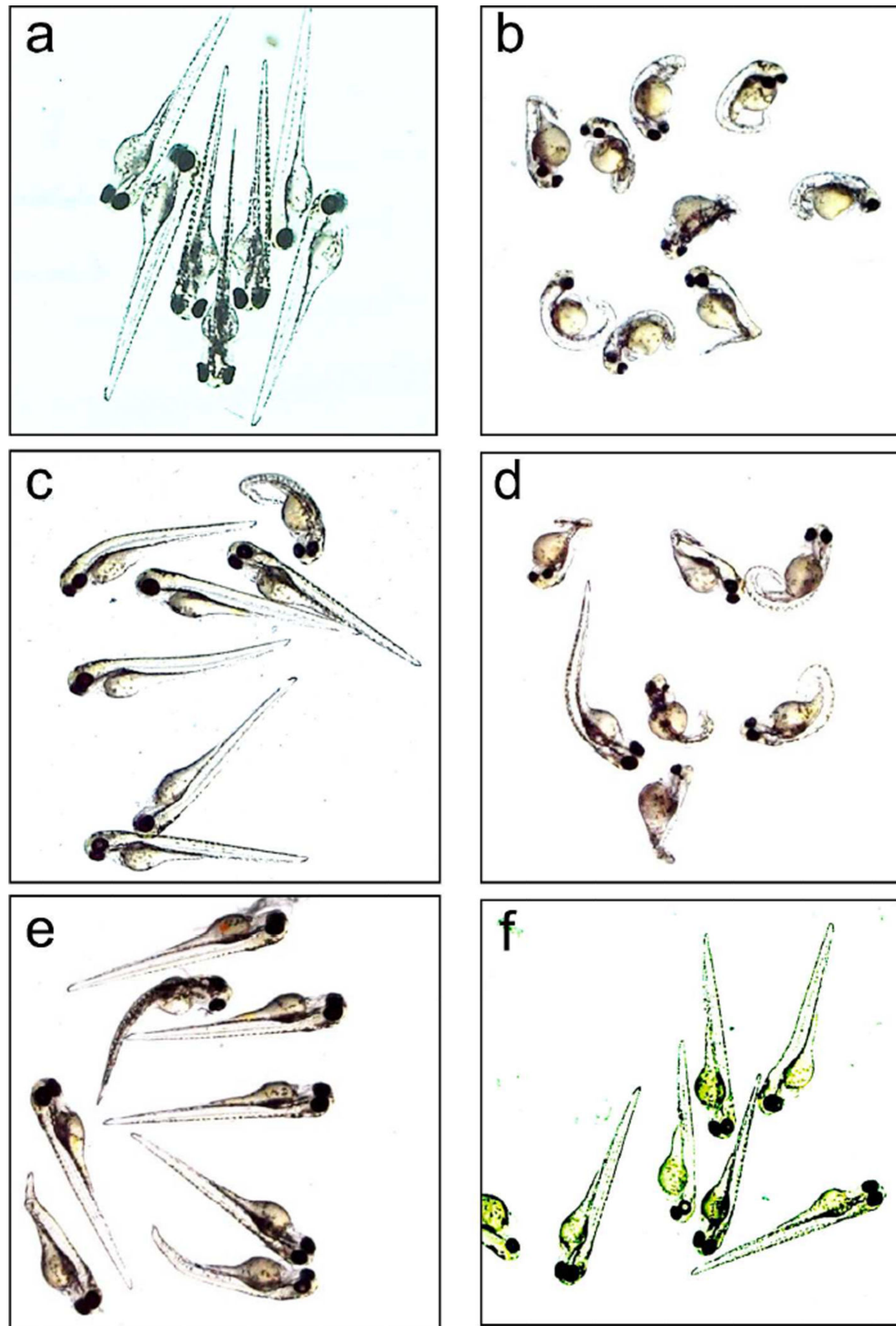

Figure 5. The zebrafish *ABCC6* mRNA rescue system

Injection of 1 to 4-cell zebrafish embryos with a morpholino targeting the *abcc6a* gene results in a profound phenotype consisting of pericardial edema and curled tail at 3 days post fertilization (b), as compared to control embryos injected with biologically inactive standard control morpholino (a). Injection of the wild-type (WT) human *ABCC6* mRNA together with the morpholino essentially rescues the phenotype (c). Similarly, injection of human *ABCC6* mRNA harboring glycine (e) or cysteine (f) at position 1141 reversed the

morpholino induced phenotype. However, injection of human *ABCC6* mRNA which harbors the p.R1141X mutation does not result in phenotypic rescue (d).

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