Development of Novel Therapeutics for all Individuals with CF (the future goes on)

Margarida D. Amaral[1] and Patrick Harrison[2]

[1]BioISI – Biosystems & Integrative Sciences Institute, Faculty of Sciences, University of Lisboa, Portugal

[2]Department of Physiology, BioSciences Institute, University College Cork, Cork, Ireland.

Correspondence to: mdamaral@fc.ul.pt or p.harrison@ucc.ie

Keywords: CFTR; Cystic Fibrosis; theratypes; genome editing

Contents

Abstract

Despite the major advances and successes in finding and establishing new treatments that tackle the basic defect in Cystic Fibrosis (CF), there is still an unmet need to bring these potentially curative therapies to all individuals with CF.

Here, we review aspects of what is still missing to treat all individuals with CF by such approaches. On the one hand, we discuss novel holistic (high-throughput) approaches to elucidate mechanistic defects caused by distinct classes of mutations to identify novel drug targets. On the other hand, we examine therapeutic approaches to correct the gene in its own environment, i.e., in the genome.

Abbreviations: CF, Cystic Fibrosis; CFTR, CF transmembrane conductance regulator; EMT, epithelial-mesenchymal transition; HC, high-content HT(S), high-throughput (screen).

Introduction

Although there are now highly effective CFTR modulator therapies (HEMT) available for clinical use, namely the triple combination tezacaftor/ elexacaftor/ ivacaftor that rescues F508del-CFTR (people with CF with either one or both CFTR alleles with this mutation are eligible), and other mutations have also been progressively demonstrated to be rescued by these and previous drugs (reviewed in 1, 2). However, it is estimated that among all individuals with CF worldwide (estimated to be ~162,000 in total [3]) still 15-20% have rare mutations which are not targeted by those drugs.

Moreover, although HEMT are 'game changers', individuals who take this drug are not completely 'free' of CF symptoms and some individuals have little or no benefit. Besides unknown long-term effects, some individuals are intolerant to these drugs and cannot take them. Importantly, it is estimated that worldwide only 12% of eligible individuals for these drugs are actually taking it [3], due to their high cost [4].

There is thus a need for alternative drugs based on the fundamental defect caused by distinct mutations, so as to improve and optimize the clinical benefit of not only people with CF, but also of individuals with CFTR-related disorders (pancreatitis, recurrent bronchitis and disseminated bronchiectasis, among others). In parallel to drug discovery to rescue the defective protein, as stated below advancements are in progress to change the genome to get rid of the gene defect i.e., the mutation (Fig.1). The ultimate goal is to identify novel therapies that not only treat every CFTR variant but also at an affordable cost so as to reach every individual with CF.

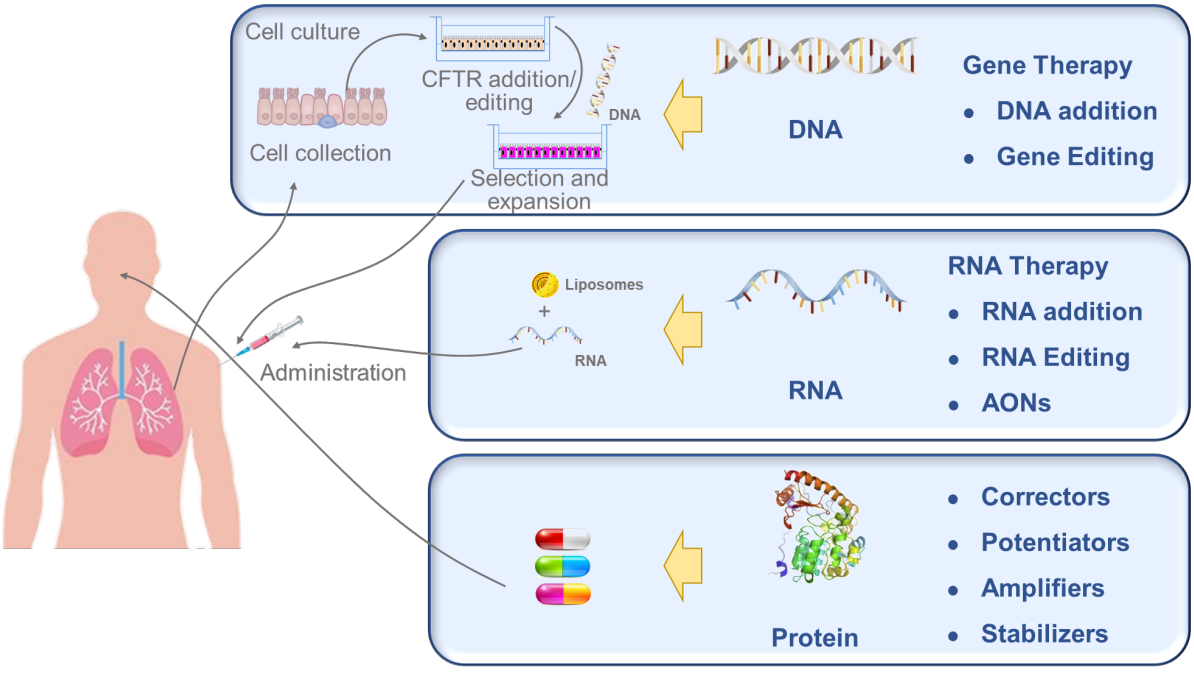

Figure 1. Strategies to rescue CFTR DNA, RNA or protein[1]

Theratyping

So far >2,100 reported variants [5] have been reported to occur in CFTR gene and they bring about very diverse molecular defects in the cell. Importantly, of these only 20% (i.e., a total of 485 variants) are annotated on the CFTR2 website [6]. Accordingly, to facilitate the need to promptly find feasible and efficient therapeutic strategies tackling the basic CF defect in individuals with rare mutations who are not eligible for currently available HEMT, CFTR mutations have been classified into seven functional classes [7]. These have evolved into theratypes based on the expectation that mutations within the same group can be tackled by the same molecular/cellular strategy, if not necessarily by the same drug.

Theratype I (formerly class IA [8,9]) refers to stop mutations (as for example G542X), i.e., those introducing a premature stop codon (PTC), which lead to shorter, non-functional proteins and induce degradation of the respective mRNA by a mechanism termed nonsense-mediated decay (NMD) [10].

Theratype II encompasses mutations (like F508del) that impair plasma membrane (PM) traffic by which the protein fails to reach the cell surface, due to a folding defect, being thus retained at the endoplasmic reticulum (ER) by the cellular mechanism termed ER quality control (ERQC) [11, 12].

Theratype III includes mutations impairing CFTR gating, i.e., the opening of the channel in response to agonists, as for example G551D [13].

Mutations in theratype IV (e.g., R334W) cause a defect in the channel conductance, i.e, the number of ions that traverse the channel pore per unit time [13].

Theratype V includes mutations that drastically reduce CFTR protein levels, in general due to creation/abolition of an alternative splicing site (e.g., 3272-26A>G), resulting in the presence of both species of aberrant and normal CFTR mRNA [14].

Theratype VI comprises mutations destabilising CFTR protein at the PM (e.g., F508del-CFTR after PM rescue), either by promoting its internalization (via endocytosis) or by impairing its return to the PM after internalization, or even by relaxing its cytoskeleton tethering [15].

[1] Although multiple organs are affected in CF, only the lung is shown in this diagram as airway delivery and targeting is the major focus of current gene-based approaches.

A novel theratype – VII (formerly class IB [8, 9]) – was proposed [7] to include CFTR mutations, such as large deletions (e.g., CFTRdele2,3), causing frameshift (e.g., 2183AA->G), or disrupting canonical splice sites (e.g., 621+1G->T), which cannot be rescued by small-molecule drugs [7]. Accordingly, these mutations are termed 'unrescuable'.

Importantly, we are still lacking a global view of mechanisms and pathways involved in CF pathophysiology. Indeed, although CF is caused by a defective anion channel, CFTR has also been implicated in other cellular processes, namely epithelial cell differentiation [16] and, when dysfunctional, cancer, particularly high for digestive tract cancers [17, 18] which was even reported among CF carriers [19].

The lab of one of us (MDA) developed several cell-based high-throughput (HT) assays and used them either in functional genomics approaches (siRNA screens) or compound screens for both a global mechanistic characterization of defects underlying CFTR mutations from different functional classes and the identification of putative drug targets. Approaches were designed for: class I (stop mutations); class II (trafficking defect); and class VII ('unrescuable' CFTR).

For class I mutations, we introduced G542X into a CFTR mini-gene assay [7] to identify novel genes and compounds suppressing premature termination codon (PTC) mutations and inhibiting nonsense-mediated decay (NMD) of PTC-mRNAs.

For class II mutations, we applied a HT microscopy F508del-CFTR traffic assay in human bronchial epithelial cells [20] to screen ~27,000 siRNAs targeting about half of the human genome. We found key regulator genes (kinases) with additive effect to current highly effective CFTR correctors.

For class VII mutations, we applied a HT microscopy-based traffic assay for TMEM16A/Anoctamin 1 [21] to screen a library of siRNAs targeting ~700 traffic regulators [22]. About 700 genes were screened (2 siRNAs per gene) of which 262 were identified as candidate TMEM16A modulators (179 siRNAs enhanced and 83 decreased TMEM16A traffic), on the primary hit list. Twenty genes could be functionally validated as hits, among the 179 siRNAs identified as enhancing TMEM16A traffic. Further mechanistic studies revealed that downregulation of two G-protein coupled receptors (GPCRs) - ADRA2C and CXCR3 - increased TMEM16A-mediated Cl^- secretion in human airway cells, in parallel with the observation that their overexpression significantly decreased TMEM16A currents in these cells. Accordingly, inhibition of these GPCRs leading to the activation of this alternative Cl^- channel

emerge as possible drug targets to compensate for dysfunctional CFTR in individuals with CF, irrespective of the genotype.

To start tackling the delay in differentiation observed in native CF tissues and cells [23], we have designed a HT wound-healing assay and applied it to CF cells. Data from this screen led to the identification of key transcriptional factors linking CFTR to both epithelial differentiation, namely KLF4 [24], and epithelial-mesenchymal transition (EMT), namely TWIST1 [25] and YAP1 [26].

Rescuing the gene defect

In 1989, *Science* gave us the CFTR gene, its cDNA, and details of the first CF-causing mutation, F508del [27-29], and sparked the search for gene-based treatments of genetic disorders. Over the last three decades, a growing number of options (Figure 1) have emerged starting with a proof of concept study that the CFTR cDNA could complement the F508del mutation [30], and if translated to the clinic, could in principle, work for any CF mutation. This was followed by antisense oligonucleotides (AONs) that could restore normal splicing of the deep-intronic mutation 3849+10kbC>T [31], and one of use (PTH) showed that DNA gene-editing could correct the F508del mutation [32]. Other groups have shown that CFTR mRNA (rather than cDNA) can express functional CFTR protein in transfected cells [33], that the CFTR mRNA can be directly edited [34], and that anticodon engineered tRNAs have the ability to "readthrough" certain CFTR premature termination codon (PTC) mutations [35]. And this has been coupled with dramatic improvements and options for DNA, RNA and protein delivery, a small menagerie of CF animal models [36] and a large number of cell and organoid models for testing [37]. But how close is a clinical breakthrough for CF using gene-based therapies?

There are no *a priori* limitations to develop gene-based medicines for CF, as witnessed by numerous clinical breakthroughs for a number of other diseases, with licenced cDNA therapies for spinal muscular atrophy and Leber congenital amaurosis, and there many other successful cDNA therapy clinical trials, most recently for haemophilia B [38]. With AON to induce exon skipping, there has been substantial progress for Duchenne's muscular dystrophy with four licenced AONs targeting different mutations [39]. It is seven years since the first gene-edited CAR T cells were tested in the clinic for leukaemia [40], and there nearly a dozen licenced cell therapies [41], and numerous clinical trials on-going for other inherited disorders using both virus vectors [42] and mRNA delivery [43] of a number of gene editing approaches.

For cDNA therapy in CF, there have been ~25 clinical trials, the most recent a large scale repeat dose cDNA trial delivery DNA using lipid nanoparticles. Whilst the clinical benefit was small, no serious adverse effects were observed in 62 people who received at least nine doses of the therapeutic agent. Importantly, this trial firmly established both the feasibility of recruiting a large number of people with CF to gene-based trials, and the logistics of delivering gene-based drugs on a regular basis. Plans are now in place for a new cDNA based clinical trial using a lentiviral delivery vector [44] known as BI 3720931 [45].

The direct delivery of mRNA has been shown to restore CFTR function in a number of cell and animal models in studies by several groups, and a phase I/II trial to test a clinical formulation of the mRNA (MRT5005) administered by nebulization in adults with CF is on-going. Early studies suggested improvements in FEV_1, but follow-up analysis indicated no significant change at the doses tested to date [46]; plans are in place to test higher doses, and other companies and many academic groups are actively working in this area.

Numerous gene editing strategies have been reported to correct multiple mutations through the targeted integration of CFTR superexons (partial cDNA sequences) that results in production of full length CFTR mRNA and functional protein, an approach that could be used for *in vivo* targeting of actively dividing basal cells [47], or *ex vivo* editing and engraftment of cells [48]. A major advantage of these approaches is that most or potentially all CF mutations could be corrected with one reagent.

High efficiency adenine base editing (ABE) of individual CF mutations has been reported by several groups, though further refinements will be required to remove potentially disruptive by-stander edits [49-51]. A major advantage of this approach is that editing is performed without the need for double stranded breaks, but each mutation will require the development of a separate guide RNA. Low levels of off-target deamination have been detected ABE, though a number of strategies exist to minimise this [52], and a clinical trial of ABE for another disease has recently commenced [53].

In 2019, prime editing emerged as a strategy which could potentially correct virtually any CF mutation, or even clusters of CF mutations [54]. To date, only one study of prime editing of a CFTR mutation has been described [55]. But several recent refinements to prime editing, and advances in delivery of both base and prime editing [56-58], should see greater impact of this approach in the near future.

One possibility for a small number of CF PTC mutations is direct correction of the CFTR mRNA using an RNA editing approach known as Repair, though to date this has only been described for W1282X [34].

As with all approaches targeting mRNA for correction, repeat dosing will be required, but this could actually be advantageous as it may provide more options when assessing dosing and safety for clinical trials. Another option for a wider number of PTCs could be to use anticodon engineered tRNAs. A panel of these small RNA molecules, known as ACE-tRNAs have developed to enable readthrough of three common CF-causing PTCs, G542X, R1162X, and W1282X, with concomitant restoration of CFTR function [35]. Potential advantages of ACE-tRNA therapy is that its payload is ~50-times smaller than a full length CFTR mRNA, which should simplify delivery to target cells. Moreover, CFTR protein should only be restored in cells that normally express the CFTR gene.

Finally, what about strategies to restore correct splicing for the six characterised deep intronic CF mutations? One of our labs (PTH) and others have successfully disrupted the splice sites for CF mutations 3849+10kbC>T, 1811+1.6kbA>G and 3272-26A>G by CRISPR Cas9 targeted excision [59] or Cas12a targeted ablation [60], essentially the same approach in clinical trials for LCA10 [42]. But it looks like a quicker route to clinic is by using AONs to restore splicing, at least for 3849+10kbC>T. Since the original proof of concept study [31], many academic and industry labs have continued to research this approach [61, 62], with the possibility of an antisense drug SPL84 being evaluated in a clinical trial commencing in late 2022 [63].

Concluding Remarks

The last decade has seen remarkable breakthroughs with licencing approval for small molecule drugs for the treatment of many people with cystic fibrosis, though as discussed here, not everyone can access these drugs for a variety of reasons. Thus, the continuing aim of CF research is to develop effective and accessible treatments for everyone with CF as soon as possible. Here, we have discussed two complementary areas of current research, the search for novel small molecule treatments, and on-going development of nucleic acid-based strategies.

The use of HT assays and functional genomics is leading to a greater mechanistic characterization of defects underlying CFTR mutations, and the identification of putative drug targets for theraptype classes I and II. For class VII mutations, where there is no CFTR protein produced, the search for novel TMEM16A modulator targets could lead to the identification of small molecules that could offer treatment options for a class of mutations known as “unrescuable”, and potentially be used for everyone with CF regardless of genotype.

The development of at least seven different nucleic acid-based strategies has already resulted in multiple clinical trials, and many potential therapeutic options are in the pipeline. Some strategies such as mRNA or cDNA-based treatments, and/or gene editing with very long superexons, have the potential to work for almost everyone with CF using a single set of reagents, which could simplify clinical trial co-ordination and regulatory matters. All gene-based approaches targeting an individual will however be more challenging to develop as there will be many cohorts of people, each requiring separate clinical trials and regulatory approval processes.

An updated theraptyping model may also be required for gene-based approaches, but this also raises the exciting possibility that as clinical breakthroughs emerge for a particular approach for an individual mutation, it should be possible to develop similar treatments using the same mechanism for other mutations. Indeed, this strategy has already been observed for AONs used in exon-skipping strategies for DMD [36]; the same could apply for breakthroughs in gene editing for CF.

Once a curative treatment is found for anyone with CF, then the challenge will be to translate this to everyone with CF. For example, if a base or prime editing strategy were successful for one particular CF mutation, then it should be feasible to change the guide RNA or prime editor guide RNA sequence, and then use the same delivery strategy to enable many other people with different genotypes to be treated. A clear and imaginative strategy to successfully approach this challenge in terms of genetics, logistics, regulatory and financing perspectives, has recently been proposed [64].

Acknowledgements

Work in MDA lab is supported by UIDB/04046/2020 and UIDP/04046/2020 centre grants (to BioISI) from FCT/MCTES (Portugal) and research grants (to MDA): "HIT-CF" (H2020-SC1-2017-755021) from EU; SRC 013 from CF Trust (UK); and "PTSense" (AMARAL19G0) from CF Foundation (USA).

Work in PTH lab is supported by SRC020/ALLEN20G0 from CF Trust (UK) and CF Foundation (USA); HARRIS20G0 and HARRISP21G0 from CF Foundation (USA); and 20-FFP-P-8661 from Science Foundation Ireland.

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