Experimental Eye Research 201 (2020) 108274

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Intravitreal administration of small molecule read-through agents demonstrate functional activity in a nonsense mutation mouse model

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ARTICLE INFO

Keywords:
Read-through
Premature stop codon
Dual luciferase assay
Albino mouse
Melanin
Inherited retinal disorder
Oculocutaneous albinism
Intravitreal injection

ABSTRACT

The prevalence of nonsense mutations as a class within genetic diseases such as inherited retinal disorders (IRDs) presents an opportunity to develop a singular, common therapeutic agent for patients whose treatment options are otherwise limited. We propose a novel approach to addressing IRDs utilizing Eukaryotic Ribosome Selective Glycosides, ELX-01 and ELX-06, delivered to the eye by intravitreal (IVT) injection. We assessed read-through activity *in vitro* using a plasmid-based dual luciferase assay and *in vivo* in a mouse model of oculocutaneous albinism type 2. These models interrogate a naturally occurring *R262X* nonsense mutation in the *OCA2* gene. ELX-01 and ELX-06 both produced a concentration-dependent increase in read-through of the *OCA2 R262X* mutation in the dual luciferase assay, with an effect at the top concentration which is superior to both gentamicin and G418. When testing both compounds *in vivo*, a single IVT injection produced a dose-dependent increase in melanin, consistent with compound read-through activity and functional restoration of the Oca2 protein. These results establish that ELX-01 and ELX-06 produce read-through of a premature stop codon in the *OCA2* gene both *in vitro* and *in vivo*. The *in vivo* results suggest that these compounds can be dosed IVT to achieve read-through at the back of the eye. These data also suggest that ELX-01 or ELX-06 could serve as a common therapeutic agent across nonsense mutation-mediated IRDs and help to establish a target exposure range for development of a sustained release IVT formulation.

1. Short communication

Inherited Retinal Disorders (IRDs) are a group of genetic diseases characterized by vision loss, with a high unmet medical need and a prevalence of nonsense mutations caused by single-nucleotide point mutations that introduce premature stop codons within the gene(s) of interest (Nagel-Wolfrum et al., 2014; Vázquez-Domínguez et al., 2019). These early stops, which terminate mRNA translation, result in failure to produce full-length proteins. The resulting truncated proteins often yield a dominant negative effect with protein partners, leading to disease phenotypes which can be more severe than diseases caused by missense mutations (Frischmeyer and Dietz, 1999; Miller and Pearce, 2014). Premature translation termination also fails to remove all exon junction complexes from the mRNA, which signals nonsense-mediated decay machinery to degrade the mRNA and further limit protein production (Celik et al., 2015). While gene therapy treatments are approved or in development for some IRDs, these therapies tend to be prohibitively expensive, are limited to mutations within a single gene, may not reach all the cell types present in the retina, may be subject to neutralizing antibodies, and often have insufficient cargo capacity to contain many of the large genes which are affected (Trapani et al., 2015; Vázquez-Domínguez et al., 2019; Ziccardi et al., 2019). Alternative therapies are needed in cases where these limitations prevent development or utilization of gene therapy technology.

Recently, a therapeutic approach has emerged in which novel compounds induce read-through of the premature stop codon introduced by nonsense mutations (Nagel-Wolfrum et al., 2016). One example, ELX-02, a eukaryotic ribosomal selective glycoside (ERSG), is currently being tested clinically to treat rare genetic diseases such as cystic fibrosis and cystinosis (Leubitz et al., 2019). ERSGs like ELX-02 induce translational read-through by binding to the decoding site of the ribosome small subunit, which monitors proper codon-anticodon interactions

Abbreviations: ANOVA, analysis of variance; ERSG, eukaryotic ribosomal selective glycoside; FF, firefly; IP, intraperitoneal; IRD, inherited retinal disorder; IVT, intravitreal; PBS, phosphate buffered saline; PECRS, pigment epithelium, choroid, retina, sclera; RNL, renilla; SD, standard deviation.

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https://doi.org/10.1016/j.exer.2020.108274
Received 12 June 2020; Received in revised form 4 August 2020; Accepted 25 September 2020
Available online 2 October 2020

(Burke and Mogg, 1985; Dabrowski et al., 2018; Moazed et al., 1986; Pokrovskaya et al., 2010; Prokhorova et al., 2017; Sabbavarapu et al., 2018). This change reduces the ability of the ribosome to discriminate between termination factors and near-cognate tRNAs and increases the probability that translational read-through will permit production of a full-length protein (Fourmy et al., 1998). The potential for translational read-through by ELX-02 has been demonstrated using both *in vitro* models (Bidou et al., 2017; Brasell et al., 2019; Crawford et al., 2020; Friesen et al., 2018; Kandasamy et al., 2012; Xue et al., 2014) and *in vivo* models (Brasell et al. 2019; Xue et al., 2014). While ERSGs like ELX-02 promote premature stop codon read-through, native stop codon fidelity appears to be maintained (Crawford et al.).

Beyond subcutaneous systemic delivery of ELX-02, structural and functional analogs are under investigation for read-through potential via more localized routes of administration. The current work aims to test the applicability and read-through efficacy of two other ERSG molecules, ELX-01 and ELX-06 [also known as either NB122 and NB128 or (*S*)-**7** and (*R*)-**12**, respectively (Kandasamy et al., 2012; Nudelman et al., 2006)], for applicability in IRDs by utilizing a particular nonsense mutation mouse model. Albino mice, including the SJL/J strain, carry an endogenous nonsense mutation within a gene sometimes referred to as *p*, for pink-eyed dilution, but more commonly is known by its gene name, *OCA2*, for its connection to the human IRD oculocutaneous albinism (Grønskov et al., 2007; Shoji et al., 2015). The *R262X* mutation is a C > T point mutation in exon 7 of the *OCA2* gene resulting in a CGA→TGA (UGA) codon conversion. The subsequent premature stop in translation results in a lack of Oca2 channel protein responsible for establishing pH in the melanocyte, and thus, failure to produce pigmented melanocytes. This leads to hypopigmentation of eyes, skin, and hair. Read-through of the *R262X* premature stop codon should then restore pigmentation. With this in mind, we hypothesized that the SJL/J mouse strain represents an animal model which can be used to evaluate compound-induced read-through at the back of the eye following IVT injection.

Before testing ELX-01 and ELX-06 in this *in vivo* model, we ran an *in vitro* dual luciferase assay (Loughran et al., 2017; Sherf et al., 1996), which permits an early assessment of read-through potential against disease-specific mutations (e.g., the mouse *OCA2 R262X* nonsense allele). Briefly, short sequences that include either the wild-type sequence or the gene-specific nonsense mutation and its stop codon context were cloned into the linker region between Firefly (FF) and Renilla (RNL) luciferase reporters of the pSGDluc plasmid (Loughran et al.). Plasmids were then transiently transfected into HeLa cells (CCL-2, ATCC, Manassas, VA) to evaluate compound read-through. HeLa cells were incubated at 37 °C in DMEM (41,966–029, Thermo Fisher, Carlsbad, CA), supplemented with 10% of non-heat-inactivated fetal bovine serum (F2442-500 mL, Sigma-Aldrich, St. Louis, MO) and 1% PenStrep (15,140–122, Thermo Fisher, Carlsbad, CA). Compound treatments were performed in the same medium, but without PenStrep addition. ELX-01 and ELX-06 were tested using a six-point concentration response (0.09–3 mM). Vehicle (1% water) was run on the plate as a negative control. Gentamicin (4.0 mM; G1264-250 MG, Lot # 048M4758V, Sigma-Aldrich, St. Louis, MO) and G418 (0.75 mM; A1720-1G, Lot # SLBZ0665, Sigma-Aldrich, St. Louis, MO), which are well established comparators for *in vitro* read-through experiments (Bidou et al.), were run as positive controls. The concentrations of these positive controls were determined during initial assay optimization efforts and chosen based on their effect window and reproducibility.

HeLa cells were plated in a 96 well format (white with clear bottom, #655098, Greiner BioOne, Monroe, NC) at a density of 7500 cells per well. Cellular transfection occurred one day later using 0.1 μL Turbofect (R0531, ThermoFisher, Carlsbad, CA) and 25 ng DNA (pSGDluc plasmid with insert sequences of either AGA CGG CGG **CGA** CCA CAG CAG for *R262 wild-type* or AGA CGG CGG **TGA** CCA CAG CAG for *R262X*; GenScript, Piscataway, NJ). Compound treatment (in vehicle) was initiated 24 h post transfection. FF and RNL luciferase measurements were made 24 h after compound treatment using the Dual Luciferase kit (E2920/2940, Promega, Madison, WI) as per the manufacturer supplied protocol. Briefly, media was removed, and wells were washed with phosphate buffered saline (PBS; TMS-012-A, Sigma-Aldrich, St. Louis, MO). Cells were then lysed by adding 25 μL of Passive Lysis Buffer to each well and incubated at room temperature for 45 min on a rocking platform. Next, 10 μL of lysate was transferred to a secondary plate and 25 μL of Luciferase Assay Reagent II was added, and the plate was shortly centrifuged (200×*g*, 1 s). White seals were applied to the bottom of the plate and then FF activity was measured on an Envision plate reader (PerkinElmer, Waltham, MA). Finally, 25 μL of Stop & Glo reagent was added to each well and the plate was shortly centrifuged (200×*g*, 1 s), followed by RNL activity measurements in the Envision plate reader. For each well, raw FF were normalized to raw RNL to obtain FF/RNL ratios. FF/RNL ratios of mutant plasmids were then normalized to wild-type plasmids. Data were then graphed and analyzed using Prism software (v7, GraphPad, San Diego, CA). The percent increase in read-through activity was derived by calculating the difference between vehicle and compound-induced read-through, then dividing that value by the read-through value for vehicle and expressing the result as a percentage.

The mean ± standard deviation (SD) basal read-through (vehicle) observed for the mouse *OCA2 R262X* mutation was 0.59 ± 0.07% (Fig. 1). Positive controls gentamicin and G418 demonstrated a consistent increase in read-through, resulting in 2.94 ± 0.08% and 8.94 ± 0.38% read-through and representing increases over basal activity of 402% and 1426%, respectively. ELX-01 demonstrated a concentration-dependent increase in read-through, with the top concentration of 3 mM eliciting 11.81 ± 0.58% read-through, which is a 1916% increase over basal activity. ELX-06 also demonstrated a concentration-dependent increase in read-through, with the top concentration of 3 mM eliciting 11.67 ± 1.22% read-through, which is a 1891% increase over basal activity. The read-through effects of these compounds were significantly higher than basal read-through ($p < 0.0001$ for each compound; unpaired, two-tailed *t*-test) and read-through by both positive control compounds ($p < 0.0001$ for each compound when compared to gentamicin; $p = 0.0006$ and $p = 0.0174$ for ELX-01 and ELX-06 when compared to G418).

With this *in vitro* read-through evidence in mind, we then executed an *in vivo* study testing for melanin production as a measure of *OCA2 R262X* read-through induction. These *in vivo* experiments were carried out in accordance with the NIH guide for the care and use of laboratory animals. Female SJL/J or C57Bl/6 J mice (#000686 or 000664, Jackson Laboratories, Bar Harbor, ME) were 8 weeks old at the time of their arrival to the test facility. Mice were housed in groups of 3–5 in large cages kept in ventilated shelves under standard animal care conditions,

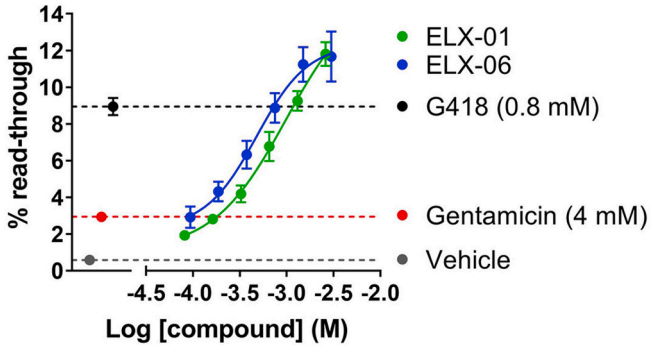

Fig. 1. ELX-01 and ELX-06 induce concentration-dependent *OCA2* mouse *R262X* nonsense allele read-through in the dual luciferase system. Data represent mean ± SD values from n = 5 wells (vehicle, ELX-01, and ELX-06). Gentamicin and G418 were tested as positive controls in n = 4 and n = 6 wells per plate, respectively. When SD at a given concentration is sufficiently small, then no error bars are shown for that point.

including 12 h light/12 h dark (except when room lights were turned on/off during the normal cycle to accommodate study procedures). Mice had *ad libitum* access to PicoLab Rodent Diet 20 (5053, #0007688, LabDiet, St. Louis, MO) and water was available to each cage via an automatic watering device. Refuge, gnawing, and nesting material were also present in every cage. Mice were acclimated for one week prior to the initiation of the study. Animals received an ear tag with a 5-digit ID number for tracking and were randomly assigned to study groups on the day of test article administration.

For IVT administration, mice were anesthetized with ketamine/xylazine applied via intraperitoneal (IP) injection at 85 mg/kg and 14 mg/kg, respectively. Pupils were dilated with topical administration of Cyclomydril® (MedHealth Choice, Tampa, FL). Following sedation and dilation, a total volume of 1 μL per eye was injected into the vitreous at the pars plana using a Hamilton syringe (Hamilton Company, Reno, NV) and a 33-gauge needle. Mice received bilateral injections of either vehicle (PBS), gentamicin sulfate (G1914-250 MG, Lot # 028M4831V, Sigma-Aldrich, St. Louis, MO), ELX-01, or ELX-06. Test agents were filtered using a 0.22 μm polyvinylidene difluoride filter prior to dosing. Given that the upper limit of human exposure tolerability for gentamicin is in the range of 100–200 μg (Zachary and Förster) and that the volume of the mouse vitreous is roughly 1:1000 that of the human vitreous, we decided to test a dose amount that was proportionally lower (1:1000 of 200 μg = 0.2 μg). The four doses of ELX-01 and ELX-06 were chosen to correspond to that same top dose and then lower doses using a half-log scale drop (0.006–0.2μg/eye).

Animals were anesthetized with ketamine/xylazine 48 h after IVT administration and euthanized by intracardial administration of Euthasol (pentobarbital, Virbac Corporation, Fort Worth, TX) at 100 mg/kg. Eyes were individually dissected to collect the pigment epithelium, choroid, retina, sclera (PECRS) tissue. The dissected tissue was placed in a sterile screw cap microfuge tube, snap frozen in liquid nitrogen, and then stored at −80 °C. For processing, the dissected PECRS tissue was removed from −80 °C and placed on ice. Triton X-100 (1%; T8787, Sigma-Aldrich, St. Louis, MO) in PBS was added to the tissue at a volume of 300 μL and homogenized in a bullet blender using 0.9–2.0 mm beads at a setting of 8 for 3 min. The homogenate was transferred to a new tube and centrifuged at 17,000×*g* for 20 min. The supernatant (soluble fraction) was then removed for Bradford analysis, while the insoluble fraction was dissolved in 100 μL of 1 N NaOH (pH 12.0) with 10 μL dimethyl sulfoxide by incubating at 80 °C for 60 min, followed by centrifugation at 12,000×*g* for 10 min and transfer to a new tube. The solubilized melanin was quantified by optical density at 470 nm and normalized to a standard curve of synthetic melanin (0–200 μg/mL; M8631, Sigma-Aldrich, St. Louis, MO). Data were graphed and analyzed using Prism software (v6.1, GraphPad, San Diego, CA). The data is presented as the ratio between total melanin and total protein (melanin: protein) and is also referred to as the melanin content.

The melanin in the PBS-administered pigmented C57Bl/6 J mouse line was measured to have a mean melanin:protein ratio of 0.016 (Fig. 2). The mean ratio detected in SJL/J mice treated with PBS was 0.004, which was significantly lower than the C57Bl/6 J ratio ($p \leq 0.001$; unpaired two-tailed *t*-test). Administration of the positive control, gentamicin at 0.2μg/eye, significantly increased melanin content in the PECRS tissue compared to PBS administration ($p \leq 0.001$; unpaired two-tailed *t*-test). Administration of ELX-01 in SJL/J mice showed a dose-dependent increase melanin content. When analyzing the melanin: protein ratio, ELX-01 delivered at 0.06 or 0.2μg/eye significantly increased melanin content compared to PBS administration ($p \leq 0.05$ or $p \leq 0.001$; One-way ANOVA with Dunnett's post-test), while there was no difference in melanin content between PBS administration and either 0.006 or 0.02μg/eye administration of ELX-01. When analyzing the melanin:protein ratio for ELX-06, 0.2μg/eye significantly increased melanin content compared to PBS administration ($p \leq 0.0001$; One-way ANOVA with Dunnett's post-test). There was no difference in melanin content between PBS administration and either 0.006, 0.02, or 0.06μg/eye administration of ELX-06.

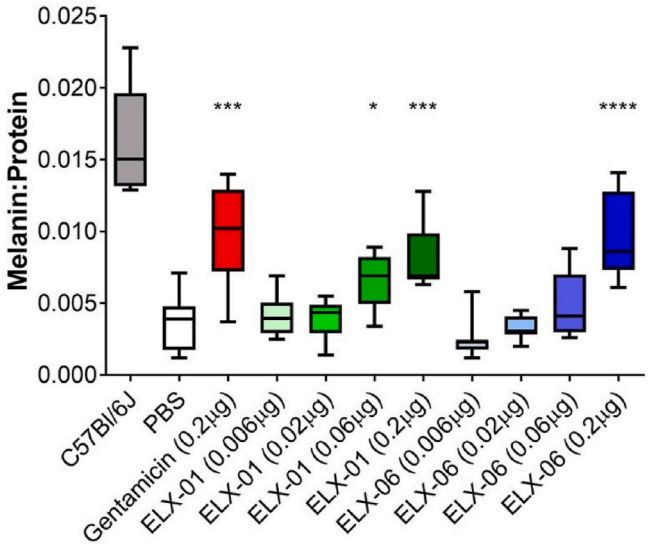

Fig. 2. IVT administration of either ELX-01 or ELX-06 induces a dose-dependent increase in melanin production in the SJL/J mouse. The total melanin as a ratio to total protein is presented as a measure of *OCA2 R262X* read-through induction and melanin production in the PECRS. The median and quartiles for each group are shown in the box plot, respectively. n = 8 mice per group; *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ by One-way ANOVA with Dunnett's post-test vs PBS control.

The highest doses of ELX-01 and ELX-06 tested in this experiment produced melanin:protein ratios that were not significantly different from the positive control gentamicin (p = 0.8504; One-way ANOVA). Gentamicin has previously shown the potential to induce translational read-through, resulting in functional protein and clinical efficacy (Clancy et al., 2001; Wilschanski et al., 2003). However, traditional aminoglycoside antibiotics like gentamicin cannot be used as a systemic, long-term therapy due to nephrotoxicity and ototoxicity following uptake and accumulation in renal proximal tubules and cochlear hair cells, respectively (O'Sullivan et al., 2017; Prayle et al., 2010). When injected locally into the eye (IVT), gentamicin is associated with retinal toxicity (Brown et al., 1990; Conway et al., 1989; Hancock et al., 2005; May et al., 1974; Zachary and Förster, 1976). Therefore, for effective long-term treatment of nonsense mutation diseases, compounds are needed which both augment premature stop codon read-through and are tolerable for long-term use. ELX-01 and ELX-06 demonstrate the ability to read-through premature stop codons, as shown here in both *in vitro* and *in vivo* assays. ELX-01 and ELX-06 have improved LC50 values relative to gentamicin as measured in HEK-293 cells (Kandasamy et al.), indicative of higher concentrations of compound required to produce cell death. In vivo toxicological assessment of ELX-01 and ELX-06 are ongoing which will help confirm the *in vitro* findings of superior tolerability of ELX-01 and ELX-06 compared to gentamicin and indicate whether the therapeutic index meets the requirements of a retinal pharmacotherapy (Penha et al., 2010).

Collectively, these results establish that ELX-01 and ELX-06 produce read-through of a premature stop codon in the *OCA2* gene *in vitro* and *in vivo*. The current data suggest that ERSGs, including ELX-01 and ELX-06, can be dosed IVT to achieve read-through at the back of the eye. As a channel, we hypothesized that small amounts of restored Oca2 protein would have a disproportionate functional impact on the production of melanin within organelles of the cell and the evidence collected support this. While the production of melanin in SJL/J mice by a single IVT injection of ELX-01 or ELX-06 did not achieve amounts measured in pigmented C57Bl/6 J mice, these results help to establish a target exposure range for development of a sustained release IVT formulation which should provide consistent compound exposure over a longer

period of time and trigger even greater protein production in the cell. IVT dosing is a safe, well tolerated, routine procedure that allows for diffuse, global accessibility to the retina for optimal efficacy potential. It is considered a subspecialty skill, increasingly performed in the office of ophthalmologists, especially for the treatment of common eye diseases (Avery et al., 2014; Myers (Provencher) et al., 2015). With an acceptable dosing regimen, this route of administration is suitable for long-term chronic administration. In addition, we demonstrated the utility of the *OCA2* nonsense allele in the common SJL/J mouse, which we have determined to be a reproducible and efficient model to evaluate read-through potential. Ongoing and future experiments across IRD models will continue to explore the potential for ELX read-through molecule therapeutic potential across nonsense mutation-mediated IRDs.

Declaration of competing interest

All authors were employed by or consultants of either Eloxx Pharmaceuticals, Inc. or EyeCRO while the described work was conducted.

Acknowledgements

The authors would like to thank Sabrina de Munnik, Sanne Holt, and Folkert Verkaar at Charles River Laboratories for their efforts running the dual luciferase assay. The authors would also like to thank Rafal Farjo at EyeCRO for his expertise and assistance in experimental design related to the *in vivo* work.

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