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Combining Translation Readthrough Inducing Drugs and Nonsense Mediated Decay Pathway Inhibition to Rescue of CFTR^W1282X in Cystic Fibrosis Cell Model System

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Abstract: Nonsense mutations affect about 11% of patients with Cystic Fibrosis and produce a premature termination codon in Cystic Fibrosis Transmembrane Conductance Regulator (_CFTR)_ mRNA causing early termination of the polypeptide translation. A potential therapy for nonsense mutations allows small molecules to overcome the premature termination codon (PTC) by a readthrough mechanism leading to the synthesis of a complete CFTR protein. However, the efficiency of the readthrough molecules is limited by a quality control cellular pathway known as Nonsense-mediated mRNA Decay (NMD). This pathway provides the degradation of the mRNA harboring a premature termination codon, to prevent the production of altered polypeptides. In contrast, the activity of this pathway interferes with the effectiveness of the readthrough drugs, limiting the CFTR mRNA amount to the rescue. In this work, we reduced the activity of the NMD pathway, silencing the Upf1 protein, one of the main components of the pathway. Our strategy was to increase the amount of the mRNA target to improve the efficiency of readthrough of TRIDs (Translational Readthrough Inducing Drugs) molecules in cells that ectopically express the W1282X-CFTR mRNA. The reduction of the Upf1 expression/activity was correlated with the increase of the efficiency of readthrough and so the increase of CFTR protein rescue.

Keywords: premature termination codon; nonsense mutation; Nonsense-mediated decay; translational readthrough inducing drugs; oxadiazoles

1. Introduction

With 70.000 affected people and a mortality rate of 90%, Cystic Fibrosis (CF) is one of the most widespread lethal genetic diseases [Rafeeq M.M., 2017]. The clinical manifestations of this monogenic disorder are caused by mutations of a single gene, encoding for a channel protein named CFTR (Cystic Fibrosis Transmembrane Conductance Regulator). This protein is expressed on the apical surface of epithelial cells of secretory organs, where it regulates the chloride ion transport and subsequently the osmotic water passage, needful to fluidify the mucus layer of these tissues [Farinha C.M., 2017].

Almost two thousand different mutations of the CFTR gene have been identified as the cause of structural and/or functional alterations of the channel. They are associated to a more or less severe dysfunction of the chloride flux, inducing duct obstruction and tissue damage [Quon B.S., 2016].

Much progress has been made in recent years in the treatment of CF with correctors or potentiators of the CFTR channel. However, these molecules act respectively by correcting the

folding, so driving the channel to the plasmatic membrane, and increasing the activity of the channel; therefore correctors and potentiators, in order to be able to act, require the presence of the protein. Nevertheless, 11% of the population affected by CF presents a premature termination codon (PTC) that causes the synthesis of an incomplete and non-functional CFTR protein, on which therefore, correctors or potentiators have no effects [Quon B.S., 2016].

A potential therapy for nonsense mutations aims to reduce the fidelity of the translation termination process using small molecules named Translational readthrough-inducing drugs (TRIDs) [Campofelice A., 2019; Samanta A., 2019]. TRIDs molecules allow the insertion of a near cognate tRNA (nc-tRNA), that, even if is not perfectly complementary to the PTC in the A site, allows the ribosome to skip the PTC and thus leads to the formation of a full-length protein [Dabrowski M. 2015].

In this context, a promising molecule is a synthetic aminoglycoside ELX02; it showed the rescue of the CFTR channel activity in human organoids from CF patients [Kerem E., 2020], but respect these promising results it *did not achieve statistical significance for efficacy endpoints in Phase 2 study in Class 1 CF mutations* [NCT04135495]. Currently, Ataluren or PTC124 is the only TRID approved by the FDA, and it has been tested in phase II and III trials both for CF and Duchenne Muscular Dystrophy (DMD, another genetic disorder caused by nonsense mutations) [Ardicli D., 2019]. However, the conflicting results in Cystic Fibrosis led to the stop of the trials for this disease and the approval of Ataluren only for the treatment of DMD [Richardson R., 2017]. For this reason, it is important to identify new readthrough molecules with low toxic effects and high efficiency of action.

Many efforts have been devoted to the search for new TRIDs [Pibiri I., 2015; Pibiri I., 2016, Pibiri I., 2018] and the investigation of their mechanism of action [Tutone M., 2019; Tutone M, 2020]. In our recent work [Pibiri I., 2020] we identified three molecules with interesting readthrough activity (NV848, NV914, and NV930) on the two most frequent Cystic Fibrosis stop mutations: G542X and W1282X mutations. Despite the demonstrated efficacy of these molecules, the efficiency of the readthrough mechanism depends on different features, including of course the genetic contest of the mutation but also inter-individual genetic variability [Loughran G., 2014]. Indeed, different patients, characterized by the same mutation, show a different response to the therapy, proving the involvement of mutation-independent factors affecting CFTR mRNA expression [Pranke I., 2019; Bongiorno R., 2021].

Among these factors, a significant role is performed by the Nonsense-Mediated decay (NMD) pathway. It is a pathway with an extremely efficient variability in population and responsible for the decay of mRNA with a PTC, to reduce the production of truncated proteins that could be toxic for the cell, due to their dominant negative function [Chang Y., 2004; Keeling K., 2013; Karousis E.D., 2016].

A prominent role in the NMD pathway is played by the up-frameshift factor 1 (Upf1), the protein that, in a phosphorylated state and the presence of a PTC, can interact with the termination factors eRF1 and eRF3, causing the recruitment of endonucleases inducing mRNA decay [Popp M.W., 2018]. The mechanisms by which Upf1 discriminates its target are mainly two: exon junction complex (EJC)-dependent and EJC-independent.

The EJC is a large protein complex recruited upstream of the splicing site during mRNA maturation and normally removed by the ribosome during translation.

According to the EJC-dependent NMD mechanism, an EJC present after a stop codon will not be removed, representing so a signal for NMD activation [Neu-Yilik G., 2017].

The EJC-dependent mechanism has been considered the only way for NMD activation for a long time. However, NMD has been reported to have a role not only in targeting PTC-containing mRNA but also in fine quality control of the transcriptome, implying a mechanism independent of EJC presence [Lou C.H., 2016]. Indeed, mRNAs without introns can similarly be recognized by NMD [Hogg L., 2010; Bongiorno R., 2021]. An alternative model, "faux 3'UTR", proposes that the distance from the termination codon to the poly (A) tail provides for the targeting of NMD due to the competitive binding of the major NMD regulator, Upf1, and cytoplasmic poly (A) binding protein 1 (PABPC1) with eukaryotic polypeptide chain release factor 3 (eRF3), which interacts with eRF1 and recognizes termination codons including the PTC [Sato H., 2021].

Several pieces of evidence demonstrate that NMD can also act on transcripts not containing an EJC [Hogg L., 2010, Metze S., 2013].

In the EJC-independent mechanism, the recognition of a PTC by NMD is possible thanks to a competition mechanism depending on the PTC position. Indeed, eRF3 can either interact with Upf1, so activating the NMD pathway or cooperate with the polyA binding protein (PABP), then inhibiting Upf1 recruiting [Ivanov I. P. 2008, Peixeiro I., 2012; Fritz S., 2020, Sato H., 2021] (Figure 1).
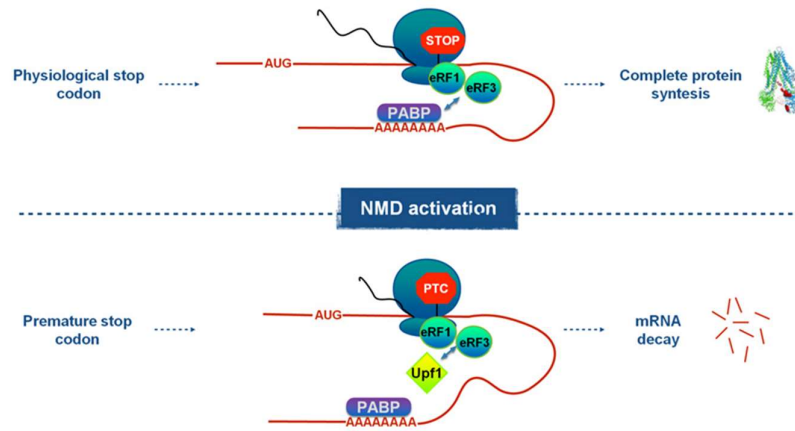

Figure 1. Schematic representation of the competition mechanism that regulates premature stop codon recognition by the NMD pathway.

In general, the NMD pathway is a limiting factor for TRIDs action, thus a combined use of readthrough agents and factors that minimize the EJC-dependent and the EJC-independent nonsense mRNA decay can be considered a promising strategy for nonsense mutation treatment. In this regard, post-transcriptional silencing of Upf1 or inhibition of Upf1 phosphorylation, by using caffeine, could be possible strategies to stabilize the CFTR mRNA and so increase the readthrough effects of TRIDs [Roy B., 2016; Lentini L., 2019].

Fisher rat thyroid (FRT) cell model system, transfected with a vector harboring the CFTR cDNA with the W1282X stop mutation was used to demonstrate the potentiality of these combined treatments and in particular to contrast the effect of the EJC-independent nonsense mRNA decay and to promote TRIDs activity.

2. Materials and Methods

TRIDs used in this study have been synthesized as reported in the literature [Pibiri I.,2020; Lentini L., 2014], and all reagents and solvents were purchased from commercial sources. All synthesized compounds were first purified by silica flash chromatography and analyzed (see supplementary material) by IR, NMR, and HPLC/MS, assessing their purity grade (>95%).

2.1. Cell Cultures

FRT YFP+/+CFTR[WT]: rat epithelial thyroid cells transfected with both pcDNA3.1 and pTRACER-CFTR-WT(pTCF-wt) plasmid vectors. So these cells express CFTR wt protein.

FRT YFP+/+CFTR[W1282X-opal]: rat epithelial thyroid cells expressing the YFP protein and transfected with pTRACER-CFTR-W1282X plasmid vector, that we obtained by replacing the tryptophan UGG codon, in 1282 position, with the UGA *opal* stop codon, through site-specific mutagenesis of pTCF-wt.

2.2. Condition of Culture and TRIDs Treatment

The culture medium is prepared by dissolving the Nutrient Mixture Coon's F-12 Ham and sodium bicarbonate (2,68 g) in sterile water for cell culture (1l). The solution is filtered and then added with fetal bovine serum (10%, Corning, USA) and glutamine, penicillin and streptomycin (1%) (removed before TRIDs treatment). Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. TRIDs were used at 12µM for 24 hours.

2.3. Real-Time RT-PCR

Total RNA was extracted from cells by using the RNAeasy Mini kit according to the manufacturer's instructions (Qiagen, Milano, Italy). RNA was reverse-transcribed in a final volume of 20 μl using the High Capacity cDNA Archive kit (Applied Biosystems, Monza, Italy) for 10 min at 25 °C and 2 h at 37 °C. For each sample 2 μl of cDNA, corresponding to 100 ng of reverse transcribed RNA, was analyzed by real-time RT-PCR (95 °C for 15 s, 60 °C for 60 s repeated for 40 cycles), in triplicate, using the ABI PRISM 7300 instrument (Applied Biosystems, Monza, Italy). Real-Time RT-PCR was done in a final volume of 25μl comprising 1x Master Mix SYBR Green (Applied Biosystems, Monza, Italy) and 2μM of forward and reverse primers for CFTR (Fwd: 5'-GCACCATTAAGAAAATATCATCTT-3'; Rev:5'-TTGTCTTTCTCTGCAAACTTGG-3') and Upf1 (Fwd:5'-CAGACTACCCTTCCCAACAG-3'; Rev:5'AGGGCTGGCTCATGGACACA-3'). RNA level was determined by using the SDS software version (Applied Biosystems, Monza, Italy) according to the 2−ΔΔCt method (Fernandes P., 2021) and Ct values was normalized to the internal control actin.

2.4. Western Blotting

Proteins (30 μg) were separated by 3–8% SDS-PAGE (Bolt, Life Technologies) containing 0.1% SDS and transferred to PVDF membranes (Thermo Scientific, Rockford, USA) by electroblotting. For CFTR detection the membrane was incubated with an anti–CFTR antibody (Abcam 1:1000), and HRP-conjugated anti-mouse (Thermo Scientific Rockford, USA, 1:5000). The target protein was detected by the use of SuperSignalWest Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, USA). We used an anti-β-tubulin antibody (mouse; Sigma-Aldrich 1:10.000) to confirm equal protein loading. Gel bands were quantified by ImageJ software. WB assays were repeated at least two times.

2.5. Immunofluorescence Microscopy

FRT pTRACER-CFTR-WT and FRT pTRACER-CFTR-W1282X-opal cells have been plated on rounded glass coverslips and suitably treated. Cells were then fixed with methanol and blocked with 0.1% BSA for one hour at room temperature. The coverslips were incubated with a mouse monoclonal antibody against the first extracellular loop of human CFTR (Abcam, 1:500) overnight at 4°C, followed by a goat polyclonal anti-mouse Alexa-Fluor-488 (Abcam, 1:1000) secondary antibodies for 1 h at 37 °C. The cell membrane and Golgi apparatus were stained by the wheat germ agglutinin Alexa 594 conjugated Alexa 594 antibody (Thermo Scientific, USA 1:1000) while nuclei were visualized with DAPI. Cells were observed under a fluorescence microscope (Carl Zeiss) at 63X magnification.

2.6. Functionality Assay: Quenching of Fluorescence of YFP Protein

The functionality of the CFTR channel has been evaluated, on pTRACER-CFTR-WT e FRT pTRACER-CFTR-W1282X cells, through both quantitative (50.000) and qualitative (250.000) assays. After the respective times of treatment with caffeine, siRNA, and TRIDs, the fluorescence was evaluated after the 60s and 120s from the addition of PBS NaI solution added with Forskolin (5μM). The fluorescence of YFP protein has been quantitative measured through the use of a fluorometer and qualitative evaluated thanks to a fluorescence microscope (Zeiss Observer.D1).

2.7. Reduction of NMD Pathway Activity

The reduction of NMD pathway activity has been done with two different approaches:
caffeine treatment for 24h at a concentration of 0,75 mM to inhibit the kinase of Upf1.

post-transcriptional silencing of Upf1 by use of a specific siRNA against Upf1 (Silencer Select Pre-designed siRNA product, Ambion) (5' – CUACCUCGGUGAUGAGUUUtt – 3'), for 72h at a concentration of 100nM. For this treatment, pTRACER-CFTR-W1282X cells (250.000) have been transfected with siRNA by using lipofectamine 2000 (5μl) and Optimem (24μl).

Non-specific siRNA, consisting in the experimental control, siGFP (Eurofins, 5'-GGCUACGUCCAGGAGCGCAdTdT – 3') was used with the same condition of siUPF1.

3. Results

3.1. NMD Pathway Inhibition Increases CFTR mRNA Level Favoring TRIDs Readthrough Action

Recently we identified three potential translational readthrough-inducing drugs named NV848, NV914, and NV930 [Pibiri I., 2020]. To increase the nonsense CFTR mRNA levels and to optimize the TRIDs readthrough effect, we used two different approaches aimed at NMD pathway inhibition. The first approach was based on the use of caffeine, an inhibitor of the Upf1 phosphorylation. The second one, relying on post-transcriptional silencing of Upf1 mRNA, was designed to reduce Upf1 activity and to evaluate the possible increase of the readthrough effect by our TRIDs.

FRT cells stably transfected with the pTRACER-CFTR^{W1282X} vector were treated with 0.75 mM caffeine for 24 hours, in parallel FRT pTRACER-CFTR^{W1282X} were transfected with siUpf1 and siGFP (used as not specific siRNA) for 72 hours to reduce the Upf1 expression, thus weakening the nonsense-mediated decay pathway. The use of 0.75mM caffeine was justified by previous results, showing the increase in CFTR mRNA expression without toxic effects [Lentini L., 2019].

Real-time RT PCR analysis performed after 72 hours from siRNA transfection revealed that Upf1 mRNA was reduced by 0.4 folds in siUpf1 transfected cells compared to siGFP-transfected cells (Figure 2A).
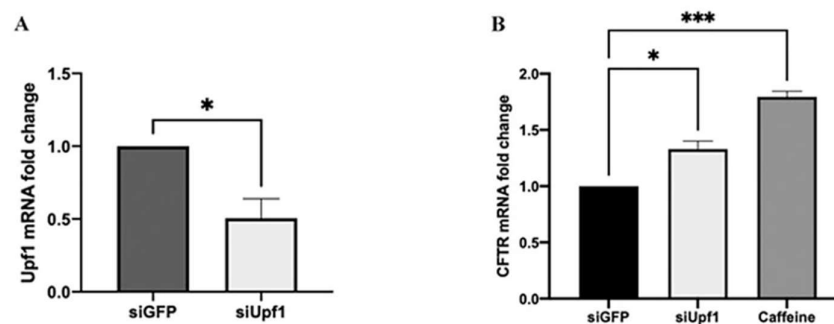

Figure 2. A) Real-time RT PCR analysis of Upf1 transcripts on FRT-CFTR^{W1282X} cell transfected with siRNA against Upf1 and GFP (non-specific siRNA). **B)** Real-time RT PCR analysis of CFTR transcripts on FRT CFTR^{W1282X} cells treated with caffeine and siUpf1. Images are representative of two different independent experiments done in triplicate. Data were analyzed by GraphPad Prism 6 software, expressed as mean values ± standard error of the mean (SEM). Symbol (*) represents the statistical significance of treatment with siUpf1 versus treatment with siGFP (p< 0.05); Three symbols (***) represent the statistical significance of caffeine treatment versus siGFP treated sample (p < 0.005) (Unpaired t-test).

To evaluate the CFTR mRNA expression levels, real-time RT PCR was performed on total mRNA extracts of siUpf1 silenced FRT pTRACER-CFTR^{W1282X} cells and of FRT pTRACER-CFTR^{W1282X} cells treated for 24 hours with caffeine (Figure 2-B). We observed that the reduction of Upf1 expression by siRNA induced a significant effect on CFTR expression. Indeed, FRT pTRACER-CFTR^{W1282X} cells, where Upf1 mRNA expression was partially silenced, showed an increase of 38% in CFTR transcript respect to siGFP transfected cells (Figure 2-B). On the other hand, caffeine treatment showed a 1.87-fold change (87%) in CFTR mRNA level (Figure 2-B).

3.2. Combined Treatment of the NMD pathway Inhibition and Readthrough Induction with NV914 or NV848 TRID Molecules Increases CFTR Expression

To increase the expression of the CFTR protein in FRT pTRACER-CFTR^{W1282X} cells, we combined the two described approaches, by treating the cells with TRIDs (NV848 and NV914) [Pibiri I., 2020] and the NMD pathway inhibitors (siUpf1 and caffeine). The choice of these molecules was made based on our previous results, showing that both NV914 and NV848 molecules were able to rescue CFTR expression and functionality in FRT pTRACER-CFTR^{W1282X} cells [Pibiri I., 2020].

FRT pTRACER-CFTR^{W1282X} cells were treated with 0.75 mM caffeine and 12 µM of NV914 or NV848 for 24 hours. In the alternative, FRT pTRACER-CFTR^{W1282X} cells were transfected with Upf1 siRNA for 72 hours with the addition of the NV914 or NV848 in the last 24 hours. CFTR expression was evaluated by immunofluorescence and Western blot analyses (Figures 3 and 4).
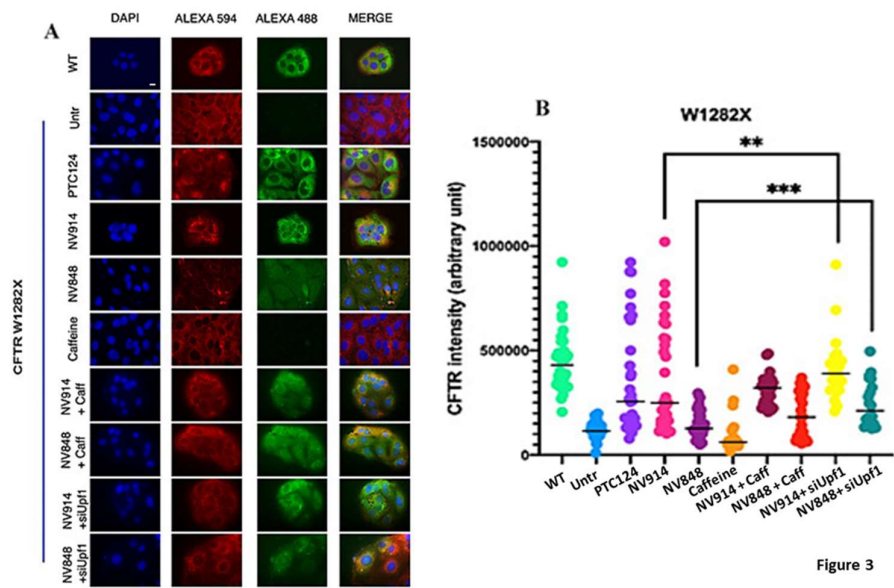

Figure 3. Immunofluorescence analysis of CFTR protein on FRT pTRACER-CFTR^{WT} (positive control) FRT CFTR^{W1282X} untreated (negative control) or treated as indicated. Images are representative of three independent experiments and Quantification of CFTR expression was done manually by using ImageJ software. The background was subtracted and the integrated signal intensity in the selected area (one cell) was measured. Data were analyzed by GraphPad Prism 6 software, expressed as mean values ± standard error of the mean (SEM). Two symbols (**) represent the statistical significance of treatment with siUpf1 and NV914 versus treatment with only NV914 ($p < 0.005$); Three symbols (***) represent the statistical significance of treatment with siUpf1 and NV848 versus treatment with only NV848 ($p < 0.0005$)(Unpaired t-test).
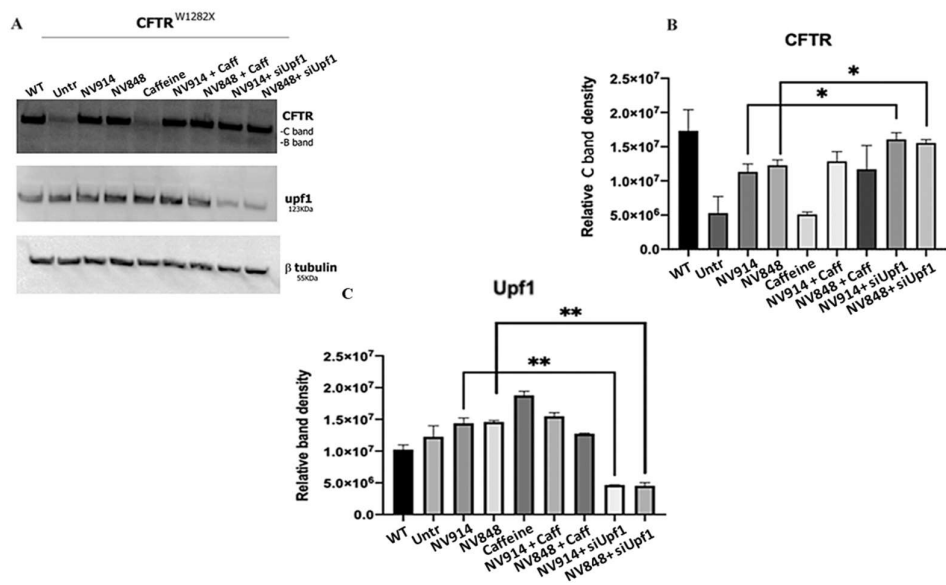

Figure 4. Western blot analysis of CFTR and Upf1 proteins on FRT pTRACER-CFTR^{WT} (positive control) FRT CFTR^{W1282X} untreated (negative control) or treated as indicated. Images are representative of two independent experiments; β-tubulin was used as a control for the protein loading. The experiment was performed in duplicate and data was normalized by β-tubulin expression, and analyzed by GraphPad Prism 6 software. Data are

expressed as mean values ± standard error of the mean (SEM). Symbols (*) represent the statistical significance of treatment with siUpf1 and NV914/NV848 versus treatment with only NV914/NV848 (p< 0.05). Two symbols (**), p < 0.005 (Unpaired t-test).

We detected that FRT pTRACER-CFTR^{W1282X} cells showed increased CFTR expression in comparison with the untreated sample after 24 hours of NV914 and NV848 treatment. Moreover, the combined treatment with TRIDs/siUpf1 for 72 hours induced a further increase in CFTR expression (Figures 3 and 4).

The caffeine treatment did not induce CFTR protein expression. Combined TRIDs/caffeine treatment, although it seems to increase CFTR rescue, failed to achieve significance compared to samples treated only with TRIDs.

Western Blot analysis of the UPF1 protein was made to verify UPF1 silencing efficiency (Figure 4).

33. Functional Analysis of CFTR Channel after TRIDs Treatment

Aiming to verify if TRID administration associated with NMD pathway inhibition can improve rescuing the CFTR channel functionality, in addition to the protein expression recovery, an indirect CFTR functionality assay has been performed.

Taking advantage of the expression of the YFP fluorescent protein in FRT cells, [Galietta L., 2001], this assay is based on the YFP characteristic of being sensitive to halides (as I$^-$), due to the presence of a binding pocket closely approached to the YFP chromophore. When these ions enter cells through the CFTR channel, they bind to YFP protein so inducing fluorescence quenching.

Consequently, a progressive quenching of green fluorescence is indicative of channel functionality.

To evaluate CFTR functionality, FRT pTRACER-CFTR^{W1282X} cells were treated with caffeine and NV914 or NV848 for 24 hours. In the alternative, FRT pTRACER-CFTR^{W1282X} cells were transfected with siUpf1 for 72 hours with the addition of the NV914 or NV848 in the last 24 hours.

After 24h, cells were pre-stimulated with Forskolin, a CFTR channel activator, and observed by microscopy before and after 60 seconds from the addition of PBS NaI (Figure 5-A). YFP fluorescence quenching was quantitatively measured after 60 seconds from the addition of PBS NaI through the use of a fluorimeter (Figure 5-B). The observation of the YFP fluorescence was further extended to 120 seconds.
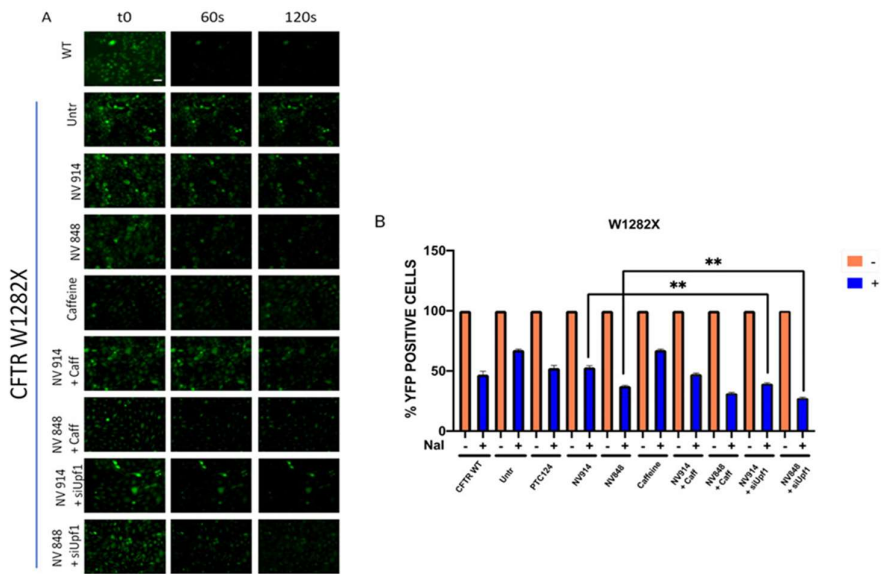

Figure 5. CFTR functionality in FRT cells measured by the yellow fluorescent protein (YFP) assay on FRT pTRACER-CFTR^{WT} (positive control) FRT CFTR^{W1282X} untreated (negative control) or treated as indicated. Images

were taken before (t^0) and after (t^{60}, t^{120}) the addition of NaI (**A**). Percentages of YFP-positive cells before (− dark green bars) and after NaI addition (+ blue bars); data were analyzed by GraphPad (n = 3) and expressed as mean values ± standard error of the mean (SEM) (**B** Data are expressed as mean values ± standard error of the mean (SEM) and were analyzed by GraphPad Prism 6 software (n = 2) comparing treatment with siUpf1 and NV914/NV848 versus treatment with only NV914/NV848. Two symbols (**) represent statistical significance ($p < 0.005$) (Unpaired t-test).

These analyses showed that combined treatment of siUpf1 with NV914 or NV848 determines a higher recovery of CFTR channel functionality than the sample treated with only NV914 or NV848 (Figure 5). In contrast, TRIDs/Caffeine treatment, although increasing CFTR rescue, failed to achieve significance compared to the samples treated only with TRIDs.

4. Discussion

In order to overcome the presence of PTC, in the last years, different molecules allowing ribosomes to readthrough the premature stop codon have been identified.

Despite the good results obtained from the readthrough approach, TRIDs efficiency is considerably reduced by the poor amount of target transcript, that is the mRNA containing the PTC. Indeed, the readthrough does not occur on the totality of the target transcript because of its degradation mediated by the NMD.

Being the NMD pathway a limiting factor, the identification and the use of approaches that induce nonsense mRNA stabilization could be a strategy to improve the readthrough efficiency of TRIDs.

In this work, we alternatively reduce Upf1 expression, through post-transcriptional silencing, and limit Upf1 activity, by using caffeine treatment in a Cystic Fibrosis cell model. In both cases, the association of the inhibition of NMD and treatment with NV914 and NV848 causes an increase of $CFTR^{W1282X}$ mRNA level followed also by the rescue of CFTR expression and functionality in FRT pTRACER-$CFTR^{W1282X}$ cells. However, unexpectedly, despite the higher $CFTR^{W1282X}$ mRNA level in caffeine-treated samples, both expression and functionality of CFTR rescue obtained by this treatment are slightly lower than the recovery achieved by Upf1 silencing.

These observations led us to hypothesize the existence of a positive feedback mechanism in which, the increase of $CFTR^{W1282X}$ mRNA level by caffeine treatment, could determine the transcriptional activation of the Upf1 gene and induce Upf1 protein expression (Figure 4). This could be an attempt of the cell to reduce the level of nonsense transcripts, which could potentially have deleterious dominant-negative effects. However, this high level of Upf1 cannot activate the NMD pathway because of the presence of caffeine. In this vision, in which both Upf1 and his transcript target ($CFTR^{W1282X}$) result high, a competition between Upf1 and our TRIDs for the interaction with nonsense mRNA could be present, thus resulting in a reduction of readthrough efficiency. Furthermore, the presence of this positive feedback mechanism could also explain the usual difficulties that we encountered in Upf1 silencing and the high concentrations of siRNA used in different works to reduce UPF1 expression [Salvatori F. 2018; Sharma N., 2018].

Conclusions

Although the sole NMD pathway inhibition cannot be considered as a solution to nonsense pathologies, our results indicate that modulation of the NMD pathway, although still to be optimized, could be a promising approach in combination with TRIDs, to rescue expression and functionality of CFTR protein. Indeed, the synergy between these two approaches, NMD modulation, and readthrough, allows greater response to treatment, increasing the quantity of produced protein and boosting its activity.

5. Patents

L.L., I.P., R.M., and A.P. are co-inventors of the patent WO2019101709 "Oxadiazole derivatives for treating genetic diseases due to nonsense mutations".

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

Conflicts of Interest: L.L., I.P., R.M., and A.P. are co-inventors of the patent WO2019101709 "Oxadiazole derivatives for treating genetic diseases due to nonsense mutations". All other authors declare no conflict of interest.

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