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PHARMACOLOGICAL APPROACHES FOR TARGETING CYSTIC FIBROSIS NONSENSE MUTATIONS

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Abstract

Cystic fibrosis (CF) is a monogenic autosomal recessive disorder. The clinical manifestations of the disease are caused by ~ 2,000 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. It is unlikely that any one approach will be efficient in correcting all defects. The recent approvals of ivacaftor, lumacaftor/ivacaftor and elexacaftor/tezacaftor/ivacaftor represent the genesis of a new era of precision combination medicine for the CF patient population. In this review, we discuss targeted translational readthrough approaches as mono and combination therapies for CFTR nonsense mutations. We examine the current status of efficacy of translational readthrough/nonsense suppression therapies and their limitations, including non-native amino acid incorporation at PTCs and nonsense-mediated mRNA decay (NMD), along with approaches to tackle these limitations. We further elaborate on combining various therapies such as readthrough agents, NMD inhibitors, and corrector/potentiators to improve the efficacy and safety of suppression therapy. These mutation specific strategies that are directed towards the basic CF defects should positively impact CF patients bearing nonsense mutations.

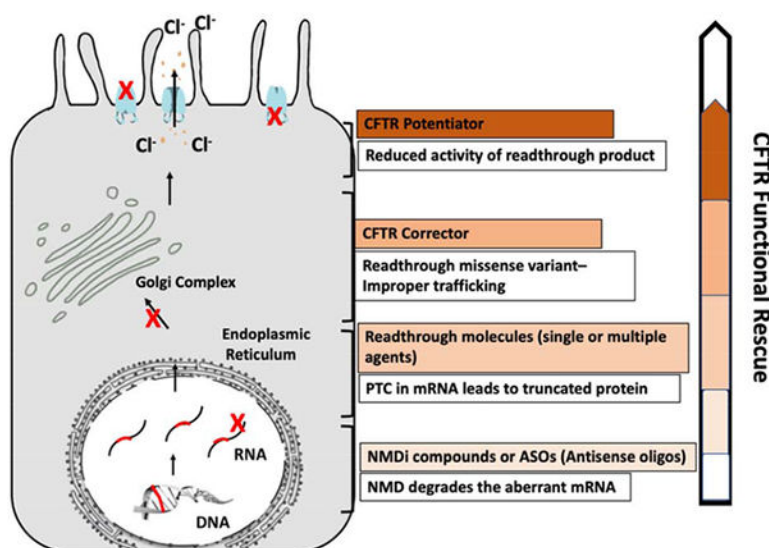
Graphical Abstract

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Targeted interventions that can be combined to rescue maximum CFTR function

Keywords

CFTR; nonsense mutations; translational readthrough; combination therapy

Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disease that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1–3). This gene encodes a cAMP-regulated anion channel that mediates Cl⁻ and HCO₃⁻ ion transport in the epithelia of airways, pancreatic ducts, and other tissues (4, 5). The CFTR protein is a member of the ATP-binding cassette class C (ABC-C) family. Consistent with other proteins in this class, it contains two membrane-spanning domains (MSD1 and MSD2) and two nucleotide-binding domains (NBD1 and NBD2) that require ATP binding for channel gating. In addition, CFTR exhibits a unique feature not found in other ABC family members, an intracellular regulatory domain (R domain) that normally requires phosphorylation for channel opening (6, 7). Improper CFTR channel function leads to dysfunction of epithelial tissues in the lung, gastrointestinal tract, pancreas, and reproductive system.

More than 2,000 *CFTR* gene variants have been identified, many of which are confirmed to be disease-causing (8). Due to the large number of known CFTR mutations, they have been categorized into six different classes based on their effect on CFTR protein expression and function: class I (no protein production), class II (defective protein processing and trafficking), class III (abnormal channel gating), class IV (reduced ion conductance), class V (reduced protein expression through aberrant splicing), and class VI (faulty recycling) (1, 9, 10). While CFTR mutation classification is a useful framework for devising therapeutic approaches to repair molecular defects in CFTR (see Figure 5 in Rowe et al (1)) it is imperfect since many mutations exhibit more than one feature (10) and some molecular

approaches extend beyond one specific therapeutic class. The quantity and the function of these disease-causing CFTR variants are the primary determinants of the clinical severity in individual CF patients.

A number of therapeutic compounds called ‘CFTR modulators’ have been identified that restore the expression or function of CFTR (3, 11). Among CFTR modulators, CFTR ‘correctors’ fundamentally target the cellular processing of mutant CFTR protein to ensure its proper folding, maturation, and trafficking to the cellular membrane (12–15). CFTR ‘potentiators’ enhance CFTR channel activity at the cell surface by improving open channel probability, thereby augmenting channel gating (3, 16–18). CFTR modulators are often used in combination to address more than one CFTR defect in a given mutation; the seminal example of this is co-administration of correctors and potentiators to restore activity to F508del CFTR, which exhibits both folding and trafficking abnormalities as well as defective channel gating. Together, these CFTR modulators have demonstrated eminent clinical success in CF patients with mutations that maintain residual CFTR function (class II, III, IV, and others) (19, 20). It is estimated that correctors and potentiators are likely to provide a therapeutic benefit in up to 90% of CF patients, as F508del, gating, and conductance mutations have all exhibited clinical efficacy (21–23).

While current modulator compounds represent a viable treatment option for many CF patients, no pharmacological options are available to individuals who carry nonsense mutations, a subtype of class I mutations that abrogate CFTR protein expression. Nonsense mutations represent single nucleotide point mutations that generate an in-frame premature termination codon (PTC) in the open reading frame (ORF) of *CFTR* mRNA, causing cessation of translation and preventing the synthesis of full-length protein (24). Additionally, a PTC often triggers nonsense-mediated decay (NMD), a conserved eukaryotic mRNA surveillance pathway that degrades aberrant mRNAs containing a PTC, reducing the pool of PTC-containing transcripts for translation and further limiting the synthesis of truncated or full-length CFTR protein (25–32). These PTC-mediated mechanisms lead to a complete absence of CFTR protein function and thus, with few exceptions, do not respond to the current CFTR modulator compounds that require at least partial CFTR protein expression. Therefore, despite tremendous efforts by the CF research community, there remains an unmet need for the CF population that bears nonsense mutations. In this review, we will discuss the potential of using small-molecule pharmacological agents to suppress PTC mutations in CF patients who carry nonsense mutations.

Potential Treatments for Class I CFTR Mutations

Class I mutations completely abrogate CFTR function and affect more than 11% of the CF population (33). The majority of these Class I lesions are comprised of nonsense mutations (e.g. Y122X, R553X, G542X, and W1282X) (www.cff.org), with the remaining mutations represented by major gene disruptions such as chromosomal deletions, insertions, and other frameshift mutations, including canonical splice site mutations (which overlap with Class V variants) (33). Since 12% of all known disease-causing gene lesions are nonsense mutations according to the Human Gene Mutation Database (34, 35), the prevalence of nonsense mutations in CF patients is not unusual.

Similar to other genetic diseases (24, 36), nonsense mutations are often associated with the most severe form of CF due to a complete absence of CFTR protein. This loss of CFTR can be attributed to premature translation termination as well as nonsense-mediated mRNA decay (NMD), which reduces mRNA stability (37). To address protein deficiencies attributable to in-frame premature termination codons (PTCs) arising from nonsense mutations, a number of different approaches are being explored to suppress translation termination specifically at PTCs, with the aim of restoring sufficient protein expression and function to alleviate genetic diseases. One approach to restore expression of CFTR (or deficient proteins in the setting of other genetic diseases) is translational readthrough, where termination at PTCs is suppressed by the insertion of aminoacyl-tRNAs at a PTC by incorporation into the ribosomal A-site. At a low frequency, near-cognate tRNAs can outcompete the translation termination factor, eRF1 (eukaryotic release factor 1) for binding to A-site bound PTCs (Figure 1). This leads to the incorporation of an amino acid into the polypeptide chain at the site of the PTC, allowing the translation elongation to continue in the correct reading frame until the next in-frame stop codon is encountered (38–40). This readthrough mechanism is being exploited for therapeutic purposes and has shown some success in preclinical studies for CFTR nonsense mutations (38, 41, 42). There are other approaches that target PTCs, including suppressor tRNAs, RNA-directed pseudouridylation, NMD inhibition and gene editing, but these therapeutic strategies are beyond the scope of this review (34, 43, 44).

Translation Termination Overview

Translation termination is a highly efficient process, with >99% efficiency at natural termination codons (NTCs). Translation termination is triggered by the entry of a stop codon (UAA, UAG, or UGA) into the ribosomal acceptor site (A-site). Unlike the decoding of sense codons by tRNAs, stop codons are decoded by a complex of proteins called the termination complex. The termination complex is minimally composed of the eukaryotic release factors 1 (eRF1) and 3 (eRF3). eRF1 binds directly to stop codons located in the ribosomal A site, akin to tRNA binding of conventional codons. The overall three-dimensional structure of eRF1 is similar to that of a tRNA (45). eRF1 bears several conserved elements, including the Asn-Ile-Lys-Ser (NIKS), Gly-Thr-Ser (GTS), and YxCxxxF (YCF) motifs that participate in stop codon recognition with high specificity (46, 47). eRF3, a GTPase, binds to eRF1 to facilitate stop codon recognition (48). eRF1 also contains a unique Gly-Gly-Gln (GGQ) motif that extends into the peptidyl transferase center and promotes liberation of the polypeptide upon GTP hydrolysis by eRF3 (49–53). These structural motifs represent potential targets for structural-based drug design.

This mechanism of stop codon recognition is similar at both natural and premature stop codons. However, the frequency of readthrough at PTCs is approximately 10-fold greater (0.01–1%) than the readthrough rate at NTCs (0.001–0.1%) (24, 54–56). Various factors likely mediate this difference in termination efficiency at PTCs. For instance, the interactions between the cytoplasmic poly(A) binding protein (PABPC1) and eRF3, which facilitates efficient translation termination, are likely to be less efficient at a PTC due to its increased distance from the 3'-UTR (3'-untranslated region) (40, 57, 58). Furthermore, ABCE1/RLI1, an essential member of the ABC family of proteins, plays an important role

in translation initiation, termination and ribosomal recycling. ABCE1/RLI1 stimulates eRF1-mediated peptidyl hydrolysis and the dissociation of the post-translation complex into free 60S subunits, mRNA-, and tRNA-bound 40S subunits for use in subsequent rounds or translation (59). The efficiency of ABCE1/RLI1 binding to eRF1 at PTCs may also be subverted due to the increased distance of PTCs from the 3' UTR, altering otherwise favorable stoichiometry for this process to occur (60). This distinction in translation termination efficiency at normal termination codons as compared to PTCs lays the foundation for pharmacologic approaches for diseases caused by PTC mutations.

Potential Therapeutic Targets for Readthrough

Recent studies that have examined the molecular interactions occurring during translation termination have identified subtle changes in the spatial orientation of various factors associated with the termination complex (61). While eRF1 is the main catalytic factor for translation termination, other factors have also been found to play important roles in mediating termination efficiency (Figure 1). For example, the cytoplasmic poly(A)-binding protein (PABPC1) binds to the poly(A)-tail of mRNAs and interacts with the translation initiation factors eIF4E and eIF4F to circularize the mRNA during translation, which increases mRNA stability and enhances translation rates (62–64). However, it has been shown recently that PABPC1 enhances translation termination efficiency through its interaction with the termination complex (65–67), specifically *via* eRF3. Thus interfering with these interactions could represent an approach to augment translational readthrough, particularly if it could be designed in such a way as to preferentially block binding at PTCs as compared to NTCs.

As another example, several translation initiation factors recently have been shown to be important contributors to the translation termination process, elucidating a stringent association between various phases of translation (68–70). Deletion of HCR1 (a subunit of eIF3 that has a principle role in ribosomal recycling) increases readthrough activity by promoting GDP-eRF3 release from the ribosomes (70, 71). In addition, eIF3j is known to bind at the ribosomal decoding center (72), which serves a proofreading function during both elongation and termination (73, 74).

Studies in *Saccharomyces cerevisiae* have identified additional factors that play significant roles in translation termination, and may also be relevant to PTC readthrough in mammalian cells (75). The ATP-dependent DEAD-box RNA helicase Dbp5 (homologous to DDX19 in mammals) is generally associated with the nuclear pore complex and is involved in transfer of mature mRNA/protein complexes into the cytoplasm. During the formation of the translation termination complex, the mRNA export factor Gle1p, in association with inositol hexakisphosphate (IP₆), directs Dbp5 to properly position eRF1 at stop codons in ribosomal A site. Dbp5 also recruits eRF3 to the termination complex (69, 75–77). Discovery of these and other novel aspects of the translation termination machinery suggests the potential for exploring these pathways to identify new ways to stimulate readthrough at PTCs.

Importance of Local PTC Sequence Context for Readthrough Efficiency

Biochemical and genetic studies, and more recently, ribosomal profiling, have shown that the stop codon and the mRNA sequence context surrounding the stop codon significantly contribute to translation termination efficiency (55, 78–80). Differences in readthrough susceptibility have been found based on the identity of the stop codon. In general, UGA is most susceptible to readthrough followed by UAG and UAA. The nucleotide directly following a stop codon (the +4 nucleotide) also has a strong effect on readthrough susceptibility, where the presence of a cytidine downstream of a stop codon generally correlates with more permissive readthrough (55, 56).

Extended mRNA context may also affect readthrough efficiency. At least 6 nucleotides of local upstream and downstream of a stop codon has been shown to affect the efficiency of translation termination (81–84). Cridge *et. al* recently showed that the +5, +6 and +8 nucleotides downstream of the UGA stop codon has a strong influence on translation efficiency and fidelity (78). In addition, the presence of two adenines at two positions 5' of stop codon are associated with a high frequency of readthrough, irrespective of the stop codon or downstream mRNA sequences (85). More distant sequence context may also affect readthrough efficiency. For example, approximately 30 nucleotides are present within the ribosomal mRNA channel, which could affect the efficiency of translation termination or the recruitment of near-cognate aminoacyl-tRNAs (78).

The sequence context has been shown to affect the clinical severity of genetic disorders by affecting the frequency of spontaneous readthrough of a PTC and therefore, the amount of residual protein function. In a patient with junctional epidermolysis bullosa due to nonsense mutations in the *LAMA3* gene (R943X/R1159X) (41, 86), the R943X allele encoded a hypothetical consensus sequence (AGU-UGA-CUA) that is thought to be amenable to readthrough. In addition, the *LAMA3* mRNA levels were relatively preserved in this patient. This resulted in a sufficient amount of spontaneous readthrough to produce full length laminin $\alpha 3$ protein, ameliorating disease severity (86). This important finding shows that the PTC context may help predict phenotypic severity, and is likely to be important in selecting a readthrough treatment for specific PTC mutations to maximize the potential for efficacy, especially if the competition between readthrough and termination is only modestly affected by treatment.

Readthrough as a Therapeutic Approach for CF and Duchenne's muscular dystrophy

Nearly two decades ago, suppressing translation termination at nonsense mutations was first described as a potential therapeutic approach for genetic diseases caused by nonsense mutations, with CF being the first disease model in which this approach was examined (87). Multiple studies have shown that some aminoglycoside antibiotics promote translational readthrough in cell (56, 57, 87) and animal models (88–90). By binding to the ribosomal decoding site, aminoglycosides can inhibit ribosomal proofreading and enhance the frequency of near-cognate tRNA accommodation at A-site bound PTCs (73, 74). This allows rescue of full-length protein synthesis, with the potential of restoring at least partial function

depending on the sensitivity of the protein product to the amino acid inserted at the PTC (73, 90).

In a subset of Duchenne muscular dystrophy (DMD) patients carrying nonsense mutations, the aminoglycoside, gentamicin, increased dystrophin expression and reduced serum creatinine kinase levels (91, 92). Similarly, pilot clinical trials in which gentamicin was administered to CF patients showed restoration of CFTR protein and function in nasal epithelium in approximately half of the patients (93, 94). However, brief proof-of-concept studies in CF patients (93–95) found that neither amikacin nor gentamicin restored enough functional CFTR protein to impart long-term clinical benefit (96). These studies indicate that aminoglycosides can restore full-length functional protein *via* readthrough of PTCs, but only in a fraction of patients. In addition, administration of aminoglycosides for lifelong genetic diseases is not feasible due to their association with off-target sites such as mitochondrial ribosomes (56, 90, 97) and lysosomal membranes (98, 99), leading to the renal and ototoxicity (99, 100).

To improve the efficiency of aminoglycosides to promote readthrough while also reducing their toxicity, a medicinal chemistry approach was used to synthesize aminoglycoside derivatives that bind more tightly to cytoplasmic ribosomes and less tightly to mitochondrial ribosomes (101, 102). ELX-02 (formerly NB124), was developed by Eloxx Pharmaceuticals (Herzliya, Israel) to bring this approach to fruition (chemical structures of gentamicin, amikacin and ELX-02 in Figure 2). ELX-02 is less toxic than conventional aminoglycosides and more efficiently promotes readthrough than gentamicin in mammalian cells (103, 104). For example, in human bronchial epithelial cells, gentamicin did not restore CFTR function, while NB-124 and similar derivatives restored ~7% of wild-type CFTR activity (103, 105). ELX-02 established a better safety and pharmacokinetics profile than conventional aminoglycosides as observed in two Phase 1a, randomized, double-blind placebo- controlled studies in healthy volunteers (106). ELX-02 is currently advancing to Phase 2 clinical trials for both CF and cystinosis patients who carry the G542X nonsense mutation (101, 106–109). Given gentamicin improved sweat chloride in patients with particularly susceptible nonsense codons (110), and the strong association of sweat chloride response and clinical outcome in other mutations of CF (111), this represents a critical near-term effort for people with CF caused by nonsense mutations. ELX-02 as part of multi-modal CFTR therapy could also be a consideration for future studies.

In addition to the structural modification of aminoglycosides, co-administration of anionic poly amino acids and antioxidants have been shown to reduce aminoglycoside toxicity while maintaining their ability to promote readthrough (112–114). For example, the co-administration of poly-L- aspartic acid with gentamicin was shown to significantly increase the duration of CFTR functional rescue via readthrough compared to gentamicin treatment only (115). Other treatments that reduce the toxicity of aminoglycosides are also in development and could be employed for chronic therapy (116–118).

In an effort to identify more potent, safe, and orally bioavailable readthrough agents for CF and other genetic diseases, PTC Therapeutics, Inc. performed a high throughput screen (HTS) of ~800,000 small molecules using firefly luciferase-based readthrough reporters.

From this screen, a hit was identified that was developed into the lead compound, ataluren (also known as PTC124 or translarna, Figure 2) (119). Preclinical studies demonstrated an acceptable safety profile, good oral bioavailability, and an absence of antibacterial activity, distinguishing the agent from aminoglycosides (120, 121). Ataluren was shown to restore dystrophin protein expression in human primary muscle cells and in the mdx mouse model of Duchenne muscular dystrophy (119). Further, ataluren has also been found to restore the CFTR expression and function in a humanized CFTR-G542X transgenic mouse model (122). However, recent randomized, double-blind, placebo-controlled Phase 3 studies showed that ataluren was unable to restore enough CFTR function in CF patients to improve lung function. A confounding factor in these trials is that the readthrough activity of ataluren is significantly inhibited by tobramycin, an aminoglycoside often administered to CF patients for acute and chronic use (39, 123). However, subsequent evaluation of ataluren efficiency in CF patients without tobramycin administration did not lead to significant improvements in lung function or other clinical endpoints, terminating further development of ataluren as a therapeutic for CF (123). The approach to optimize the compound during preclinical development may also have been impacted by stabilization of the luciferase reporter (124). A likely conclusion from these studies is that we are still lacking an effective readthrough therapy for the CF population bearing nonsense mutations and that more effective and safer readthrough agents are needed. Primary human bronchial epithelial (HBE) cells or organoids served as preclinical models to determine the efficacy of potentiators and correctors of CFTR modulator therapies, which advanced in parallel with the development of ataluren. These novel CF models provided an opportunity to retrospectively evaluate ataluren in novel CF model systems. For example, in rectal organoids derived from CF patients harboring nonsense mutations, ataluren was unable to restore CFTR function (125) and our group generated similar unpublished results in primary HBE and HNE cells from CF donors. Future studies may benefit from the use of CF primary cell models and organoids as a benchmark to measure the efficacy of readthrough agents to restore CFTR function to therapeutic levels.

Hurdles to Implementing Readthrough Therapy as a Treatment for CF

Multiple challenges stand in the way of achieving efficient readthrough for treating CF in patients who carry a nonsense mutation. For example, the abundance of mRNA encoding nonsense mutations available for translation and subsequent readthrough is often reduced by the NMD pathway (Figure 3). For the fraction of PTC-containing mRNAs that remain to be translated, the local mRNA context surrounding a PTC influences the pattern of amino acid incorporation at a PTC, affecting the protein stability, folding and/or function of the readthrough product (39, 80). The amino acids incorporated during G418-mediated readthrough of the CFTR G542X mutation (a TGA stop codon) consisted of cysteine, tryptophan, or arginine (39). In contrast, readthrough of the CFTR W1282X mutation (also a TGA) with G418 led to the incorporation of leucine, cysteine or tryptophan (80). Thus, only a fraction of the full-length protein generated by readthrough contained the native amino acid, with the other proteins generated by readthrough contained a non-native amino. Thus, the local sequence surrounding a PTC can alter the amino acids incorporated at a PTC during readthrough. Furthermore, the different variant proteins resulting from readthrough

showed differences in CFTR channel maturation, stability and function (80), depending on the amino acid inserted and its physiochemical properties as compared to the native codon (see the graphical abstract Figure). These barriers to readthrough therapy have led to investigations to identify novel approaches to further enhance readthrough efficacy, including the combination of multiple treatments to overcome these challenges, as described below.

NMD Inhibition to Augment mRNA Substrate for Readthrough

Decreased abundance of PTC-containing CFTR transcripts due to their sensitivity to NMD limits the efficacy of nonsense suppression therapy (126, 127). Approaches for inhibiting NMD to enhance the availability of mRNA substrates for readthrough therapy include using pharmacological inhibitors (e.g. NMDI-1, NMDI-14, (Figure 2) and SMG1i) (128, 129), small interfering RNAs (siRNA), and antisense oligonucleotides (ASOs) that target NMD factors, attenuating NMD activity (43, 129–131). The potential interconnection between NMD attenuation and readthrough has been shown *in vitro* and *in vivo*, including human subjects (24, 89, 127, 129). Patients with higher transcript levels have repeatedly been found to respond better to readthrough therapy because of the availability of more substrate available for translation. This association was also seen in CF patients bearing the W1282X mutation, where improvements in sweat chloride and nasal potential difference measurement were observed following gentamicin treatment in patients with higher levels of *CFTR* mRNA abundance due to less efficient NMD (127). Similar associations between mRNA levels and readthrough efficacy were also observed in the initial trials of ataluren (132, 133).

Pharmacologic approaches have been attempted to recapitulate the association between increased mRNA abundance and improved readthrough efficacy. Co-administering gentamicin with the NMD inhibitor NMDI-1 generated increased efficacy in an Mucopolysaccharidosis I-Hurler (MPS I-H) mouse model by enhancing the restoration of α -L-iduronidase activity, leading to greater reduction of glycosaminoglycan storage in mouse tissues (129). ASOs that specifically inhibited the Upf3b NMD factor, combined with G418, also increased efficacy in restoring full-length functional hFIX protein in hemophiliac mice bearing the R29X nonsense mutation (130). Furthermore, inhibiting NMD using an ASO directed against SMG1 showed significant rescue of CFTR protein and function in an immortalized primary human bronchial epithelial cell line modified by CRISPR/Cas 9 methodology to express the CFTR W1282X mutation (16HBEge cells) (134, 135). In total, these approaches indicate that inhibition of NMD may improve the therapeutic benefits of readthrough therapy. However, caution must be taken, in addition to degrading PTC-bearing mRNAs, NMD regulates the levels of many physiological transcripts (136–138), plays an important role in embryonic development (137, 139), and appears to influence multiple cellular pathways (140, 141). Thus, additional work is needed to determine whether NMD inhibition can be safely implemented as a therapeutic approach. Even if suitable at low levels in adults, approaching the pathway in children may raise particular safety issues.

CFTR Modulators to Augment Readthrough Therapy for CF

CFTR modulators, which have received US Food and Drug Administration (FDA) approval, primarily consist of two classes of compounds: 1) CFTR correctors, which facilitate protein processing and trafficking, and 2) potentiators, which activate the CFTR channel (142–144). These agents have been approved as a combination therapy to treat CF patients with responsive mutations (2). The CFTR potentiator ivacaftor (formerly VX-770, Figure 2) is effective with multiple CFTR missense mutations where adequate levels of CFTR is present at the cell surface (145). In an single patient study, an individual homozygous for the W1282X mutation showed significant benefits with ivacaftor treatment, where lung function was stabilized and improvements in pulmonary exacerbation frequency, BMI, and insulin requirement were observed (146). The beneficial effects of ivacaftor are largely due to the presence of sufficient levels of truncated W1282X CFTR protein being present at cell surface (146), which can be activated even in the truncated state (147). Haggie et.al has also identified an novel series of small molecules, correctors and potentiators, with potentially greater efficacy for CFTR W1282X mutation (148). These may also be applicable to other terminal (i.e. 3') mutations when sufficient CFTR mRNA is present (149, 150). However, CFTR correctors and potentiators, alone or in combination, have not exhibited efficacy for CFTR nonsense mutations where cell surface expression of truncated protein is absent, such as G542X, or NMD eliminated protein expression (151).

Beyond the efficacy for CFTR mutations, when combined with readthrough agents, CFTR modulators (correctors and potentiators) have shown improvements in CFTR cell surface expression and function in various *in vitro* studies (80, 103, 146) by acting on the full-length CFTR protein restored *via* readthrough; this may be particularly important for missense CFTRs generated upon readthrough (152). We have recently shown that adding CFTR corrector and potentiator treatments doubled the amount of CFTR activity rescued after PTC suppression of the CFTR G542X and W1282X mutations compared to readthrough treatment alone (80). Altogether, these studies suggest the importance of CFTR modulator treatments in combination with PTC suppression therapies is likely applicable for multiple nonsense mutations to augment the function of the readthrough product.

Beyond the CFTR modulators that are already clinically available, alternative CFTR modulators that are specifically designed for PTC readthrough products may have particular advantages, whether as single agents or as part of combination treatments. ASP-11, a novel potentiator for the CFTR W1282X mutation, significantly enhanced CFTR channel activity in combination with ivacaftor in cells derived from a G542X/W1282X patient (153). Additional CFTR modulators are currently being developed by Galapagos/Abbvie, Flatly Discovery, and Vertex Pharmaceuticals that may also increase the functionality of proteins generated by readthrough (154). As a prime example, Trikafta, a combination of three CFTR modulators (elexacaftor and tezacaftor are CFTR correctors; ivacaftor, a CFTR potentiator, Figure 2) has been shown to be beneficial in 90% of CF population who carry at least one F508del allele (21–23). However, its efficacy for the CF population bearing a nonsense mutation remains to be determined, and those with a nonsense mutation in trans with an F508del allele did not have an appreciably different response to elexacaftor/tezacaftor/ivacaftor (22).

Dual Readthrough Agent Combination Therapy

Multi-agent corrector therapy has been used to augment F508del processing and trafficking (15, 155, 156). A similar approach could be used to augment readthrough efficacy (157) by combining readthrough agents with distinct mechanisms of action. There are multiple factors involved in translation termination, representing potentially distinct therapeutic targets (Figure 1). Studies in *Saccharomyces cerevisiae* has shown that translation termination efficiency is dependent on the levels of eRF1 and eRF3 (158). Overexpression of eRF1 increases translation termination efficiency (159); conversely, reduced rates of eRF3 GTP hydrolysis diminishes translation termination efficiency (48). These studies lay the foundation for exploring potential small molecule therapies that can alter the levels of eRF1 or eRF3 to prolong ribosomal pausing at PTCs, providing sufficient time for near-cognate tRNAs to become accommodated at a PTC to promote readthrough. As explained earlier, other factors such as ABCE1 (59), PABPs (65), eIF3 (71) and eIF3j (72) could be exploited for developing novel nonsense suppression therapeutic targets.

Alternatively, phthalimide derivatives are a class of small molecules that have recently been shown to act as enhancers for aminoglycoside-mediated readthrough. The use of enhancer molecules may enable the administration of aminoglycosides at much lower doses in order to minimize their toxicity while also enhancing their readthrough ability for nonsense mutations (160, 161). However, the discovery of how these small molecules function is needed to understand their therapeutic efficacy and to further optimize them. This further raises the hope for combination therapies, where readthrough agents that work by different methods of action can be used to produce the level of translational readthrough that is needed to benefit the CF population bearing nonsense mutations.

Summary

Lessons learned from clinical experiences, significant heterogeneity in response based on characteristics of the nonsense allele, and an improved understanding of challenges of nonsense suppression therapy at the mechanistic level has opened a new horizon for combining therapies to treat CF patients harboring nonsense mutations. The therapeutic paradigm for developing CF modulator combination therapies and the magnificent success of drugs that target mutation-specific molecular defects of CFTR (21, 162, 163) raises the hope for combination treatment therapies that treat CFTR nonsense mutations. Combinations of small molecules that address CFTR protein synthesis, stability, folding and transportation may help enhance the therapeutic levels of CFTR function restored by readthrough. The high therapeutic threshold (estimated as high as 30–35% of normal CFTR function) needed for correction makes it challenging to attain adequate efficacy by nonsense suppression monotherapy, but this will need to be balanced with the risk of toxicity and drug-drug interactions associated with combination therapies (105). Beyond additional treatment options, future research should focus on early identification and selection of appropriate cell or animal models that best recapitulate the disease phenotype to identify the appropriate combination approaches.

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Highlights

- Translational readthrough therapy for CFTR nonsense mutations
- Translation termination factors are crucial targets for nonsense suppression therapy
- NMD inhibition approaches may be effective to enhance the substrates for nonsense suppression therapy
- Combine various approaches to enhance the efficacy for nonsense suppression therapy

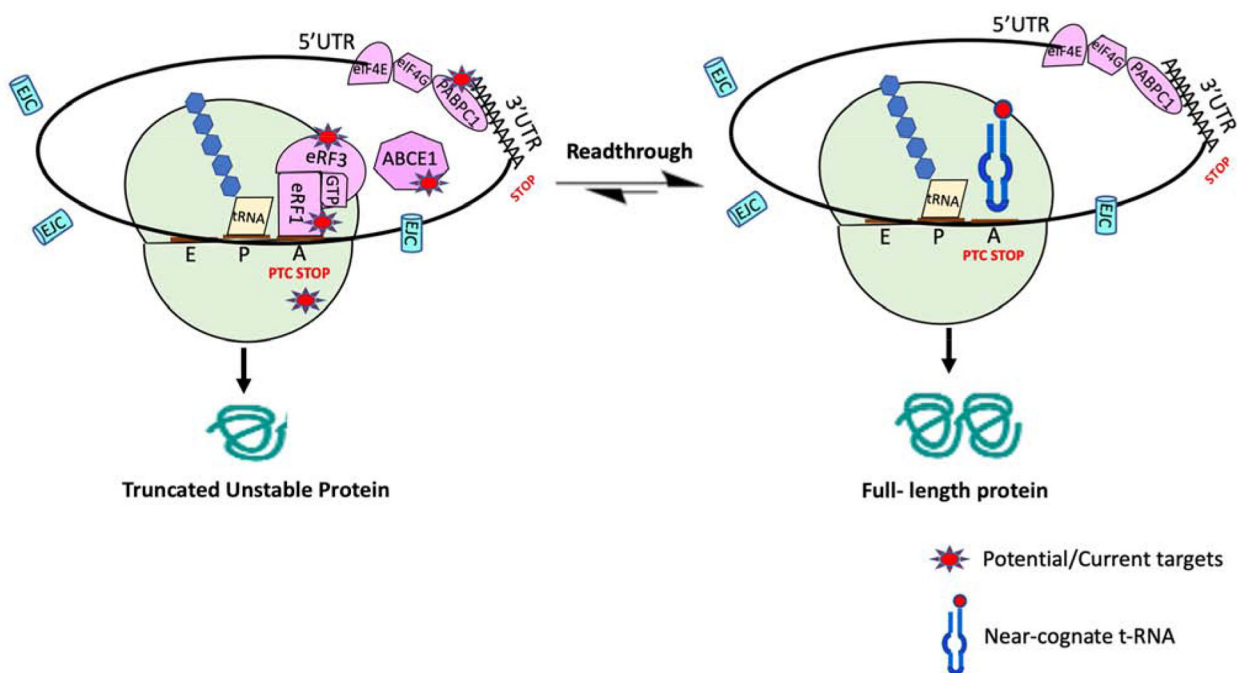
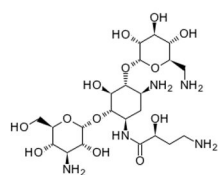
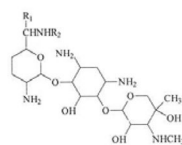


Figure 1. Translation termination versus readthrough at PTCs:

eRF1 and eRF3 bind to a stop codon when it enters the ribosomal A site. PABPC1 binds to eRF3 and promotes efficiency termination. ABCE1 assists in polypeptide release and mediates ribosomal recycling. When a PTC enters the A site, a truncated polypeptide is generated. At a low frequency, near-cognate tRNAs compete with the eRF1/eRF3 complex for binding to A-site bound PTCs, allowing translation elongation to continue in the original ribosomal reading frame to produce a full-length protein. Several therapeutic approaches are attempting to skew this competition more toward elongation in order to generate more full-length protein.

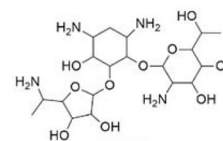
Readthrough Agents:

Amikacin

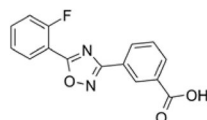


R₁ = R₂ = CH₃ Gentamicin C₁
 R₁ = CH₃ R₂ = H Gentamicin C₂
 R₁ = R₂ = H Gentamicin C_{1a}

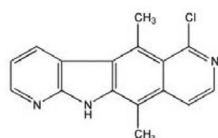
Gentamicin



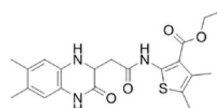
ELX-02



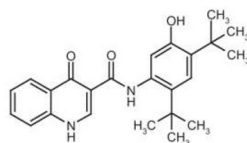
Ataluren

Nonsense Mediated Decay Inhibitors:

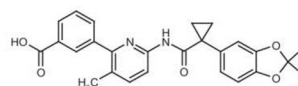
NMDI-1



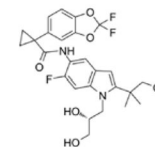
NMDI-14

CFTR Modulators:

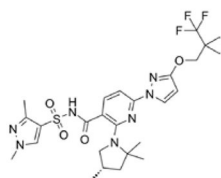
Ivacaftor



Lumacaftor



Tezacaftor



Elexacaftor

Figure 2.
 Chemical structures of translational readthrough agents, potential nonsense mediated decay inhibitors, and CFTR modulators.

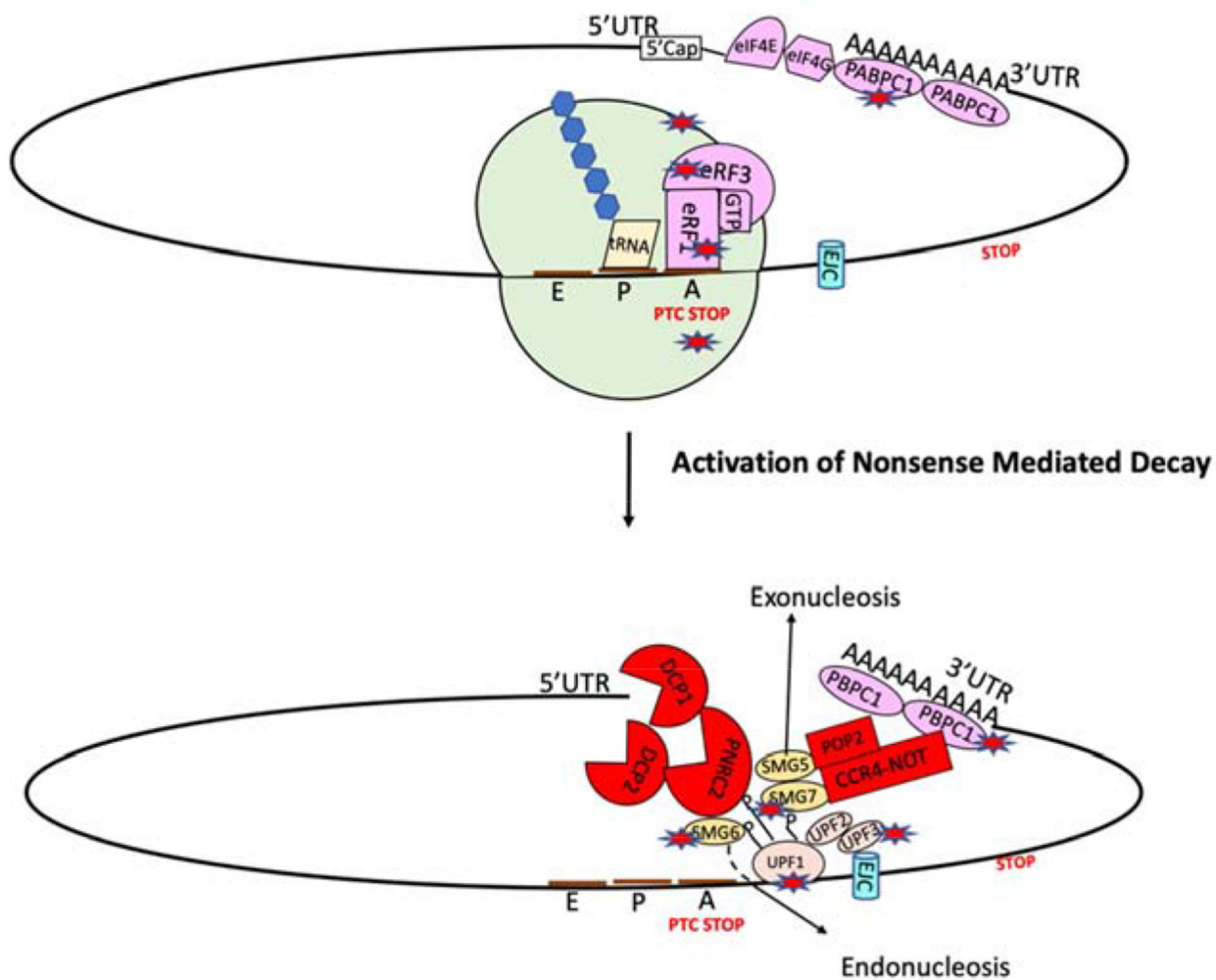


Figure 3. Schematic illustration of the aberrant translation termination resulting in the activation of nonsense-mediated mRNA decay (NMD).

When a ribosome encounters a PTC, the termination factors eRF1 and eRF3 are recruited to the PTC. UPF1 then binds to the termination complex and becomes phosphorylated, allowing an interaction with UPF2 and/or UPF3. This association recruits decay factors, including the SMG6 endonuclease and the SMG5–7 heterodimer, which subsequently recruit the CCR4/NOT complex to promote decay of the mRNA. Star represents the potential therapeutic target.