Beneficial Read-Through of a *USH1C* Nonsense Mutation by Designed Aminoglycoside NB30 in the Retina

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Purpose. The human Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness. USH is clinically and genetically heterogeneous, assigned to three clinical types. The most severe type is USH1, characterized by profound inner ear defects and retinitis pigmentosa. Thus far, no effective treatment for the ophthalmic component of USH exists. The p.R31X nonsense mutation in *USH1C* leads to a disease causing premature termination of gene translation. Here, we investigated the capability of the novel synthetic aminoglycoside NB30 for the translational read-through of the *USH1C*-p.R31X nonsense mutation as a retinal therapy option.

Methods. Read-through of p.R31X by three commercial, clinically applied aminoglycosides and the synthetic derivative NB30 was validated in vitro, in cell culture, and in retinal explants. Restoration of harmonin functions was monitored in GST pull-downs (scaffold function) and by F-actin bundling analysis in HEK293T cells. Biocompatibility of aminoglycosides was determined in retinal explants by TUNEL assays.

Results. In vitro translation and analyses of transfected HEK293T cells revealed a dose-dependent read-through by all aminoglycosides. In addition, gentamicin, paromomycin, and NB30 induced read-through of p.R31X in mouse retinal explants. The read-through of p.R31X restored harmonin protein function. In contrast to all commercial aminoglycosides NB30 showed good biocompatibility.

Conclusions. Commercial aminoglycosides and NB30 induced significant read-through of the *USH1C*-p.R31X nonsense mutation. However, the observed read-through efficiency, along with its significantly reduced toxicity and good biocompatibility, indicate that the novel derivate NB30 represents a better choice than commercial aminoglycosides in a read-through therapy of USH1C and other ocular diseases. (*Invest Ophthalmol Vis Sci.* 2010;51:6671–6680) DOI:10.1167/iovs.10-5741

The human Usher syndrome (USH) is the most frequent cause of combined hereditary deaf-blindness. Based on the heterogenic clinical course of the disease, USH is subdivided into three clinical types (USH1-USH3).[1] The most severe form of the disease is USH1, characterized by profound prelingual hearing loss, vestibular areflexia, and prepubertal onset of retinitis pigmentosa (RP).[2,3] Although the auditory deficit can be successfully treated with cochlear implants,[4] to date there is no effective treatment for the retinal phenotype of USH.

In the present study, we focused on the *USH1C* gene, which encodes the PDZ domain (named after its presence in *P*SD-95, *D*LG, *Z*O-1) containing scaffold protein harmonin.[5] Our previous molecular analyses revealed harmonin as one of the key organizers in the protein interactome related to USH, by binding all known USH1 proteins and most USH2 proteins.[2,6–8] Harmonin-b isoforms additionally bind directly to actin filaments, introducing prominent actin filament bundles.[9] The *USH1C* gene consists of 28 coding exons, of which eight are differentially spliced, resulting in numerous alternative transcripts of harmonin grouped in three subclasses (a-c).[5] In the retina, harmonin a1 and b4 transcripts are the most prominent.[10] However, thus far which isoform is essential for the maintenance or restoration of retinal function has remained elusive.[2] This, in combination with the numerous splice variants, makes *USH1C* a difficult target for conventional gene addition approaches. Although truncating mutations, including nonsense mutations in *USH1C*, cause USH1, certain missense mutations in the gene lead to nonsyndromic deafness not associated with RP.[2,11,12] These observations suggest that partial or low-level activity of harmonin may be sufficient for correct retinal function, though not for normal hearing.

Aminoglycosides are commonly used as antibiotics, but during the past few years they attracted much attention because of their ability to read-through nonsense mutations.[13] This dual function is based on aminoglycoside-mediated reduced ribosomal proofreading, which alters the fidelity of mRNA translation into protein (antibiotic function) but which can also challenge the read-through of nonsense mutations and the generation of full-length protein (read-through function).[13–15] In the latter case, the amino acid inserted at the position of the stop codon is not necessarily the one present in

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the wild-type protein, but the resultant protein may still be fully or at least partially functional.[13,16] The read-through of nonsense mutations by aminoglycosides has been demonstrated for several genetic diseases[17–22] that already paved the way for clinical trials.[23,24] Nevertheless, the degree of read-through varies significantly and depends on several factors, including the nature of the stop mutation, the neighboring DNA sequence context, and the chosen read-through drug.[13,25,26] Therefore, examination of the read-through efficiency of specific nonsense mutations and applied aminoglycoside is necessary.

The most critical factor that limits the potential of aminoglycosides for read-through therapy is their insufficient biocompatibility; their systemic application can have drastic adverse effects, namely nephrotoxicity and ototoxicity.[27,28] Although at low dosages aminoglycosides are commonly used as intravitreal antibiotics in the eye, their application was found to be associated with retinal toxicity.[29,30] Our hypothesis is that novel aminoglycoside derivatives that maintain high read-through activity while exhibiting better biocompatibility can overcome this dilemma.[22,31,32]

In the present study, we focused on a specific mutation in *USH1C* (p.R31X).[33] This point mutation at position 91 changes a cytosine to a thymine and creates a premature translation stop codon (TGA) in the mRNA translation of the *USH1C* gene (Fig. 1A). Because the mutation is located in the constitutively transcripted exon 2, the transcript lacks all domains necessary for harmonin function. Here we demonstrated that several commercial clinically applied aminoglycosides, including geneticin (G418), gentamicin, paromomycin, and the novel synthetic variant of paromomycin NB30, recently developed by us,[31,32] induced the read-through of the p.R31X nonsense mutation. We also demonstrated the restoration of harmonin scaffold and actin filament bundling function. Furthermore, we showed that in retinal explants, among all aminoglycosides evaluated, the synthetic derivative NB30 exhibits the best retinal biocompatibility while maintaining substantial read-through activity. These findings have important implications for the development of targeted therapeutic strategies that are effective for patients with nonsense mutations in *USH1C* and other ocular and nonocular diseases.

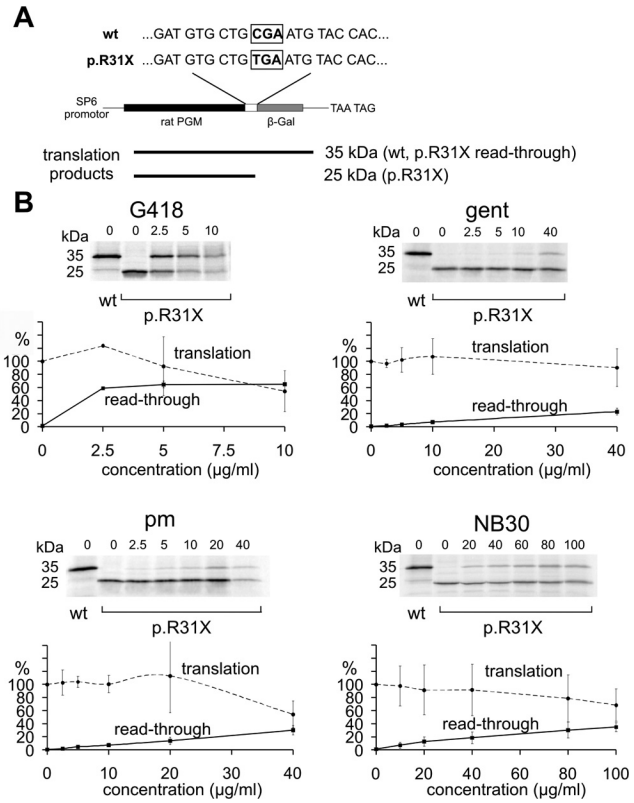


Figure 1. Read-through of the *USH1C*-p.R31X mutation by aminoglycosides in vitro. (**A**) Schematic representation of reporter plasmid for in vitro translation. The reporter construct consists of a SP6 promotor control, the *phosphoglucomutase* (rat PGM) gene (25-kDa polypeptide), harmonin a1 cDNA, including either the wt sequence or the p.R31X nonsense mutation, and the α-complementation region of β-galactosidase (10-kDa polypeptide). Resultant products are wt or read-through of p.R31X, 35 kDa and p.R31X, 25 kDa. (**B**) Dose-dependent read-through of p.R31X in vitro. Translation was performed in a reticulocyte system in the absence or increasing concentrations of aminoglycosides G418, paromomycin (pm), gentamicin (gent), and NB30. *Solid line*: percentage of read-through, calculated as relative proportion of the 35-kDa product out of total reaction products (35-kDa and 25-kDa polypeptides). *Dasbed line*: percentage of translation efficiency, calculated as the sum of total reaction products (35-kDa and 25-kDa polypeptides) in the presence of each compound and compared with a reaction with no compound added. All aminoglycosides tested mediated read-through of the p.R31X mutation.

Materials and Methods

Aminoglycosides

Commercial aminoglycosides were purchased from Sigma-Aldrich (paromomycin and gentamicin; Diesenhofen, Germany) and Invitrogen (G418; Carlsbad, CA). NB30 was synthesized and previously characterized.[22,31] Paromomycin, gentamicin, and NB30 were in their sulfate salt forms and diluted in water. G418 was obtained as an aqueous solution.

In Vitro Translation Assay

DNA fragments derived from *USH1C* harmonin isoform a1, including the p.R31X stop mutation or the corresponding wild-type (wt) codon, and six upstream and downstream flanking codons were created by annealing the following couples of complementary oligonucleotides: wt, 5′-GATCCTATCTCTATGATGTGCTGCGAATGTACCACCAGACCATGGAA-3′ and 5′-AGCTTTCCATGGTCTGGTGGTACATTCGCAGCACATCATAGAGATAG-3′; p.R31X, 5′-GATCCTATCTCTATGATGTGCTGTGAATGTACCACCAGACCATGGAA-3′ and 5′-AGCTTTCCATGGTCTGGTGGTACATTCACAGCACATCATAGAGATAG-3′. Fragments were inserted into the pDB plasmid.[25] In vitro translation was performed as previously described.[22]

Antibodies and Dyes

Monoclonal mouse antibodies to actin (clone C4) and to FLAG were obtained from Seven Hill Bioreagents (Cincinnati, OH) and Sigma-Aldrich, respectively. Polyclonal rabbit antibodies against harmonin (H3) were used as previously described.[10] Actin filaments were visualized by rhodamine-phalloidin (Sigma-Aldrich). Secondary antibodies conjugated to Alexa488 (Molecular Probes, Leiden, Netherlands) and DAPI (Sigma-Aldrich) for the visualization of the nuclear DNA were applied.

Cell Culture

For Myc-tagged harm_a1 and harm_a1-p.R31X, the murine cDNA of harmonin a1 was amplified and inserted into the pCS2+MT vector.[22] For S-FLAG-tagged harm_b3 and harm_b3-p.R31X, the human cDNA of harmonin b3 was subcloned and inserted into the pDest/C-SF-TAP vector.[34] The p.R31X mutations were generated with a mutagenesis kit (QuickChange Lightning Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA). HEK293T cells were grown in Dulbecco's modified Eagle's medium with media (D-MEM GlutaMAX; Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO_2. Transfections were performed, with reagents (Lipofectamine LTX and PLUS; Invitrogen) according to the

manufacturer's protocol. After 7 hours, the medium was changed to fresh medium, and the cells were incubated with aminoglycosides for 48 hours.

Western Blot Analyses

Harvesting of cells and Western blot analyses were performed as previously described.[35,36] Band intensities were obtained using LI-COR (Lincoln, NE) software. To quantify aminoglycoside-induced read-through of the p.R31X mutation, the optical density of the 80-kDa band was ascertained and normalized to the loading control actin. The fold over basal was calculated as the ratio of appropriate untreated p.R31X-transfected cells to p.R31X-transfected aminoglycoside-treated cells. The percentage of restored harmonin protein was calculated as the ratio of p.R31X-transfected aminoglycoside-treated cells to the appropriate wt control. Different control experiments were run in parallel to every drug trial. Consequently, small variations within the control groups have led to asymmetries in the percentage increase over wt. The presented data are an average of two to four independent repeats of the experiment.

GST Pull-Down Assay

GST (glutathione S-transferase) pull-downs were performed as previously described.[7,36] To quantify aminoglycoside-induced expression of functional harmonin, the optical density of the 80-kDa band was ascertained and was normalized to 5% of the input. The percentage of functional protein was calculated as the ratio of untreated wt harmonin a1-transfected cells to p.R31X-transfected aminoglycoside-treated cells.

Animals

C57BL/6J mice were maintained on 12-hour light (200 lux)/12-hour dark cycle, with food and water ad libitum. The ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and national and institutional guidelines for animal care were followed.

Organotypic Retina Culture

Mouse retinas were cultured as previously described.[37] Cultures were incubated with various concentrations (1, 2, or 4 mg/mL) of the aminoglycosides in DMEM-F12 for 48 hours. Subsequently, retina cultures were fixed and embedded for microscopic analyses.

Immunofluorescence Microscopy

Immunofluorescence analyses were carried out on HEK293T cells seeded on coverslips, followed by fixation with methanol, and proceeded as previously described.[9,38] Cultured retinas were sectioned and stained as previously described.[37] Specimens were analyzed with a fluorescence microscope (DMRB; Leica Microsystems, Bensheim, Germany). Images were obtained with a charge-coupled device camera (ORCA ER; Hamamatsu, Herrsching, Germany) and were processed with image editing software (Photoshop CS; Adobe Systems, San Jose, CA).

Electroporation of Retinal Explants

The harm_a1-mRFP and harm_a1-p.R31X-mRFP plasmids were generated by inserting the murine cDNA coding for harmonin a1 into the pTER-mRFP vector.[39] Retinas of postnatal day 5 mice were dissected and treated as described.[37] Subsequently, harmonin plasmids were transferred into the explants by electroporation (ECM 830; BTX Harvard Apparatus, Holliston, MA) as described.[40] Retinas were cultured for 24 hours before the addition of aminoglycosides into fresh medium for 72 hours. Read-through efficiencies were determined by counting mRFP-positive cells in randomly chosen 10,000 μm^2 squares of retinal whole mounts.

TUNEL Assay

TUNEL (TdT-mediated dUTP nick end labeling) has been described[41] using a kit for measuring and quantitating cell death (In Situ Cell Death Detection Kit; Roche, Mannheim, Germany). For the quantification of apoptotic cells, TUNEL-positive cells were counted and normalized to DAPI-stained nuclei. The fold over basal was calculated as the ratio of the number of apoptotic cells in treated retinal explants compared with the number of apoptotic cells in untreated retinal explants.

Statistical Analysis

Statistical significance of difference between untreated and aminoglycoside treated samples was analyzed using one-way *t*-test analysis.

Results

Read-Through of the p.R31X Nonsense Mutation of *USH1C* by Aminoglycosides In Vitro

In the present study, we focused on the p.R31X nonsense mutation in the *USH1C* gene.[33] First, the read-through of the p.R31X mutation by aminoglycosides was analyzed by in vitro translation (Fig. 1A).[25] In vitro translation of the wt construct resulted in the production of a 35-kDa polypeptide, whereas a 25-kDa polypeptide was produced from the p.R31X construct. Addition of G418, gentamicin, paromomycin, and NB30 to the translation reactions of the p.R31X construct increased the intensity of the 35-kDa band in a dose-dependent manner, indicating a partial read-through of the nonsense mutation. The highest read-through activity of 65% was achieved with G418. Read-through rates of the other analyzed aminoglycosides were milder and in the range of 23% for gentamicin, 30% for paromomycin, and 35% for NB30 (Fig. 1B; Table 1). However, in parallel to their read-through capacity, a dose-dependent inhibition of the general translation efficiency was observed after aminoglycoside application (Fig. 1B). The total translation rate was reduced to approximately 50% by 10 μg/mL G418 and 40 μg/mL paromomycin. In contrast, the translation efficiency was nearly unaffected in the presence of gentamicin and NB30, indicating reduced cytotoxicity of these latter compounds.

Table 1. Read-Through Capacity, Restoration of Protein Function, and Biocompatibility of Aminoglycosides

	Read-Through Capacity			Restoration of Harmonin Function		
Aminoglycoside	**In Vitro**	**Cell Culture**	**Retinal Explants**	**Pull-Down**	**Active Harmonin**	**Biocompatibility**
G418	64% (10 μg/mL)	8.9 × (2 mg/mL)	0 (1 mg/mL)	75% (2 mg/mL)	16.4% (2 mg/mL)	22.9 × (2 mg/mL)
Gentamicin	23% (40 μg/mL)	3.4 × (2 mg/mL)	3.4 × (2 mg/mL)	73% (2 mg/mL)	3.8% (2 mg/mL)	5.0 × (2 mg/mL)
Paromomycin	30% (40 μg/mL)	1.6 × (2 mg/mL)	3.1 × (2 mg/mL)	ND	ND	4.8 × (2 mg/mL)
NB30	18% (40 μg/mL)	2.0 × (2 mg/mL)	7.2 × (2 mg/mL)	75% (2 mg/mL)	1.9% (2 mg/mL)	1.9 × (2 mg/mL)
	34% (100 μg/mL)	2.2 × (4 mg/mL)				3.9 × (4 mg/mL)

Read-Through of the p.R31X Nonsense Mutation of *USH1C* by Aminoglycosides in Cell Culture

We next tested the read-through of the p.R31X nonsense mutation by aminoglycosides in HEK293T cells. For transfections, cDNAs encoding the murine harmonin a1 isoform (harm_a1-wt) or for the harmonin a1 isoform carrying the nonsense mutation (harm_a1-p.R31X) were used. After the transfection of HEK293T cells, aminoglycosides were added to the culture media of harm_a1-p.R31X-transfected cells, and 48 hours later induced read-through was analyzed by indirect immunofluorescence and Western blot analysis using the H3 antibody against harmonin (Figs. 2, 3A).

Immunofluorescence analyses demonstrated that transfections with harm_a1-wt plasmid resulted in approximately 35% of harmonin-expressing cells (Fig. 2). In cultures transfected with p.R31X, <1% of the cells yielded a positive harmonin staining that probably resulted from the spontaneous read-through of the p.R31X nonsense mutation. However, after G418, paromomycin, gentamicin, or NB30 treatment of harm_a1-p.R31X-transfected cells, a dose-dependent increase in the number of harmonin a1-expressing cells was observed, indicating induced read-through of p.R31X (Fig. 2).

In Western blot analyses with anti-harmonin of lysates from cells transfected with harm_a1-wt, a band of approximately 80 kDa was detected, corresponding to the calculated molecular weight of 6 myc-tags (6 kDa) and full-length harmonin a1 construct (73 kDa) (Fig. 3A). In Western blot analysis of untransfected and harm_a1-p.R31X-transfected cells, no band at 80 kDa was detected, indicating that harmonin a1 was not expressed. In contrast, in lysates of harm_a1-p.R31X-transfected cells treated with aminoglycosides, the band of 80 kDa appeared, demonstrating aminoglycoside-induced restoration of full-length harmonin a1. It is noteworthy that in addition to this 80-kDa band, a fade band at approximately 73 kDa was consistently found in all HEK293T lysates because of intrinsic harmonin a1 expression.[10] Very weak bands with lower molecular weights were detected by H3-antibodies in harm_a1-p.R31X-transfected cells, indicating either degradation products or incomplete read-through of harmonin a1.

To quantify aminoglycoside-induced read-through of the p.R31X mutation, the optical densities of the 80-kDa bands were ascertained and normalized to the loading control actin (Fig. 3B; Table 1). This quantification revealed the maximal read-through of 8.9-fold over the basal level in harm_a1-p.R31X-transfected cells treated with 2 mg/mL G418. This represents 22% restored harmonin protein compared with the harmonin level in wt harmonin a1-transfected cells. The effect of the three other aminoglycosides tested was milder, and the maximal read-through of the p.R31X mutation compared with the basal level ranged from a 1.6-fold increase for paromomycin (2 mg/mL; 2.5% compared with wt), a 2.2-fold increase for NB30 (4 mg/mL; 5.0% compared with wt), and a 3.4-fold increase for gentamicin (2 mg/mL; 5.2% compared with wt).

Aminoglycoside-Mediated Restoration of Harmonin Protein Function

We have previously characterized harmonin as one of the major scaffold proteins in the USH protein interactome, binding all known USH1 and USH2 proteins through its PDZ domains.[2,6–8] For testing protein function of recovered harmonin, we took advantage of the specific interaction between the harmonin PDZ1 domain and the PBM (PDZ-binding motif) in the cytoplasmic tail of the USH2a isoform b[7] and of the actin filament-bundling capacity of the harmonin b isoform.[9] The scaffolding function of harmonin was investigated in GST pull-down assays. Recombinant GST-tagged USH2a tail was incubated with protein lysates of HEK293T cells transfected with

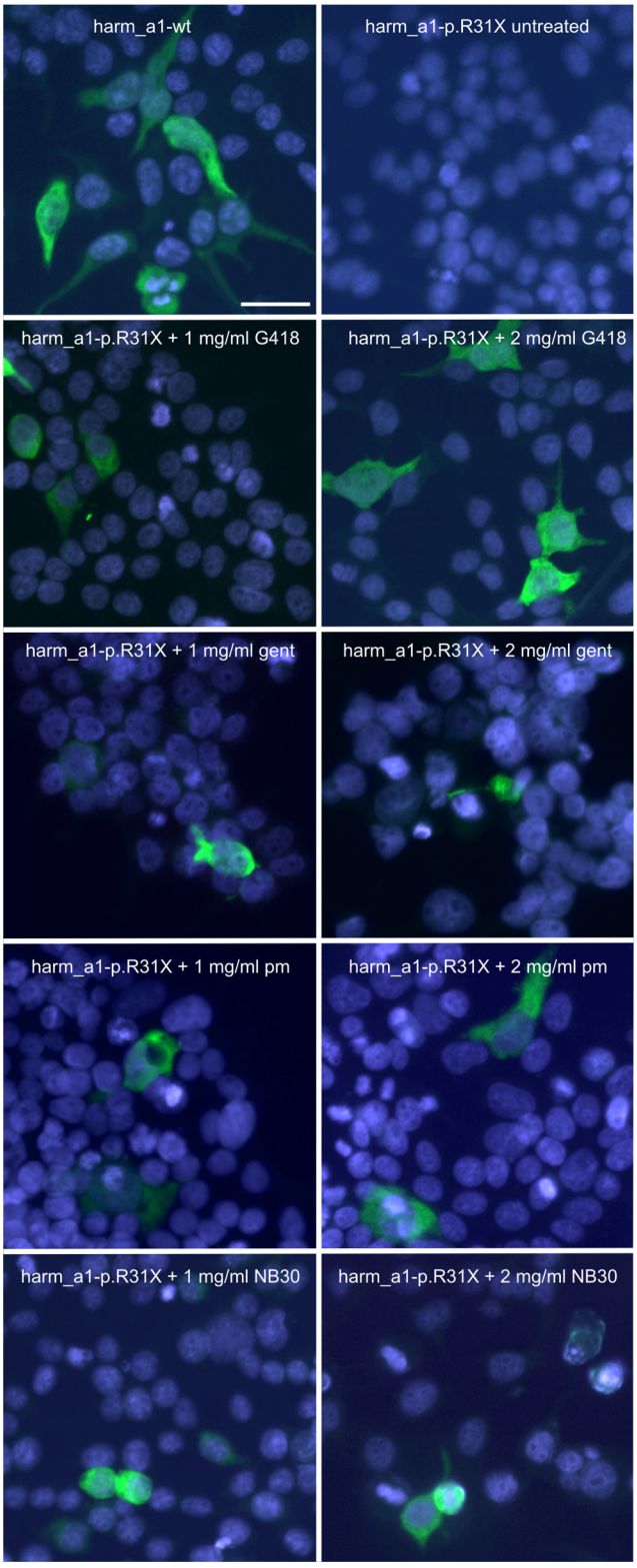


FIGURE 2. Immunofluorescence analyses of read-through of the p.R31X nonsense mutation by aminoglycosides in HEK293T cells. HEK293T cells were transfected with wt harmonin or harm_a1-p.R31X. Indirect immunofluorescence of anti–harmonin antibodies revealed harmonin a1 expression (*green*) in harmonin a1-transfected but not in untreated harm_a1-p.R31X-transfected cells. Aminoglycoside treatment restored harmonin a1 expression (*green*) in p.R31X-transfected cells. Cell nuclei are counterstained by DAPI (*blue*). Scale bar, 20 μm.

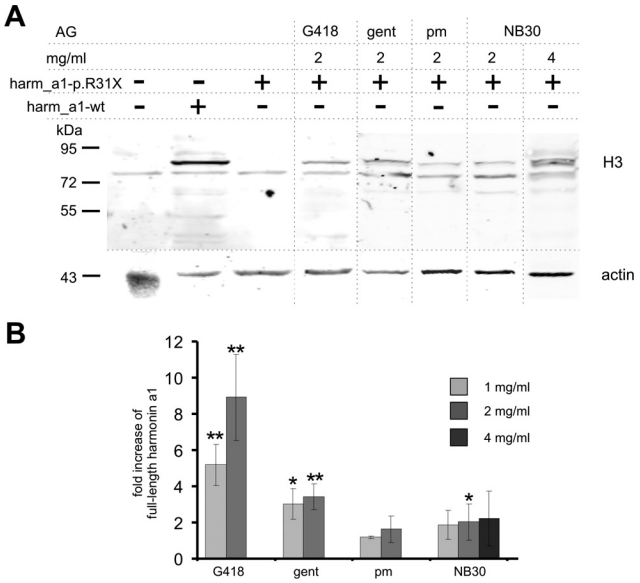


FIGURE 3. Western blot analyses of the read-through of the harm_a1-p.R31X nonsense mutation by aminoglycosides. (**A**) Representative Western blot analysis of HEK293T cells transiently transfected with the wt harmonin or the harm_a1-p.R31X, grown in the presence or absence of aminoglycosides. Cell lysates were subjected to Western blot analyses with anti-harmonin (H3) and anti-actin antibodies (loading control). Harmonin a1 expression (~80 kDa) was detected in harm_a1-wt–transfected cells but not in untransfected or untreated harm_a1-p.R31X–transfected HEK293T cells. Treatment with aminoglycosides restored full-length harmonin a1 expression in p.R31X–transfected cells. (**B**) Quantification of read-through activity of aminoglycosides. For quantification of aminoglycoside-mediated read-through of the harm_a1-p.R31X mutation, the optical densities of the 80-kDa bands representing full-length harmonin a1 were ascertained and normalized to the appropriate loading control. Fold increase of full-length harmonin a1 compared with the untreated was calculated. $^{**}P < 0.01$; $^{*}P < 0.05$.

wt harmonin a1 or harm_a1-p.R31X. As expected, harmonin a1 was pulled down by the GST-tagged USH2a tail from wt harmonin a1-transfected HEK293T cells but was not recovered in pull-downs from p.R31X-transfected cells (Fig. 4A). However, harmonin a1 was pulled down from p.R31X-transfected cells treated with aminoglycosides, indicating a restoration of harmonin expression and its scaffolding function (Fig. 4A; Table 1). Quantification of the bound harmonin a1 to the USH2a tail in the pull-downs revealed that the read-through of p.R31X mediated by G418, gentamicin, and NB30 restored approximately 75% of binding activity of the wt protein. Because of the low intensity of the harmonin a1 band restored by paromomycin, the quantification of its binding activity was not possible. Furthermore, we tested the actin filament-bundling capacity of recovered harmonin b3 in cells transfected with FLAG-tagged harm_b3-p.R31X. Double labeling with anti-FLAG antibodies and rhodamine-phalloidin revealed actin filament bundling in aminoglycoside-treated cells (Fig. 4B), as observed in FLAG-tagged harm_b3 controls, but not in untransfected or untreated FLAG-tagged harm_b3-p.R31X control cells (data not shown). In summary, the translational read-through of the p.R31X nonsense mutation of *USH1C* by aminoglycosides, including the novel designed NB30, not only recovered the expression of full-length harmonin, it also restored the cellular function as a scaffold protein and the characteristic actin filament-bundling capacity of harmonin b.

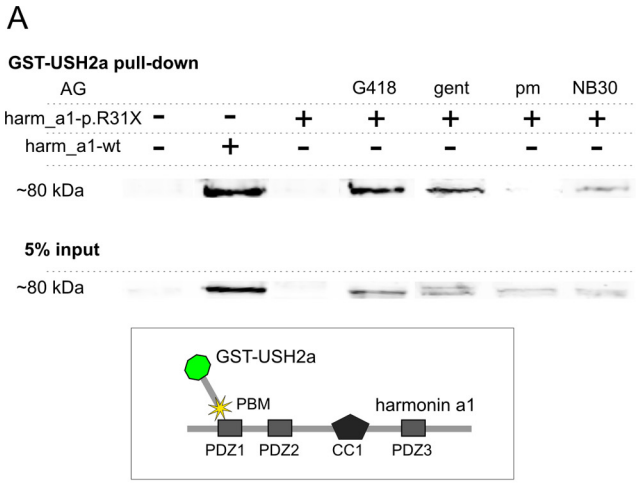
Effect of Aminoglycosides on Retinal Cells

Next we tested the biocompatibility of aminoglycosides on murine retinal cells. The toxicity of aminoglycosides was previously obtained primarily as nephrotoxicity and ototoxicity but was only occasionally studied in the retina.[27–30] Here we analyzed the retinal biocompatibility of G418, paromomycin, gentamicin, and NB30 by applying these aminoglycosides to murine retinal explants. The suitability of the organotypic mouse retina culture for drug treatments was recently demonstrated.[36,42,43] To determine the retinal biocompatibility of aminoglycosides, mouse retina explants at postnatal day 10 were incubated with various concentrations of the four aminoglycosides. Subsequently, TUNEL assays were performed for the visualization of the nuclei of apoptotic cells in the retina.

Epifluorescence microscopy of cryosections through untreated retinal cultures demonstrated low numbers of TUNEL-stained nuclei in the inner and the outer nuclear layers of the retina (Fig. 5). G418 induced a marked increase of apoptotic retinal cells, whereas the toxicity of gentamicin, paromomycin, and NB30 was notably lower. Quantification of the apoptotic nuclei in the control revealed <2% TUNEL-stained nuclei because of naturally occurring apoptosis during retina maturation and the process of cultivation. To quantify the number of apoptotic cells after aminoglycoside treatment, the ratio of TUNEL-labeled nuclei to DAPI-stained nuclei was calculated, and the increase compared with untreated retina cultures was estimated (Fig. 6; Table 1). In G418-treated organotypic retina cultures, a 22.9-fold (1 mg/mL) and a 24.2-fold (2 mg/mL) increase of TUNEL-positive nuclei compared with the untreated culture was observed. The number of TUNEL-positive nuclei increased 3.4-fold for 1 mg/mL and 5.1-fold for 2 mg/mL after gentamicin treatment and 3.2-fold for 1 mg/mL and 4.8-fold for 2 mg/mL after paromomycin treatment. In contrast, the application of 1 mg/mL or 2 mg/mL NB30 resulted in a 1.9-fold increase of TUNEL-positive nuclei. Treatment with 4 mg/mL NB30 induced a 3.9-fold increase of TUNEL-positive nuclei. In conclusion, these results demonstrate that the derivate NB30 has the best biocompatibility for the retina even in a 2.0-fold higher concentration than the commercial aminoglycosides.

Read-Through of the p.R31X Mutation in Retinal Explants

We next tested the read-through potential of aminoglycosides in tissue, namely in the retina. However, to date no mouse model for the translational read-though therapy in *USH1C* exists. Unfortunately, the recently published *Ush1c216AA* knock-in mouse contains a frameshift mutation, making it unsuitable for evaluation of translational read-through of *USH1C*.[44,45] To overcome this dilemma, we analyzed the ability of aminoglycoside-mediated read-through of the p.R31X mutation of USH1C in vivo in electroporated retinal explants of mice. For this purpose, reporter constructs coding for the monomeric red fluorescent protein (mRFP) molecule fused to the C terminus of the harmonin a1 (harm_a1-mRFP) or the mutated p.R31X (harm_a1-p.R31X-mRFP) were used. After electroporation of the wt reporter construct, strong expression of the harm_a1-mRFP fusion protein was observed in retinal explants (Fig. 7). In explants transfected with harm_a1-p.R31X-mRFP, a small number of cells showed weak expression of the fusion protein, indicating spontaneous read-through of the p.R31X nonsense mutation. In whole mounts of the harm_a1-p.R31X-mRFP-transfected and G418 aminoglycoside-treated retinas, no red fluorescent mRFP-positive cells were detected. The latter is most probably the result of the high toxicity of G418 in retinal cells. After the application of gentamicin, paromomycin, and NB30 to p.R31X-transfected explants, the number of mRFP-expressing cells increased compared with untreated



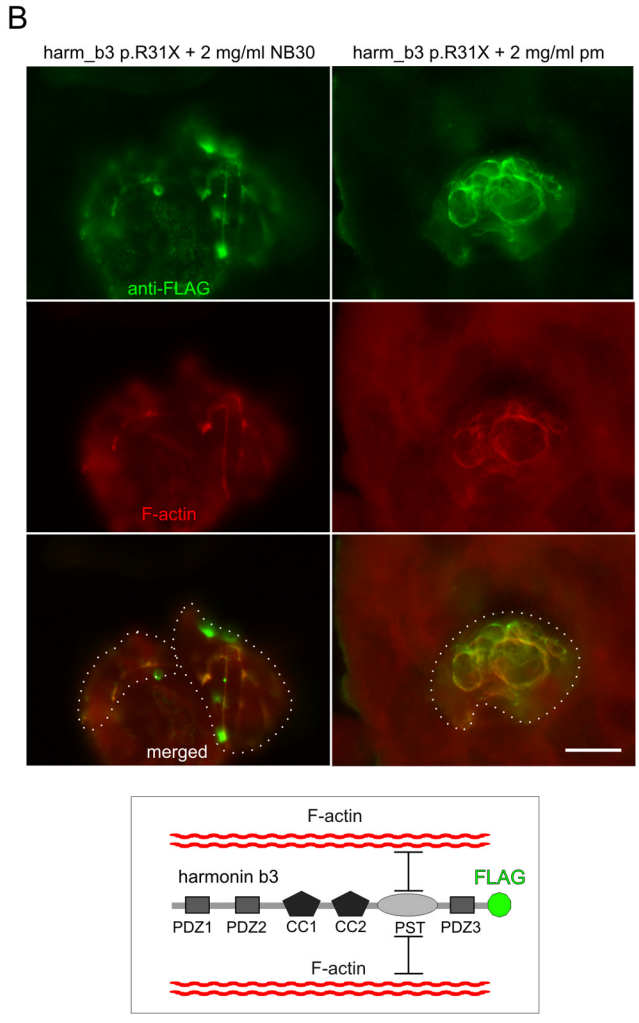


FIGURE 4. Functional analyses of restored harmonin proteins. (**A**) GST pull-down. HEK293T cells transfected with harm_a1-wt or harm_a1-p.R31X, grown in the presence (+) or absence (−) of aminoglycosides, were incubated with immobilized GST-tagged USH2a tail. Pull-downed proteins were subjected to Western blot analyses using antibodies against harmonin (H3). Treatment of harm_a1-p.R31X–transfected HEK293T cells with G418, gentamicin, or NB30 restored the binding of harmonin a1 to the USH2a tail. Input of GST pull-downs. Five percent of the HEK293T-lysates were analyzed by Western blot analysis using H3 antibodies. (**B**) Representative immunofluorescence analyses of HEK293T cells transiently transfected with harm_b3-p.R31X, grown in the presence of aminoglycosides. Costaining with anti-FLAG and rhodamine-phalloidin for F-actin revealed colocalization of harmonin with F-actin bundles[9] in read-through–positive cells (*broken lines*). Aminoglycosides mediated read-through–induced recovery of harmonin b3 F-actin bundling capacity. Scale bar, 10 μm.

explants. To quantify aminoglycoside-induced read-through of the p.R31X mutation in the whole mounts, the number of the red fluorescent mRFP-positive cells was determined in seven random 10,000 μm^2 regions on a whole mount and displayed as a fold increase of harmonin a1 expression (Fig. 8; Table 1). Our quantification revealed the maximal read-through (7.2-fold increase) in transfected retinal explants treated with NB30. The effects of paromomycin (3.1-fold) and gentamicin (3.4-fold) were milder. These findings highlight the potential of NB30 for read-through therapies to enable the formation of a functioning protein in patients with genetic disorders caused by a nonsense mutation.

DISCUSSION

Aminoglycosides were previously suggested as potential therapeutics for the read-through of nonsense mutations leading to various genetic diseases.[13,16] Here we demonstrate the ability of a series of aminoglycosides to induce read-through in the disease causing p.R31X nonsense mutation in USH1C both in vitro and in cell culture assays along within retinal cells. We observed substantial differences in read-through efficiency between in vitro translation assays and cellular systems that have previously been reported for various other systems.[13,16] Possible reasons for these differences are the cytotoxicity of the tested compound and the different length of the recovered polypeptide in the test systems. With regard to the latter, in the present in vitro experiments a short 35-kDa reporter polypeptide is recovered, whereas in cell culture experiments an 80-kDa full-length tagged harmonin a1 was recovered. Nevertheless, in both cases the highest rate of read-through was induced with G418, followed by gentamicin, NB30, and paromomycin. These data are consistent with the previously reported data gathered on other mutations.[20–22,25,46] It is noteworthy that the read-through efficacy of the novel designed aminoglycoside NB30 corresponds to the data recently obtained for the investigational new read-through–inducing drug PTC124 in cell culture and retinal explants (Goldmann et al., manuscript submitted). Based on the promising preclinical results, the latter drug is currently applied in clinical trials for the treatment of patients with cystic fibrosis caused by nonsense mutations.[47] The impact of the tested aminoglycosides by the read-through of the *USH1C*-p.R31X nonsense mutation is further demonstrated by showing restoration of the harmonin scaffold function. The recovered harmonin protein after the treatments with G418, gentamicin, and NB30 exhibited 75% binding activity to the tail of USH2a molecules compared with the wt harmonin protein. Considering the observed read-through efficiencies of 22% by G418 and 2.6% by NB30 (Table 1), the binding activity data correlate to a restoration of approximately 16.5% and 2% activity of harmonin scaffolding function in the cell by G418 and NB30, respectively (Table 1). The aminoglycoside-induced production of 1% to 3% full-size normal protein in a recessive disease may be sufficient to stop or slow down the progression of the disease,[19,48] particularly in USH1C, in which protein expression approaches zero and in which even such a low protein level is sufficient to restore a near-normal or at least a clinically less severe phenotype.[26,49,50] Interestingly, nonsense mutations in *USH1C* leading to the expression of truncated harmonin cause combined deaf-blindness (USH1), whereas certain missense mutations introducing a punctual incorrect

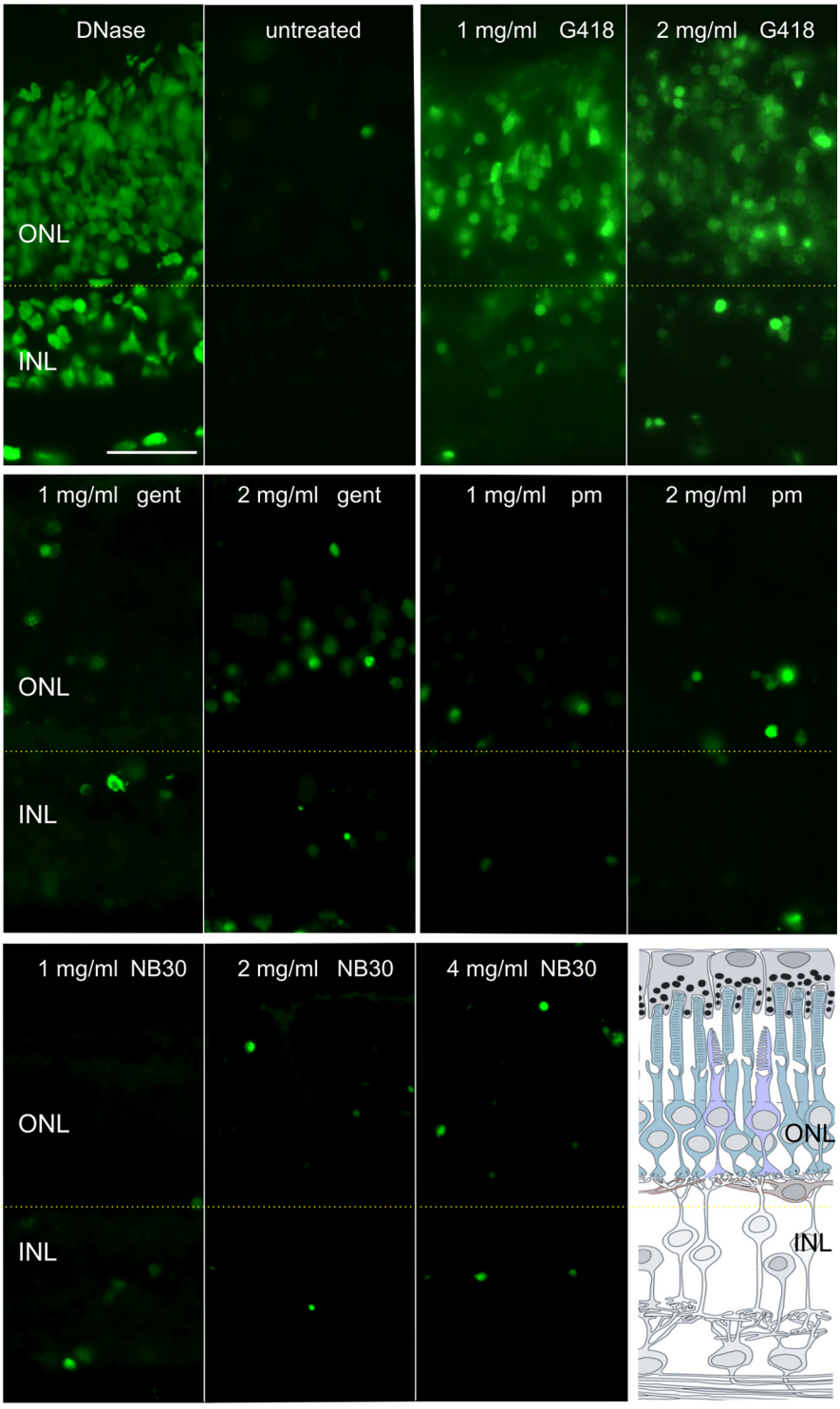


FIGURE 5. Retinal biocompatibility of aminoglycosides. Epifluorescence microscopy of TUNEL-stained cryosections of retina cultures. Murine retinas were cultured in the absence (untreated) or presence of various concentrations of aminoglycosides (G418; gentamicin [gent]; paromomycin [pm]; NB30) in the culture medium. Cryosections were stained for apoptotic nuclei by TUNEL. DNase-treated cryosections served as positive control. In contrast to the G418 application of gentamicin, paromomycin and NB30 caused little increase of apoptotic cells in the mouse retina. *Bottom right*: schematic representation of the vertebrate retina. ONL, outer nuclear layer; INL, inner nuclear layer. Scale bar, 10 μm.

amino acid in the protein sequence lead to nonsyndromic deafness not associated with RP.[2,11,12] The transcriptional read-through of a nonsense mutation such as the *USH1C*-p.R31X mutation can introduce a correct or an incorrect amino acid. In any case, the read-through–induced restoration of full-length harmonin of NB30 should be sufficient for retinal function.

Furthermore, we hypothesized that because of its improved biocompatibility, NB30 can be applied at significantly higher concentrations as commercial aminoglycosides. To prove this hypothesis, we tested the comparative read-through of the p.R31X nonsense mutation in retinal tissue by commercial aminoglycosides and NB30. Although G418 did not induce any read-through in tissue, NB30 showed twofold higher read-through efficiency than paromomycin or gentamicin at the same concentration tested (summarized in Table 1). Recent data demonstrated that systemic application of gentamicin to rodent animal models harboring the rhodopsin nonsense mutation resulted in a slight amelioration of the retinal phenotype.[51] Given that NB30 induces significantly higher read-through than gentamicin or other commercial aminoglycosides in retinal tissue, it is apparent that NB30 should also ameliorate retinal phenotypes in organisms.

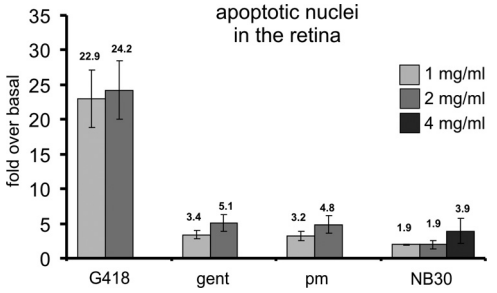


FIGURE 6. Quantification of retinal biocompatibility. Quantification was calculated as the ratio of TUNEL-positive to DAPI-stained nuclei. Increase of apoptotic cells after aminoglycoside application is shown in relation to the untreated control (fold increase). Application of G418 to the culture medium induced a dramatic increase of apoptotic nuclei. The influence of the remaining aminoglycosides was milder compared with G418. The lowest retinal toxicity was achieved by NB30. Error bars represent SD.

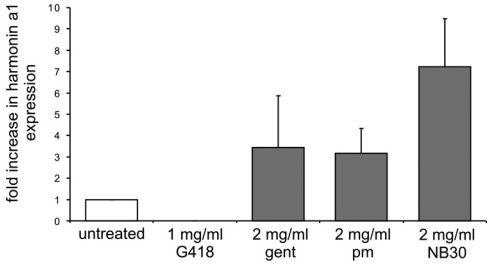


FIGURE 8. Quantification of aminoglycoside-mediated read-through in retinal explants. Quantification of fold increase of harmonin a1–expressing cells in harm_a1-p.R31X-mRFP–transfected whole mounts. The maximal read-through of p.R31X was obtained by NB30, whereas the effects of paromomycin and gentamicin were milder.

In the present study, the biocompatibility of aminoglycosides was assessed by TUNEL assay as an approved sensitive method for visualizing apoptotic cells in tissue. Of the four aminoglycosides tested, a large difference for their relative retinal biocompatibility was observed. The enormous increase of apoptosis in retinal cells when the explants were treated with G418 is in agreement with previous data.[22,51–54] The observed reduced biocompatibility of G418 along with its well-known high cytotoxicity[22,53] further explains the lack of read-through activity of G418 in transfected retinal explants and prohibits its use as a potential treatment for retinal diseases. In contrast to G418, applications of paromomycin, gentamicin, or NB30 were much better tolerated by retinal cells, indicating the improved retinal biocompatibility of these drugs. The observed best retinal biocompatibility of NB30, along with its significantly elevated read-through activity of p.R31X nonsense mutation in retinal tissue and the recently observed reduced ototoxicity,[32] places NB30 on top among the four aminoglycosides tested for potential use in translation therapy of USH1.

In conclusion, the newly developed aminoglycoside NB30 exhibits both appreciably higher read-through efficiency and

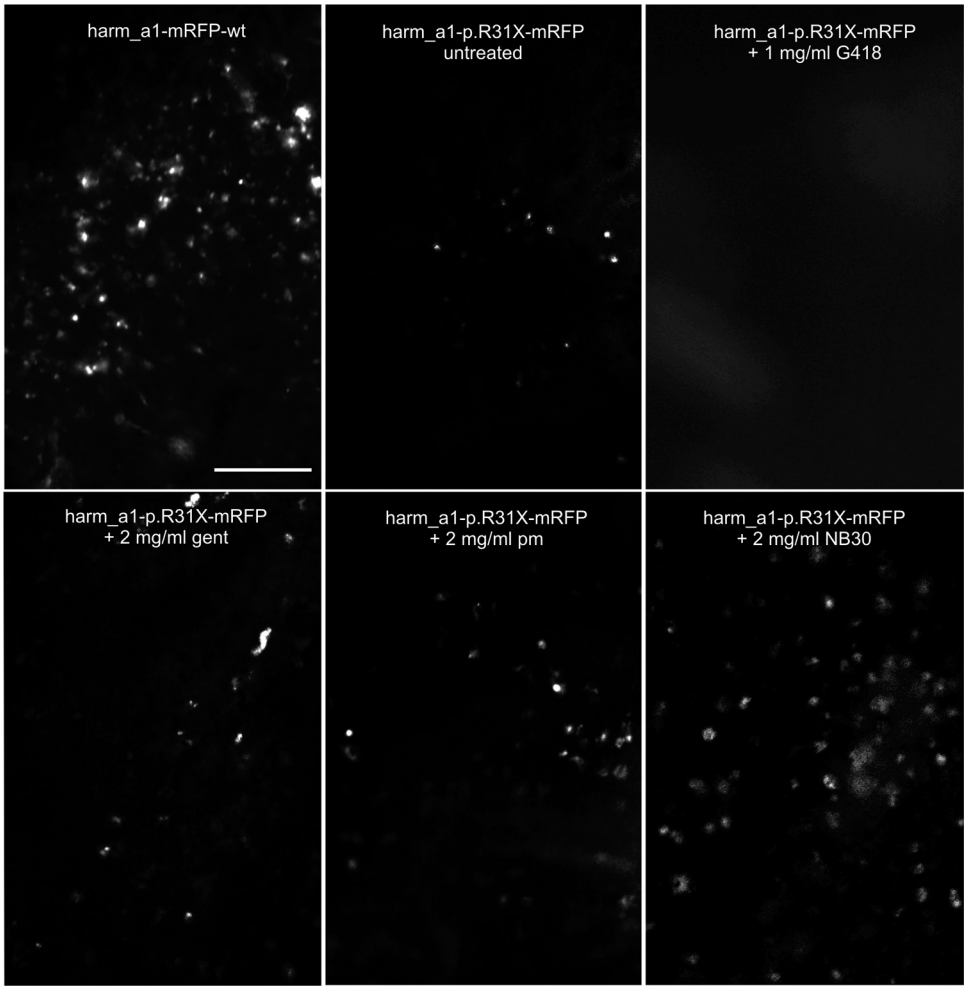


FIGURE 7. Aminoglycoside-mediated read-through of p.R31X in retinal explants. Epifluorescence microscopy of whole mounts of transfected retinal explants. Murine retinas were electroporated either with wt harmonin a1 (harm_a1-mRFP) or the p.R31X (harm_a1-p.R31X-mRFP) and subsequently were cultured in the absence or presence of aminoglycosides. Analysis by fluorescence microscopy revealed a substantial increase of mRFP-positive cells after gentamicin, paromomycin, and NB30 treatments. No fluorescence was visible in G418-treated retinas. Scale bar, 100 μm.

excellent biocompatibility compared with the clinical drug gentamicin. The most compelling evidence for the superior read-through efficiency of NB30 over that of gentamicin was demonstrated in retinal tissue on the p.R31X nonsense mutation representing the underlying cause of the genetic disease USH1. This achievement has several important implications. First, the observed results prove the principle that the fine-tuning of aminoglycoside structure can lead to new compounds with improved read-through efficiency. Here we demonstrate that our recently developed derivative, NB30, is substantially more efficient than gentamicin for the rescue of functional protein from the mutant p.R31X in retinal tissue. Second, the observed elevated biocompatibility of NB30 over that of gentamicin most likely resulted from the significantly reduced toxicity of NB30 compared with G418, paromomycin, and gentamicin. Despite its proven high toxicity, the clinical aminoglycoside antibiotic gentamicin is frequently used for proof-of-concept in various disease models and in clinical trials.

Finally, these data show that NB30 is an excellent translational read-through drug for the treatment of USH1C in the eye, raising hope for future clinical trials. Nevertheless, the potential of NB30 has to be investigated in suitable mammalian animal models carrying a nonsense mutation. Our data support the feasibility of testing NB30 in vivo to evaluate its therapeutic potential for USH1 or RP.

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