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Pharmacophore-based design of new chemical scaffolds as translational readthrough-inducing drugs (TRIDs)

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ABSTRACT: Translational readthrough-inducing drugs (TRIDs) rescue the functional full-length protein expression in genetic diseases, such as cystic fibrosis, caused by premature termination codons (PTCs). In this context, small molecules have been developed as TRIDs to trick the ribosomal machinery during recognition of the PTC. Herein we report a computational study to identify new TRIDs scaffolds. A pharmacophore approach was carried out on compounds that showed readthrough activity. The pharmacophore model applied to screen five different libraries containing more than 87000 compounds identified four hit compounds presenting scaffolds with diversity from the oxadiazole lead. These compounds have been synthesized and tested using the Fluc reporter harboring the UGA premature stop codon. Moreover, the cytotoxic effect and the expression of the CFTR protein were evaluated. All these compounds showed to be potential new lead compounds as TRIDs, boosting further efforts to address the optimization of the chemical scaffolds.

In recent years many efforts have been dedicated to personalized medicinal approaches to genetic disease. In this context, being Cystic Fibrosis a largely diffused genetic pathology, researchers have focused to the therapy of the basis genetic defect. Approximately 10%-15% of the cystic fibrosis (CF) cases are due to nonsense-mutations in the cystic fibrosis trans-membrane conductance regulator gene (CFTR). The presence of a nonsense mutation, giving rise to a PTC in the mRNA, produces a truncated protein that is rapidly degraded, so the patients lack the functional protein and suffer a hardest form of the disease. Therapies targeting this specific genetic defect have been addressed and, besides aminoglycosides, heterocyclic scaffolds play the main roles.[1–5]

Concerning patients with nonsense mutations, in the last ten years, the only pharmaceutical option was the translational readthrough of the premature termination codon (PTC) in order to bypass the PTC, and restore to sufficient extent the expression of a functional protein. Aminoglycosides (e.g. gentamicin, tobramycin, paromomycin) had been previously studied to this aim, as suppressing the normal proof-reading function of the ribosome these allow the translation and lead to the insertion of a near-cognate amino acid at the PTC site.[6,7] However, severe side-effects have been reported[8] by prolonged treatments with aminoglycosides including renal, auditory, and vestibular toxicities, that have limited their widespread clinical use as TRIDs. In this context, recently a new aminoglycoside ELX-2, showing potential activity as TRID and less side effects, has been launched in phase II clinical trials.[9]

Previously, in 2007 Ataluren (aka PTC-124) was proposed by PTC Therapeutics as able to promote the readthrough of premature but not normal termination codons.[10] The small molecule Ataluren, is a diaryl-1,2,4-oxadiazole, is less toxic than aminoglycosides, and has been suggested as a potential treatment of genetic disorders caused by nonsense mutations, particularly those involving the UGA premature codon.[11] Results of the phase II and III trials showed improvement in markers of CFTR function, but no improvements in sweat chloride levels or nasal potential difference.[12] PTC Therapeutics concluded phase III Clinical trials in 2014 for CF and Duchenne Muscular Dystrophy (DMD), in order to evaluate long-term safety of Ataluren. At the conclusion of this study, Ataluren has been approved for DMD while for CF it was evidenced that, although cystic fibrosis patients who received this treatment had beneficial effects, patients taking chronic inhaled tobramycin did not show the same benefits; allowing the researchers to hypothesize that the two drugs were competing at the level of the ribosome.
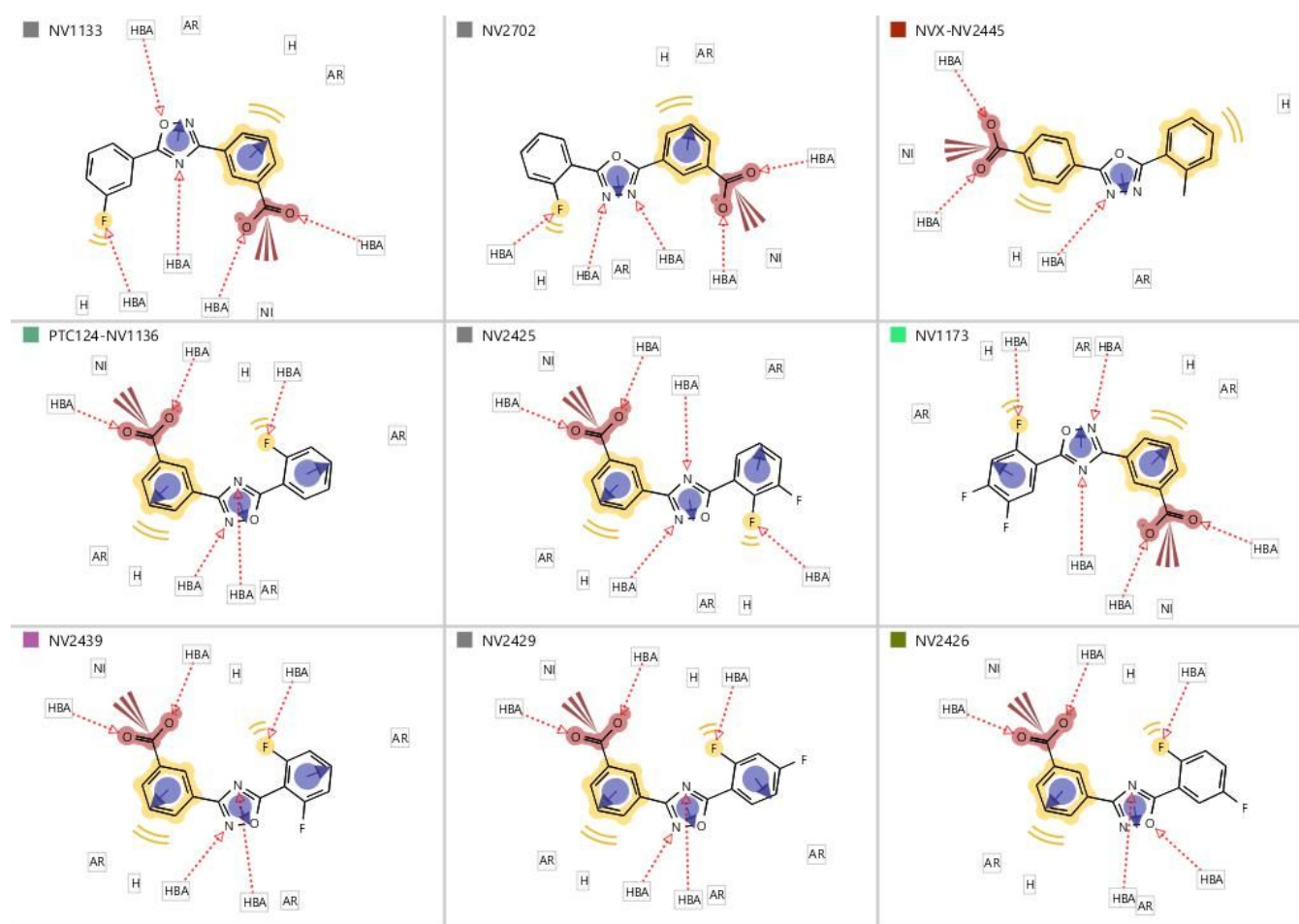

Figure 1. Pharmacophore features for the actives dataset: HBA, H-bond acceptor; AR, aromatic; H, hydrophobic; NI, negative ionic.

Additional confirmation Phase III clinical study resulted in the approval of the drug under the trade name Translarna for DMD patients, while the trial for CF patients was suspended in April 2017 due to conflicting results.[12,13]

Considering Ataluren's failure to be the prospective lead compound as TRID, and being its biological target still not clear[14] prompted us to further studies on the topic in order to find a valuable sostitute to Ataluren and to understand its mechanism of action.

In this context, we identified a set of small molecules containing an oxadiazole heterocyclic core by means of virtual screening on public and *in-house* libraries and cell-based

experimental studies.[15,16] The compound NV2445 (Figure 1) showed the most promising activity both in FLuc assays and in cells expressing a nonsense-CFTR-mRNA.[17,18] Notwithstanding these efforts with the aim to identify an effective alternative to Ataluren, new and more potent TRIDs are necessary for the treatment of the disease. In this paper, we performed an *in silico* design focusing on the identification of new chemical scaffolds not related with 1,2,4 or 1,3,4-oxadiazoles. We carried out a pharmacophore-based modeling study exploiting available experimental data on our previous identified compounds.[15–17,19] As starting point of our analysis, we considered the 24 active compounds in FLuc assay over the 61 synthesized compounds. Nine of the most promising compounds have a carboxylic group (Ataluren and NV2445, included), for this reason we decided to identify these nine compounds as the active dataset (Figure 1).

Pharmacophore models have been generated using LigandScout 4.3 by Inte:Ligand GmbH.[20,21] The dataset compounds have been randomly split into a training and a test set. Before models generation, conformers' generation and alignment have been performed. The pharmacophore features have been identified in a ligand-based mode, and ten different pharmacophore models have been identified. The better one (Model 1) showed a score of 0.90 out of 1 and for each compound matched 7 to 10 features. Model 1 consists of three aromatic features, two hydrophobic features, 5 H-bond acceptor features and one negative ionic feature (Figure 2).
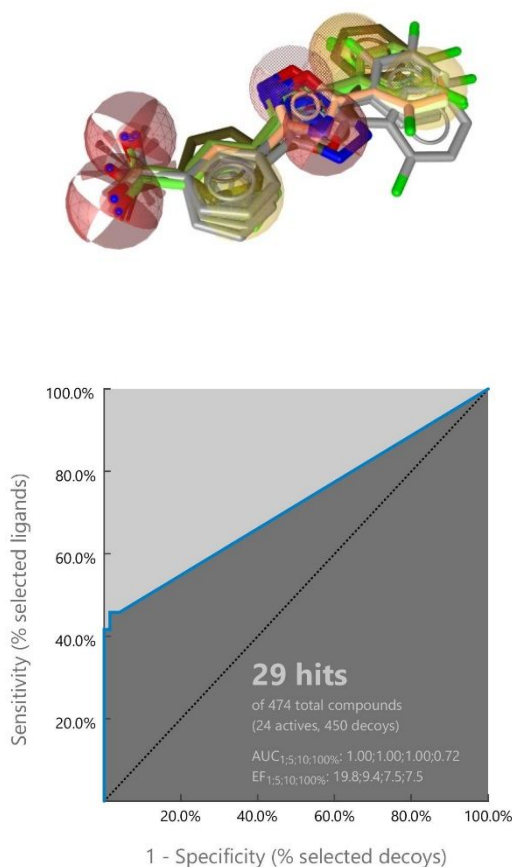

Figure 2. 3D representation of the best model (Model 1), up; ROC curve for the Model 1, bottom.

In order to refine and validate the model we employed the dataset of active compounds previously identified by us and a dataset of 450 decoys generated using the DUD-E tool.[22,23] For the validation of the pharmacophore model related its insight power, we considered the enrichment factor (EF) and the area under the curve (AUC) of the Receiver Operating Characteristic (ROC) curve. The validation test identified 29 hits, all the twenty-four actives and five decoys. The Figure 2 (bottom) displays the ROC plot, the AUC and the EF factor values at 1%, 5%, 10% and 100% of the best pharmacophore model. As shown in Figure 2, the early enrichment (EF 1%) is equal to 19.8 with an AUC value of 1.00 demonstrating that our pharmacophore model was able to discriminate between active and inactive compounds. Overall, the model has a preference for active compounds with an AUC value of 1 in the first 10% and AUC = 0.72 at the 100%, and EF value of 7.5 in the first 10% until the 100% showing a good accuracy for new hits identification.

In the end, this model was used as a query to screen 5 different databases: 1) an OTAVA commercial library containing 2775 potential mRNA binders; 2) the Drugbank library updated to January 2018 containing 8721 compounds; 3) the Maybridge hit discover library containing 52146 compounds, 4) an *in-house* library of 1829 small molecules designed by the Almerico group[24–27]; 5) an *in-house* library of about 26000 small molecules designed by the Pace and Pibiri group. The overall used library contained about 87000 compounds. The search yielded 23 hits compounds. Of these, four hits were chosen among the most synthetically accessible not containing the oxadiazole core (Figure 3).
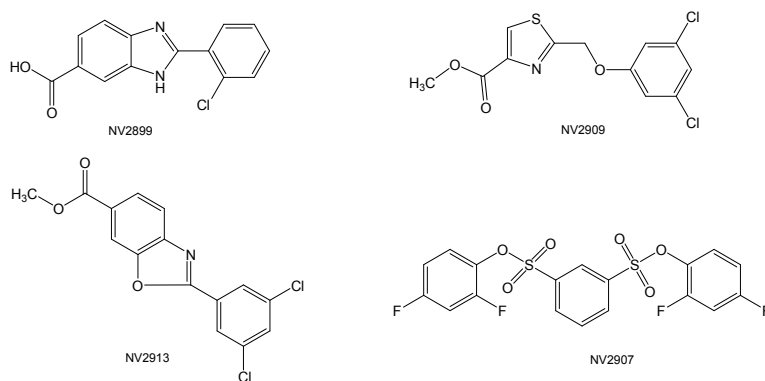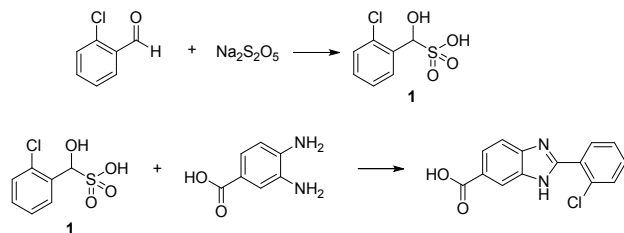

Figure 3

The hit compounds were easily obtained in one or two steps from commercially available compounds according to Schemes 1-4. Thus the benzimidazole derivative NV2899 was prepared by cyclocondensation in DMF between 3,4-diaminobenzoic acid and **1**, obtained in turn from chlorobenzaldehyde and metabisulfite (Scheme 1).
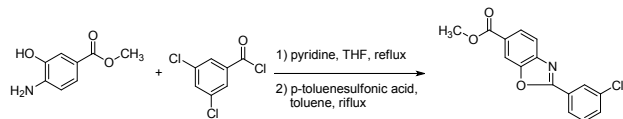
Scheme 1

NV2909, a 2-(3,5-dichlorophenoxymethyl)-thiazole derivative, was synthetized in one step from the corresponding chloromethyl-thiazole and 3,5-dichlorophenol in acetonitrile in presence of a base (Scheme 2).
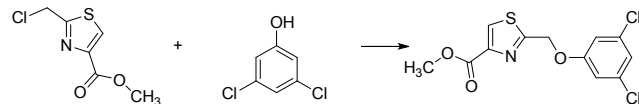
Scheme 2
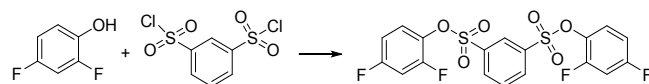

Also, the 2-(3,5-dichloro-phenyl)-benzoxazole NV2913 was prepared from a 3,5-dichlorophenyl derivative (an acyl chloride) and the suitable aryl ester, likely through a not isolated amido intermediate (Scheme 3).

Scheme 3

As shown in Scheme 4, NV2907 was obtained from 1,3-disulfonyl chloride and 2,4-difluorophenol, in DCM and carbonate.

Scheme 4


To evaluate the activity of the newly synthesized small molecules in promoting the readthrough of PTCs we used the FLuc cell- based assay.[15,16] To this aim HeLa cells were transfected transiently with the plasmids pFLuc-WT (control) and pFLuc-opal (UGA stop mutation).[28] After transfection, Hela cells were treated for 24 hours with the compounds (NV2907, NV2909, NV2899, NV2913) and subsequently the activity of the Fluc protein was measured by a luminometer. HeLa cells transfected with the pFluc-WT plasmid were used as positive control and showed high levels of luciferase activity (Figure 4). As negative control pFluc-opal transfected HeLa cells were used and these cells did not show any activity. Moreover, when these pFluc-opal cells were treated with Ataluren and the four different compounds we observed an increase of luciferase activity (Figure 4).
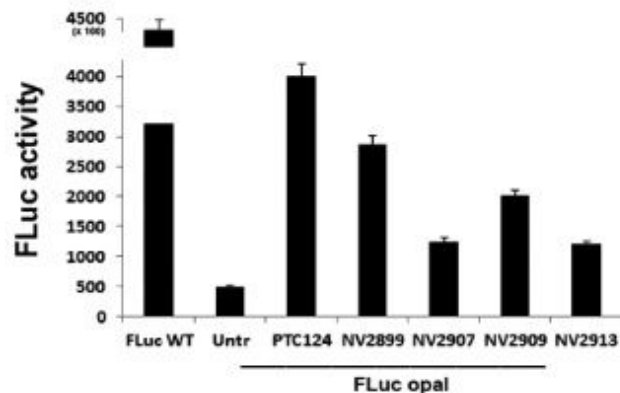
**Figure 4.** Histogram showing luciferase (FLuc) activity after 24 h of exposition to PTC124 and its analogues (all at the concentration of 12 μM) in HeLa FLuc-opal transfected cells

FRT CFTR wild type cells were used to determine the possible cytotoxic effects of NV2907, NV2909, NV2899, NV2913 and to visualize the impact on cell proliferation. In the graphs in Figure 5 are reported the percentages of dead cells and proliferating cells after treatment with 12 μM NV2907, NV2909, NV2899, NV2913. PTC124 at 12 μM was used as control. Our results showed similar increase in dead cells at 24-48-72 hours in all samples analyzed (Figure 5 up). We also evaluated the expression of the CFTR protein after treatment with the selected compounds. FRT cells stably transfected with the pTracer vectors containing either the WT-CFTR(CFTR-WT) or the G542X-CFTR human cDNA were used. The expression of the CFTR WT and G542X-CFTR after treatment with 12 μM of NV2899, NV2907, NV2909 and NV2913 was detected by immunofluorescence microscopy (Figure 6). After 24 hours of treatment with the two compounds NV2899 and NV2909, we observed CFTR expression in G542X-CFTR FRT cells. In contrast CFTR expression was not revealed in G542X-CFTR FRT untreated cells (negative control) (Figure 6).
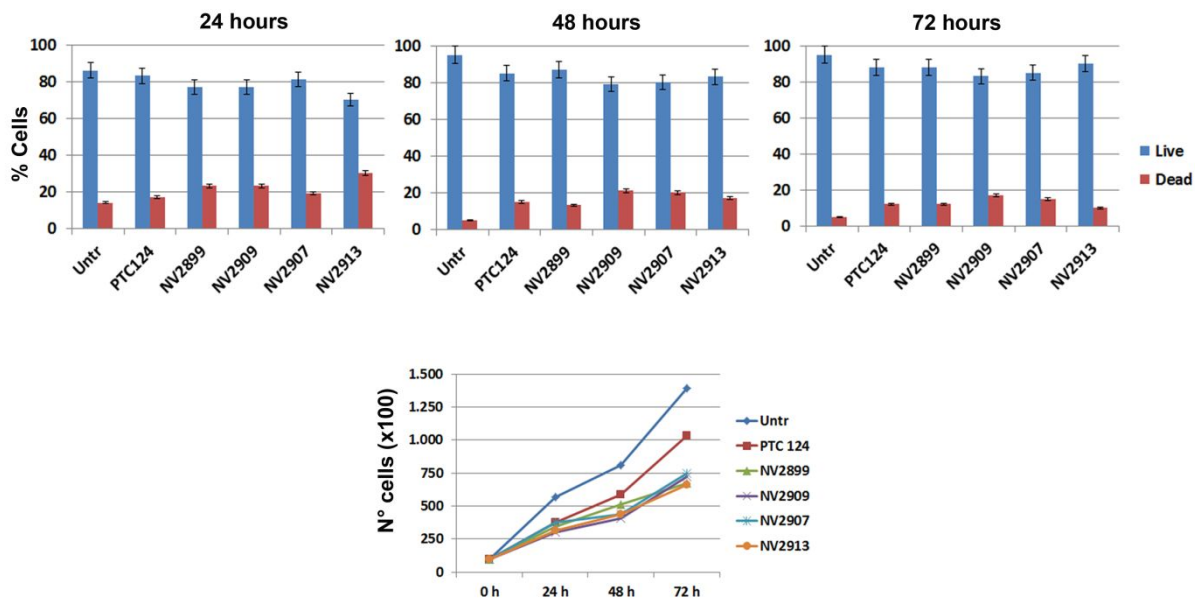
Figure 5 Histograms showing live and dead cells at 24-48-72 hours post treatment with the indicated compounds (up). Graphs showing cell proliferation at the same time intervals (24-72 hours) (bottom).
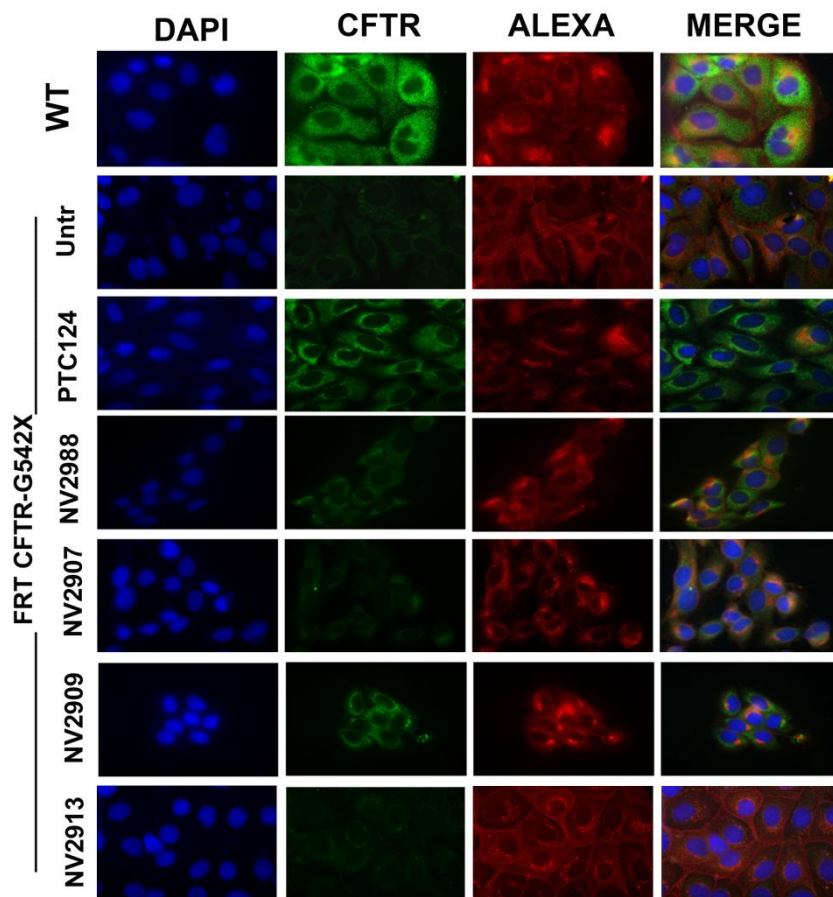
Figure 6 Immunofluorescence analysis to visualize CFTR (green-Ab570) expression in G542X-CFTR FRT cells treated with the indicated compounds for 24 hours. Cell membrane was stained in red and nuclei in blue with DAPI.

In conclusion, our studies, with the aim to identify a valuable substitute of Ataluren for the treatment of nonsense mutation in CFTR gene, allowed us to find out new promising chemical scaffolds as TRIDs. Following a ligand-based pharmacophore approach together to a virtual screening protocol, several small molecules capable to allow the *in vitro* expression of the gene pFLuc-opal (UGA stop mutation) were discovered. These compounds showed low cytotoxic effect and displayed the expression CFTR protein in transfected cells. We envision that these molecules could to be potential lead compounds as TRIDs and further studies will be performed in order to optimize these scaffolds.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Material and methods (Supplementary Information.pdf)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

TRIDs, Translational Readthrough Inducing Drugs

PTC, Premature Termination Codon

CF, Cystic Fibrosis

CFTR, Cystic Fibrosis Transmembrane Conductance Regulator

FLuc, Firefly Luciferase

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