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REVIEW

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Stopcodon readthrough approach as a therapeutic approach in genetic diseases

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ABSTRACT • Approximately one-third of human genetic diseases are caused by alleles carrying premature termination codons, which lead to the production of truncated proteins that result in loss of function and are often accompanied by low levels of mRNA transcripts. Some chemicals referrred to as stopcodon readthrough drugs (or translational read-through-inducing drugs) including aminoglycosides and non-aminoglycoside small molecules restore full-length functional protein by inducing 'stopcodon readthrough' of premature termination codons. During stopcodon readthrough (or translational readthrough), the translational machinery recognizes the stop codon of the premature termination codon as a triplet coding for an amino acid resulting in the translation of a full-length protein from mutant messenger RNA. Considering that >1,800 distinct genetic disorders are caused by premature termination codons, the readthrough of primary premature termination codons has potential for the treatment of a wide range of genetic diseases mainly diseases with neurological symptoms for which enzyme replacement is not an option. Especially it is likely to have an impact on disease pathology for lysosomal storage diseases since even a minor increase in enzyme activity is sufficient to prevent storage. The success of stopcodon readthrough therapy has been shown for many diseases by using in vitro and animal disease models and is currently in clinical trials for treatment of several genetic diseases caused by premature termination codons.

INTRODUCTION

Approximately 30% of human disease causing alleles are premature termination codons (PTCs), which lead to the production of truncated proteins (1). PTCs can arise from various types of mutations in germ or somatic cells including nonsense mutations that change a sense codon to an in-frame premature termination codon (PTC), insertion or deletions that alter the reading frame, and mutations that lead to mRNA splicing defects (2). Stopcodon readthrough therapy, also called translational readthrough or nonsense suppression therapy, is an approach aimed at treating or alleviating the phenotypic consequences of a wide range of genetic diseases caused by in-frame PTC or nonsense mutations (3). Some compounds can induce read through of PTCs by reducing proofreading of codon-anticodon recognition in the ribosome and result in translation of full-lenght protein. The induced protein might gain function although it carries a missense amino acid but it may have a

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reduced half-life due to the post-translational surveillance system such as endoplasmic reticulum associated degradation (ERAD) but the recovered enzymatic activity might allow improvement of the biochemical phenotype (4). As a general rule glutamine or tryptophan is inserted at premature UAG/UAA or UGA codons, respectively (5). The best characterized of these drugs are the aminoglycosides (6).

Aminoglycosides act as antibiotics in high doses by inhibiting protein synthesis, where they bind to a region of the 16S ribosomal RNA in the bacterial ribosome called the decoding center (7). By binding to the complementary sequences 1404–1412 and 1488–1497, respectively at the decoding center, aminoglycosides displace non-complementary adenines and locking them into a 'flipped out' configuration, which results in reduced discrimination between cognate and near-cognate tRNA:mRNA complexes and hence reducing translational fidelity with an end result of nonfunctional truncated proteins and final cell death (8). Due to the fundamental differences in the nucleotide sequences that is necessary for hydrogen bond formation (A2408 and G1491 in bacteria, G1408 and A1491 in mammalian cells), the interaction between aminoglycosides and human 18S rRNA is less stable but sufficient to reduce the proofreading to cause the insertion of a near-cognate aminoacyl-tRNA into the ribosomal A site that is subsequently incorporated into the polypeptide chain (8). Studies in eukaryotic cells have found that aminoglycosides that bind to the eukaryotic ribosome do not appear to induce significant misreading at sense codons, but can induce low levels of misreading at PTCs (9).

The first demonstration that aminoglycosides could suppress PTC in a defective gene was carried out in cystic fibrosis (10). Since then stopcodon readthrough has been reported in cell and animal models of different disorders including cystic fibrosis (11), Duchenne muscular dystrophy (12), phenylketonuria (13), Rett syndrome (14), ataxia-telangiectasia (15), xeroderma pigmentosum (16), mucopolysaccharidosis type I-Hurler syndrome (17,18), Nieman–Pick A/B, mucopolysaccharidosis type IIIB, mucopolysaccharidosis type II (4), mucopolysaccharidosis VI (19), Usher syndrome (20), methylmalonic academia (21), proximal spinal muscular atrophy (22), and Stüve-Wiedemann Syndrome (23). Several pilot clinical trials with patients carrying nonsense mutations with cystic fibrosis (24,25) and Duchenne muscular dystrophy (26,27) have shown the partial restoration of full-length functional protein to a variable extent with gentamicin administration. However, the toxicity of most aminoglycosides in mammals has greatly restricted their potential as readthrough drug (28). Therefore, efforts have been spent to develop aminoglycoside derivatives with reduced toxicity and enhanced activity. NB30, NB54, and NB84 are among these aminoglycoside derivatives with lower toxicity and exhibiting higher readthrough activity (29,30).

PTC therapeutics described an efficient nonaminoglycoside readthrough compound, PTC124 (Ataluren™), which was developed synthetically by screening >800,000 chemicals and analogues using a luciferase-based high-throughput screening (HTS) assay (31,32). A phase-I clinical study in cystic fibrosis confirmed that PTC124 is generally well tolerated and appears to have more efficient readthrough activity than aminoglycosides (32). PTC124 was initially shown to suppress nonsense mutations associated with Duchenne muscular dystrophy (DMD) and cystic fibrosis in mouse models (2).

The success of suppression therapy to provide a therapeutic benefit in various individuals depends on many factors. One particularly important factor is the threshold of correction for a particular disorder that varies upon the function of the factor and the tissues where the protein is expressed. For example, for some disorders that result from an

enzyme deficiency such as mucopolysaccharidosis type I-Hurler (MPS I-H), as little as 1% of wild-type enzymatic activity can significantly alleviate the disease phenotype (33).

FACTORS AFFECTING THE RESPONSE TO READTHROUGH

Several factors were reported to affect the efficiency of stopcodon readthrough treatment including the identity of the PTC, the sequence context around the PTC and nonsense mediated decay (NMD). UGA stop codon exhibit the highest readthrough efficiency, followed by UAG and, to a lesser extent, UAA (5,34). Regarding the effect of sequence context, the fourth position of the tetranucleotide also plays a role in determining the efficiency of readthrough; however, its effect depends on largely to the PTC itself (4,5). For example, the UGA C was shown to exhibit a three-to sixfold higher level of readthrough than the other UGA (N) signals. The relative order of susceptibility to readthrough as a function of the fourth base was C>A, G>U. However, readthrough of the UAG C and UAA C signals was not significantly higher than the readthrough observed at the other UAG (N) and UAA (N) signals, respectively (5).

Gentamicin treatment of C2C12 mouse myoblast cells transfected with PTC-bearing dual luciferase vector resulted in ~8% readthrough for UGA C while little measurable increase in readthrough was observed for UAA A (*mdx*) premature stop codon for Duchenne's muscular dystrophy (DMD) and Becker's muscular dystrophy (BMD). Gentamicin-induced readthrough levels for eight PTCs identified in DMD and BMD patients varied between approximately 1 and 10% and roughly paralleled as a function of the fourth base (UGA>UAG>UAA; +4 C>U>G≥A) (35).

Chemical composition of aminoglycosides is another factor affecting the response to readthrough. When compared in human cells expressing reporter constructs, gentamicin and paromomycin were most effective at inducing readthrough while tobramycin and neomycin showed lower readthrough efficiency than gentamicin, amikacin and paromomycin (35).

One factor leading to low readthrough efficiency is the low amount of PTC-bearing transcripts caused by nonsense mediated decay (NMD) which degrades mutated mRNAs (36). NMD is a surveillance system detecting and commiting PTC-bearing transcripts to rapid decay to prevent the synthesis of unstable proteins that might be deleterious for the cell (37). NMD is an evolutionary conserved mechanism to be implicated in surveillance and regulation of gene expression in all eukaryotes (38). NMD downregulates not only PTCs but also one-third of alternatively spliced mRNAs, certain selenoprotein mRNAs, some mRNAs that have upstream open reading frames, and some mRNAs that contain an intron within the 3´ untranslated region (39,40).The NMD process could be relevant in terms of the phenotypic presentation of human diseases caused by nonsense mutations. In some cases, the lack of a mutant protein due to NMD could result in a milder phenotype since the deleterious effect of the aberrant protein is partially abolished. In other cases, the NMD could eliminate a partially active mutant protein and produce a more severe phenotype (41,42). PTCs in mammalian systems are targeted for NMD when located more than 50-54 nucleotides upstream the last exon-exon junction whereas PTCs located downstream of this boundary are not. Recognition of PTC- mRNAs and their targeting for degradation requires a set of conserved NMD effectors, which include the Up-frame shift (UPF) proteins UPF1, UPF2 and UPF3B and some exon junction complex (EJC) proteins (2). The EJC complex was shown to constitute a binding platform for the NMD effectors UPF2 and UPF3 (up-frameshift) (37). When the ribosome reaches a PTC, interaction of the release factors eRF1 and eRF3 with downstream EJCs bridged by the UPF proteins triggers the

phosphorylation of UPF1 and subsequent degradation of the mRNA (for detailed information about NMD, see the reviews (37,43,44,45).

PTC readthrough compounds may increase the stability of mutant RNA by limiting NMD. Infact, several papers have reported that gentamicin and other readthrough agents inhibit NMD and increase the amount of PTC-containing RNAs (4,46,47). In an effort to determine the correlation between the recovery residual enzymatic activity and mRNA expression in response to gentamicin treatment, mRNA expression levels of about 20-40% that of controls for SMPD1 (Nieman-Pick A/B disease) and NAGLU (MPS III disease) genes except IDS gene (MPS II disease) was observed (4). Although these levels did not reach those obtained after treatment with cycloheximide, which is a general translation inhibitor used to assay for the occurrence of NMD, suggesting that gentamicin readthrough was not totally efficient and some mRNA was still being degraded. Interestingly, the gentamicin treated culture of MPS II (Hunter) disease presented mRNA expression levels similar to controls, which is explained by the location of the relavant PTC in IDS gene where it is located in the last exon, so that the resulting mRNA might elude the NMD surveillance mechanism resulting in normal mRNA levels (4). In the same study, gentamicin treatment of two different MPS III patients, one with p.W168X and p.R234C and another with p.W168X and Q566X resulted in approximately 20% and 40% increase in NAGLU gene expression, respectively. Although one of the alleles of MPS III patient (p.Q566X) is located in the last exon of NAGLU gene, mRNA expression in fibroblasts was low (20% of control values), which was explained by the presence of p.W168X mutation that did not elude the NMD surveillance (4).

In a study investigating the readthrough effects of gentamicin, G148 (geneticin) and five non-aminoglycoside compounds (PTC124, RTC13, RTC14, BZ6 and BZ16) on fibroblasts from one patient with MPS IIIB (Sanfilippo B) harbouring p.W168X-/p.Q566X, and one with MPS IIIC (Sanfilippo C) harbouring p.R384X/c.1542+dupA mutations, it was found that although no recovery was detected for relevant enzyme activities, mRNA recovery was observed in both cases, nearly a two-fold increase for Sanfilippo B fibroblasts with G418 and around 1.5 fold increase for Sanfilippo C cells with RTC14 and PTC124 (48).

Strategies have been developed to inhibit NMD and hence increase the expression of PTC containing mRNAs by using small molecules. Through a high-throughput screening, amlexanox was found to inhibit to increase the amount of PTC-bearing mRNAs in cell lines from patients suffering from nonsense-mutation mediated lung cancer, Duchenne muscular dystrophy (DMD) or cystic fibrosis (CF) (49). In addition to acting as NMD inhibitor, amlexanox leads to the readthrough of mutated mRNA and results in the synthesis of full-lenght protein (49). Amlexanox was found to be as potent as G418 and PTC124 and more effective than combinations of them at higher concentrations due to the combined function of amlexanox in both NMD and readthrough processes (49).

CONCLUSION

Premature termination codons (PTCs) or nonsense mutations account for about 11% of all described gene defects that cause inherited diseases. Stopcodon readthrough, also called nonsense suppression, hold promise as a therapeutic strategy for the treatment of a broad range of genetic diseases caused by nonsense mutations. An advantage of stopcodon readthrough therapy is that it can be applied to any disease provided that the molecular cause is a primary nonsense mutation in which the PTC results directly from a point mutation in the DNA. In the case of neurological lysosomal storage diseases, additional advantage is the potential penetrance through the blood brain barrier. The fact that a slight recovery of protein levels could

be enough to alleviate disease phenotype, and potential penetrance of readthrough drugs through the blood brain barrier makes the stopcodon readthrough therapy an ideal treatment method mainly in diseases with neurological symptoms that are caused by nonsense mutations and for which enzyme replacement is not an option. As the resulting readthrough proteins contain a different amino acid that might cause misfolded protein, combined application of readthough drugs with pharmacological chaperones or proteostasis regulators should be considered.

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