

PTC124-Mediated Translational Readthrough of a Nonsense Mutation Causing Usher Syndrome Type 1C

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Abstract

We investigated the therapeutic potential of the premature termination codon (PTC) readthrough-inducing drug PTC124 in treating the retinal phenotype of Usher syndrome, caused by a nonsense mutation in the *USH1C* gene. Applications in cell culture, organotypic retina cultures, and mice *in vivo* revealed significant readthrough and the recovery of protein function. In comparison with other readthrough drugs, namely the clinically approved readthrough-inducing aminoglycoside gentamicin, PTC124 exhibits significant better retinal biocompatibility. Its high readthrough efficiency in combination with excellent biocompatibility makes PTC124 a promising therapeutic agent for PTCs in *USH1C*, as well as other ocular and nonocular genetic diseases.

Introduction

POINT MUTATIONS resulting in in-frame nonsense mutations, also called premature termination codon (PTCs), are frequent, responsible for ~12% of all mutations causing human genetic diseases (Kellermayer, 2006). Translation of mRNAs containing PTCs often results in truncated, mostly nonfunctional polypeptides (Fig. 1A). Pharmacogenetic agents that mediate translational readthrough of nonsense mutations and thereby stimulate the expression of full-length functional proteins are promising therapeutics (Fig. 1A) (Kellermayer, 2006; Linde and Kerem, 2008). In comparison with gene augmentation, translational readthrough-inducing drugs have significant advantages; as no DNA is transferred, it cannot randomly integrate. Furthermore, the targeted gene remains under normal endogenous control tissue specificity and timing, and duration of expression as well as alternative splicing are not issues.

In earlier work aminoglycosides were shown to promote readthrough of PTCs, but also to induce severe side effects at therapeutically relevant concentrations (Linde and Kerem, 2008). In screens for readthrough agents with better biocompatibility, PTC124, structurally not related to aminoglycosides or any other drug, was identified (Welch *et al.*, 2007). Chemical footprinting studies indicate binding of PTC124 to the 60S ribosomal subunit and thereby inducing readthrough of nonsense mutations (Finkel, 2010). The readthrough efficiency of PTC124 was successfully demonstrated in animal

models of Duchenne muscular dystrophy (DMD) and cystic fibrosis (CF) (Welch *et al.*, 2007; Du *et al.*, 2008). Phase I and IIa trials demonstrated the effectiveness of PTC124 treatment combined with no drug-related serious side effects in children and adults (Hirawat *et al.*, 2007; Kerem *et al.*, 2008; Sermet-Gaudelus *et al.*, 2010), paving the way for further clinical trials in, for example, patients with DMD, CF, and hemophilia.

Here we have investigated the readthrough efficacy of PTC124 on PTCs causing retinal diseases, in particular on the retinal degenerations related to Usher syndrome (USH), the most frequent cause of combined hereditary deaf-blindness (Reiners *et al.*, 2006). Although the auditory deficit can be treated with cochlear implants (Damen *et al.*, 2007), to date there is no effective treatment for the retinal phenotype of USH (Reiners *et al.*, 2006). Approximately 12% of all USH cases are accounted for by PTCs (Baux *et al.*, 2008). As proof of principle for USH-related PTCs, we focused in the present study on the p.R31X nonsense mutation in *USH1C* (p.R31X), which is one of the three known nonsense mutations in the *USH1C* gene (Zwaenepoel *et al.*, 2001; Ahmed *et al.*, 2002; Ebermann *et al.*, 2007) (https://grenada.lumc.nl/LOVD2/Usher_montpellier/home.php?select_db=USH1C). Whereas two of these nonsense mutations are present in the French Canadian population, p.R31X has been found in a German family with Huguenot background. The p.R31X nonsense mutation leads to a premature stop (UGA) in the translation of the *USH1C* gene.

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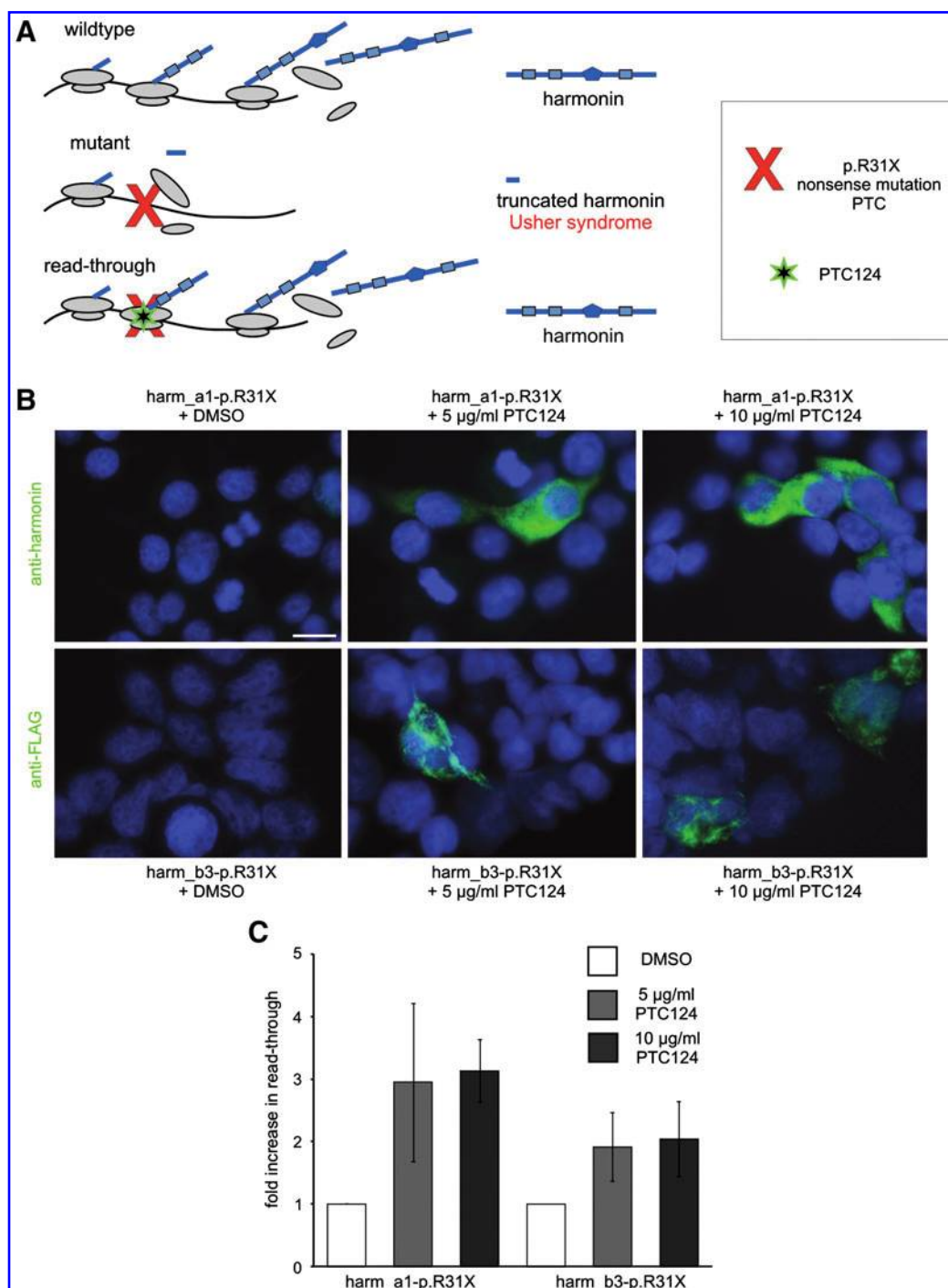


FIG. 1. Indirect immunofluorescence analyses of PTC124-mediated readthrough of the *USH1C* nonsense mutation p.R31X in HEK293T cells. **(A)** Scheme of drug-induced readthrough of nonsense mutations. Translation of mRNA results in the generation of functional full-length protein. The p.R31X nonsense mutation introduces a premature termination codon (PTC; red X) in the mRNA, which results in truncated, nonfunctional harmonin protein. PTC124 promotes the incorporation of an amino acid at the PTC of the mutant mRNA and, therefore, the generation of full-length harmonin. **(B)** PTC124-mediated readthrough in transiently transfected HEK293T cells analyzed by indirect immunofluorescence with anti-harmonin and anti-FLAG antibodies. PTC124 treatments restored harmonin a1 and b3 expression (green) in p.R31X-transfected cells. Nuclear DNA was stained with DAPI (blue). **(C)** For quantification of PTC124-mediated readthrough of the p.R31X mutation, either harmonin-positive stained cells for isoform a1 or FLAG-positive cells for isoform b3 were counted and normalized to the total amount of cells. The increase in readthrough is shown as the fold increase over DMSO-treated cells. Error bars represent the standard deviation. Scale bar in **(B)**: 10 µm.

The *USH1C* gene encodes the PDZ (postsynaptic density 95/disc large/zonula occludens-1)-containing scaffold protein harmonin, which is expressed as numerous alternative spliced variants (Verpy *et al.*, 2000; Reiners *et al.*, 2003). Harmonin isoforms a–c are classified by their modular composition of protein-interacting domains, namely PDZ, coiled-coil, and PST (proline, serine, and threonine-rich) domains. Their numerous interactions include the binding to all other USH1 and USH2 proteins, which specifies harmonin as a key organizer in the protein interactome related to USH (Reiners *et al.*, 2006). Furthermore, the harmonin b isoforms directly bind to actin filaments inducing filament bundling (Boëda *et al.*, 2002) and thereby link the USH protein network to the actin cytoskeleton (Reiners *et al.*, 2006). In rodent and primate retinas, harmonin isoforms are expressed in both the outer and inner segments as well as the synapses of photoreceptor cells (Reiners *et al.*, 2003; and M. Becker, T. Goldmann, K. Nagel-Wolfrum, N. Fuhrmann, M. Faust, C. Müller, J. Vetter, and U. Wolfrum, unpublished data). The p.R31X mutation of the *USH1C* gene introduces a PTC at the 5' end (Zwaenepoel *et al.*, 2001; Ahmed *et al.*, 2002), which affects all known harmonin isoforms and results in a truncated harmonin polypeptide (~3.4 kDa) lacking all scaffold function domains (Fig. 1A). The lack of harmonin scaffold function is thought to disrupt the USH protein network in photoreceptor cells, thereby leading to retinal degeneration (Reiners *et al.*, 2005, 2006). Therefore, therapeutic attempts to treat USH1C in the retina should target the photoreceptor cells.

We demonstrated PTC124-mediated translational readthrough of the *USH1C* p.R31X mutation in cell culture, retinal explants, and *in vivo*. The recovered harmonin expression restored harmonin scaffolding function and actin filament-bundling activity. Furthermore, in comparison with the clinically approved readthrough-inducing aminoglycoside gentamicin, PTC124 revealed much better retinal biocompatibility. Our data conclusively demonstrate the capacity of PTC124 to treat the retinal phenotype of USH1C and other ocular and nonocular diseases.

Materials and Methods

Human material

Human donor eyes were obtained from the Department of Ophthalmology of the University Medical Center (Johannes Gutenberg University Mainz, Mainz, Germany). The guidelines of the Declaration of Helsinki were followed.

Animals

C57BL/6J mice were maintained on a 12 hr/12 hr light (200 lx)/dark cycle, with food and water *ad libitum*. Association for Research in Vision and Ophthalmology (ARVO, Rockville, MD) statements and institutional guidelines for animal care were followed.

Chemicals

PTC124, kindly provided by V. Maslenko (Exchemistry, Moscow, Russia), was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Deisenhofen, Germany). Gentamicin was purchased from Sigma-Aldrich. Monoclonal mouse antibodies to actin (clone C4) and to FLAG were obtained from Seven Hills Bioreagents (Cincinnati, OH) and Sigma-Aldrich,

respectively. Polyclonal rabbit antibodies against harmonin (H3) were used as previously described (Reiners *et al.*, 2003). Affinity-purified rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP) were obtained from Dako (Glostrup, Denmark). Rabbit antibodies against calbindin (Swant, Bellinzona, Switzerland) and protein kinase C (PKC)- α (Sigma-Aldrich) were previously characterized for retinal applications (Haverkamp and Wässle, 2000). Monoclonal rat antibodies against red fluorescent protein (RFP) were obtained from ChromoTek (Martinsried, Germany). Actin filaments were visualized with rhodamine-phalloidin (Sigma-Aldrich). Secondary antibodies conjugated to Alexa 488 were from Molecular Probes (Leiden, The Netherlands). 4',6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used for the visualization of nuclear DNA.

Plasmid clonings

The murine cDNA of harmonin a1 was amplified and inserted into the pCS2+MT vector, encoding Myc tags (Rebibo-Sabbah *et al.*, 2007); the pDest47 vector, encoding green fluorescent protein (GFP) (Invitrogen, Karlsruhe, Germany); or the pTER-mRFP vector, encoding monomeric red fluorescent protein (mRFP). cDNA of human harmonin b3 was subcloned into the pDest/C-SF-TAP vector, encoding S-FLAG (Gloeckner *et al.*, 2007). All introduced tags were located at the C terminus of the harmonin isoforms. The p.R31X mutation was generated in all plasmids with a QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Cell culture and protein function analyses

HEK293T cells were grown in Dulbecco's modified Eagle's medium with GlutaMax supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin–streptomycin (Invitrogen) at 37°C and 5% CO₂. Transfections were performed, with Lipofectamine LTX with PLUS reagent (Invitrogen), according to the manufacturer's protocol. Immunofluorescence analyses were done on methanol-fixed HEK293T cells as previously described (Overlack *et al.*, 2008). For the analysis of harmonin b3-induced actin filament bundling, cells were incubated with rhodamine-phalloidin. Harvesting of cells and Western blot analyses were performed as previously described (Nagel-Wolfrum *et al.*, 2004; Maerker *et al.*, 2008). Western blots were analyzed with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Glutathione S-transferase (GST) pull-downs were performed as previously described (Reiners *et al.*, 2005; Maerker *et al.*, 2008).

Retina culture and electroporation

Murine and human retinas were cultured as previously described (Reidel *et al.*, 2006). Terminal deoxynucleotidyl-transferase dUTP nick end labeling (TUNEL) was performed according to the protocol of the manufacturer (*In Situ* cell death detection kit; Roche, Mannheim, Germany). Plasmids were transferred into retinal explants by electroporation (ECM 830; BTX Harvard Apparatus, Holliston, MA) as previously described (Matsuda and Cepko, 2004). Retinas were cultured for 24 hr before adding the pharmacological compounds in fresh medium for an additional 72 hr.

In vivo electroporation and subretinal PTC124 injection

Retinas of postnatal (PN) day 0 mouse pups were electroporated as previously described (Matsuda and Cepko, 2004). Briefly, plasmids were injected subretinally followed by electroporation, using a pair of tweezer-type electrodes across the eyes. After 6 weeks, mice were anesthetized and 1 μ l of PTC124 (2.5 μ g/ μ l in DMSO) or DMSO as solvent control was injected subretinally. Three days after the injection, animals were killed. For fluorescence analyses, the eyes were removed, fixed with 4% paraformaldehyde, and embedded for fluorescence microscopy analysis. For quantification, confocal images were taken, applying the same set of imaging parameters. Fluorescence intensities of different regions of photoreceptor cells and fluorescence plot profiles were quantified with ImageJ software (National Institutes of Health, Bethesda, MD; <http://imagej.nih.gov/ij/>). Five images were chosen from each sample for quantification of photoreceptor regions. For intensity profile plots, fluorescence intensity was averaged over 5- μ m bins. For Western blot analyses, retinas were removed through a slit in the cornea, homogenized (Reiners *et al.*, 2003), and processed as described previously.

Microscopy

Light microscopy analyses of immunofluorescence and epifluorescence samples were performed with a Leica DM6000 B or a Leica SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) and images were processed with Adobe Photoshop CS (Adobe Systems, San Jose, CA).

Statistical analysis

One-way *t* test analysis was applied to determine statistical significance.

Results and Discussion

PTC124-induced translational readthrough of p.R31X nonsense mutation in USH1C in cell culture

In the human *USH1C* mutation p.R31X, the CGA codon of an arginine (R) is altered to the premature stop codon UGA (X). Here, we analyzed the readthrough of this premature stop codon (PTC) exemplarily for the most abundantly expressed harmonin isoform a1 and the longer retinal harmonin isoform b3. For this, HEK293T cells were transfected with cDNAs encoding the mutated harmonin a1 (harm_a1-p.R31X) and mutated harmonin b3 (harm_b3-p.R31X), respectively. After adding PTC124 to the culture medium a dose-dependent increase in harmonin a1 and b3 expression was observed by immunofluorescence and Western blots (bands at 79 kDa for harmonin a1; 95 kDa for harmonin b3) applying anti-harmonin (H3) and anti-FLAG antibodies, respectively (Figs. 1B and 2A). In contrast, low harmonin expression was detected in DMSO-treated control cells (Figs. 1B and 2A), which probably results from spontaneous readthrough of the p.R31X nonsense mutation, previously reported for other correlated nonsense mutations (Rebibo-Sabbah *et al.*, 2007).

Quantification of the number of immunofluorescently stained cells revealed a 3.1-fold increase in readthrough in

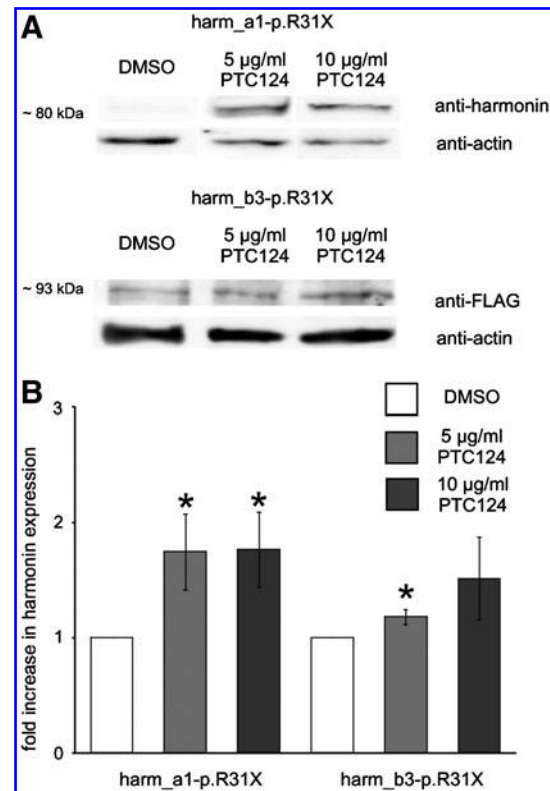


FIG. 2. Western blot analyses of PTC124-mediated readthrough of the *USH1C* nonsense mutation p.R31X in HEK293T cells. (A) PTC124-mediated readthrough in transiently transfected HEK293T cells analyzed by Western blotting. Treatment with PTC124 restored full-length harmonin a1 (~80 kDa) and harmonin b3 (~93 kDa) in p.R31X-transfected cells. Actin staining (~42 kDa) was used as loading control. (B) For quantification of PTC124-mediated readthrough of the p.R31X mutation, the optical densities of harmonin a1 bands (~80 kDa), stained with anti-harmonin antibodies, and harmonin b3 bands (~93 kDa), stained with anti-FLAG antibodies, were ascertained and normalized to the appropriate loading control. The increase in readthrough is shown as the fold increase over DMSO-treated cells. Error bars represent the standard deviation. **p* < 0.05.

harm_a1-p.R31X-transfected PTC124-treated cells and a 2.0-fold increase for harm_b3-p.R31X compared with control cells. The readthrough level obtained in Western blots revealed a 1.8-fold increase of readthrough in harmonin a1 p.R31X-transfected PTC124-treated cells and a 1.5-fold increase for harmonin b3 compared with control cells. The percentage of restored harmonin protein was calculated as the ratio of harmonin expression in p.R31X-transfected PTC124-treated cells to its expression in appropriate wild-type harmonin-transfected cells. This calculation indicated that PTC124 treatment induced 2.5% of the harmonin expression. Although the critical level of harmonin protein that is necessary to rescue retinal function in patients with *USH1C* is unknown, the PTC124-induced recovery of functional active harmonin might be sufficient to stop or at least slow the progression of the autosomal recessive *USH1* disease, reestablishing a near-normal or at least clinically less severe retinal phenotype.

Translational readthrough might introduce an incorrect amino acid in the recovered harmonin protein at the p.R31X mutation (Linde and Kerem, 2008), which may interfere with protein function. To assess the restored properties of harmonin we analyzed the binding capacity of harmonin a1 to the PDZ-binding motif (PBM) in the cytoplasmic tail domain of USH2a isoform b (Reiners *et al.*, 2005) and the actin filament-bundling activity of harmonin b (Fig. 3) (Boëda *et al.*, 2002). In pull-down assays with GST-tagged USH2a cytoplasmic tail and cell extract of PTC124-treated cells, clear and single sustained harmonin a1 bands were visible, whereas from extracts of DMSO-treated control cells only faint bands were present (Fig. 3A).

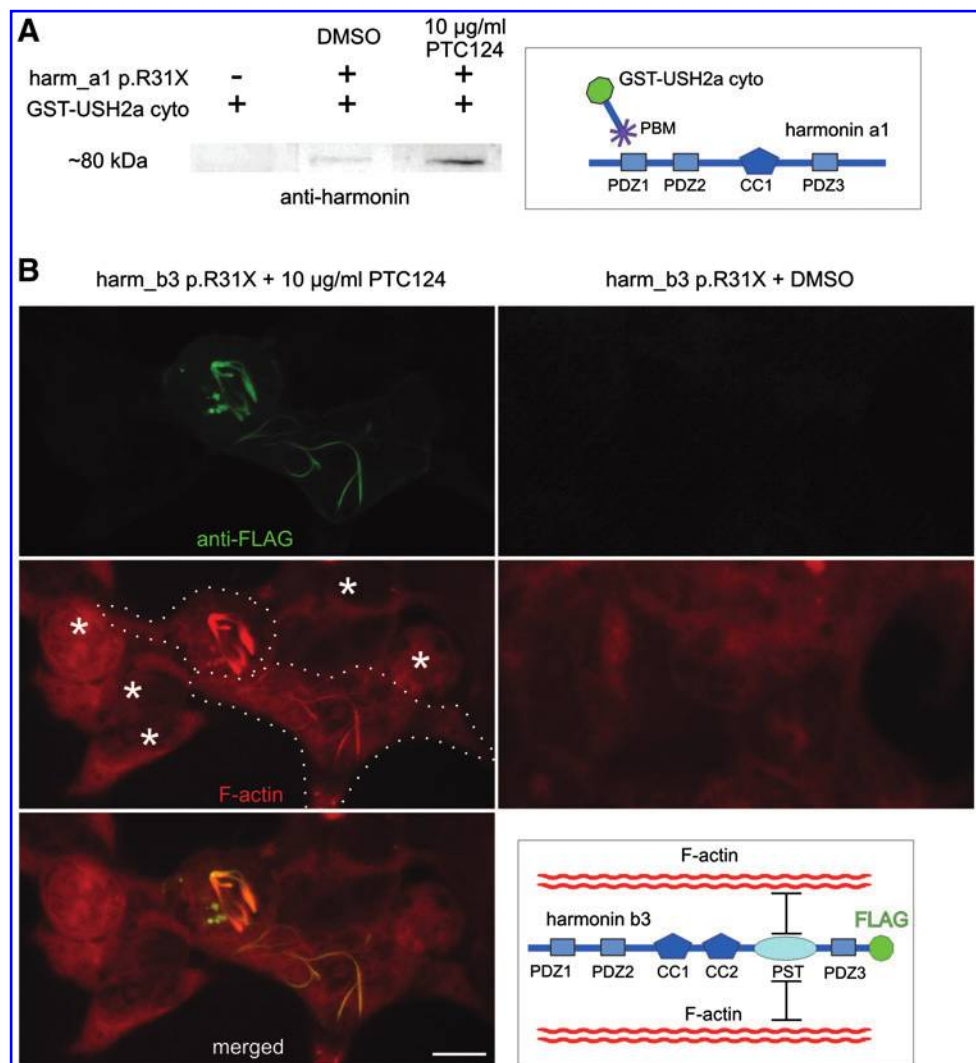
The actin filament-bundling capacity of recovered harmonin b3 was tested in cells transfected with FLAG-tagged harm_b3-p.R31X. Double labeling with anti-FLAG antibodies and rhodamine-phalloidin revealed actin filament bundling in PTC124-treated cells but not in DMSO controls (Fig. 3B). In summary, the translational readthrough of the p.R31X nonsense mutation of *USH1C* by PTC124 not only recovers the expression of full-length harmonin but also restores its cellular function as scaffold and actin filament-

bundling protein. Thus functional recovery occurs although the randomly introduced amino acid at the stop codon is not necessarily the arginine of wild-type harmonin. These data are in line with previous genotype-phenotype correlations, which demonstrate that missense mutations in *USH1C* introducing a single incorrect amino acid in the protein sequence do not result in retinitis pigmentosa but cause nonsyndromic isolated deafness (Ahmed *et al.*, 2002; Ouyang, *et al.*, 2002; Reiners *et al.*, 2006). In contrast, the p.R31X nonsense mutation in *USH1C* leading to the expression of truncated harmonin causes USH characterized by combined deaf-blindness. Therefore, PTC124-mediated readthrough of the p.R31X mutation in harmonin most probably will be sufficient for retinal function.

Readthrough of p.R31X mutation in organotypic retina cultures

We next investigated the readthrough potential of PTC124 in the retina, where harmonin isoforms are predominantly expressed in the photoreceptor cells (Reiners *et al.*, 2003). For this, we electroporated murine retinal explants, introducing

FIG. 3. PTC124-mediated restoration of harmonin protein function. (A) Restoration of harmonin a1 scaffold function assayed by GST pull-down. Lysates of harm_a1-p.R31X-transfected and PTC124-treated HEK293T cells were incubated with the immobilized GST-USH2a cytoplasmic tail. Pulled-down proteins were subjected to Western blot analyses, using anti-harmonin antibodies. PTC124 treatment of harm_a1-p.R31X cells restored binding of harmonin to the USH2a cytoplasmic tail. (B) Readthrough induced restoration of harmonin b3 actin filament (F-actin)-bundling capacity. HEK293T cells were transfected with FLAG-tagged harm_b3-p.R31X before treatment with PTC124. Costaining with anti-FLAG and rhodamine-phalloidin for F-actin revealed colocalization of harmonin with F-actin bundles in readthrough-positive cells (dotted lines) but not in untransfected cells (asterisks) or DMSO-treated controls. Scale bar: 5 μ m. PBM, PDZ-binding motif.



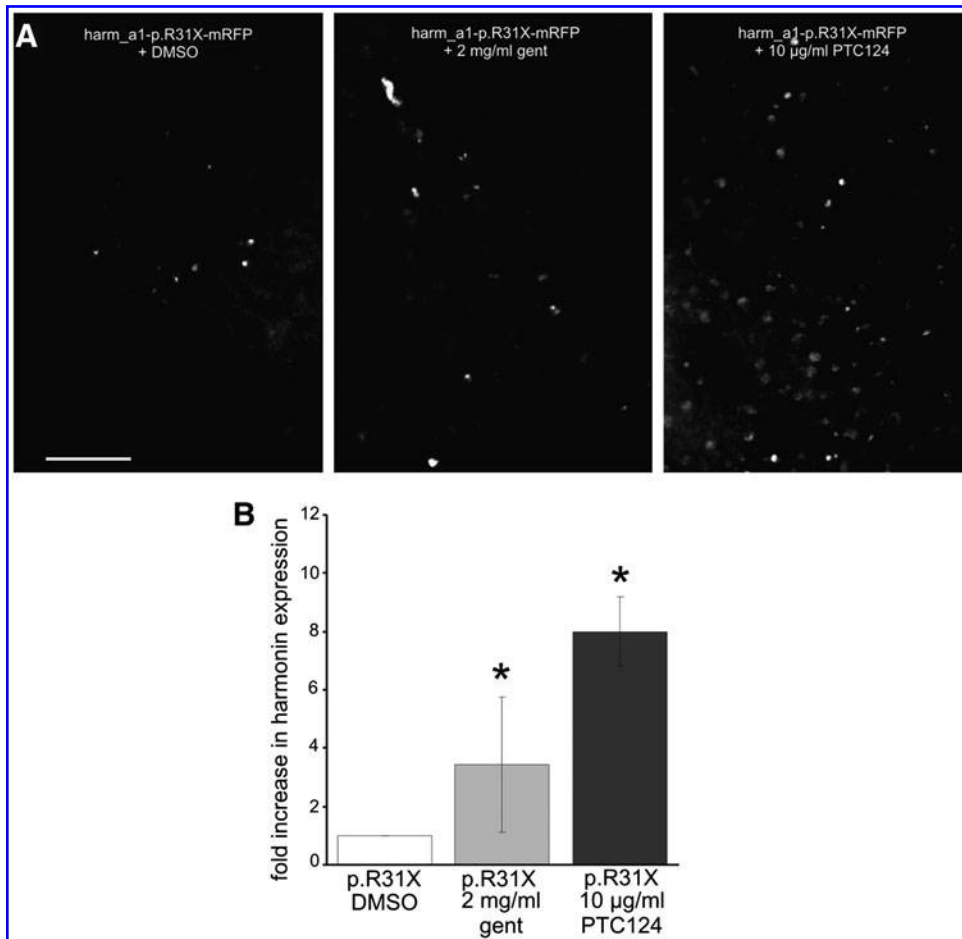


FIG. 4. PTC124-mediated readthrough of the *USH1C* nonsense mutation p.R31X in organotypic retina cultures. **(A)** PTC124-mediated readthrough in p.R31X-transfected murine retinal cultures. Retinas were transfected with harm_a1-p.R31X-mRFP; subsequently cultured in the presence of DMSO (control), gentamicin, or PTC124; and analyzed by fluorescence microscopy. **(B)** Quantification of mRFP-positive cells in retinal whole mounts revealed a substantial increase after gentamicin and PTC124 treatments. Scale bar in **(A)**: 100 μ m. Error bars represent the standard deviation. * $p < 0.05$.

the mutated harmonin a1 C-terminally fused to a reporter encoding red fluorescent protein (harm_a1-p.R31X-mRFP). In DMSO-treated controls only a small number of mRFP-positive cells were detected in retinal explants transfected with harm_a1-p.R31X-mRFP (Fig. 4). These results were in accordance with our data on cultured cells, indicating spontaneous readthrough of the harm_a1-p.R31X nonsense mutation. In contrast, in retinal explants transfected with the mutated harm_a1-p.R31X-mRFP, treatment with PTC124 resulted in a significant increase in the number of mRFP-expressing cells. It was even higher than in explants treated with gentamicin (Fig. 4), a readthrough-inducing aminoglycoside previously adapted in animal experiments and clinical trials (Wilschanski *et al.*, 2003; Guerin *et al.*, 2008; Linde and Kerem, 2008; Moosajee *et al.*, 2008).

To quantify drug-induced readthrough of the harmonin p.R31X nonsense mutation, mRFP-positive cells in seven randomly chosen regions of the whole mount were counted and calculated as fold increase over mRFP-expressing cells in DMSO-treated harm_a1-p.R31X-mRFP-transfected explants (Fig. 4B). This quantification revealed an 8.0-fold increase in readthrough of harm_a1-p.R31X-mRFP in PTC124-treated retinal explants, whereas the increase in gentamicin was lower (3.4-fold). These results indicate that PTC124 is twice as efficient in inducing readthrough of the harmonin p.R31X nonsense mutation in the retina than gentamicin. Previous

studies demonstrated that systemic gentamicin treatment induced readthrough of a nonsense mutation in rhodopsin and thereby slightly improved retinal function (Guerin *et al.*, 2008). Extrapolating the results from the latter study to the present data suggests that PTC124 treatment induces sufficient amounts of harmonin protein to combat retinal degeneration in patients with USH1.

Retinal biocompatibility of PTC124

An important concern for readthrough therapy is the compatibility of readthrough compounds in tissues and the organism (Linde and Kerem, 2008). Although systemic application of PTC124 has been shown to be nontoxic in animals and humans (Hirawat *et al.*, 2007; Welch *et al.*, 2007), retinal compatibility of PTC124 has not yet been evaluated. Here we analyzed the safety profile of PTC124 in the retina. We monitored potential histopathologies and retinal integrity by means of cell-specific molecular markers and possible cell death, applying TUNEL assays, a reliable tool with which to analyze any occurring cell death (Bramall *et al.*, 2010). For this, organotypic retina cultures were incubated with various concentrations of PTC124 or the reference readthrough drug gentamicin.

Nuclei were stained with DAPI to monitor possible changes in the well-defined layered organization of the ret-

ina. Comparisons of PTC124-treated retinas with control treated retinas revealed no difference in the DAPI staining pattern, either in the outer nuclear layer containing the nuclei of photoreceptor cells or in the inner nuclear layer containing nuclei of the secondary retinal neurons (Fig. 5A). Furthermore, we did not find any decrease in the thickness of the outer nuclear layer, indicating that there is no effect of PTC124 treatments on the number of photoreceptor cells, often regarded as an indicator of retinal degeneration.

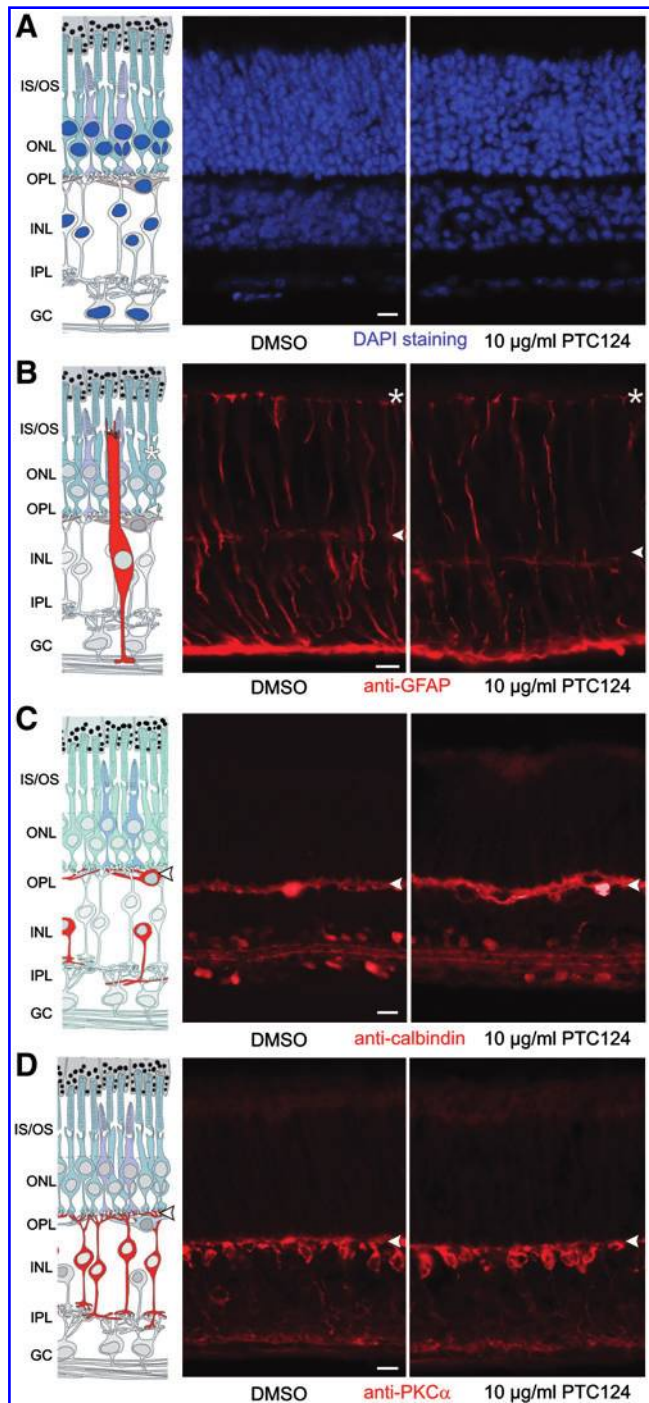
Effects of PTC124 treatment on specific retinal cells were monitored by indirect immunofluorescence, applying well-characterized antibodies as cell-specific molecular markers, namely anti-GFAP for Müller glia cells, anti-calbindin for horizontal cells and amacrine cells, and anti-PKC- α for bipolar cells. Our analyses revealed no apparent differences in the cell number and spatial organization of these retinal cell types of PTC124-treated compared with DMSO-treated retinas (Fig. 5B–D) or untreated retina cultures (Reidel *et al.*, 2006; Orisme *et al.*, 2010). In conclusion, these data indicate that PTC124 treatment does not alter the structural integrity of the neuronal retina and has no cytotoxic effect on retinal neurons and glia cells.

In addition, cell death was analyzed by TUNEL assays in treated retinas. Quantification of TUNEL-positive nuclei revealed only minor increases in cell death in murine retinal cultures treated with PTC124 compared with DMSO-incubated control cultures (Fig. 6A) or untreated retina cultures (data not shown). These data on murine retina cultures indicate that PTC124 is even more biocompatible than the novel designed aminoglycoside NB30, introduced as a potent investigational readthrough drug for retinal applications (Hainrichson *et al.*, 2008; Goldmann *et al.*, 2010). In contrast, quantification of TUNEL-positive nuclei revealed a more than 3.4-fold increase in dying cells after gentamicin treatment compared with control cultures (Fig. 6B). In human retinal explants cultured from donor eyes postmortem we observed 1.2% TUNEL-stained nuclei in DMSO-treated controls (Fig. 6), which is in line with cell death levels in untreated cultures (data not shown). Whereas the application

of gentamicin slightly increased cell death (1.9-fold), nearly no difference from control cultures was found after PTC124 treatment of human retinal cultures (Fig. 6B).

These results clearly favor PTC124 as a readthrough-inducing drug for retinal applications compared with the clinically introduced reference gentamicin. Because of the dissatisfying safety profile of gentamicin in the retina we excluded the drug in further investigations. However, the excellent biocompatibility of PTC124 for retina further

FIG. 5. Histocompatibility and cytocompatibility of PTC124 in the retina. Retinal integrity and cellular composition of PTC124- and DMSO-treated murine retinal cultures were analyzed in cryosections. (A) DAPI staining of nuclear DNA visualized the well-defined layers of the neuronal retina. No alteration in the characteristic layer organization and thickness of these layers was observed after PTC124 treatment. (B–D) Indirect immunofluorescence of retinal cell types, using cell-specific molecular marker antibodies: Müller glial cell extensions, which span from the ganglion cell (GC) layer through the inner nuclear layer (INL) and outer nuclear layer (ONL) to the membrana externa limitans (asterisk), were stained with anti-GFAP (B). Horizontal cells and amacrine cells were stained with anti-calbindin (C) and bipolar cells were stained with anti-PKC- α (D). The staining patterns of specific cell markers did not change after PTC124 administration, indicating no apparent effect of PTC124 on retinal cells, underlining the high biocompatibility. DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; GFAP, glial fibrillary acidic protein; IPL, inner plexiform layer; IS/OS, inner/outer segment; OPL (arrowhead), outer plexiform layer; PKC- α , protein kinase C- α . Scale bars: 10 μ m.



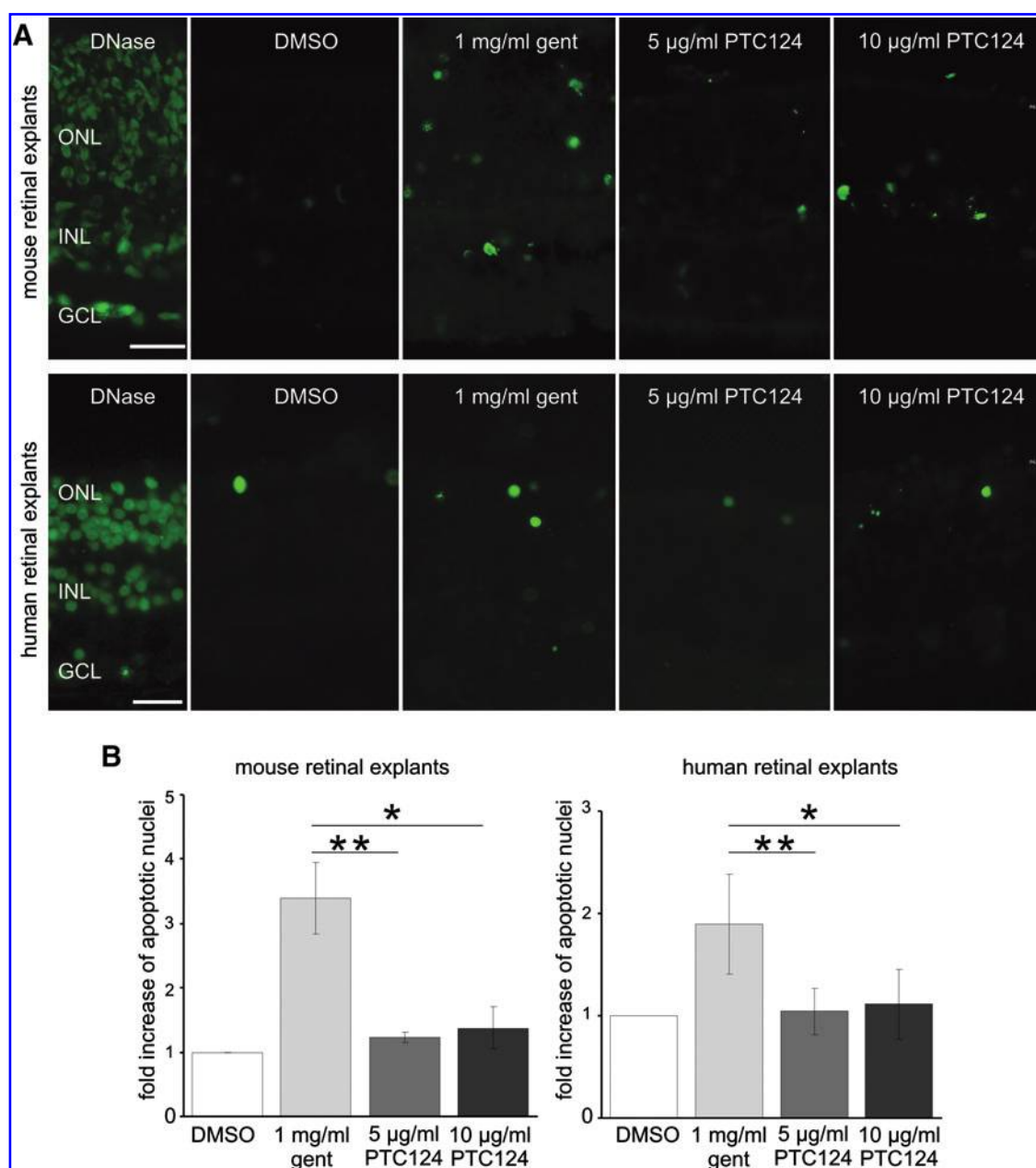


FIG. 6. Biocompatibility of PTC124 in mouse and human organotypic retina cultures. **(A)** Fluorescence microscopy analyses of TUNEL-stained sections through mouse retina explants and human retinal explants treated with DMSO control, gentamicin (gent), and two different concentrations of PTC124. DNase-treated cryosections served as positive control. In contrast to gentamicin, PTC124 treatments cause only a little increase in TUNEL-positive apoptotic cells in mouse and human retinas. **(B)** Quantification was based on the ratio of TUNEL-positive to DAPI-stained nuclei, representing the percentage of apoptotic cells. The increase in apoptotic cells after drug treatment is shown in relation to the DMSO control. In contrast to gentamicin, both concentrations of PTC124 cause only slight increases in apoptotic cells in mouse and human retinas. Error bars represent the standard deviation. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: 10 µm. * $p < 0.05$; ** $p < 0.01$.

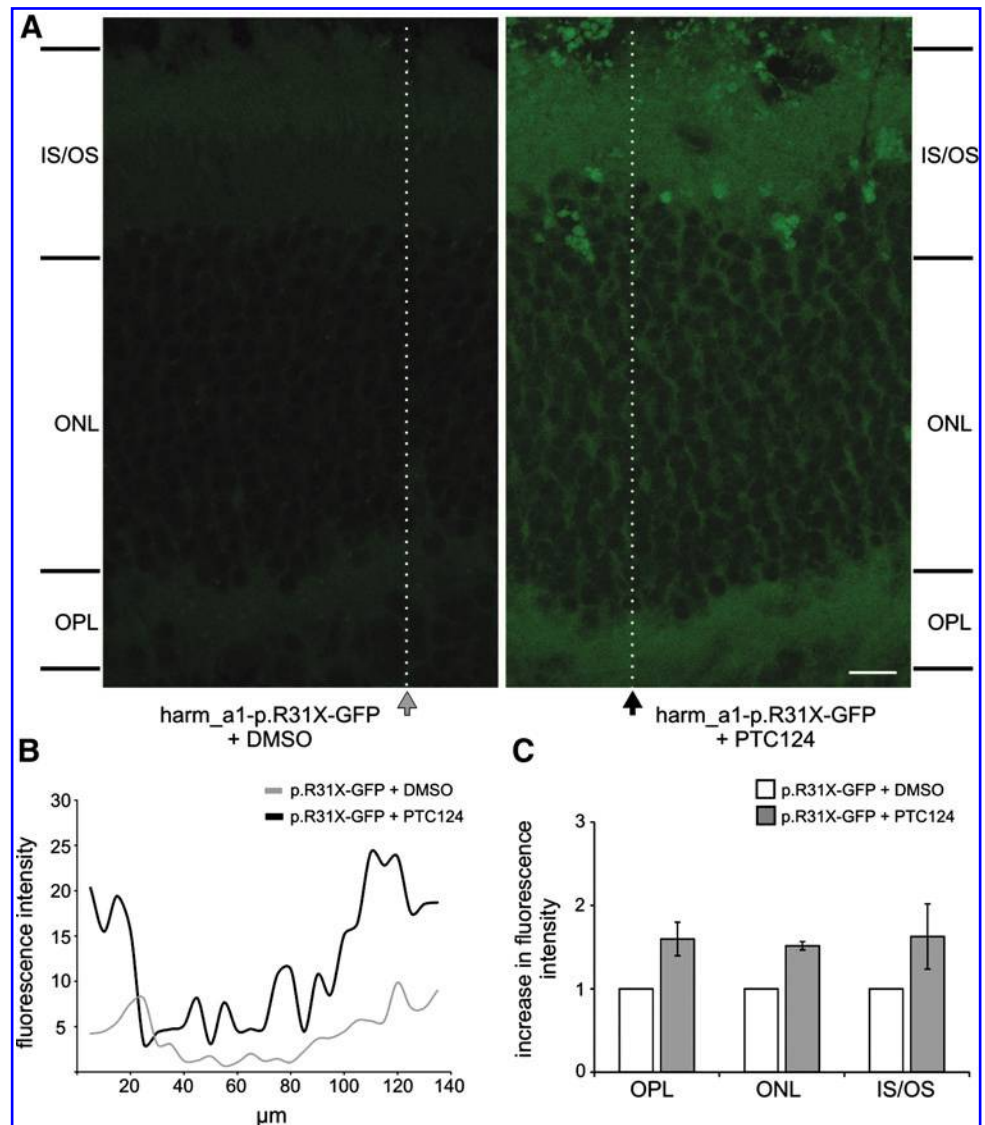
supports the previously stated high safety profile of PTC124 in humans (Hirawat *et al.*, 2007).

Readthrough of *p.R31X* mutation in retina *in vivo*

Because application of PTC124 can restore harmonin expression in cell culture and *ex vivo* in organotypic retina

cultures, we next tested the readthrough performance of PTC124 in the organism *in vivo*. For this we adopted an *in vivo* electroporation technique introduced by Matsuda and Cepko (2004) to investigate the functionality of PTC124 in the context of a mouse eye. We transferred harm_a1-p.R31X reporter constructs into the retina of newborn mice via electroporation. After 6 weeks PTC124 was injected subretinally

FIG. 7. Confocal analyses of PTC124-mediated readthrough in retinas *in vivo*: retinas of harm_a1-p.R31X-GFP-electroporated mice. **(A)** Comparison of representative cryosections of DMSO- and PTC124-treated retinas revealed harmonin a1-GFP expression in photoreceptor cells of PTC124-treated retinas. **(B)** Intensity profiles of GFP fluorescence at the dotted lines in the images of the retinas in **(A)**. DMSO- and PTC124-treated retinas are represented by gray and black lines, respectively. **(C)** Quantification of GFP fluorescence intensity in selected regions of photoreceptor cells, namely the outer plexiform layer (OPL), outer nuclear layer (ONL), and inner and outer segment (IS/OS) in DMSO-treated (gray columns) and PTC124-treated (solid columns) retinas, respectively. GFP fluorescence intensity was higher in all compartments of photoreceptor cells in PTC124-treated retinas compared with DMSO-treated retinas, indicating that PTC124 induced readthrough of the p.R31X mutation *in vivo*. Scale bar in **(A)**: 10 μm .



into the electroporated mouse eye and readthrough was assessed by fluorescence microscopy analyses and Western blot analyses (Figs. 7 and 8). Fluorescence microscopy revealed hardly any GFP expression in the retinas of DMSO-injected control eyes. In contrast, in retinas treated with PTC124, apparent harmonin_a1-GFP expression was observed (Fig. 7A). Quantification of the fluorescence intensity along photoreceptor cells showed an overall increase in GFP intensity after PTC124 treatment (Fig. 7B). In addition, separate measurements of photoreceptor compartments revealed a clear enhancement of fluorescence in the outer plexiform layer (photoreceptor synapses) and the inner and outer segments of photoreceptor cells (Fig. 7C), the subcellular compartments of the cell where harmonin is expressed in wild-type photoreceptor cells (Reiners *et al.*, 2003). Our Western blot analyses demonstrated that PTC124 treatment recovered full-length harmonin a1 expression in harm_a1-p.R31X-mRFP-electroporated retinas (Fig. 8A). Quantification of the readthrough level obtained in the Western blot analyses revealed a 1.4-fold induction of readthrough in harmonin a1 (Fig. 8B). Present data prove in principle that

PTC124-mediated readthrough of nonsense mutations is suitable to restore retinal protein expression in the organism. However, the specific mode of application of PTC124 into the eye will be reserved for further studies. These findings confirm the readthrough ability of PTC124 *in vivo* and highlight the potential for PTC124 as a treatment option for the p.R31X mutation and other retinal diseases. The relatively late onset of retinal degeneration in USH leaves a broad window for pharmacological interventions, compared with early-onset defects in the inner ear.

Concluding remarks

The results obtained in this proof-of-principle study demonstrate the capacity of PTC124 for readthrough therapies of PTCs in retinal diseases as well as in other genetic disorders. PTC124 appeared to be highly biocompatible in the retina, in comparison with the aminoglycoside gentamicin. These gathered data emphasize the enormous potential of PTC124 as a readthrough therapy in the retina to combat nonsense mutation-based retinal disorders and other groups

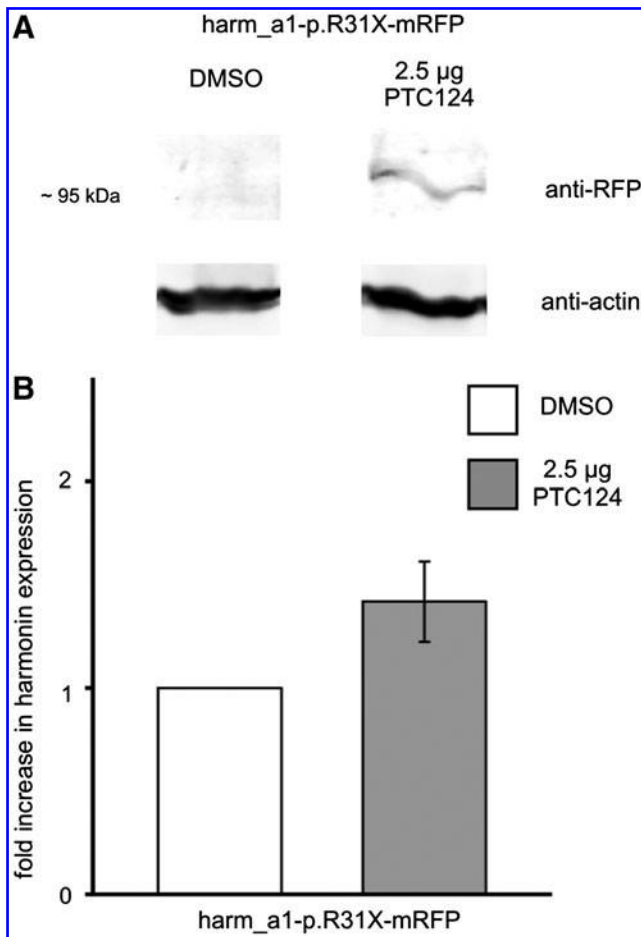


FIG. 8. Western blot analyses of PTC124-mediated readthrough in retinas *in vivo*: retinas of harm_a1-p.R31X-RFP electroporated mice. **(A)** Treatment with PTC124 restored full-length harmonin a1-mRFP. Actin staining (~42 kDa) was used as loading control. **(B)** For quantification of PTC124-mediated readthrough of the p.R31X mutation, the optical densities of harmonin a1-mRFP, stained with anti-RFP antibodies, were ascertained and normalized to the appropriate loading control. The increase in readthrough is shown as the fold increase relative to DMSO-treated mice.

of genetic disorders with limited or no current therapeutic options, and raise hope for future clinical trials.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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