



## Caffeine boosts Ataluren's readthrough activity

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### ABSTRACT

The readthrough of nonsense mutations by small molecules like Ataluren is considered a novel therapeutic approach to overcome the gene defect in several genetic diseases as cystic fibrosis (CF). This pharmacological approach suppresses translation termination at premature termination codons (PTCs readthrough) thus restoring the expression of a functional protein. However, readthrough might be limited by the nonsense-mediated mRNA decay (NMD), a cell process that reduces the amount/level of PTCs containing mRNAs. Here we investigate the combined action of Ataluren and caffeine to enhance the readthrough of PTCs. IB3.1 CF cells with a nonsense mutation were treated with caffeine to attenuate the Nonsense-Mediated mRNA Decay (NMD) activity and thus enhance the stability of the nonsense (ns)-CFTR-mRNA to be targeted by Ataluren. Our results show that NMD attenuation by caffeine enhances mRNA stability and more importantly when combined with Ataluren increase the recovery of the full-length CFTR protein.

### 1. Introduction

Nonsense mutations are responsible up to 70% of the individual cases of most inherited diseases, including cystic fibrosis (CF), Duchenne muscular dystrophy (DMD) and a variety of other genetic disorders and cancer (Baker and Parker, 2004; Maquat, 2005). Besides gene therapy, pharmacological approaches aiming at modifying gene expression by promoting the ribosomal readthrough of nonsense mutations have gained interest in these years (Rowe and Clancy, 2009; Goldmann et al., 2012; Lentini et al., 2014; Pibiri et al., 2015; Roy et al., 2016; Dabrowski et al., 2018; Tutone et al., 2019).

Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). So far, 27 different nonsense mutations, corresponding to 10% of cystic fibrosis (CF) cases worldwide have been identified (Kerem et al., 2008).

A potential treatment for these patients makes use of drugs that selectively suppress translation termination at in-frame premature termination codons (PTCs). Among these aminoglycosides were previously employed to suppress the normal proof-reading function of the ribosome (Prayle and Smyth, 2010) leading to the insertion of a near-cognate amino acid at the PTC site, thus allowing the translation of a full-length protein. This "translational readthrough" of premature stop

codons has been shown to partially restore protein function in a number of preclinical settings (Goldmann et al., 2012; Harmer et al., 2012; McElroy et al., 2013, Aslam et al., 2017). However, severe side-effects caused by prolonged treatments with aminoglycosides have been reported limiting their widespread clinical use for this purpose (Sarkar et al., 2011; Wilschanski et al., 2011; Kerem et al., 2014).

On the contrary, the compound PTC124 (Ataluren) reported to promote the readthrough of premature but not normal termination codons does not possess the toxicity of aminoglycosides and has been suggested as a potential treatment for genetic disorders caused by nonsense mutations, particularly those involving the UGA premature codon (Welch et al., 2007; Lentini et al., 2014, Lentini et al., 2015).

Although some studies (Auld et al., 2009; McElroy et al., 2013; Kerem et al., 2014) questioned whether Ataluren is able to promote readthrough, several independent publications demonstrated its efficacy (Goldmann et al., 2012; Lentini et al., 2014; Pibiri et al., 2015, 2016, 2018; Roy et al., 2016). Moreover, Ataluren clinical trials showed the efficacy in the readthrough of premature stop codon (Wilschanski et al., 2011; Finkel et al., 2013; Ng et al., 2018). However, the amount of readthrough can be limited by the nonsense-mediated mRNA decay (NMD) pathway, a conserved eukaryotic cellular pathway that targets PTC-containing mRNAs for degradation (Keeling et al., 2013; Linde et al.,

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2007). Several drugs such as SMG1 kinase inhibitors have been found to attenuate the NMD pathway by inhibiting the Upf1 and the Upf1 protein phosphorylation (Keeling, 2016). Caffeine attenuate NMD by blocking the UPF1 phosphorylation cycle by inhibiting the SMG1 kinase that phosphorylates UPF1 (Keeling et al., 2013). Inhibition of NMD may enhance the effect of nonsense suppression drugs to restore protein function by increasing steady-state mRNA abundance (Keeling et al., 2013).

Here we report new evidences about the enhancement of premature stop codon readthrough of the CFTR mRNA by Ataluren when associated with caffeine an inhibitor/attenuator of the NMD response providing new insights for Ataluren therapeutic improvement.

## 2. Materials and methods

### 2.1. Cell culture conditions and caffeine/PTC124 treatment

The IB3.1 cell line, gifted by Dr. Paola Melotti, Azienda Ospedaliera Universitaria Integrata, Verona, Italy, is a bronchial cell line derived from a cystic fibrosis patient with a  $\Delta F508/W1282X$  CFTR genotype. IB3.1 cells were cultured in DMEM supplemented with FBS 10% (GIBCO) in a humidified atmosphere of 5% CO<sub>2</sub> in air at 37 °C. Caffeine treatment was performed for 24h at different concentrations (0.75, 1.5, 3 mM), while Ataluren/PTC124 and G418 were added in the cell medium for 24 or 72 hours at 12  $\mu$ M and 430  $\mu$ M, respectively.

### 2.2. Trypan blue dye exclusion cell viability test

The number of dead (blue stained) and live (clear cytoplasm) cells after caffeine treatment was quantified using trypan blue staining. The cells were detached from the plate surface and then diluted in 0.4% trypan blue stain (v:v) (Gibco by Life Technologies) and counted in a Burkler chamber. The percentage of viable cells was calculated by dividing the number of live cells by the total number of cells, multiplied by 100. Three replicates were performed for each condition.

### 2.3. Immunofluorescence microscopy

To visualize the CFTR protein cells were grown on rounded glass coverslips and fixed with cold methanol for 2 min. The cell membrane and Golgi apparatus were stained by the Wheat Germ Agglutinin (WGA) Alexa 594 1:1000 (Life Technologies). For CFTR-detection cells were blocked with 0.1% BSA (60 min) at RT and cover slips 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 to mouse Alexa-Fluor-488 (Abcam, 1:1000) secondary antibodies for 1h at 37 °C. Nuclei were visualized with DAPI. Cells were examined under a Zeiss Axioskop microscope equipped for fluorescence.

### 2.4. Real-time RT-PCR

Primers used in real-time RT-PCR experiments were designed with

Primer Express software (Applied Biosystems, Monza, Italy) or based on Kerem et al., (2008) (5). Amplicons size was of approximately 70–100 bp. When not already tested the selected sequences were blasted against public databases using BLAST to confirm the identity of the genes. Total RNA was extracted from cells by using the RNeasy Mini kit according to manufacturer's instruction (Qiagen, Milano, Italy). RNA was reverse-transcribed in a final volume of 50  $\mu$ l 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  $\mu$ l 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 quadruplicate, using the ABI PRISM 7300 instrument (Applied Biosystems, Monza, Italy). Real-Time RT-PCR was done in a final volume of 20  $\mu$ l comprising 1x Master Mix SYBR Green (Applied Biosystems, Monza, Italy) and 0.3 $\mu$ M of forward and reverse primers for: CFTR (Fwd: 5'-CTACATGGAACACATACCTTCG-3'; Rev: 5'-GGTGATAACTACTGCATAGC-3') (29); non F508del-CFTR (Fwd: 5'-GCACCATTAAAGAAAATATCATCTT-3; Rev:5'-TTGTCTTTCTCTGCAAACCTGG-3') (Kerem et al., 2008), GAPDH (Fwd: 5'-CTCATGACCACAGTCCATGCC-3'; Rev: ;5'-GCCAATCCACAGTCTTCTGGGT-3'). Data were analyzed by averaging quadruplicates Ct (cycle threshold). Levels of RNA were determined by using the SDS software version (Applied Biosystems, Monza, Italy) according to the 2- $\Delta\Delta$ Ct method and Ct values were normalized to the internal control GAPDH.

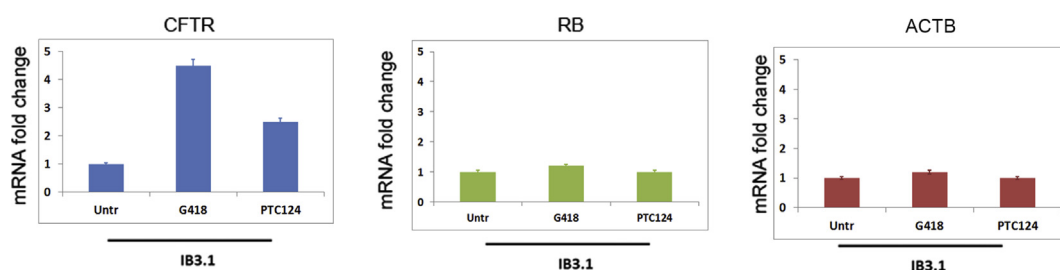
### 2.5. Western blotting

Proteins (50  $\mu$ g) were separated by 3–8% SDS-PAGE (Bolt, Life Technologies) containing 0.1% SDS and transferred to Hybond-C nitrocellulose membranes (Amersham Life Science) by electroblotting. For CFTR detection the membrane was incubated with a goat polyclonal anti-CFTR antibody (C-19, Santa Cruz 1:500) raised against a peptide mapping near the C-terminus of human CFTR, and HRP-conjugated anti-goat (Abcam, 1:5000). The target protein was detected by ECL reagent (Pierce). We used an anti- $\beta$ -tubulin antibody (mouse; Sigma-Aldrich 1:10.000) to confirm equal proteins loading. Gel bands were quantified by Image Lab software (BioRad). WB assays were repeated at least three times and were reproducible within 5 % variations.

## 3. Results

### 3.1. Prolonged treatment with Ataluren increases the CFTR-transcript

It has been reported that different readthrough-promoting RNA compounds have similar capacities to inhibit the nonsense-mediated mRNA decay (NMD) pathway and that the level of translational read-through required to skip NMD depends on how distant is the PTC from the end of the mRNA (Baker and Hogg, 2017; Midgley, 2019). We used the IB3.1 cells to investigate if Ataluren (indicated as PTC124 in figures) is able to stabilize the mRNA containing nonsense mutations in addition to the suggested readthrough activity. Cells were treated also with G418 as a control since it affects mRNA stabilization of genes containing PTCs



**Fig. 1.** CFTR mRNA was increased in IB3.1 cells treated with PTC124 (Ataluren). Real-Time RT-PCR of CFTR, RB and actin genes in IB3.1 cells treated or left untreated (G418 and PTC124) for 24 hours.

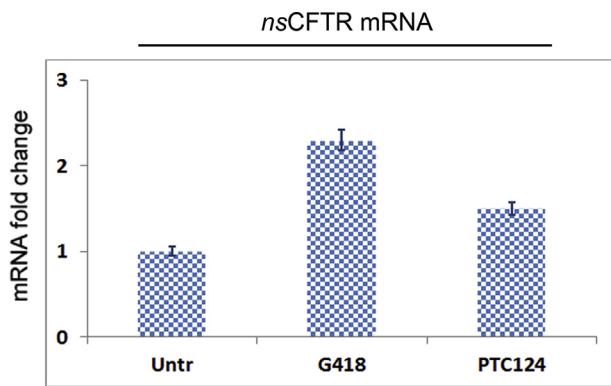


Fig. 2. The increase of CFTR mRNA level after treatment of IB3.1 cells with Ataluren/PTC124 was specific for the nonsense mutated mRNA. Real-Time RT-PCR with primers specific for the nsCFTR-mRNA after 24 hours of treatment with G418 and PTC124.

(Salvatori et al., 2009). Real-Time RT-PCR showed that G418 and Ataluren increased 5 folds and 3 folds the CFTR transcript, respectively (Fig. 1). To exclude a general effect on mRNA stabilization caused by G418 and Ataluren we looked at the expression levels of two unrelated genes: RB (Retinoblastoma) and ACTB ( $\beta$ -actin). Real-Time RT-PCR did not show an increase of RB and ACTB mRNAs, indicating that the mRNA stabilization was specific for the CFTR transcript with PTC (Fig. 1).

To exclude that the observed increase of the CFTR mRNA was attributable to the  $\Delta$ F508-CFTR allele we used two primers specifically designed to amplify only the not- $\Delta$ F508 CFTR transcripts (Kerem et al., 2008). The Real-Time RT-PCR done with these primers (Fig. 2) shows that after the treatment with G418 and PTC124 (Ataluren) the IB3.1 cells still show increased levels of the CFTR mRNA (mainly nonsense CFTR mRNA) confirming the results of the previous analysis.

3.2. Low doses of caffeine do not affect cell viability and stabilize the nsCFTR mRNA

Ataluren might increase the nonsense CFTR transcript by impairing the nonsense-mediated mRNA decay (NMD) pathway. This surveillance

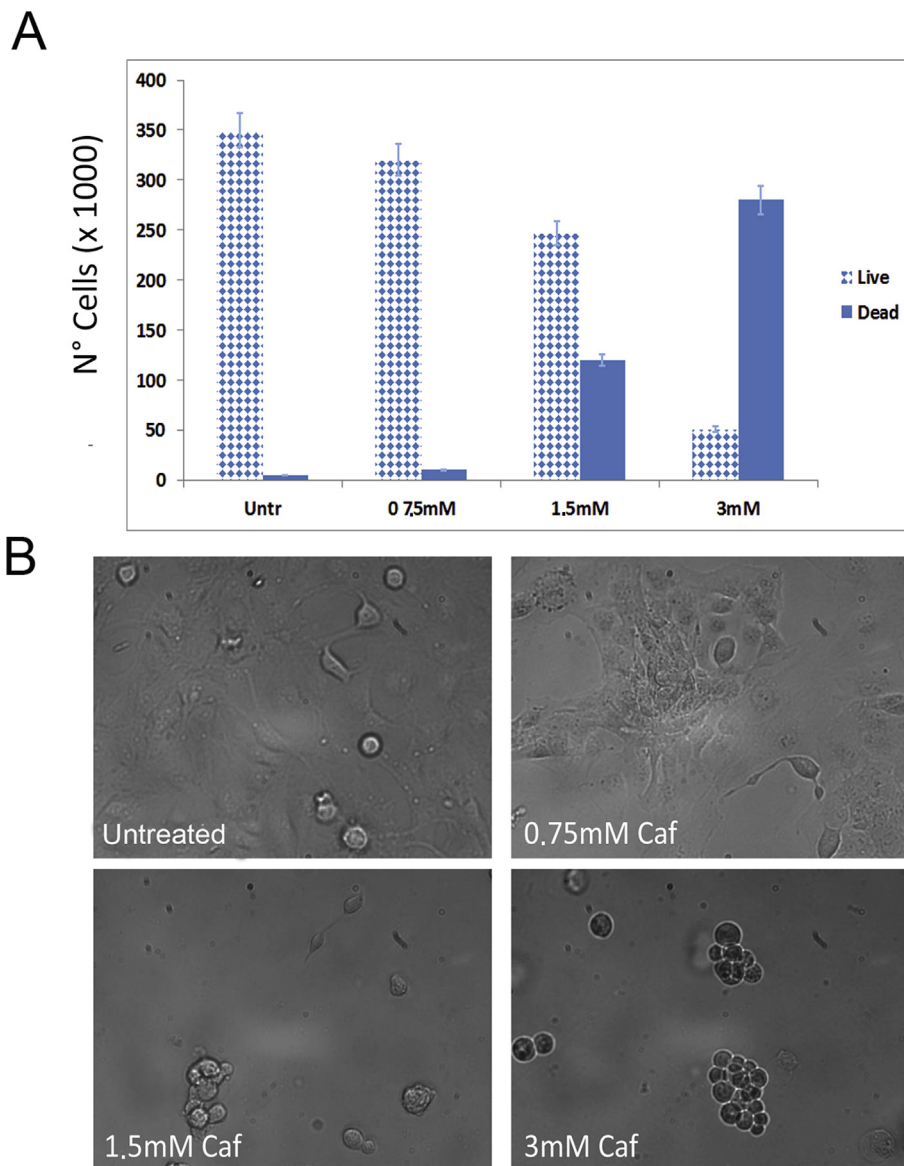
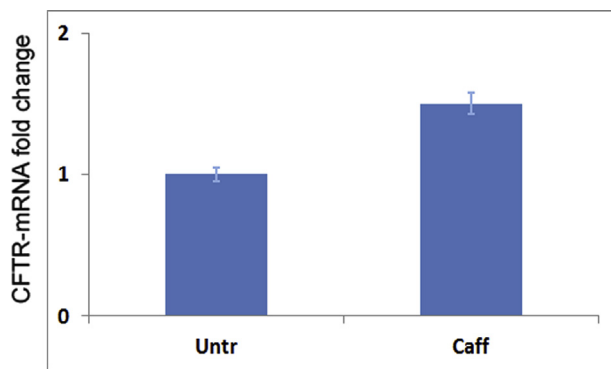


Fig. 3. Cell viability of IB3.1 cells after caffeine treatment. A) Graph shows the number of live or dead cells after 24 hours of caffeine treatment. B) Images of IB3.1 cells untreated or treated with 0.75–1.5–3 mM caffeine for 24 hours.



**Fig. 4.** Caffeine treatment increases CFTR mRNA levels in IB3.1 cells. Real-Time RT-PCR shows increased levels of the CFTR mRNA after 24 hours treatment with 0.75mM caffeine.

system degrading the mRNA harboring PTCs clearly disadvantages Ataluren's readthrough (Keeling et al., 2013). Thus, we hypothesized that the use of caffeine hampering the NMD (Shi et al., 2008) might increase the mutated (nonsense) CFTR mRNA boosting Ataluren readthrough effect. To this aim, the IB3.1 cells were treated with caffeine and Ataluren separately or in combination and the levels of the CFTR mRNA and protein were evaluated.

Caffeine has been reported to cause a different degree of toxicity according to the cell type used (Shi et al., 2008). We performed then a cell viability assay in order to choose the optimal dose of caffeine that allows the IB3.1 cells to survive to the treatment. To this aim, cells were treated for 24 hours with different concentrations (0.75–1.5 and 3 mM). We observed that 0.75 mM of caffeine treatment did not affect significantly cells viability and cell morphology (Fig. 3-A, B) and this concentration was used for further experiments.

We evaluated then the CFTR mRNA levels by Real-Time RT-PCR after treatment with 0.75 mM of caffeine. The analysis revealed a 1.5 fold change of the CFTR mRNA compared to the untreated cells (Fig. 4).

### 3.3. Combined effect of Ataluren and caffeine induced CFTR protein expression in cystic fibrosis cells

To favor the increase of the CFTR mRNA levels in the attempt to enrich the *target* for the Ataluren mediated readthrough, we performed a combined treatment of the two molecules: caffeine and Ataluren. IB3.1 cells were treated with 0.75 mM caffeine for 24 hours, the medium was then changed and Ataluren was added at the concentration of 12  $\mu$ M for additional 24 hours.

Real-Time RT-PCR analysis in IB3.1 cells confirmed the partial CFTR mRNA increase/stabilization after caffeine (1.5 folds) and Ataluren (2.5 folds) treatment in comparison to untreated control cells (Fig. 5-A). Moreover, the Real-Time RT-PCR analysis showed the additive effect on the CFTR mRNA level following the caffeine and Ataluren combined

treatment (3.8 folds) (Fig. 5-A).

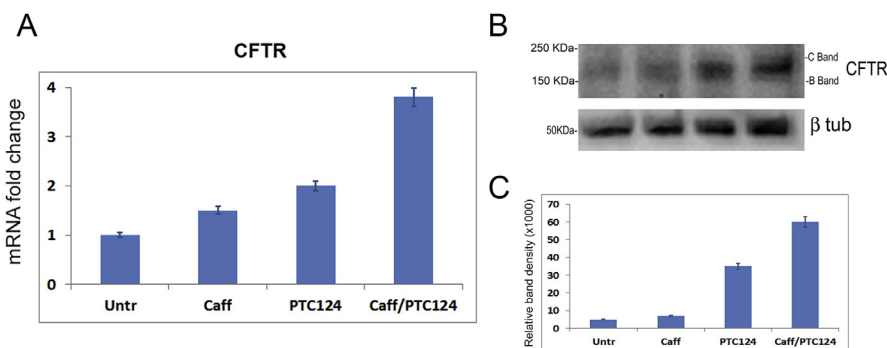
As expected the increase in the nonsense-CFTR mRNA after caffeine treatment did not result in the increase of the CFTR protein levels (Fig. 5-B). In contrast, Ataluren induced also the increase of the CFTR protein (Fig. 5-B). Interestingly, the combined treatment of caffeine and Ataluren induced a significant increase of both CFTR mRNA and protein levels, suggesting an additive effect of the two molecules (Figure 5 A-B).

To assess whether the full-length CFTR rescued protein was properly localized to the plasmatic membrane, immunocytochemistry analysis was performed using a CFTR antibody that recognizes the first external loop of the channel. As positive control we used CFBE cells that express the CFTR cDNA ectopically. The results show that the CFTR protein was properly localized to the plasmatic membrane after the combined treatment (Fig. 6).

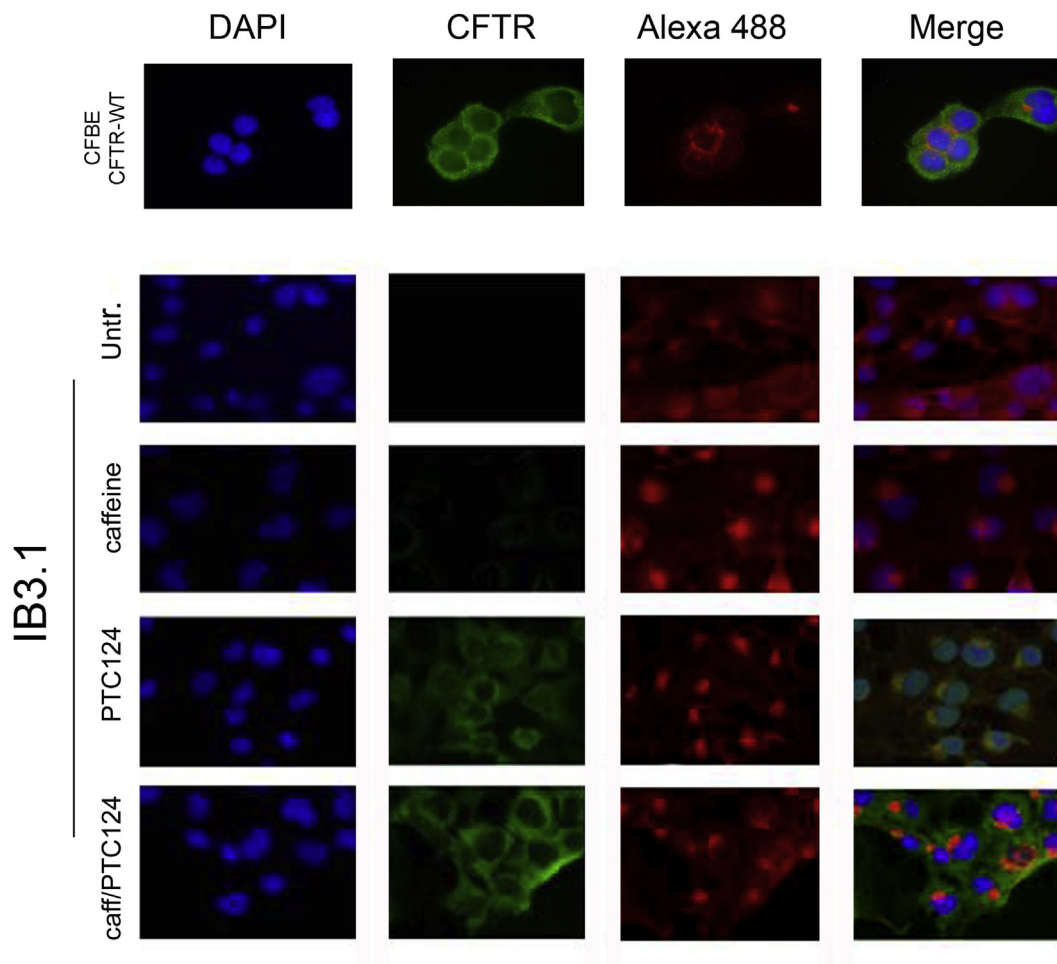
## 4. Discussion

The readthrough approach could be an excellent method to restore the expression of an mRNA harboring premature stop codon, however it results in controversial response on the basis of the genetic context. The different response to Ataluren observed in different genetic diseases could be attributed to several factors including the tissue physiology (protein turnover, NDM functionality, etc.) or the minimum amount of full-length protein required to perform its function. It is possible that in a particular tissue/cell context a small amount of protein rescued by the readthrough is sufficient to supply the complete absence of the mutated/absent protein. In other tissue/cell, it could be necessary recover at least the 50 % or more of the wild type protein.

Therefore, the approach based on the readthrough of the premature stop codons remains a good strategy for recovering the full-length protein, but it may need some adjuvants. From this point of view it is crucial the amount of the "target": the nonsense mutated mRNA. These mRNAs are frequently eliminated by the nonsense-mediated mRNA decay (NMD). By reducing the amount of PTC-containing mRNAs the NMD is thought to act as a protective mechanism for the cell by reducing the expression of truncated proteins that could potentially have deleterious, dominant-negative functions. Reducing the pool of mRNAs available for translation the NMD surveillance mechanism negatively impacts the therapeutic suppression of PTCs. In order to perform the readthrough of the premature stop codon it is of fundamental importance the presence of a sufficient amount of mutated mRNA and this is possible only if the NMD pathway is attenuated, inhibited or shielded. On the basis of these observations, it is clear that the use of a drug (as caffeine) that induces nonsense-mRNA stabilization could be a strategy to amplify the readthrough effect of Ataluren or others readthrough agents. Our results showed that Ataluren induced, in light measure, the accumulation of a little amount of nonsense-mRNA. The mechanism is not clear, we hypothesize that Ataluren induces (in addition to the readthrough activity) directly or indirectly this mRNA stability/accumulation. In a recent work it was reported that molecules containing a central aromatic heterocycle having two or three substituents including Ataluren could have multi-site



**Fig. 5.** The combined treatment of caffeine and Ataluren shows an additive effect on CFTR mRNA and protein levels. A) Real-Time RT-PCR evaluation of CFTR mRNA levels in IB3.1 cells untreated and treated for 24 hours with: 0.75mM caffeine (Caff), 12  $\mu$ M Ataluren (PTC124), and with a combination of the two (Caff/PTC124). B) Western blot showing CFTR protein levels in IB3.1 cells untreated (lane 1) or treated with the indicated molecules (lane 2: 24h caffeine 0.75 mM; lane 3: 24h PTC124 12  $\mu$ M; lane 4: 24h caffeine/24h PTC124). C) Histogram of the densitometry of the Western blot bands. A primary antibody raised against the C-terminus of CFTR was used (see also Supplementary Fig.1 and 2).



**Fig. 6.** CFTR protein is localized to the cell membrane after the combined treatment of caffeine and Ataluren. Immunofluorescence assay showing the CFTR protein in IB3.1 cells after 24h of treatment with caffeine, Ataluren (PTC124) and combined caffeine and PTC12. Nuclei were stained by DAPI (bleu), CFTR localization was detected by a primary antibody that recognizes the first external loop of the protein (as secondary antibody, Alexa 488-green). Membrane and Golgi apparatus were stained by WGA-Alexa 594 antibody.

binding to the protein synthesis machinery (Ng et al., 2018). In our previous work we observed by molecular dynamic simulation that Ataluren binds near the CFTR-G542X-UGA stop codon in a 33 nucleotides strand of CFTR mRNA (Lentini et al., 2014). These observations suggest an interaction Ataluren/mRNA that might influence the mRNA stability/accumulation.

Finally, our results indicate that the CFTR protein rescue is dependent by the readthrough of the PTCs mediated by Ataluren and that this effect increases when Ataluren disposes of a more abundant substrate (mRNA).

## 5. Conclusions

Our results show that the combined treatment of caffeine and Ataluren is able to enrich the target for the readthrough of the UGA premature stop codon, most probably by reducing the rate of CFTR mRNA degradation, thus increasing the expression levels of CFTR protein properly localized to the membrane of human IB3.1 cells. Our experiments evidenced that the readthrough strategy to restore the translation of full-length proteins could be a very good approach although it probably requires a further improvement in terms of nonsense-mRNAs target recovery and could find application, besides CF, in several genetic diseases due to nonsense mutations.

## Declarations

### Author contribution statement

Laura Lentini: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Raffaella Melfia, Patrizia Cancemi: Performed the experiments; Analyzed and interpreted the data.

Ivana Pibiri: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Aldo Di Leonardo: Analyzed and interpreted the data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2019.e01963>.

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