Design of Next-Generation CFTR mRNA Therapeutics (MRT) for the Treatment of Patients with Cystic Fibrosis

L Boeglin, A Dias, K Tran, P Nanduri, C Guenard, N Kaushal, J Abysalh, J Skaleski, C Woo, K Lane, F DeRosa, J Androsavich, A Barbier, R Wooster

Translate Bio, Lexington, MA

Introduction

- Though recent advances in modulator therapy have increased treatment options to a large percentage of patients with cystic fibrosis, there remains a significant portion of the patient population that is unable to benefit from the use of these therapeutics.
- A clinical study with the first inhaled mRNA therapeutic, MRT5005, yielded encouraging initial data, and additional innovations to optimize the overall drug candidate profile of successive therapeutics are being explored.
- We have employed rational protein engineering and mRNA design to develop novel CFTR mRNA candidates coding for wild-type and functionally variant CFTR proteins.
- Three areas were identified to implement this approach- mimicking phosphorylation of the R domain via phosphomimetic substitutions to increase frequency of channel opening, modulation of ATP-dependent channel gating through targeted single amino acid changes, and regulation of cellular trafficking via changes in ubiquitination patterns.

Methods

- mRNAs coding for wild-type and engineered CFTR protein were designed using proprietary algorithms and manufactured using optimized processes.
- In vitro chloride-ion channel activity measurements were conducted in Fischer rat thyroid (FRT) cells to assess sensitivity to forskolin-activated stimulation, peak activity, persistence of channel function and presence of forskolin-independent chloride transport.
- Standard analysis of maximum chloride current was evaluated by subjecting the cells to Amphotericin B to permeabilize the basolateral membrane, followed by stimulation by forskolin, modulation using VX-770, and finally inhibition using CFTRinh-172. Assessment of forskolin sensitivity was conducted by instead using a sequential addition of increasing concentrations of forskolin followed by CFTRinh-172 in order to calculate the resulting EC-50 values.
- Trafficking and expression in HEK293 cells were assessed by Western blot using UNC #596 antibody and detectionand quantification of expression via LI-COR Odyssey.

Messenger RNA provides a tool for rational protein design

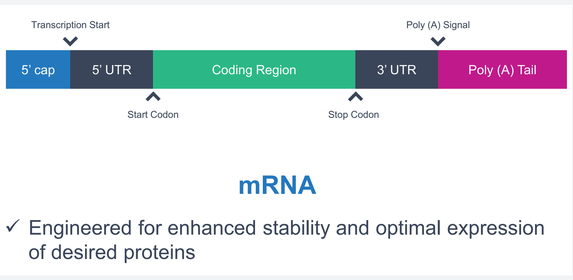

Figure 1: Translate Bio's messenger RNA structure

Utilizing Translate Bio's universal mRNA structure, targeted amino acid changes can be programmed into the coding region to express engineered CFTR protein variants.

Results

- Optimized wild-type or engineered CFTR proteins demonstrated four distinct profiles of channel activity in FRT cells. Each engineered variant showed an enhanced ability to move chloride as measured by Isc in Ussing assay, reaching as high as 200% activity compared to wild type channels.
- In addition to increased activity under maximum activation conditions, some channels showed increased sensitivity to forskolin, with up to a 10-fold reduction in forskolin EC50 values.
- Significant forskolin-independent current was observed in select constructs, consistent with the predicted effects of the functionally engineered changes.
- Analysis of trafficking in HEK293 cells demonstrated increased C-band expression for all three engineered CFTRs as measured by Western blot, with the 13E phosphomimetic channel producing 2-fold greater C-band expression as compared to wild type.

Phosphomimetic substitutions lead to CFTR channels with altered activity profiles in vitro

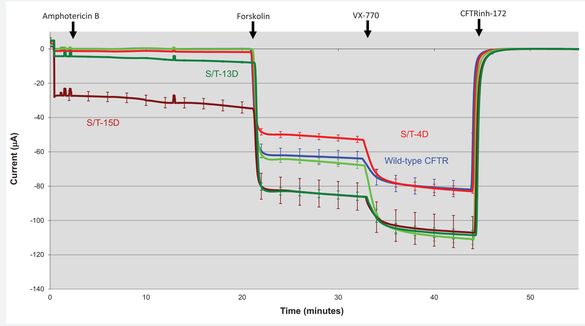

Figure 2: Chloride channel activity in Fischer rat thyroid (FRT) cells after transfection with phosphomimetic mRNAs as measured by Ussing chamber assay

Combinations of S/T to D mutations in the R domain of CFTR impart alterations to channel function. 4D and 6D channels demonstrate greater responsiveness to VX-770, while 13D and 15D display forskolin-independent chloride transport, as well as increased overall activity as compared to wild-type CFTR channels.

Glutamic acid substitutions produce superior activity in Ussing chamber

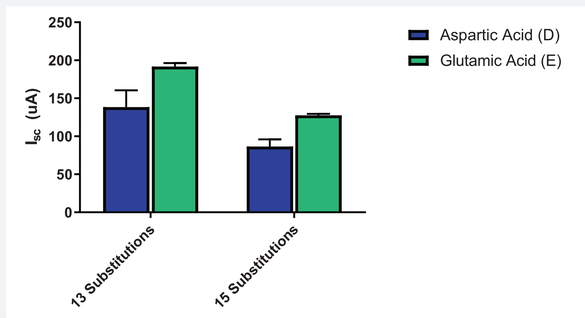

Figure 3: Comparison of phosphomimetic amino acids

Modeled in CFTR containing 13 or 15 phosphomimetic changes, glutamic acid-containing proteins demonstrate higher chloride conductance in FRT cells as compared to channels containing aspartic acid.

Gating mutations produce CFTR proteins with altered activity profiles

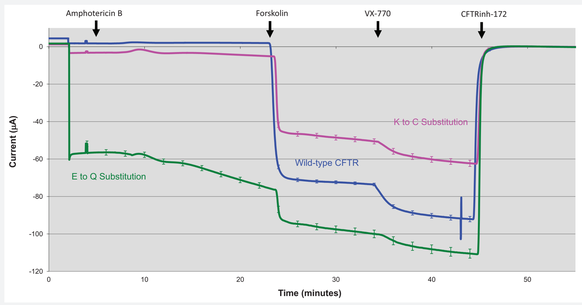

Figure 4: Manipulation of ATP-dependent channel gating

Chloride channel activity in Fischer rat thyroid (FRT) cells after transfection with gating variant mRNAs demonstrates two distinct effects. Substitution of cysteine for a particular lysine (K to C) in cytosolic loop 3 yielded a channel with overall reduced activity as compared to wild-type CFTR, though it did demonstrate slight conductance prior to forskolin addition. In contrast, replacing a specific glutamic acid with glutamine (E to Q) in NBD-2 produced significant pre-forskolin conductance, as well as overall increased activity in FRT cells.

Altering ubiquitination increases activity in selected constructs

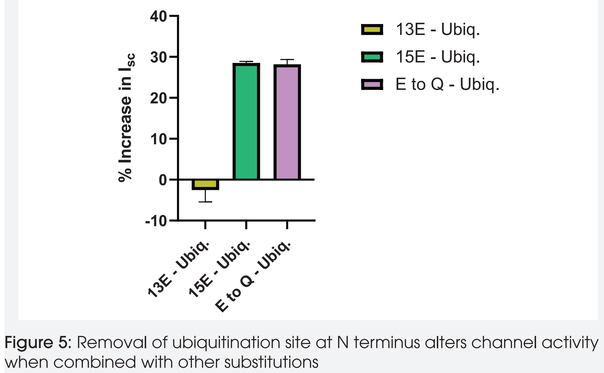

Figure 5: Removal of ubiquitination site at N terminus alters channel activity when combined with other substitutions

Three engineered CFTR proteins were further designed to alter ubiquitination patterns in the N terminus. The additional substitution had no effect on the chloride conductance when combined with the 13E phosphomimetic protein, in contrast with the 15E and E to Q gating variant proteins, both of which showed >25% increase in activity.

Phosphomimetic substitutions reduce the threshold for forskolin-mediated activation in a dose-dependent manner

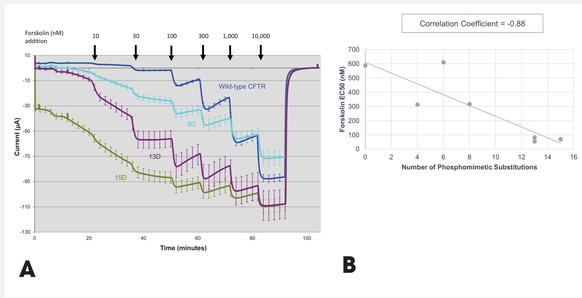

Figure 6: The number of phosphomimetic substitutions correlates to a decrease in forskolin EC50 concentration

(A) Modifying the typical Ussing chamber procedure to include successive escalating doses of forskolin demonstrates varying forskolin sensitivity of engineered CFTR variant channels. (B) A negative correlation was observed between increasing number of phosphomimetic substitutions and reduced requirements for forskolin-mediated channel activity.

Alanine variant protein demonstrates that phosphorylation is necessary for wild-type levels of channel activity

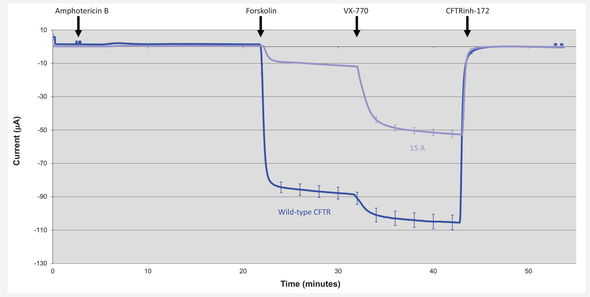

Figure 7: Alanine substitution at phosphomimetic sites reduces forskolin-induced chloride conductance in FRT cells

An engineered CFTR was produced containing alanine residues at 15 known phosphorylation sites. Analysis in Ussing chamber shows 85% reduction in short circuit current after forskolin addition when compared to wild type CFTR, consistent with the known role of phosphorylation in pka-mediated channel activation.

Engineered CFTRs show increased translation and trafficking in HEK293 cells

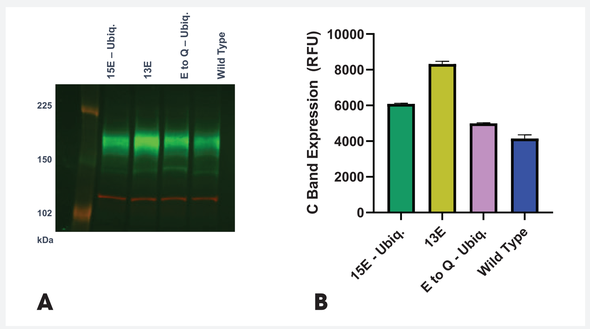

Figure 8: Expression and quantification of CFTR protein in HEK293 cells

(A) Western blot showing expression patterns for variant and wild type CFTR mRNAs (B) Quantification of Western blot results demonstrates increased C-band expression from three variant CFTRs, with one showing a greater than 2-fold increase in protein.

Conclusion

- Design of novel mRNA sequences encoding rationally engineered CFTR protein was shown to produce highly active ion channels and enhanced protein expression when compared to a reference sequence encoding the native protein.
- Channels with greater activity or enhanced translation could potentially allow for lower dosing requirements to achieve the same level of correction when compared to a messenger RNA encoding a wild type channel protein.
- These novel constructs, when combined with efficient delivery systems such as lipid nanoparticles, could possibly represent a potent messenger RNA therapeutic for the treatment of patients with cystic fibrosis, regardless of underlying mutation.

Acknowledgements

Translate Bio's Research and Development, Translational Development and Business Development teams

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