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Abstract

Between 5-70% of genetic disease is caused by in-frame nonsense mutations, which introduce a premature termination codon (PTC) within the disease-causing gene. Consequently, during translation, non-functional or gain-of-function truncated proteins of pathological significance, are formed. Approximately 50% of all inherited retinal disorders have been associated with PTCs, highlighting the importance of novel pharmacological or gene correction therapies in ocular disease. Pharmacological nonsense suppression of PTCs could delineate a therapeutic strategy that treats the mutation in a gene- and disease-independent manner. This approach aims to suppress the fidelity of the ribosome during protein synthesis so that a near-cognate aminoacyl-tRNAs, which shares two of the three nucleotides of the PTC, can be inserted into the peptide chain, allowing translation to continue, and a full-length functional protein to be produced. Here we discuss the mechanisms and evidence of nonsense suppression agents, including the small molecule drug ataluren (or PTC124) and next generation 'designer' aminoglycosides, for the treatment of genetic eye disease.

Key words: genetic eye disease; premature termination codon; nonsense mutation; nonsense suppression therapy; readthrough; translational bypass; aminoglycosides; ataluren

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1. Introduction

An estimated 11.2% of human genetic disease is caused by in-frame, nonsense mutations,[1] resulting in the premature introduction of a termination codon, (UAA, UAG or UGA), in the mRNA transcript. When translated, this generates a truncated, often dysfunctional polypeptide through premature ribosome dissociation. The resulting abrogated protein can exert a knock-out, dominant-negative or gain-of-function effect on gene function, dependent on the gene affected.[2] With this in mind, the efficiency of translation termination has been described as a therapeutic target for human pathologies resulting from premature termination codons (PTCs).

The classical approach involves gene replacement therapy to restore protein production by the introduction of gene cassettes. For example, adeno-associated viral (AAV) vectors allow localised and systemic delivery of the desired gene carrying the correct sequence to the target tissue. Although several clinical trials have demonstrated encouraging results, a number of issues surround the efficacy of such treatments,[3] for example, the possibility of donor DNA integration into unwanted sites in a patient's genome which could cause harmful off-target effects.[4, 5] Additionally, many large genes exceed the current packaging limits of viral vectors, for example, *USH2A,* responsible for Usher syndrome type II (see section 6.4), encodes two mRNA transcripts, the largest of which is 18 Kb.[6] Furthermore, difficulties may arise in accurately engineering the expression of tightly regulated disease-causing genes, for example transcription factors, where different transcript levels can encourage target tissue-specific phenotype variability.

A novel approach known as nonsense suppression therapy or translational bypass readthrough is under investigation to treat PTC-derived diseases, based on the discovery that certain compounds, namely aminoglycosides, can promote a low level of eukaryotic ribosomal readthrough of PTCs.[7-9] For many recessive, loss of function disorders, especially metabolic disease, only small amounts of functional protein can be therapeutically relevant in improving function and halting disease progression.[10]

Nonsense suppression therapy allows the affected gene to remain under the control of natural regulatory mechanisms and there is also no gene size limitation. To date, most notably, nonsense suppression has been employed in the treatment of cystic fibrosis (CF) and Duchenne muscular dystrophy (DMD), for which effective curative

therapies are currently lacking.[7, 11-13] In ophthalmology, the first phase II clinical trial for aniridia using Translarna™ (also known as ataluren or PTC124) has commenced (NCT02647359). In-frame PTCs contribute to approximately 30% of genetic eye disorders and recent preclinical research indicates that topical and systemic administration of readthrough compounds can ameliorate nonsense-mediated ocular disease phenotypes. In this review we discuss the mechanisms governing nonsense suppression and their relevance to ocular genetic disorders, providing evidence for the application of nonsense suppression therapy as a viable therapeutic option for untreatable genetic eye diseases and multi-system disorders with ocular involvement.

2. Mechanism of nonsense suppression therapy

During normal translation, free tRNAs are sampled at the aminoacyl (A)-site of the eukaryotic ribosome. Both cognate aminoacyl-tRNAs (perfectly matched base pairs) and near-cognate aminoacyl-tRNAs (containing two of the three base pairs) can interact with the A-site. Once a cognate aminoacyl-tRNA is detected and accommodated to the empty A-site, enzymatic attachment of the amino acid to the polypeptide chain can occur.[14] This results in translocation of the tRNA-polypeptide complex into the P-site, hence vacating the A-site and allowing the next codon in the mRNA sequence to be sampled and translated (Figure 1). When a ribosome encounters a stop codon in the mRNA sequence, there is no cognate aminoacyl-tRNA that can bind to the sequence. Instead, interplay between eukaryotic release factors (eRFs), eRF1 and eRF3 facilitates peptidyl-tRNA hydrolysis and release of the newly formed polypeptide by forming a 'termination complex.' eRF1 recognizes and binds the stop codon at the A-site instead of a cognate aminoacyl tRNA, thereby altering the activity of the ribosomal peptidyl transferase. The GTPase eRF3, stimulates peptide release by eRF1 in a GTP-dependent manner.[15-17]

Near-cognate aminoacyl-tRNAs are able to compete with eRFs for binding of the stop codon in the A-site.[18] This leads to the incorporation of the corresponding amino acid into the growing polypeptide chain and translocation of this tRNA-peptide complex into the P-site, effectively allowing 'readthrough' of the stop codon (Figure 1C). Each stop codon can be translated through nonsense suppression using a specific group of near-cognate amino acids (Table 1). In humans, under normal conditions, readthrough of PTCs occurs in <1% of translation events and suppression of natural termination codons occurs at a frequency of <0.1%.[19] Basal

levels of readthrough of nonsense mutations are too low to retrieve functionality, however, further increases in functional protein may rescue disease pathology.[10] Several compounds that increase the readthrough of PTCs have demonstrated potential in the treatment of genetic disorders caused by nonsense mutations, where the recovery of small amounts of functionally active protein can be therapeutic particularly in loss-of-function recessive disorders.[20]

3. Nonsense suppression compounds

3.1 Aminoglycosides

Aminoglycosides are bactericidal antibiotics composed of small, di-/tri- saccharide units joined by glycosidic linkages (Figure 2). They bind to the adenosine nucleotide in the rRNA sequence at position 1408 in the prokaryotic A-site, resulting in ribosomal infidelity, poor codon recognition and the production of jumbled, non-functional proteins leading to bacterial cell death. Eukaryotic ribosomes contain a guanosine nucleotide in this position, which can confer resistance to certain aminoglycosides as proper Hydrogen bonding between amino and hydroxyl functional groups of aminoglycosides and RNA bases in the A-site are not formed. For example, 6'-NH_2 containing aminoglycosides, such as kanamycin A demonstrate lower affinity for the eukaryotic rRNA sequence than the prokaryotic equivalent.[18, 21-23]

The relationship between readthrough efficacy and the induction of A-site structural changes during aminoglycoside-mediated nonsense suppression is not well understood.[21, 24, 25] It has been hypothesized that the decoding mechanism by which the ribosomal machinery deciphers the mRNA code in the A-site is conserved between pro- and eukaryotes, but the mechanism of drug action appears to differ.[21] A number of aminoglycosides have demonstrated nonsense suppression activity including: gentamicin, paromomycin, geneticin (G418), streptomycin, lividomycin, and tobramycin, in multiple tissues and disease models, *in vivo* and *in vitro*, highlighting a promising clinical application to alleviate the consequences of a multitude of hereditary disorders.[7-9, 26] Of the aminoglycosides described, G418, gentamicin and paromomycin are capable of the broadest and most efficacious PTC readthrough activity.[21, 27-29] Readthrough efficacy not only varies between genes, but is also dependent on the type and location of the termination codon in the gene of interest and on the surrounding mRNA sequence context. Some stop codons are more susceptible to readthrough than others; a predilection for UGA>UAG>UAA, has

been described,[18] although one study described no correlation between stop codon identity and response to gentamicin-treatment.[30]

The efficiency of translational readthrough of PTCs can vary as a consequence of mRNA secondary structures, for example downstream stem-loop structures can induce ribosomal pausing. Additionally, nucleotide context of the PTC seems important in determining readthrough efficiency.[30] Cytosine residues at position +4 (immediately downstream from the stop codon), facilitated a higher level of readthrough than uracil, guanine or adenine (C>U>G≥A) either in the presence or absence of aminoglycosides.[18, 30] The presence of a uracil residue in the -1 position, was associated with higher levels of gentamicin-induced readthrough than any other nucleotide. Although the chemical properties of the nascent polypeptide chain have been reported to modulate translational readthrough in these investigations, these rules are not definitive in predicting readthrough efficacy, for example, the order of hierarchy of the +4 residue may rotate depending on which stop codon is employed[18] and combined effects between specific residues in different positions have been noted for induced-readthrough.[30]

3.1.1 Aminoglycosides – Clinical delivery

Although aminoglycosides have demonstrated considerable potential for therapeutic application of the readthrough mechanism, problems persist. Aminoglycosides such as gentamicin are not lipid soluble and have difficulty penetrating cells when administered systemically.[31] Most notably, challenges of effective systemic delivery to the retina or cerebrospinal fluid, where limited penetration across the blood-retinal or blood-brain barrier remain. Liposome-encapsulation of aminoglycosides has been attempted to achieve intracellular delivery therefore optimizing therapeutic efficacy by increasing delivery of the drug to the cytoplasm of the cell.[32] Amikacin accumulated at higher concentrations and persisted longer in tissues of mice treated with liposome-encapsulated drug when compared to administration of the free compound at an equivalent dose, hence prolonging exposure to the pharmacological effects of the drug.[33] Importantly, mice that received a dose of free amikacin excreted most of the administered dose in the urine within the first day of treatment.

3.1.2 Aminoglycosides – Toxicity

Severe drug toxicity at therapeutically relevant levels limits the clinical utility of long-term aminoglycoside treatment.[18, 34, 35] Aminoglycosides are nephrotoxic and ototoxic due to the enriched presence of endocytic megalin receptors, the primary mediator of aminoglycosidic uptake, on the surface of epithelial cells of the renal proximal tubules and hair cells of the inner ear.[36] Additionally, aminoglycosides possess neuromuscular blocking activity.[27]

The gentamicin complex is naturally produced by the bacterium *Micromonospora*, as a mixture of five structurally related active compounds or congeners (C1, C1a, C2, C2a and C2b). Each congener exhibits different readthrough efficacy and toxicity. Initial studies suggested that gentamicin nephrotoxicity was largely attributable to the C2 congener. Conversely, more recently the isolated C2 congener exhibited low cellular toxicity in *in vitro* mouse cytotoxicity assays and showed reduced nephrotoxicity in rats *in vivo*, when compared to treatment with the native gentamicin compound.[37] A further study suggested the C2 congener induced the strongest ototoxic effects, whilst C1 and C1a were the least severe in rats treated intratympanically with the isolated congeners.[38] Understanding the relative toxicity of the individual congeners would facilitate development of custom-made aminoglycosidic therapies without sacrificing nonsense suppression capacity.

Ototoxic mechanisms of aminoglycosides may also be due to the inhibition of mitochondrial protein synthesis. Mitochondria share evolutionary lineage with prokaryotic cells and aminoglycosides preferentially bind to the prokaryotic, rather than the eukaryotic, ribosomal A-site.[39] Inhibition of mitochondrial protein synthesis leads to the oxidative inactivation of mitochondrial aconitase, as a result of superoxide production and the dose- and time-dependent mobilization of free ferrous iron. Cells ultimately undergo apoptosis via the Fenton reaction, as demonstrated by the auditory sensory cell damage in aminoglycoside-treated cochlear explants and in guinea-pigs *in vivo*.[39, 40]

The co-administration of aminoglycosides with antioxidants significantly reduced oxidative damage, therefore potentiating some level of hair cell protection.[41] The mechanism of reactive oxygen species (ROS)-mediated damage to mitochondrial aconitase remains unclear, however, aminoglycosides modified to exhibit greater specificity for cytoplasmic rRNA rather than mitochondrial rRNA, have reduced ototoxic potential. For example, the aminoglycoside G418 and designer aminoglycoside NB84 exhibit similar efficacy of inhibition of cytoplasmic protein

synthesis, but G418 has a 30-fold higher propensity to inhibit mitochondrial protein synthesis. NB84 exhibits reduced ototoxic potential as a result of decreased affinity for mitochondrial rRNA.[39, 40] All studies investigating the efficacy of liposome-encapsulated aminoglycosides in animals reported a reduction in acute toxicity when compared to administration of the free drug.[33] Additionally, co-administration of calcium cation can antagonize the acute toxicity and neuromuscular blocking effects of aminoglycoside treatment, and co-administration of poly-L-aspartate or daptomycin to concentrate aminoglycosides in the cytoplasm, confers a level of renal protection, and enhances nonsense suppression.[42, 43]

Intraocular administration of aminoglycosides has been associated with retinal toxicity for the treatment of endophthalmitis, leading ophthalmologists to search for other Gram-negative targeting antibiotics for routine intravitreal injections.[44] Initial case reports demonstrated that administration of gentamicin, amikacin or tobramycin caused vision loss with a pale fundus, intraretinal hemorrhages, arteriolar narrowing and venous beading.[45] More chronic findings included neovascular glaucoma, pigmentary degeneration, optic atrophy and severe visual loss.[46] Lavage of the anterior chamber and early vitrectomy, in some cases, prevented such vision loss.[47] More recent *in vitro* and *in vivo* ERG studies in rabbits and rats showed that the b-wave in electroretinography was completely eliminated by high-dose gentamicin treatment, with diffuse disruption of the nerve fiber layer and inner plexiform layers of the eye.[48] These effects were reversible with short-term exposure to gentamicin. Despite multiple case reports of macular infarction after intravitreal injection of aminoglycosides, amikacin is still administered intravitreally with vancomycin in cases of endophthalmitis.

3.2. Designer aminoglycosides

Discovery efforts to produce new readthrough compounds with lesser toxicity than aminoglycosides have been driven by rational synthesis, focusing on the manufacture of designer derivatives of existing drugs.[28] 'Designer aminoglycosides' maintain the natural aminoglycoside backbone, but the attachment of various structural appendages allows for the selection of favourable bioactivity and toxicity properties (Figure 2). The nomenclature for designer aminoglycosides is as follows: neomycin derivatives have the prefix TC-, paromomycin derivatives the prefix NB-, and kanamycin derivatives the prefix JC-.[1]

In the case of the NB-compounds, it was suggested that a C6'-hydroxyl group was important for readthrough efficacy, given that this was conserved in both paromomycin and G418, two of the most potent natural readthrough inducers.[49] The C6'-hydroxyl group can form hydrogen bonds with the crucial 1408G residue found in eukaryotic rRNA, whereas the C6'-NH_2 group found in other aminoglycosides cannot.[22] Paromomycin also showed the lowest levels of toxicity, so selective removal of individual saccharide rings from the original paromamine backbone resulted in the production of a pseudo-trisaccharide compound which retained high readthrough capacity in its simplest form. As interactions between aminoglycosides and rRNA are largely mediated by electrostatic interactions,[23] it was reasoned that the addition of an amino group to the third ribose saccharide ring would facilitate binding of the eukaryotic ribosome, hence further enhancing the readthrough capacity of the novel compound. Direct comparison of compounds containing the additional amino group with plain ribose rings bound in the same positions confirmed this.

Importantly, when the readthrough potential of the modified pseudo-saccharide compound, namely NB30, was compared with gentamicin or paromomycin using an *in vitro* luciferase reporter assay, it exhibited higher readthrough potential than either natural aminoglycoside, perhaps due to a reduction in total positive charge of the compound.[49] Additionally, synthesized aminoglycoside analogues did not retain the anti-microbial activity of parent aminoglycoside compounds, suggesting a change in the affinity for prokaryotic ribosomal binding. Readthrough of an Usher syndrome type 1 nonsense mutation (*pR31X, CGA>UGA*) in the *USH1C* gene, using NB30 treatment, induced lower toxicity levels than gentamicin, G418 or paromomycin. However, the readthrough efficacy of NB30 was much lower than that of the natural aminoglycosides.[50, 51]

Amikacin shows much higher readthrough efficacy than kanamycin despite differing by only the addition of a (S)-4-amino-2-hydroxybutanoyl (AHB) group to its N1 position (Figure 2).[52] Addition of this group to the N1 position of NB30 conferred increased readthrough potential whilst lowering its acute lethal toxicity.[27] Additionally, N1-AHB-modified aminoglycosides exhibit increased binding affinity to the ribosomal A-site.[49, 53] This resulting second-generation compound, NB54, showed greater *in vitro* nonsense suppression activity than paromomycin or gentamicin in PTCs derived from multiple disease-causing genes including: *USH1C* (Usher syndrome), *CFTR* (CF), *Dystrophin* (DMD), and *IDUA* (Hurler Syndrome).[27, 54]

Further development of NB54, to improve readthrough efficacy, led to the production of two third-generation compounds, NB74 and NB84 which differ from NB30 and NB54, respectively, by the addition of a (R)-6'-methyl group to the glucosamine ring (ring I) (Figure 2).[21] G418, the most potent nonsense suppressor,[18, 55] is the only aminoglycoside that has a (R)-C6'-methyl group on ring I, with a secondary alcohol at position C6'. Additionally, gentamicin studies demonstrated that the inversion of an absolute configuration at a single carbon atom of the C2 congener from (S)-C6'-gentamicin to (R)-C6'-gentamicin, significantly reduced toxicity of the compound, including nephrotoxicity.[37] The presence of the (R)-6'-methyl group in NB74 and NB84 enhanced readthrough potency comparative to that of gentamicin or NB54, while its effect on toxicity was negligible.[21] Interestingly, no particular motif appeared to be indispensable for nonsense suppression, rather each individual modification was observed to cumulatively increase the capacity for readthrough when added to the basic NB30 backbone.

Of the new generation aminoglycoside-derivatives, NB84 showed the greatest readthrough potency in an *in vitro* luciferase assay. Protein assays suggested that the readthrough induced by NB84 was significantly higher than gentamicin but not than G418.[21] However, NB84 was five times less acutely toxic than G418. Novel synthetic aminoglycoside derivatives continue to be developed with ever-increasing readthrough efficacy; evaluation of their applicability in a clinical setting is on-going. Recently, a novel compound NB124 has been found to mediate the highest levels of readthrough, with significantly lower levels of ototoxicity than gentamicin, in various CF models.[56]

3.3. Non-aminoglycoside small molecule nonsense suppressors

3.3.1. Ataluren

High throughput screening of large libraries of small molecules has led to the discovery of several non-aminoglycoside candidate drugs with readthrough potential, however, controversies remain regarding their mechanism of action.[57] Ataluren (also known as Translarna™ or PTC124, Figure 3) was identified from 800,000 molecules screened by PTC Therapeutics Inc., using a luciferase-based reporter system.[58] Genes with PTCs were fused in-frame with a luciferase reporter cassette, and luciferase activity was used as a measure of readthrough of a particular stop

mutation; higher levels of readthrough resulted in increased luciferase activity. Despite initial concerns that the observed nonsense suppression activity actually reflects stabilization of the steady-state activity of the luciferase enzyme, giving an artificially high reading,[59] it is now accepted that ataluren is a potent inducer of translational readthrough of multiple PTCs across many genes, in *in vitro* and *in vivo* models of disease.[59-63] Moreover, clinical trials have demonstrated the therapeutic benefit of ataluren's readthrough activity: ataluren induced full-length functional CFTR protein production in patients with CF[12, 60, 64-67] and dystrophin protein production in patients with DMD,[68, 69] with a marked improvement in disease-associated phenotype. Encouragingly, ataluren exhibited lower toxicity than traditional aminoglycosides in several phase I/2a clinical trials.[70, 71] Ataluren is currently the first drug in its class to have received global approval for a phase III clinical trial in the treatment of DMD in ambulatory patients aged 5 years or older.[72] Only mild side-effects such as vomiting, nausea and headaches were reported, with a number of other trials on-going.

Ataluren is water-soluble, with good bioavailability, and can be delivered systemically via oral administration. However, there is some question as to the suitability of this approach for the treatment of ocular and brain disorders due to the blood-retinal/brain barrier.[73] A recent report suggested that the use of a simple delivery agent known as the START formulation (0.9% **S**odium chloride, 1% **T**ween 80, 1% powdered **A**taluren, and 1% ca**r**boxyme**t**hylcellulose), yielded vast improvements in the capacity for ataluren to cross the ocular surface and penetrate the eye. Furthermore, increased suspension viscosity allowed prolonged contact of the drug with the ocular surface, maximising absorption. Topical application of the START formulation not only rescued the retinal and lens defects observed in the *Pax*$^{Sey+/-}$ mouse model of aniridia, but also showed a marked reduction in ocular irritation when compared to application of a 1% aqueous ataluren suspension.[73]

Recent work suggests that ataluren binds the ribosome, enhancing tRNA insertion, with a tRNA selection bias favouring a distinct subset of tRNAs, generally leading to the incorporation of specific amino acids at the PTC.[63] These insertion biases are thought to arise from mRNA:tRNA mispairing at codon positions one and three. Ataluren is able to stimulate the insertion of near-cognate tRNAs that resemble those inserted endogenously, without promoting readthrough of normal stop codons, therefore producing proteins that are unlikely to be antigenic; an asset that appears to be unique to ataluren.[63] Chemical optimization of PTC124 by PTC Therapeutics,

led to the discovery of novel derivative PTC414, a compound suggested to increase plasma exposure and tissue penetration whilst maintaining the favourable properties of PTC124. Like PTC124, PTC414 demonstrated nonsense suppression ability in the choroideremia zebrafish model chmru848, restoring sufficient protein function to increase embryo survival and prevent retinal degeneration, whilst exhibiting improved pharmacokinetic properties (see section 6.1).[74]

3.3.2. Small molecule readthrough (SMRT) compounds

Readthrough compounds 13 and 14 (RTC13 and RTC14, Figure 3) are SMRT compounds identified through a high-throughput protein transcription/translation (PTT) ELISA-based assay; a luciferase-independent system that enabled the direct quantification of full-length protein levels resulting from successful readthrough events.[75, 76]

RTC13 and RTC14 exhibited readthrough potential, restoring dystrophin protein expression in myoblasts isolated from the skeletal muscles of *mdx* mouse mutants. Moreover, intramuscular injection of RTC13 resulted in recovery of full-length dystrophin expression in the muscles of *mdx* mice, at higher levels than the observed recovery with ataluren injection. Repeated systemic delivery of RTC13, not only slowed the progression of myofiber degeneration characteristic of *mdx* mutants, but also increased muscle strength and morphology.[76, 77]

Repeat screens of additional compound libraries led to the identification of the compounds GJ071 and GJ072 (Figure 3), which exhibited equivalent or increased readthrough potential than RTC13 or ataluren. GJ072 and RTC13 bear structural similarity to ataluren, sharing a common three-ring structure. However, GJ071 and RTC14 are very structurally different. Moreover, additional compounds identified in the primary drug screen, RTC204 and RTC219, share similar structural features to GJ702, suggestive of the importance of this structural feature in the prediction of readthrough efficacy and optimization of novel designer analogues.[75] SMRT compounds may eventually form the clinical basis for the treatment of disease caused by nonsense mutations. This is reinforced by the association of these compounds with low partition coefficient (cLogP) values, indicative of their ability to easily permeate tissues following administration *in vivo*.

4. Potential limitations of nonsense suppression therapy

A critical factor limiting the potential application of nonsense suppression in the treatment of ocular disease is retinal biocompatibility. NB30 showed good biocompatibility in contrast to gentamicin, paromomycin and G418 in murine retinal explants, causing little increase in apoptotic cell death upon administration.[78] In a similar study, PTC124 showed excellent retinal biocompatibility when compared to gentamicin upon administration to human retinal explants.[79] A key step to evaluate preclinical retinal biocompatibility of novel nonsense suppression compounds for human therapy requires the development of higher-order nonsense mediated animal models for *in vivo* testing. This will also enable researchers to determine the passage of drug across the blood brain barrier and determine the pharmacokinetics, including half-life, in ocular tissue.

A more general concern raised about nonsense suppression therapy was the potential effect of increasing readthrough of natural stop codons within non-diseased genes, resulting in an accumulation of misfolded, dysfunctional proteins as a consequence of continued translation into the 3'UTR of the transcript. However, subsequent research demonstrated increased termination of translation kinetics at natural stop codons, when compared to PTCs.[80] This was partly due to ribosome pausing at a PTC, rendering it susceptible to the binding of nonsense suppressors prior to termination complex-formation.[80] Termination at a natural stop codon relies on the proximity of the sequence to the 3'-poly-A tail of the mRNA transcript (Figure 4). This allows binding of the eRFs to the poly(A) binding protein (PABP), thereby increasing the efficiency of translation termination.[81] Conversely, at PTCs, the eRF termination complex cannot interact efficiently with the 3'-PABPs due to the lack of proximity between the PTC and the poly-A-tail of the mRNA transcript, where PABP binds, thereby reducing the efficiency of translation termination.[82]

When considering the efficacy of readthrough, it should also be highlighted that not all full length protein restored by nonsense suppression may be functional. Several near-cognate tRNAs, that associate with two of three nucleotides of a PTC may induce readthrough, possibly incorporating one of several amino acids. Incorporation of a nonfunctional amino acid at the site of a PTC may result in the production of a full length protein with a missense mutation which attenuates protein activity or affects protein stability.[83] Genotype-phenotype correlation must be considered, for example, there is no association in choroideremia, therefore the effect of introducing a missense mutation in place of a nonsense variant will have no effect. However, in

aniridia, it has been suggested that missense mutations lead to a milder phenotype[84] and hence this may have a slightly therapeutic effect over a loss-of-function change.

The flanking sequence of a stop codon greatly influences the efficiency of translational termination. Many genes have several, in-frame, natural, stop codons at the end of the open reading-frame (ORF) to ensure that translation does not continue into the 3'-UTR by facilitating ribosomal release.[18, 19, 85-87] Interestingly, Hsp70 levels, which are elevated in response to unfolded protein accumulation, were only slightly increased upon administration of clinically relevant doses of gentamicin, suggesting that PTC suppression does not cause large deleterious effects on global translation or natural stop codon recognition.[88] Additionally, overall translation rates remained comparable between treated and non-treated mammalian cells in culture at doses of suppression agents able to induce PTC readthrough.[34, 85]

Recent research suggests that mammalian cells use nonsense suppression to expand and/or control gene expression.[89] For example, proangiogenic vascular endothelial growth factor A (*VEGFA*) mRNA undergoes programmed stop codon readthrough to generate VEGF-Ax, a unique protein isoform, which exhibits antiangiogenic activity. A *cis* element in the 3' UTR of VEGFA promotes decoding of a UGA stop codon as a serine. Importantly, VEGF-Ax expression is depleted in adenocarcinomas.[89] Other mammalian transcripts that elicit nonsense suppression were also identified. Furthermore, the efficiency of translation termination can be altered in response to stress stimuli. Research suggests that in some cases nonsense suppression may result in the production of novel immunogenic epitopes that elicit a T-cell mediated immune response against the newly restored full length or near-full length protein, reducing the therapeutic effectiveness.[90] This highlights the importance of monitoring immunity during nonsense suppression testing on a case-by-case level.

With this in mind, it is important to consider the long-term effects of global readthrough of PTCs in a clinical context. Patients may harbour other nonsense mutations that would be susceptible to readthrough upon treatment with nonsense suppressors. This could potentiate off-target effects by production of previously absent proteins that may have deleterious effects. Given this, and the significant differences in susceptibility of mutations to readthrough by various compounds, innovations in personalized medicine are important, for example evaluation of global

readthrough by parallel individualized genomic screening, to determine the suitability of readthrough therapy.

5. Nonsense-mediated decay (NMD)

An important factor influencing the efficacy of readthrough therapies is NMD. The NMD pathway facilitates the identification and degradation of abnormal transcripts containing PTCs.[91] The mechanism of NMD is found in all eukaryotic organisms and is a highly conserved pathway among many species (Figure 4). In mammalian cells, pre-mRNA splicing triggers mobilization of the exon junction complex (EJC) approximately 20-24 nucleotides upstream of exon-exon junctions. When the ribosome encounters a PTC that is at least 50-55 nucleotides upstream of an EJC, the mRNA is marked for destruction by NMD machinery.

The kinase SMG1 binds together with NMD factor UPF1 to eRF1 and eRF3, forming the SMG-1-Upf1-eRF1-eRF3 (SURF) complex at a PTC. If an EJC is downstream of the SURF complex, as in the case of a PTC, UPF1 is able to bind UPF2, a protein component of the EJC, facilitating phosphorylation of UPF1 by SMG1, within the SURF complex. This triggers release of the eRFs and recruitment of SMG5 and SMG7. SMG5- and SMG7-mediated decay of the target mRNA occurs by deadenylation-dependent decapping with the involvement of additional NMD-factors such as the 5'-3' exonuclease hXRN1.[9, 91, 92] Although this model of NMD is widely accepted, recent studies suggest the convergence of multiple factors to promote or antagonize NMD, such as translation re-initiation.[80, 93-96]

NMD naturally reduces the number of transcripts available for translational readthrough. The level of transcripts carrying PTCs governs the efficacy of readthrough therapy in individuals; the best responders to readthrough therapy generally have the highest detectable levels of target transcript.[97] Importantly, the efficacy of NMD varies naturally between individuals, not only highlighting the importance of assaying the natural level of transcript before clinical application of nonsense suppression therapy, but also indicating that mild pharmacological inhibition of NMD efficiency would be tolerated by a wide population.

Strategies that inhibit NMD may viably increase the level of partially functional truncated polypeptides or, in conjunction with nonsense suppression agents, restore full length functional proteins, thereby alleviating disease pathology. Indeed, siRNA-

mediated inhibition of UPF1,[98] enhanced the efficacy of nonsense-mediated suppression therapy.[97, 99] Additionally, partial inhibition of NMD resulting in an increased number of target transcripts, may allow for lower therapeutic doses of readthrough compounds in the treatment of disease, with obvious benefits of reduced cellular toxicity.

Interestingly, NMD inhibition rescued the nonsense suppression activity for PTCs that had previously shown no response to treatment with readthrough agents; this has promising implications for increasing the spectrum of targets for readthrough therapy.[97] The drug amlexanox, has been described as both a putative NMD-inhibitor and a readthrough agent that is efficient in increasing the expression of full-length functional CFTR protein in human cells,[100] presenting a combined therapeutic opportunity for the treatment of patients with low levels of native transcript susceptible to NMD.

6. Nonsense suppression in ocular disease

PTCs contribute significantly to inherited eye disease, making nonsense suppression a viable therapeutic option. There are several examples of successful nonsense suppression in the treatment of eye disease, including biochemical disorders such as choroideremia, and developmental disorders, for example, aniridia.[73, 101, 102]

6.1. Choroideremia

Choroideremia is an X-linked recessive chorioretinal degeneration, with over 30% of patients harbouring nonsense mutations in the *CHM* gene.[103] A zebrafish model of choroideremia has been employed to ascertain the efficacy and safety of readthrough agents in restoring the translation of functional rab escort protein-1 (rep-1), the protein encoded by the *chm* gene.[74, 101] The rep-1 protein is responsible for prenylation and subsequent trafficking of rab GTPase family members to the cell membrane. In humans, REP-1 is globally expressed throughout the body but patients only manifest a retinal degeneration due to the REP-2 isoform compensating for the lack of REP-1 in all tissues except for the retina. Preferential binding of certain Rab proteins, such as Rab27a, with REP-1, prevents functional rescue.[104]

In contrast to humans, zebrafish only have one rep isoform, and homozygous nonsense mutations in the orthologous gene cause embryonic lethality at

approximately 5 days post-fertilisation (dpf).[105] The *chm* mutant therefore provides a robust model for testing readthrough agents, where viability of embryos beyond this time point indicates functional rescue of rep-1 protein. Mutants dosed at 10 hours post-fertilisation (hpf) with gentamicin or paromomycin showed a 1.7-fold increase in survival,[101] and treatment with PTC124 or PTC414 induced a 2-fold increase in survival.[74] Readthrough treatment with each compound prevented the onset of retinal degeneration in the mutants and eye morphology appeared normal. Rescue of full-length rep-1 protein expression and restored biochemical function was confirmed post-treatment by western blot and *in vitro* prenylation assay, highlighting the translational potential of these compounds for inherited retinal disease.

6.2. Ocular coloboma

Ocular coloboma arises from incomplete fusion of the optic fissure during weeks 5-7 of embryogenesis, potentially affecting the iris, ciliary body, zonules, retina, choroid and optic nerve. Ocular coloboma has been reported in up to 11.2% of blind children worldwide, with an estimated incidence of between 0.5–7.5 per 10 000 births.[106] Nonsense mutations in the *PAX2* gene or *CDH7* gene have been associated with renal-coloboma syndrome and CHARGE syndrome, respectively, but there are no known mutation-subtype preponderance. The *no isthmus* (*noi*^{tu29a}) zebrafish mutant has a recessive nonsense mutation in *pax2.1* which manifests in defective optic stalk formation and failed optic fissure closure. Similarly, the *grumpy* (*gup*^{m189}) mutant, displays ocular coloboma due to a recessive nonsense mutation in *lamb1*. Treatment of mutants with gentamicin and paromomycin increased readthrough efficiency of both mutations, assayed by luciferase reporter activity. Importantly, direct exposure to either aminoglycoside *in vivo* resulted in increased survival and rescued ocular phenotypes including fusion of the optic fissure by 9 dpf.[101]

6.3. Retinitis pigmentosa (RP)

RP is a group of retinal degenerative diseases characterized primarily by the loss of rhodopsin expression in the photoreceptors cells of the eye which ultimately leads to a complete loss of vision. Thus, a strategy for rescuing rhodopsin expression may restore vision.[107, 108] Aminoglycoside-treatment of the *S334ter* rat, a model of autosomal dominant nonsense-mediated RP caused by a nonsense mutation in rhodopsin (*Rho*), resulted in enhanced photoreceptor survival when compared to untreated littermates.[29] A 5% reduction of abnormal truncated protein expression was

sufficient to improve retinal histopathology and preserve retinal function in gentamicin-treated rats, indicative of effective PTC readthrough. Daily injections of gentamicin proved more effective than continuous administration via osmotic pump in *S334ter* rats, and this may prove useful in the clinical application of nonsense suppression.[29]

The X-linked *RP2*, *R120X* mutation is responsible for approximately 15% of RP cases.[109, 110] This gene is ubiquitously expressed in human tissues and does not appear to be enriched in the retina, despite patients with RP2 mutations showing retinal dysfunction but no systemic effects. The mechanism for RP2 pathology is not well understood, however, it may be involved in assembly and trafficking of membrane-associated cilia proteins within the RPE and photoreceptor cells. RP2 patient fibroblasts and induced pluripotent stem cell (iPSC)-derived RPE cells were treated with G418 (geneticin) restoring up to 20% endogenous RP2 protein.[61] Previously, aminoglycosides were unable to restore full length RP2 protein expression in *RP2* patient-derived lymphoblasts.[111] This may be attributable to the surrounding nucleotide context of the PTC, or to NMD of mutant transcripts. Interestingly, treatment of the *RP2* fibroblasts with G418 resulted in an 40% increase in *R120X RP2* mRNA levels, suggesting that this drug is able to inhibit NMD, thereby increasing the number of *RP2* transcripts available for translation.[11] Conversely, ataluren treatment, which restored up to 13% endogenous RP2 protein in fibroblasts, failed to significantly increase *RP2* transcript expression, suggestive of a different mode of action.[66]

It is important to note that aminoglycoside-mediated readthrough of PTCs cannot be predicted from genomic context of the PTC alone. Mutations in *RPE65* are associated with Leber's Congenital Amaurosis type 2 (LCA2) and RP, characterized by a severe early-onset retinal degeneration. For example, systemic aminoglycoside-treatment of the autosomal recessive *rd12* mouse, which exhibits retinal degeneration resulting from a PTC in *Rpe65*, had no effect on translational readthrough or phenotype.[29] Despite the promise of PTC-readthrough therapies, the mechanisms of translation termination and external factors, for example epigenetic effects, may dictate readthrough efficacy and require further elucidation.

6.4. Usher syndrome

Usher syndrome (USH) is the most common form of deaf-blindness worldwide with

an incidence of 3.2–6.2 per 100,000. Type I disease (USH1) is characterized by profound congenital sensorineural hearing loss, absent vestibular function and retinitis pigmentosa (RP), which manifests in late childhood. While it is possible to compensate for the loss of hearing with hearing aids and cochlear implants, no effective therapy is available for the ensuing RP.[112-114] Several genes have been associated with the three clinical types of Usher syndrome and nonsense mutations account for approximately 12 % of all USH cases.[74]

Initial cell culture studies focused on the suppression of *PCDH15* nonsense mutations associated with USH type 1F to enable partial translation of functional protein, thereby delaying the onset and/or progression of RP.[50] Treatment with G418, gentamicin, paromomycin and NB30 resulted in the production of variable full-length protein levels as a consequence of partial readthrough of *PCDH15* nonsense mutations, although the assays employed did not confirm the functionality of full length protein produced or evaluate the *in vivo* suppressive activity and toxicity.[50]

More recently, translational readthrough efficacy for the treatment of USH type I caused by the nonsense mutation *p.R31X* in *USH1C* has been investigated using a number of nonsense suppression agents including gentamicin, ataluren, NB30 and NB54. [51, 115] Significant rescue of full-length harmonin expression has been described in HEK293 cells *in vitro*, organotypic retinal cultures *ex vivo*, and in *harm_a1-p.31X* mice harbouring the same PTC observed in USH1F patients *in vivo*.

6.5. Aniridia

Aniridia is a congenital eye anomaly characterized by complete or partial iris hypoplasia, frequently associated with glaucoma, cataracts, corneal anomalies and foveal hypoplasia. It can also form part of Wilms tumour syndrome (WAGR).[73, 116, 117] Isolated aniridia is predominantly caused by mutations in *PAX6*, 50% comprise in-frame PTCs and give rise to a more severe phenotype than rare missense mutations.[118] Therefore in cases of applying nonsense suppression therapy, spurious missense transcripts may still be produced, conferring a milder, clinical phenotype.

Notably, administration of gentamicin and ataluren to the *Pax6*$^{sey+/-}$ mouse model of aniridia was not only able to inhibit the progression of the disease, but also reversed the effects of the disorder within a specified developmental time-frame.[73] Treated animals showed improved retinal histopathology and improved responses to light

stimuli. Additionally, topical application of ataluren using the START formulation resulted in spatial frequency levels that were comparable to responses seen in wild-type animals (see section 3.3.1). This work not only demonstrates a viable therapeutic strategy to reverse symptoms caused by *PAX6* mutations postnatally, but also highlights the potential of readthrough agents to treat conditions caused by dosage-sensitive genes. The first phase II clinical trial of Translarna™ for aniridia is currently underway (NCT02647359) and the results will be eagerly awaited.

7. Conclusion and future perspectives

Overall, nonsense suppression therapy provides the basis for a new era of pharmacological genetic intervention in the treatment of hereditary disorders, reaching several million patients across the globe. In some cases, the use of nonsense suppression therapy alone may not be sufficient to overcome the therapeutic threshold required to restore functional protein levels and alleviate disease phenotype. Combining NMD-inhibition compounds with nonsense suppression drugs, or in fact the discovery of dual function drugs such as amlexanox,[119] may enhance the activity of current nonsense suppression strategies, increasing the abundance of PTC-containing mRNA substrate. Where cell and tissue specific changes in NMD efficiency alter disease pathology, a more personalized approach must be employed to enhance the effectiveness of the chosen treatment, particularly with patients who have a low baseline mRNA level, and therefore, would benefit from NMD inhibition. Caution must be exercised as little is known about all the mechanisms and triggers of NMD and the consequences of its attenuation. Targeting ribosome accessory proteins, for example termination complex proteins eRF1 and eRF3, or proteins that effect NMD efficiency, for example UPF1 may be a feasible step in improving the efficiency of nonsense suppression agents. In addition, it is important to consider the bioavailability of these compounds in the target tissue and the pharmacokinetics before human application, a consideration that is particularly relevant in treating ocular disease when compounds must cross the blood-retina barrier at high enough concentrations to act effectively. Other modes of delivery must be considered as intravitreal or subretinal injections may be more suitable than exposing the whole body to potential readthrogh of PTCs elsewhere.

Finally, although nonsense suppression has demonstrated great potential in the treatment of eye disease in preclinical studies, implementation in a clinical setting is required. Phase II clinical trials using Translarna™ are underway in Ophthalmology,

with translation to numerous inherited retinal diseases in the pipeline. With the discovery of nonsense pathogenic variants associated with ocular disorders extending to corneal dystrophies, anterior segment dysgenesis, glaucoma, multiple retinal dystrophies and optic atrophies,[120-122] the development of novel designer compounds which exhibit improved safety profiles, more efficient PTC suppression and optimum delivery methods will be vital in potentiating nonsense suppression as a therapeutic strategy.

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Conflict of interest

The authors declare no conflict of interest.

ACCEPTED MANUSCRIPT

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Figure Legends

Figure 1. Mechanism of protein translation and nonsense suppression.
(A) During normal translation, free aminoacyl-tRNAs (green) diffuse into the ribosomal A-site where they bind complementary codons in a sequence-specific manner. The attached amino acid is subsequently enzymatically added to the growing peptide chain and the tRNA is translocated into the P-site. This process continues until a stop codon is encountered. (B) When the ribosome encounters a premature termination codon (PTC), no corresponding tRNAs exist. Therefore, eukaryotic release factors (eRFs), eRF1 (orange) and eRF3 (yellow) bind to the A-site and facilitate early enzymatic release of the peptide chain. This results in the synthesis of a truncated protein. (C) Binding of nonsense suppressing agents (blue) to the ribosome affects the fidelity of translation meaning near-cognate aminoacyl-tRNA codons (those with two conserved residues) can compete with eRFs for binding of the A-site. In the instances where aminoacyl-tRNAs bind to the A-site, the amino acid is added to the peptide chain as in normal translation and the PTC is bypassed. This readthrough allows the synthesis of a full-length protein by incorporating an amino acid in place of the PTC (red oval).

Figure 2. Structures of aminoglycosides used to synthesize novel designer aminoglycosides.
Structural features of natural aminoglycosides paromomycin, amikacin and G418 were combined to produce designer aminoglycosides NB30, NB54, NB74 and NB84. Designer aminoglycosides incorporated the three ring pseudo-trisaccharide backbone of paromomycin (red), ring II holds C1'-AHB of amikacin (magenta), and ring I includes C6'-methyl group of G418 (blue) to produce a number of novel compounds. NB84 has all of these structural features and has shown the most potential for nonsense suppression.

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Figure 3. Structures of non-aminoglycoside small molecule readthrough compounds. Ataluren (PTC124) has demonstrated potent capacity for readthrough and was discovered from a high-throughput luciferase assay screen of a large compound library. RTC13, RTC14, GJ071 and GJ072 were similarly identified from a high-throughput protein transcription-translation ELISA-based screen and have also demonstrated nonsense suppression. These compounds have been used to make a number of other promising analogues that may retain the capacity for readthrough.

Figure 4. Translation termination and nonsense-mediated decay (NMD).
(A) During normal translation the ribosome will move along the mRNA and displace the exon-junction complexes (EJC) (magenta) as it progresses. It will only stop when it reaches the terminal stop codon where formation of the termination complex including recruitment of release factors eRF1 (orange) and eRF3 (yellow) facilitate translational termination and release of the peptide/ribosome. This release is very rapid as eRFs bind the adjacent poly-A binding proteins (PABP) (blue) localised to the 3'-end of the mRNA, which increases the kinetics of termination at a natural stop codon. (B) When the ribosome encounters a premature termination codon (PTC) the ribosome pauses and the eRFs bind to facilitate translational termination. However, the kinetics of termination are much slower at a PTC because the distal position of the PTC does not facilitate PABP binding. eRFs recruit UPF1 (green) which in turn binds kinase SMG1 (blue) to form the SURF complex. When this complex is at least 50-55 nucleotides upstream of an EJC, UPF1 can bind UPF2 (green), a component of the EJC, to facilitate phosphorylation of UPF1 by SMG1 to (C) release eRFs and recruit two further proteins, SMG5/7 (blue). This triggers NMD via the recruitment of various factors including hXRN1 exonuclease (red), resulting in decreased mRNA levels.

Table 1. Amino acids that can replace PTCs through binding of near-cognate tRNAs during readthrough.

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UAA					
AAA	Lys	UUA	Leu	UAG	Stop
GAA	Glu	UCA	Ser	UAC	Tyr
CAA	Gln	UGA	Stop	UAU	Tyr

UAG					
CAG	Gln	UCG	Ser	UAA	Stop
GAG	Glu	UUG	Leu	UAC	Tyr
AAG	Lys	UGG	Trp	UAU	Tyr

UGA					
CGA	Arg	UCA	Ser	UGU	Cys
GGA	Gly	UUA	Leu	UGC	Cys
AGA	Arg	UAA	Stop	UGG	Trp

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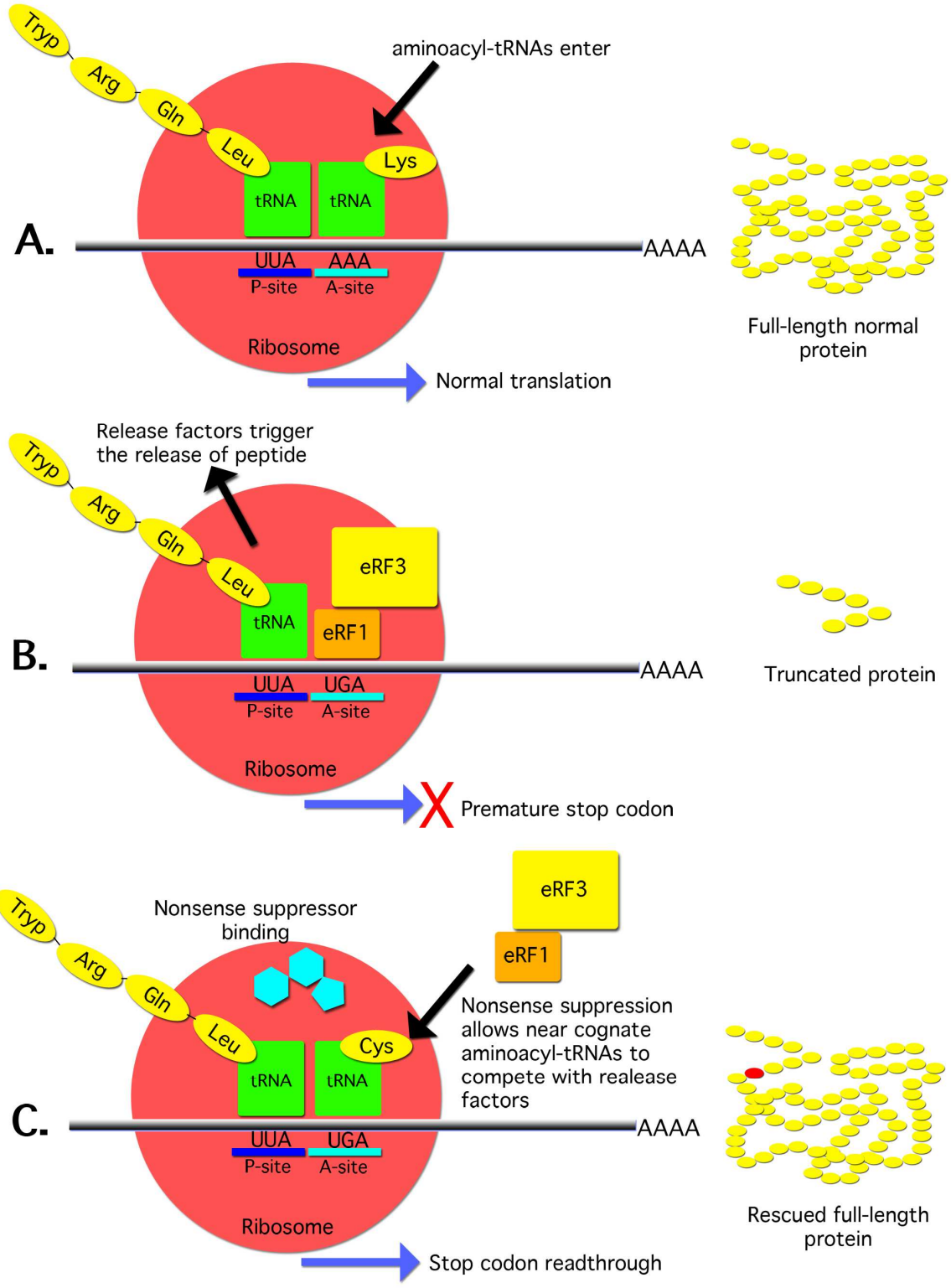
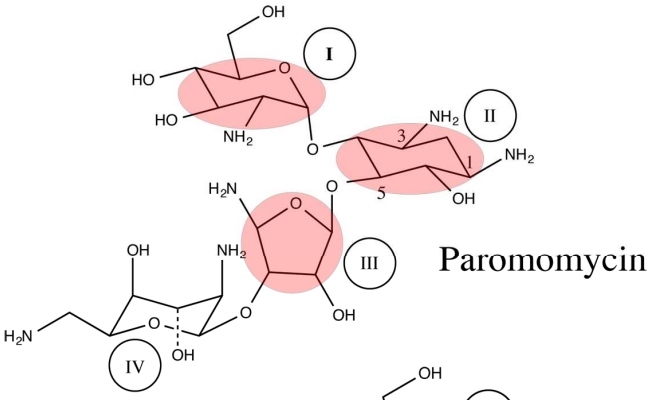
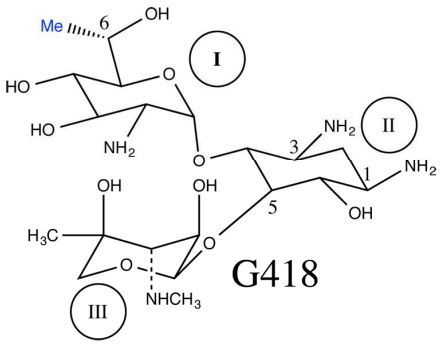
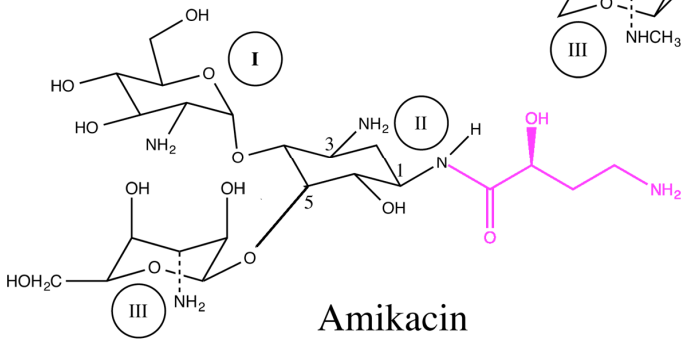

A.
Tryp
Arg
Gln
Leu
Lys
aminoacyl-tRNAs enter
tRNA
tRNA
AAAA
UUA
AAA
P-site
A-site
Ribosome
Normal translation
Full-length normal protein
B.
Release factors trigger the release of peptide
Tryp
Arg
Gln
Leu
eRF3
tRNA
eRF1
AAAA
UUA
UGA
P-site
A-site
Ribosome
Premature stop codon
Truncated protein
C.
Nonsense suppressor binding
eRF3
eRF1
Tryp
Arg
Gln
Leu
Cys
Nonsense suppression allows near cognate aminoacyl-tRNAs to compete with realease factors
tRNA
tRNA
AAAA
UUA
UGA
P-site
A-site
Ribosome
Stop codon readthrough
Rescued full-length protein



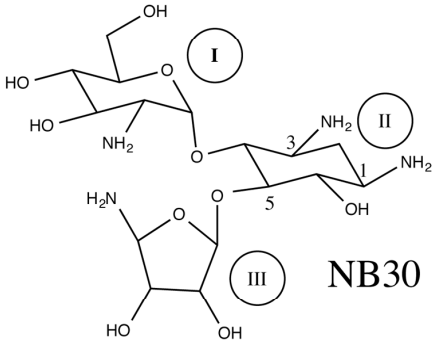
Paromomycin



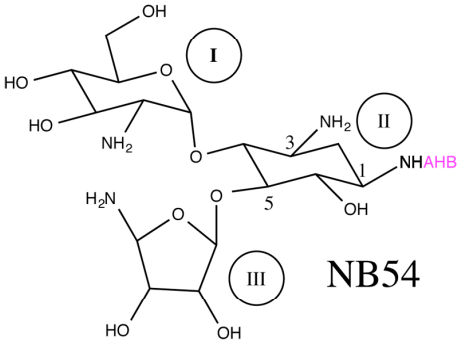
G418



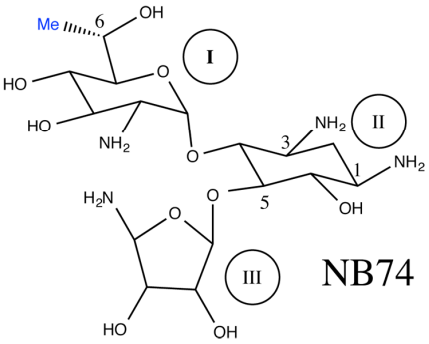
Amikacin



NB30

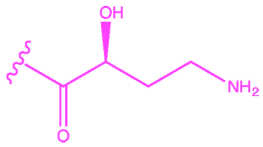


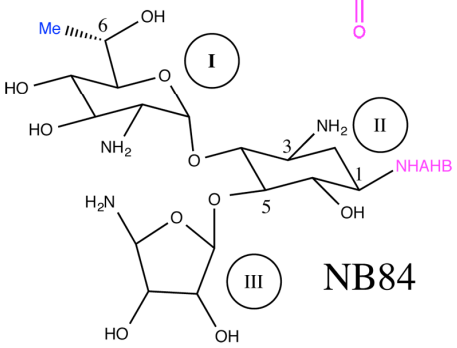
NB54



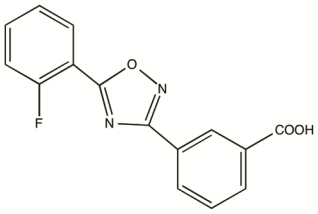
NB74

AHB =

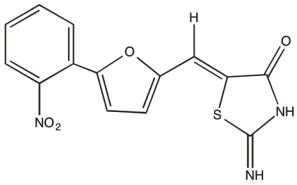




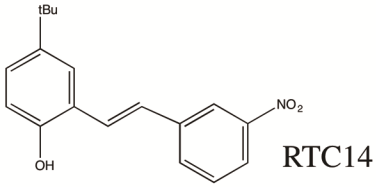
NB84



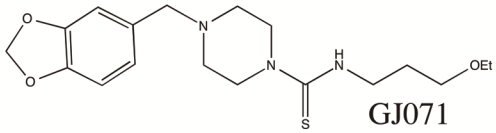
Ataluren



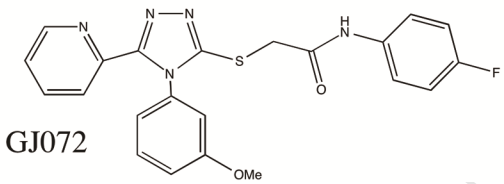
RTC13



RTC14



GJ071



GJ072

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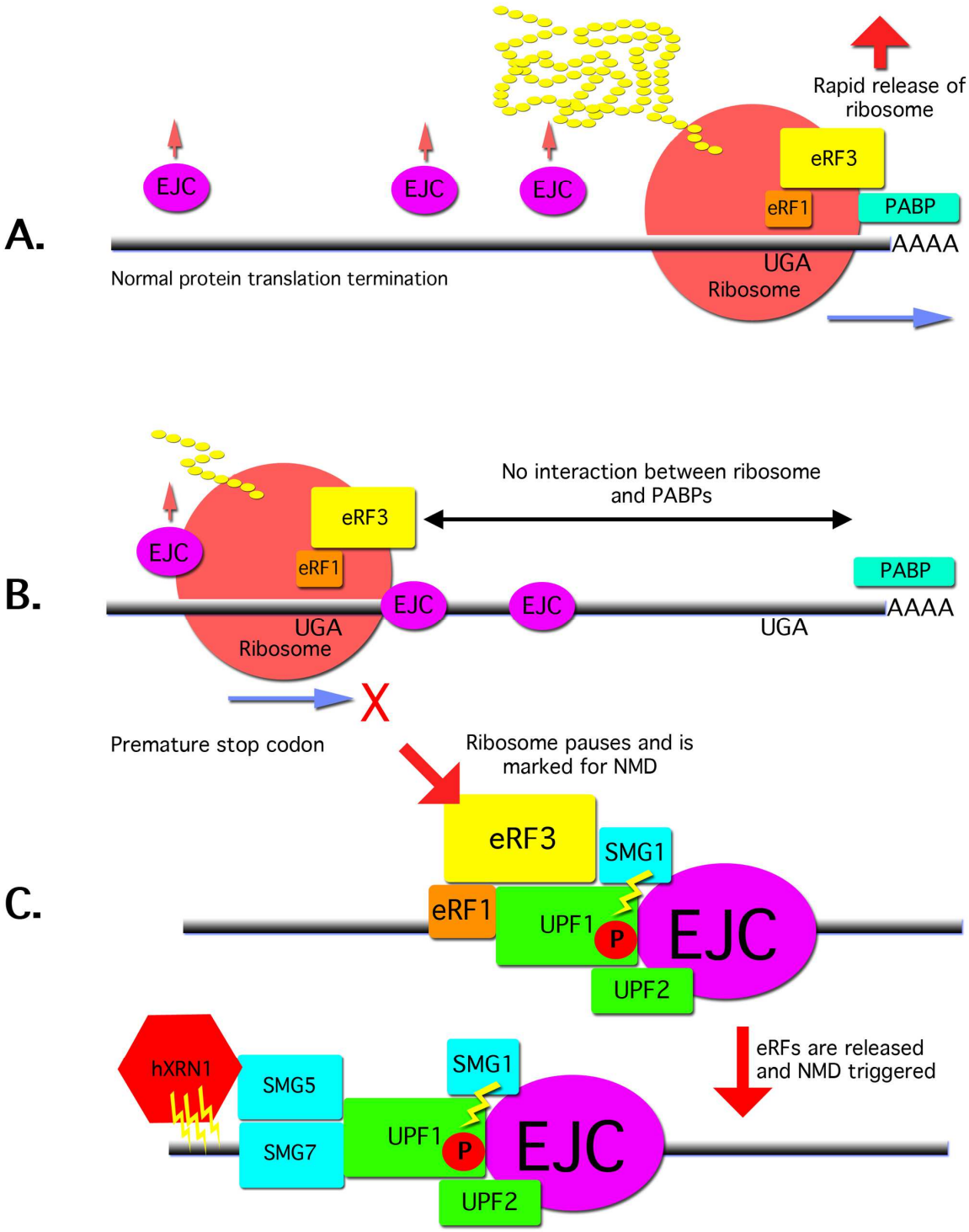
A.
Rapid release of ribosome
EJC
EJC
EJC
eRF3
eRF1
PABP
AAAA
UGA
Ribosome
Normal protein translation termination
B.
No interaction between ribosome and PABPs
eRF3
eRF1
EJC
EJC
EJC
PABP
AAAA
UGA
Ribosome
UGA
X
Premature stop codon
C.
Ribosome pauses and is marked for NMD
eRF3
SMG1
eRF1
UPF1
P
EJC
UPF2
hXRN1
SMG5
SMG7
SMG1
UPF1
P
EJC
UPF2
eRFs are released and NMD triggered

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Highlights

- Mechanism of nonsense suppression therapy
- Recent developments in pharmacological nonsense suppression
- Evidence of nonsense suppression agents in ocular disease
- Implications for the treatment of genetic eye disorders

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