Translational read-through inducing drugs for the treatment of inherited retinal dystrophies

Abstract:

Introduction:

Inherited retinal disorders (IRDs) are the most common cause of certifiable blindness in working age adults in the UK. There are currently no treatments for the majority of patients, resulting in considerable morbidity with lifelong socioeconomic implications. 12% of all genetic disease variants are nonsense mutations, which encode a premature termination codon (PTC). The resultant transcript is either degraded through nonsense mediated decay (NMD) or translated to produce a truncated protein. Nonsense suppression therapy aims to bypass and allow peptide synthesis beyond the PTC, creating a full-length protein and possible phenotypic rescue. The responsible agents, known as translational readthrough inducing drugs (TRIDs), have been in continuous development to maximise readthrough efficiency and minimise toxicity. These include aminoglycosides, aminoglycoside derivatives and non-aminoglycoside small molecule drugs and have been successfully applied to a number of disease models in recent preclinical studies.

Areas Covered:

This review provides an update in the advancements of nonsense suppression therapy in the treatment of IRDs, including an overview of this process and NMD, advancements in the development of TRIDs and barriers to clinical trials including drug developments, disease modelling and patient selection.

Expert Opinion:

Clinical trials are forthcoming for patients with IRDs to determine TRID suitability as a viable therapy option.

Keywords:

Aminoglycosides; genetic eye disease; inherited retinal disorders; nonsense mediated decay; nonsense mutations; nonsense suppression; PTC124; readthrough; small molecules; translation

Highlights:

- Mechanisms of nonsense mutations, nonsense mediated decay (NMD) and nonsense suppression therapy.
- Updates in development of translational readthrough inducing drugs (TRIDs) and NMD inhibitors.
- Recent applications to models of inherited retinal dystrophies.
- Improvements in drug delivery and clinical trial considerations.

1. Introduction

Inherited retinal disorders (IRDs) are the most common cause of certifiable blindness in working age adults in the UK [1]. There is significant clinical and genetic heterogeneity with over 250 causative genes according to RetNet (https://sph.uth.edu/retnet/sum-dis.htm). Affected individuals have a profound loss in quality of life and there are no treatments for the majority, which represents a significant economic burden for health care systems and society [2,3].

The retina is at the vanguard of genetic therapeutic development due to its accessibility, immune-privilege site and high-resolution non-invasive imaging modalities. Over the past decade there have been exciting therapeutic advancements in retinal prosthesis, stem cells and gene therapy to treat these disorders. The first retinal gene therapy, Voretigene neparvovec (LUXTURNA™), was approved by the US Food and Drug Administration

(FDA) in 2017, using the adeno-associated virus 2 (AAV2) for the treatment of biallelic mutations in the *RPE65* gene known to cause Leber congenital amaurosis type 2. Phase III clinical trial (NCT00999609) results showed significant improvements in functional vision, light sensitivity and visual function in participants for up to 3 years [4]. Despite several other clinical trials underway, there are still concerns over efficacy, repeated administration, immune response, and harmful off-target effects of viral approaches [5,6]. In addition, conventional viral vectors such as AAVs have an upper packaging capacity of 5 kb which excludes incorporation of larger genes such as *USH2A* and *EYS*, which are commonly mutated in Retinitis Pigmentosa patients [7]. Alternative methods of genetic treatments include antisense oligonucleotides, which prevent pre-mRNA splicing by binding to and inhibiting target mRNA sequences [8]. Positive preclinical results have led to 2 trials for IRD patients with *USH2A* (NCT03780257) and *CEP290* (NCT03140969) mutations [9–11], but there is uncertainty about off-target effects and, for example, the residual function of Usherin lacking exon 13 [8,12].

Advances in whole exome and genome sequencing have increased the diagnostic rate of disease-causing variants to over 70% for IRDs. It is estimated that approximately 12% of all genetic diseases are caused by nonsense mutations [13]. In some genetic eye diseases such as choroideremia and isolated aniridia, nonsense mutations account for 30% and 40% of all cases respectively [14,15]. Therapies that target nonsense mutations to bypass the PTC have therefore been widely explored; their modes of action and applications in eye diseases, and particularly in IRDs, will be the focus of this review.

2. Nonsense mutations and mRNA fate

Nonsense mutations are single in-frame nucleotide changes that result in the formation of a stop codon - UAG, UAA or UGA - termed a premature termination codon (PTC). PTCs can

also be introduced by indel frameshift mutations and splice-site variants causing defective intron removal from pre-mRNA [13,16] or via aberrant alternative splicing of mRNA [17,18], however these are usually out-of-frame and not amenable to nonsense suppression. During normal translation, the aminoacyl-tRNA complex binds the ribosomal A-site, the amino acid is added to the peptide chain and translation continues (Fig.1 A). If a PTC-containing mRNA transcript enters the ribosomal A-site, translation is prematurely terminated [19]. The mRNA then meets one of two fates. Either, it is translated into a truncated protein, which is often non-functional or can harm the cell in a number of ways including misfolding, aggregation, or binding to cellular machinery and disrupting its structure or function, or rarely causing pathological gain of function (Fig.1 B) [20,21]. Or, PTC-containing mRNA transcripts are degraded by a natural cellular surveillance mechanism called nonsense-mediated decay (NMD) [22] (Fig.2).

3. Nonsense mediated decay

NMD is an evolutionary conserved process that protects the cell from potentially harmful truncated proteins generated by PTCs. The NMD machinery must therefore correctly identify and degrade PTC-containing mRNA transcripts without affecting normal termination codons. To achieve this, three NMD models have been proposed. The “faux UTR” model describes a reduction in termination kinetics at the PTC [23]. This occurs due to a failure of association between the PTC and poly-A binding protein (PABP), which usually facilitates fast ribosomal release from the peptide chain (Fig.2 A). The exon junction complex (EJC)-dependent model suggests that upstream termination prevents ribosomal displacement of EJCs, which are protein complexes situated 20-24 bp upstream of each exon-exon junction (Fig.2 A). The combined model of NMD suggests the nondisplaced EJC and reduced termination kinetics recruit the SURF complex (SMG1/UPF1/eRF1/eRF3), consisting of the helicase and translocase UPF1, eukaryotic release factors (eRFs) 1 and 3, and the protein

kinase SMG1 (Fig.2 B). Within the SURF complex, SMG1 forms a complex with SMG8 and 9 and together with UPF2, a component of the EJC, activate UPF1 by SMG1-dependent phosphorylation (Fig.2 B) [24]. Activated UPF1 is the key NMD regulator, which clears the mRNA of proteins such as eRFs, as well as associating with phospho-binding proteins such as SMG5, 6 and 7 to recruit deadenylation, decapping and endonucleolytic enzymes that decay the mRNA [25,26] (Fig.2 B). Its importance and specificity in NMD is proven by inactivating mutations or deletion of *UPF1*, which increase the accumulation of only PTC-containing transcripts [27,28].

NMD is influenced by variations in genomic context: AUGs closer to the PTC inhibit NMD compared to those at a distance [29,30] and transcripts with longer open reading frames (ORF) are more NMD sensitive [31]. NMD efficiency also varies across different tissues in the same organism [32]. This was previously thought to be the result of differing expression of NMD proteins in these tissues, however no correlation was found between protein expression and NMD efficiency [32]. Finally, NMD efficiency varies across patients harbouring the same mutation [33,34]. For example, Sarkar *et al*. found *CHM* transcript levels ranged from 13% to 52.6% in choroideremia patients with the mutation c.715C>T; p.R239* [35]. These differences were likely due to individual factors as opposed to tissue-specific factors as there was little difference in NMD or mRNA levels between skin fibroblasts and induced pluripotent stem cell-derived retinal pigmented epithelium (iPSC-RPE) of the same patient. Inter-individual variation did not correlate with differing expression of genes encoding the NMD machinery such as *UPF1* [35]. A better understanding of the mutation, tissue and individual determinants of NMD efficiency is therefore desired as it is a critical determinant for translational readthrough; if NMD activity is high and transcript levels are low, readthrough is less effective. NMD efficiency could

therefore act as a prognostic indicator for translational readthrough therapy once reliable markers of efficiency can be discovered.

3.1 NMD inhibitors

PTC-containing transcripts are typically degraded by NMD, reducing their availability. Therefore, inhibition of NMD has been proposed as a way to increase mRNA stability and as a consequence, increase the amount of substrate for TRIDs to target. Caffeine inhibits NMD through the inhibition of SMG1 kinase [35,36] (Fig.2 C). It alone has been shown to rescue the phenotype in fibroblasts carrying a PTC seen in the muscular dystrophy Ullrich's disease, by increasing mRNA and protein levels of the defective collagen VI α2 [37]. NMDI1 is a tetracyclic compound that traps UPF1 in a hyperphosphorylated state, preventing downstream interactions with SMG5 [38] (Fig.2 D). It is specific for NMD, does not affect translation efficiency and is non cytotoxic [38]. In a mouse model of Mucopolysacchardisosis I-Hurler syndrome (MPS I-H) that carries the knock-in *Idua* p.W392* mutation, NMDI application with gentamicin resulted in greater readthrough and functional enzymatic activity than gentamicin alone [39]. However, NMDI1 synthesis is inefficient and technically difficult. In contrast, VG1, a structural analogue of NMDI1 with similar inhibition capability, is more easily produced and shows an improved yield [40]. Its mechanism of action is currently unknown but its application to IRDs is awaited. Amlexanox is a compound with both readthrough and NMD inhibition properties. It has been reported as a combined therapy option for PTCs causing cystic fibrosis (CF) and recessive dystrophic epidermolysis bullosa, showing increased levels of full length proteins compared to G418 and PTC124 alone [41,42].

The inclusion of NMD inhibitors to clinical trials is eagerly awaited. Particular attention must be paid to monitoring side effects, as there is little current literature [35,43]. In the MPS I-H

mouse model, systemic NMDI1 application carried no systemic side effects, but the study duration was only 3 days [39]. Caffeine has well known effects on bodily systems and pathways. However, the doses required to achieve NMD inhibition may prohibit systemic therapy and local administration of higher doses may be better tolerated.

4. Nonsense suppression therapy

During translation, the ribosomal A-site can interact with near-cognate codons [44]. At all termination codons there is competition between the termination complex and near-cognate aminoacyl-tRNAs. If a near-cognate tRNA outcompetes the termination complex, tRNA complex translocates to the P-site and translation continues [45,46] (Fig.1 C). This occurs endogenously but at a very low frequency (between 0.01 to 0.1%), due to the three dimensional configuration of the ribosome and key signalling proteins such as PABP [47,48]. Endogenous readthrough of a PTC is more common (increasing to <1%) as the ribosomal pausing facilitates more aminoacyl-tRNA sampling [49]. However, this is not in sufficient levels to restore protein function. Nonsense suppression therapy uses translational readthrough-inducing drugs (TRIDs) to efficiently identify and readthrough PTCs alone, resulting in the production of full-length protein.

There are several genetic factors influencing the efficacy of nonsense suppression therapy. These include the identity of the PTC, where TRIDs appear to have an affinity to UGA>UAG>UAA. The identity of the nucleotides both downstream and upstream to the codon is also important; the preference downstream is C>U>A≥G [21,50] and the presence of adenine upstream at positions -1 or -2 to a PTC encourages its readthrough by modulating mRNA structure P-site, which adjusts the competition between release factors and aminoacyl-tRNAs [51,52]. However, readthrough efficiency cannot be predicted by the

genetic composition of the PTC alone. This is shown by the failed readthrough of the *rd12* mouse carrying a theoretically readthrough-sensitive mutation in *Rpe65* (discussed below) [53]. Other factors include the availability of eRFs [54] and factors affecting translation control such as the prolyl hydroxylation of the 40S subunit ribosomal protein (RPS23) [55,56]. The future clinical significance of this is unknown and further work surrounding the factors influencing readthrough therapy is awaited.

Nonsense suppression therapies avoid a number of the issues of alternative gene therapies. As it does not interfere with endogenous gene regulation mechanisms, temporal expression, duration and alternative splicing of target genes remain intact. TRIDs specifically target PTCs, meaning the same compound can work across a variety of genes and diseases. This is particularly convenient for IRDs as the genetic heterogeneity presents a considerable cost-issue per-patient. In addition, small increases of functionally active protein achieved with translational readthrough appear to be of great benefit, particularly in loss-of-function conditions such as choroideremia and Usher syndrome [57,58].

However, TRIDs may act on non-pathogenic nonsense variants in other genes and the near-cognate aminoacyl pairing at a PTC may also introduce missense changes, affecting protein activity [59]. This effect is unpredictable but is unlikely to cause additional harm in diseases with no genotype-phenotype correlation such as choroideremia [15,35,60,61]. That being said, in some diseases such as aniridia and USH1, missense mutations result in milder disease than null variants so the introduction of missense changes following nonsense suppression may still improve the phenotype [62,63]. Nonetheless, the influence of variants must be modelled on a protein-by-protein basis, and failed rescue of phenotype highlights the importance of understanding protein tolerances to amino acid changes [64].

5. Translational readthrough-inducing drugs

5.1 Aminoglycosides

Aminoglycosides are gram-negative bactericidal antibiotics that contain amino-modified glycosidic linkages. They bind to the adenosine nucleotide in position 1408 in the rRNA sequence of the prokaryotic A-site and induce ribosomal infidelity, disrupted codon recognition, inaccurate mRNA translation and the production of aberrant proteins [65]. A number of aminoglycosides have exhibited readthrough capacity across the decades [53,66,67] but the most effective are gentamicin and G418 [53,67,68] (Fig.3 A-C). These have shown therapeutic potential in both *in vitro* and *in vivo* systems for a number of genetic diseases including CF [69] and Duchenne muscular dystrophy (DMD) [70]. However, their clinical use is prevented by considerable renal and oto-toxicity at the doses required for translational readthrough [71], as renal proximal tubule epithelial cells and inner ear hair cells preferentially uptake aminoglycosides [72]. Though this is partially alleviated by co-administration with poly-L-aspartic acid [73], this toxicity prevents its clinical use. In addition, aminoglycosides such as gentamicin are not lipid soluble and have poor penetration through the blood-brain/blood-retinal barrier and the cell membrane [74].

2.1. Aminoglycoside derivatives

To enhance readthrough capacity and minimise toxicity, aminoglycoside scaffolds were used to create “designer” aminoglycosides such as the NB compounds [68,75]. These exhibit efficient translational readthrough by conservation of the C6’-hydroxyl group, which forms hydrogen bonds with the guanosine nucleotide at position 1408 in eukaryotic rRNA [76] (Fig.3 D-H). Designer aminoglycosides lose antimicrobial properties, suggesting the interaction with prokaryotic rRNA is reduced [75]. Consequently, NB30 treatment confers a

reduced toxicity than traditional aminoglycosides [77,78]. Addition of the (S)-4-amino-2-hydrocybutanoyl (AHB) group, found on the aminoglycoside amikacin (Fig.3 B), to the N1 position of NB30 resulted in superior binding with the ribosomal A-site [75,79]. The new compound, NB54 (Fig.3 E), displayed greater *in vitro* translational readthrough for nonsense mutations in several genes, including *USH1C* causing type 1 Usher syndrome [58,80]. Further modifications resulted in NB74 and NB84, which contained a (R)-6'-methyl group increasing readthrough potency [67] (Fig.3 F,G). Similarly, NB124 (with commercial name ELX-02, Fig.3 H) exhibited a favourable readthrough-toxicity relationship across a number of CF alleles [81]. A phase I clinical trial for ELX-02 for CF showed no adverse effects [82] and a phase II trial has recently been announced for CF patients with the *CFTR* p.G542* mutation. ELOX-02 has achieved orphan drug status for the treatment of CF and mucopolysaccharidosis type 1 [83].

Commercial gentamicin is a mixture of major and minor congeners [84–86], each of which have been subject to readthrough testing. A minor component of gentamicin, X2 (Fig.3 C), has recently showed superior readthrough capacity than gentamicin, NB84 and NB124 and was only beaten in potency by the positive control G418 [87]. However, gentamicin X2 presented a superior *in vivo* safety profile in rodent models compared to G418, hence it is better suited as a therapeutic readthrough agent [87]. However, the historical side effect profile of aminoglycosides and issues with clinical delivery have forced a search for non-aminoglycoside-based small molecule drugs with readthrough capacity.

2.2. Non-aminoglycoside small molecule drugs

Non-aminoglycoside small molecules with readthrough potential have been discovered from high throughput screening using premature UGA luciferase reporters [88]. PTC124, known as

Ataluren or commercial name Translarna™ (Fig.3 I), is currently best known to promote readthrough of UGA, UAA and UAG codons, with highest activity for UGA. It also has lower therapeutic concentration ranges than gentamicin, has fewer toxic effects than the classical aminoglycosides, and retains PTC selectivity over natural stop codons [88]. The mechanism of action for PTC124 is still unknown, but it is thought to bind to the ribosome, stimulating sampling of near-cognate tRNAs [89]. It reached phase III clinical trial in cystic fibrosis (NCT02107859) but failed to achieve therapeutic benefit [90]. In a phase III clinical trial for DMD (NCT01826487), none of the children taking the drug lost the ability to walk over the 48 weeks trial period compared with 8% on the placebo (0 out of 47 compared with 4 of 52) [91]. It was predicted to delay loss of walking for up to 7 years and patient experts argued they had seen meaningful stabilisation or improvements in their child's mobility. As a result, PTC124 was approved in the UK for the treatment of DMD. No adverse effects have been noted from the DMD and CF clinical trials [82,90]. It has since been applied to eye diseases with a phase II clinical trial for aniridia currently underway (NTC02647359). Preclinical data in the aniridia mouse model *Pax6*$^{Sey+/-}$ showed reversal of the ocular malformations, improving the retinal histology and responses to light indicating post-natal developmental plasticity (discussed below) [92]. While PTC124 has minimal ocular side effects, good oral bioavailability and is water soluble, concerns still remain regarding penetration through an intact blood-retinal barrier [91].

Other small molecule drugs have shown readthrough potential. PTC414 is a derivative of PTC124 and shows comparable readthrough capabilities for all types of PTC in choroideremia *in vitro* and *in vivo* models [57]. High-throughput protein transcription/translation enzyme-linked immunosorbent (ELISA)-based assays have identified small molecule readthrough (SMRT) compounds with similar three-ring structures to PTC124 such as RTC13 and RTC14

[93] (Fig.3 J,K). RTC13 was shown to restore dystrophin expression and muscle function in mouse models of DMD [94] and efficiencies independent of the position of the PTC within the transcript [95]. GJ071 and GJ072 have similar readthrough efficiency as RTC13 or RTC14 but have greater tolerability based on patient cells with ataxia telangiectasia [93]. Amlexanox (Fig.3 L) is a FDA-approved drug for the treatment of recurrent apthous mouth ulcers [96], but it was recently shown to have dual action of NMD inhibition and nonsense suppression [42]. The use of these small molecule compounds on *in vitro* and *in vivo* models of IRDs is awaited.

6. Application in inherited retinal disorders

Several *in vitro* and *in vivo* disease models of IRDs have been tested with TRIDs including choroideremia, retinitis pigmentosa, Leber congenital amaurosis and Usher syndrome. A summary of the most relevant studies is listed in Table 1.

6.1. Choroideremia

Choroideremia (CHM) is a X-linked chorioretinal dystrophy affecting 1 in 50,000 to 100,000 males. Features include nyctalopia in childhood, followed by visual field loss during adolescence and progressive blindness by late middle age. The responsible gene is *CHM*, which encodes REP1 (Rab escort protein 1) that mediates vesicular trafficking and post-translational modification of Rab proteins [15]. Though ubiquitously expressed, systemic manifestations are thought to be mitigated by the presence of the isoform REP2. Phase II and III clinical trials of retinal gene therapy with AAV2 vectors are underway (NCT02407678) [97] but recent reports from Aleman *et al* report no difference between treated and untreated eyes at year 2 and 3 post-treatment [98]. Over a third of *CHM* mutations are nonsense variants, making them an appropriate target for nonsense suppression [15]. Zebrafish *chm*^{ru848} models of CHM carry a UAA PTC and survive for only 5 days post fertilisation due to the

presence of a single REP isoform. Following treatment with gentamicin, paromomycin, PTC124 and PTC414, an increase in survival by up to 2-fold was seen [57,99]. Patient fibroblasts with the *CHM* mutation c.126C>G, p.Y42* were treated with PTC414 and PTC124 and showed recovery of prenylation activity despite no increased protein levels detected [57]. More recently, Torriano *et al* also investigated the effect of PTC124 on patient fibroblasts and iPSC-RPE carrying a c.772A>T, p.K258* in *CHM*. However, they found no significant trend in functional rescue, despite the addition of NMD and the proteasome inhibitors cycloheximide and MG132 [64]. This failed rescue was attributed to the identity of the missense amino acid that replaced the PTC (tyrosine or glutamine instead of the original lysine), which was determined *in silico* to be damaging to REP1 function [64]. This exemplifies that a detailed understanding of protein responses to amino acid changes is needed to achieve functional rescue and avoid toxic/gain-of-function side effects. Finally, the application of the NMD inhibitor caffeine to patient *CHM* fibroblasts resulted in restoration of *CHM* mRNA to near wild-type levels, suggesting NMD inhibition could be used in conjunction with TRIDs to increase readthrough efficiency [35].

6.2. Retinitis pigmentosa

Retinitis pigmentosa (RP) is a group of IRDs affecting around 1 in 4000 people [100]. It is characterised by rod photoreceptor cell death causing nyctalopia and visual field loss leading to tunnel vision, followed by cone dysfunction and total blindness. There is significant genetic heterogeneity meaning gene-targeted approaches present a considerable cost per patient issue [101]. The development of effective mutation-dependent approaches, such as nonsense suppression, would be more cost effective and widely applicable.

The *S334ter* rat model of dominant RP has a nonsense variant in the *Rho* gene creating a gain-of-function Rhodopsin mutant. Rats treated with gentamicin showed reduced abnormal

protein expression, resulting in improvements in retinal histology and vision [53]. In contrast, gentamicin treatment of *rd12* transgenic mouse model of RPE65-associated retinopathy did not slow the retinal degeneration [53]. Around 15% of X-linked RP cases are associated with mutations in the *RP2* gene [101–103]. Application of gentamicin to patient lymphoblasts carrying a nonsense mutation of *RP2*, c.519C>T, p.R120*, previously failed to restore full length RP2 protein [102]. However, applying G418 and PTC124 to fibroblasts and iPSC-derived RPE with the same mutation resulted in restoration of 20% and 13% of full length functional RP2 protein respectively, as well as improved cellular phenotype [103]. Readthrough success due in part to novel TRIDs and a better disease model is a reflection of the advancements in the field over the past 15 years.

Deficiency in the MER receptor tyrosine kinase (MERTK) protein in the RPE leads to impaired phagocytosis and a harmful build-up of photoreceptor outer segments (POS), causing RP [104,105]. An AAV2-mediated gene therapy phase I clinical trial (NCT01482195) showed mixed results, with no ophthalmological or systemic toxicity detected at the 2-year follow up but variable clinical response of affected patients included in the study [106]. More recently, patient-derived iPSC-RPE derived from a *MERTK* compound heterozygous patient, with splice-site (c.61+1G>A) and nonsense (c.1951C>T, p.R651*) variants were treated with PTC124. Full-length functional protein was restored with a 12% increase in phagocytic function [107].

6.3. Leber congenital amaurosis

Leber congenital amaurosis (LCA) encompasses the severe early onset retinal dystrophies, affecting between 1 in 30,000 to 80,000 people, with 25 causative genes known to date [108]. LCA16 is caused by mutations in *KCNJ13* [109–111], encoding a potassium channel subunit, Kir7.1, expressed on the RPE surface [112,113]. Shahi *et al* recently administered NB84 to a

KCNJ13 c.158G>A, p.W56* patient-derived iPSC-RPE model, resulting in restored Kir7.1 protein and RPE membrane potential [114]. *In vitro* and *in vivo* toxicity studies reported good safety of NB84 [78] meaning translational therapy for LCA16 in animal and human subjects is eagerly awaited.

6.4. Usher syndrome

Usher syndrome (USH) is the most common cause of deaf-blindness worldwide, affecting 3.2-6.2 per 100,000 people. It is characterised by profound sensorineural hearing loss, variable vestibular dysfunction and RP [115]. Though the hearing loss can be partially improved with hearing aids and cochlear implants, no current treatments exist for the RP [116]. It is clinically and genetically heterogeneous, subdivided into three types (USH1-USH3) according to the clinical severity [117]. The causative genes are expressed in the retinal photoreceptors and stereocilia of the inner ear. A number of USH disease genes such as *USH2A* and *ADGRV1* are too large to package into traditional AAV vectors [118–120], therefore alternative therapies are sought. Nonsense mutations account for 20% of USH cases [121] (https://www.lovd.nl/). *In vitro* and *ex vivo* readthrough of four nonsense mutations in *PCDH15* causing USH1 was achieved by Rebibo-Sabbah *et al* with NB30 and the positive controls gentamicin, G418 and paromomycin [122]. Goldmann *et al* have displayed readthrough of the *USH1C* c.91C>T, p.R31* mutation with designer- and non-aminoglycosides NB30, NB54 and PTC124 in *in vitro* HEK293T cells, *ex vivo* retinal explants and *in vivo* mouse models [58]. The most efficient and biocompatible compounds were NB54 and PTC124, and crucially the restored scaffold protein, harmonin, was shown to be functional [58]. Recently, both gentamicin and PTC124 were also shown to increase levels of usherin from reporter constructs containing the mutation c.9424G>T, p.G3142* in *USH2A* [123]. Furthermore, in patient-derived fibroblasts with the same variant treated with PTC124,

usherin levels increased up to 4.3-fold, and treatment was shown to restore protein membrane localisation and increase cilia formation from 54% to 78% of total cells [123].

6.5. Aniridia

Aniridia, although not an IRD, is a congenital pan-ocular anomaly which has reached the most advanced clinical application with TRIDs. The condition is characterised by iris hypoplasia, cataracts, glaucoma, foveal hypoplasia and corneal anomalies [14]. It affects approximately 1.8 per 100 000 live births and presents in isolation or as part of WAGR (Wilms tumour, aniridia, genitourinary anomalies and intellectual disability) syndrome [63,124]. Non-syndromic aniridia is mostly associated with heterozygous mutations in *PAX6* leading to haploinsufficiency of this transcription factor, which has a pivotal role in ocular development [14]. Nearly half of patients with *PAX6* mutations carry nonsense variants [14,125], making this disease an ideal target for nonsense suppression approaches. The *Pax6*$^{Sey+/-}$ small eye mouse model of aniridia contains a c.580G>T, p.G194* PTC and typically presents with a thickened cornea, underdeveloped lens as well as a thickened and infolded retina [126]. Systemic treatment of *Pax6*$^{Sey+/-}$ with PTC124 (and gentamicin) increased lens size, improved retinal infolding and photoreceptor density but had limited effect on the cornea thickening [92]. In fact, corneal improvement was best achieved with topical administration of PTC124, which restored PAX6 to 90% of wildtype levels in both cornea and retina of affected animals [92,127]. A phase II clinical trial for the treatment of aniridia with oral suspension of PTC124 (Translarna™) is underway (NCT02647359).

7. Current limitations of translational readthrough-inducing drugs

The availability of PTC-containing transcripts is what most directly limits the efficiency of current nonsense suppression approaches. This availability is dependent on the NMD process

as outlined previously. Attempts to better understand NMD regulators must account for species-specific differences in genomic context and epigenetic influences, both of which must be better understood [32,128]. For example, the human *CHM* gene contains an open reading frame (ORF) between -20 of the main AUG and a termination codon at +32. These are initiation codons in the 5'UTR that are out of frame to the main coding sequence. By helping identify termination codons as premature they trigger NMD [129]. The zebrafish ortholog *chm* lacks an upstream ORF, meaning NMD may be less effective in this model compared to humans. Differences like this may change the response to translational readthrough seen. For example, PTC124 reportedly does not affect NMD [88,103,130], however Moosajee *et al* found increased levels of mRNA following dosing of *chm*^{ru848} zebrafish with PTC124 suggesting reduced NMD efficiency [57]. There is therefore a need to understand species-specific NMD and nonsense suppression mechanisms. The identity of the substituted amino acid is also important to target protein function. This must be modelled on an individual basis in preclinical models, even in those where missense mutations derive milder disease phenotype [62,125].

7.1. Drug delivery

Methods of TRID delivery to the retina requires further development. Though the blood ocular barrier is disrupted in many retinal dystrophies, its presence reduces systemic drug absorption of TRIDs into the retina (though this is molecule dependent) [74]. Fortunately, the retina can be approached with local administration, either topical or intraocular. This administration would reduce systemic side effects, allowing for higher therapeutic doses to be delivered to the eye. The START formulation is a topical Ataluren mixture designed to effectively deliver Ataluren as a topical therapy without ocular irritation. The formulation of 0.9% **S**odium chloride, 1% **T**ween 80, 1% powdered **A**taluren, and 1%

carboxymethylcellulose was found to maximise particle dispersion and increase suspension viscosity. It had much reduced ocular irritation compared with a 1% aqueous Ataluren suspension [92]. As described in section 6.5, treatment of the *Pax6*$^{Sey+/-}$ mouse with topical PTC124 using the START formulation restored PAX6 levels to 90% of wild-type, which was not achieved by systemic administration [92,127]. The penetration of topical PTC124 to the retina suggests that treatment of retinal dystrophies with similar non-invasive deliveries is an exciting possibility. The current aniridia trial is with oral PTC124 (Translarna™) suspension but further clinical trials of local TRID administration (topical and intraocular) are awaited

Another approach to improve localised delivery and cell membrane uptake of TRIDs is their encapsulation within a liposome [131]. The slower release of these formulations was thought to reduce PTC suppression as aminoglycosides deliver peak-driven readthrough capacity [132]. Injecting gentamicin within lysosomes into a mouse model of DMD found increased readthrough compared to gentamicin alone [133]. Finally, to allow for sustained drug delivery to the retina, nonsense suppression therapies may in the future utilise delivery devices such as nanoporous film devices, which have been developed for use in more common retinal diseases such as diabetic retinopathy or age-related macular degeneration [134,135].

8. Conclusion

Nonsense suppression therapy is a method of pharmacological gene therapy that could help millions of patients across the globe. IRDs are a prime target for nonsense suppression due to their preponderance for nonsense mutations and the devastating blindness they currently cause. This review highlights the exciting preclinical promise of nonsense suppression therapy for the treatment of IRDs. Though Translarna™ has reached clinical trial

(NCT02647359) for aniridia, the preclinical developments described create anticipation for more clinical trials, including for other compounds.

9. Expert's Opinion

This review presents extensive evidence supporting TRIDs as a promising therapeutic approach for the treatment of genetic eye disorders caused by nonsense mutations, with special focus on IRDs. Over the next five years, we expect to see the translation of preclinical data into clinical trials. Current limitations of TRIDs such as readthrough efficiency, toxicity and delivery methods are also expected to improve, helped by the development of novel TRIDs alongside more sensitive detection and formulations in representative human disease models.

Advances in next generation sequencing, such as whole genome sequencing, are increasing our genetic diagnostic rates for patients. Using information such as PTC identity and genomic context in patient-specific *in vitro* models may predict response to nonsense suppression therapy or provide indicators as to which small molecule drug may be more suited for the individual, as a form of personalised medicine. Patient derived iPSC-RPE or 3D retinal organoids are an accurate model of human disease, which provide valuable insights on diseases mechanisms and can be powerful platforms for drug development. In fact, patient-specific retinal organoids have been used as proof-of-concept models for other therapeutics including CRISPR/Cas9 gene correction, antisense oligonucleotides and AAV delivery [136–138].

Preclinical studies must also pay close attention to study endpoints. Some studies of TRIDs have demonstrated functional benefits without a detectable increase in protein levels in several models, such as increased rab prenylation in human nonsense-mediated

choroideremia fibroblasts with PTC124 [57] and reduced glycosaminoglycan accumulation in the *Idua* MPS I-H mouse model with NB84 [139]. This may be due to reduced sensitivity of existing antibodies, hence functional endpoints/assays are important.

The exact mode of action for some TRIDs, such as PTC124, remains unknown. Further work is required to determine this and may provide an explanation of why some drugs work better on PTCs. Another factor to consider when improving TRIDs efficiency is NMD. Better understanding of why there appears to be varied NMD efficiency across patients with the same variant, between patients with mutations in the same gene, and even across different tissues within the same patient is required. Recent evidence points to the use of NMD inhibitors, such as caffeine, in parallel with TRIDs to further improve readthrough efficiency [39,41]. Further developments on this strategy or even the identification of more compounds with double action, like amlexanox, are expected and their progress into clinical trials for IRDs is eagerly awaited.

The systemic administration of both TRIDs and NMD inhibitors may increase the number of harmful truncated proteins and off-target effects. Local delivery to the eye may be safer for patients. Topical formulations have already been shown to penetrate to the posterior segment and may become the administration method of choice for IRDs, perhaps aided by nanotechnology-based developments [134,140]. If both eyes are being treated simultaneously, we require a detailed understanding of the natural history of each IRD, with reliable biomarkers and outcome metrics to assay the response to drug treatment.

In conclusion, patient pre-screening may be required to determine the optimal nonsense suppression therapy for best prognostic outcome dependent on PTC, genomic context,

baseline transcript levels/NMD activity. Localised administration to the eyes would further maximise the therapeutic effect in IRD patients, by increasing drug availability and reducing off target effects.

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