Review Article

Molecular basis of cystic fibrosis: from bench to bedside

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Abstract: Cystic fibrosis (CF), is an autosomal recessive disease affecting different organs. The lung disease, characterized by recurrent and chronic bacterial infection and inflammation since infancy, is the main cause of morbidity and precocious mortality of these individuals. The innovative therapies directed to repair the defective CF gene should account for the presence of more than 200 disease-causing mutations of the CF transmembrane conductance regulator (*CFTR*) gene. The review will recall the different experimental approaches in discovering CFTR protein targeted molecules, such as the high throughput screening on chemical libraries to discover correctors and potentiators of CFTR protein, dual-acting compounds, read-through molecules, splicing defects repairing tools, CFTR "amplifiers".

Keywords: Cystic fibrosis (CF); personalized medicine; cystic fibrosis transmembrane conductance regulator correctors (CFTR correctors); CFTR potentiators

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Introduction

Cystic fibrosis (CF), the most common life-threatening rare disease among Caucasians, is an autosomal recessive genetic disease occurring in approximately one in 3,000–4,000 live birth as based on neonatal screening (1). Although several organs are involved, manifestation of CF disease in the airway tract is the main cause of mortality and morbidity in these patients (2). From the first description of a disease of the exocrine pancreas associated with lung symptoms in 1938 (3), survival of CF patients increased to a median age of 40 years, thanks to antibiotic therapies and correcting the intestinal malabsorption (4,5). After the identification of defective CF transmembrane conductance regulator (*CFTR*) gene, in 1989 (6-8), therapeutic approaches on the management of symptoms had a turning point which opened many hopes towards *CFTR* gene-targeted strategies (9), a field of investigation full of promises in steady progress.

Role of CFTR protein

CF disease is due to the defect of the *CFTR* gene located on chromosome 7 (6). *CFTR* gene encodes a protein encompassing the cellular membrane with two membrane-spanning domains (MSD), each constituted by six alpha-helices, two cytoplasmic domains, each binding one ATP molecule, termed nucleotide binding domains (NBD), a regulatory (R) domain with several consensus sequences for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC). CFTR protein belongs to the family of ATP-binding cassette (ABC) transmembrane proteins (10). It is a chloride ion transporter localized at the apical membrane of several polarized epithelia (11,12),

although other small molecules seem to be transported by CFTR (13), including ATP (14-17). As a chloride transporter, CFTR plays a critical role in the hydration of the mucus at the surface of the airway tract (18). Moreover, it favors mucus tethering and detachment through alkalinization with bicarbonate (19,20). De-hydration of airway surface fluid is a critical feature in the onset of the neutrophil dominated inflammatory and infective milieu of CF airways, which begins in the early months of life (21). CFTR-mediated ion transport requires binding of ATP on NBFs and phosphorylation of the R domain by protein kinase A (22-25) and protein kinase C (26-28).

Lung disease in CF

Defective ion transport mediated by CFTR reduces airway surface liquid hydration, which impairs mucociliary clearance, one of the basic innate immune defense mechanism of the respiratory tract (18,21). CF lung disease is characterized by an exaggerated inflammatory response accompanied by a huge number of neutrophils in the lumen of bronchi (21). However, these neutrophils are unable to completely clear bacteria; thus, repeated infections, mainly by *Haemophilus influenzae* and *Staphylococcus aureus*, pave the way to a chronic settlement of *Pseudomonas aeruginosa*. In addition, neutrophils release proteases, mainly elastases, reactive oxygen species and neutrophil extracellular traps thus worsening respiratory function and progressive tissue destruction and ultimately leading to respiratory insufficiency, reduced quality and expectancy of life (21,29-34). Experiments performed in different model systems *in vitro*, *ex vivo* and *in vivo* animal models have not yet clarified whether recruitment of neutrophils in the bronchial lumen, precedes or follows bacterial infection (35-37). To combat respiratory insufficiency, CF patients are treated with antibiotics and anti-inflammatories and soon or later, they undergo lung transplantation, which provides a dramatic improvement in the quality of life and some extension of survival (38-40).

The *CFTR* gene mutations

The 250-kb gene, located in chromosome 7, is structured in 27 exons. An International Worldwide Consortium of laboratories of molecular genetics extensively analyzed sequence variants and to date, over 2,000 sequence variations have been reported, at least 200 of them being associated with the disease (see the Cystic Fibrosis Mutation Database of the Cystic Fibrosis Gene Analysis Consortium, www.genet.sickkids.on.ca/cftr/) (41,42). Deletion of the phenylalanine in position 508 of the polypeptide chain, known as Phe508del or F508del, is the most common *CFTR* mutation, affecting from 50% to 90% of the chromosomes of CF patients along different geographical areas (43). Besides F508del *CFTR*, most CF causing mutations are missense variants (42%), nonsense (10%), frameshift (15%), splicing (13%), in frame deletion/insertion (2%) and promoter (0.5%) mutations (42,43).

The molecular defects of CFTR protein

F508del *CFTR* mutation (8) leads to the synthesis of an immature, non-glycosylated protein unable to localize on the plasma membrane (44). In-depth studies on the consequences of the different mutations on CFTR protein have allowed to simplify functional defect mechanisms (45), now schematized into the six classes (2), as shown in *Figure 1* and described as follows:

Class I—"No protein"

These mutations affect protein synthesis, due to stop-codon (nonsense) mutations in which the CFTR mRNA is degraded through a process termed nonsense-mediated decay. This class includes G542X mutation (common in Mediterranean coastal area), R1162X (common in North-eastern Italy and Catalonia), W1282X (affecting about 40% chromosomes in Ashkenazi Jews).

Class II—"No traffic"

These mutations affect CFTR protein processing, due to protein misfolding, which is recognized by the endoplasmic reticulum (ER) quality control machinery leading to protein degradation. This class includes the most common F508del mutation, N1303K, R560T, A561E and R1066C.

Class III—"No function"

These mutations, also termed as "gating defect", affect the activation of ion transport function, although CFTR is correctly glycosylated and located at the plasma membrane.

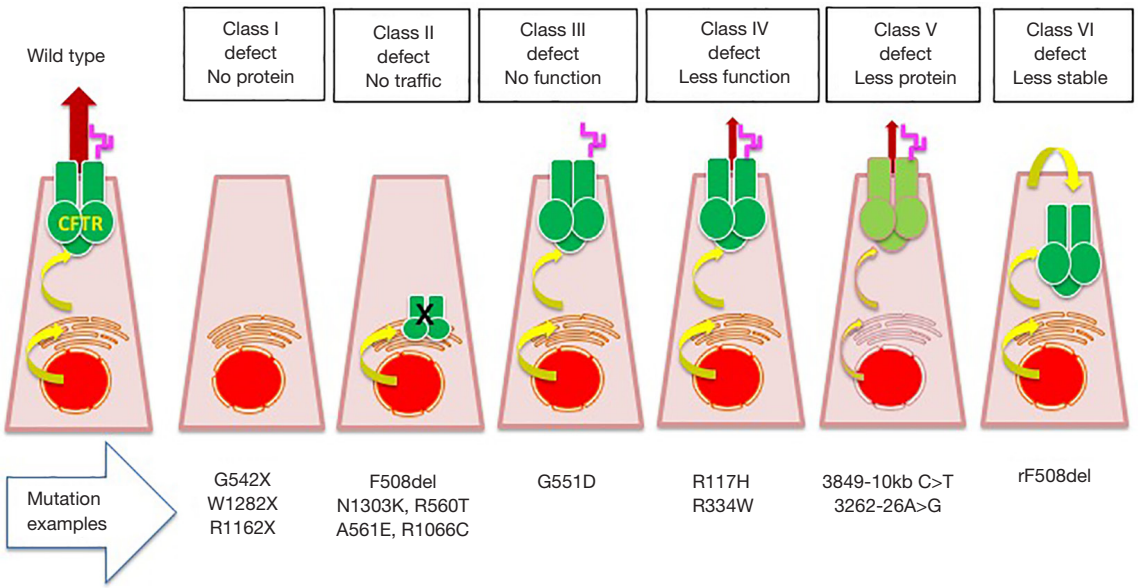

Figure 1 The molecular defects of CFTR protein. Paradigmatic CF gene mutations are reported in association to the schematic representation of six classes of cellular and molecular defects of CFTR protein, as described in the text. CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis.

This class includes G551D mutation.

Class IV—"Less function"

These mutations reduce chloride ions transported through pore channel, due to mutations the arginine located in the MSDs, which are involved in the flow of chloride through the plasma membrane. This class includes R117H and R334W.

Class V—"Less protein"

These mutations significantly reduce the amount of wild-type CFTR protein at the plasma membrane, mainly due aberrant splicing of RNA, leading to a non-functional protein. This class includes 3849-10 kb C>T and 3262-26A>G.

Class VI—"Less stable"

These mutations affect the stability and/or anchoring of CFTR protein at the plasma membrane. This class includes F508del CFTR rescued (rF508del) by correctors.

Notably, many *CFTR* causing mutations are not classified in one of these six classes and in some cases, mutations present more than one class defect, e.g., F508del mutation has a processing defect (class II), a gating defect (class III) and a reduced stability at the plasma membrane after being rescued (class VI). Despite simplistic, this classification has focused research of novel drugs towards different protein defects thus allowing development of personalized medicine, i.e., specific treatments tailored on CF genotype (46).

High throughput screening (HTS) in search of new CFTR protein targeted molecules

In search of CFTR modulators, large scale chemical libraries comprising thousands of compounds were tested. Initial challenge was to set up simple and rapid technological tools to study the effect of each molecule on chloride channel activity. In this respect, three different HTS assays have been developed, as reviewed and depicted in *Figure 2* (50). Starting from the SPQ molecule, whose emission intensity is modulated by intracellular collisional quenching, other halide-sensitive fluorescent probes have been developed, such as MQAE, a membrane permeable dye retained inside the cells by cleavage of acetyl ester residues (47,51-53). A second assay, based on membrane depolarization dependent on chloride channels activation under proper experimental conditions has been set up. In this assay, membrane depolarization can be detected by measuring variations of fluorescence of membrane-potential sensitive dyes, due to

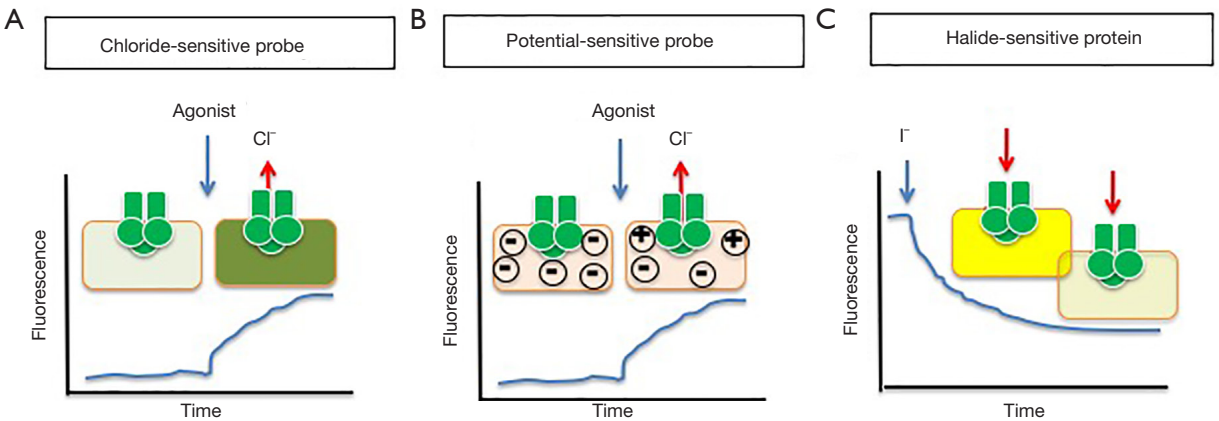

Figure 2 Fluorescence-based assays for high-throughput screening of chemical libraries to detect functional CFTR protein. (A) Chloride-sensitive fluorescent probes (SPQ, MQAE) undergoing collisional quenching upon interaction with chloride ion or other halides (e.g., iodide). Agonists activating efflux of chloride ion from inside to outside the cell reduce the quenching of the fluorescent probe (47); (B) membrane potential-sensitive fluorescent molecules partition from extracellular space to the plasma membrane as a function of chloride efflux-dependent depolarization. Partition of the probe inside the less polar plasma membrane dramatically increases quantum yield and consequently the emission of fluorescence (48); (C) yellow fluorescent protein (YFP) made sensitive to collisional quenching to halides reduces the emission of light as a function of entry of chloride or iodide inside the cell (49). CFTR, cystic fibrosis transmembrane conductance regulator.

the quantum yield change upon different polarity of the cellular environment (54,55). We developed the membrane-potential sensitive probe bis-oxonol to detect CFTR correction after transferring with viral vectors the wild type CF gene in CF bronchial epithelial cells (48). This assay was then accomplished by an HTS of more than 100,000 compounds that lead to the discovery of the first two small molecules became drugs for CF patients: VX-770 and VX-809 (56-58). A third tool, based on dynamic quenching of a yellow-fluorescent protein (YFP) made sensitive to intracellular chloride-ion concentration was set up (59) and further improved by mutations that render YFP very sensitive to chloride ion (49,60). F508del CFTR correctors and G551D CFTR potentiators were discovered by this assay (61-64). Interestingly, also a potent CFTR-specific inhibitor (65), currently used to inhibit CFTR function *in vitro* assays, was discovered and proposed to target the hyper-secretory diarrhea mediated by hyper-functional CFTR protein, induced by cholera toxin (65).

The first molecules reaching the chemist's bench

CFTR correctors are the molecules able to rescue the class II defective CFTR, e.g., F508del CFTR and CFTR potentiators those activating the chloride transport in Class III gating-defective CFTR, e.g., G551D (2). This terminology allows to define the effect of each molecule and recalls the experimental conditions utilized in the screening. As a matter of fact, HTS for discovery correctors is performed in F508del CFTR expressing cells, incubated for 24–48 hours with the testing molecules whereas HTS for potentiators is carried out in G551D CFTR expressing cells, acutely treated with such compounds (50). Firstly, CFTR modulators were identified by academic groups (61-65), however these molecules did not undergo a pharmaceutical development from preclinical to clinical trials. Importantly, the biotech company Vertex Pharmaceuticals published its first "CFTR corrector" VX-809 (Lumacaftor) (56,58) and its first "CFTR potentiator" VX-770 (Ivacaftor, trade name Kalydeco) (57), few years later.

These molecules underwent a quick drug development passing from *in vitro* assays (56-58) directly to clinical trials in CF patients. Food and Drug Administration approved VX-770 in 2012 and VX-809 in 2015, for the treatment of CF patients carrying specific *CFTR* mutations.

VX-770 has proven excellent efficacy in children over six years of age and adults with G551D mutation in at least one allele (66-68), as demonstrated by an average 10% increase of forced expiratory volume in 1 second (FEV_1), decrease of pulmonary exacerbations, weight increase and normalization of sweat electrolytes (67), also patients with

very low residual lung function (e.g., FEV_1 <40%) (69,70) or carrying class III mutations other than G551D (71). Unfortunately, the advantages of this drug are limited to very few CF patients as G551D mutation is very rare (43,72).

On the contrary, treatment with VX-809 in F508del *CFTR* homozygous patients did not produce any improvement of FEV_1 (73). These disappointed results led to development of VX-770 and VX-809 combined formulation, named Orkambi that was tested in CF patients homozygous for the F508del *CFTR* mutation, providing some benefits in lung function (74). These data were reproduced in a large international multicentric clinical trial showing a 2–3% increase of FEV_1 in respect to placebo after 24 weeks of treatment with Orkambi (75), although benefits were less evident in compound heterozygous CF patients carrying F508del *CFTR* in one allele (76). Different investigations *in vitro* have been pursued in order to understand these clinical limitations. For instance, it has been found that VX-770 negatively interacts with the rescued F508del CFTR protein by VX-809, thus reducing plasma membrane stability (77,78). How this interaction translates in CF patients is presently debated (79,80).

Molecules for class I "No protein" defects

As mentioned above, class I mutations cause CFTR mRNA degradation through nonsense-mediated decay. Discovery of molecules able to read-through the premature stop codons for treating CF patients carrying class I mutations started after the observation that aminoglycoside antibiotics can correct this defect (81,82). In this respect, the aminoglycoside gentamycin, previously used for the treatment of bacterial infections (83-86) was investigated. In order to avoid toxicity of aminoglycosides, gentamycin was then replaced by the analogue ataluren, produced by PTC Therapeutics (87-89). Unfortunately, a long-term placebo-controlled double-blind phase 3 study showed no improvement in the primary endpoint FEV_1 in CF patients, despite initial promising findings in several clinical trials (90). This led PTC Therapeutics discontinuing the development of ataluren in CF, leaving wide open the need of compounds targeting class I mutations (91).

Molecules for class II "No traffic" defects

Several correctors to rescue the class II defective CFTR, e.g., F508del CFTR, have been discovered by different academic groups in the United States and Europe (64,92-115). However very few of them underwent pharmaceutical development. Thus, different pharmaceutical companies are investing their own resources in pre-clinical discovery of new correctors.

The encouraging advancements obtained with VX-809 prompted Vertex Pharmaceuticals to explore new correctors, such as VX-661 (Tezacaftor) in association with VX-770 (116-118). F508del *CFTR* homozygous patients, treated with this combination ameliorated lung function (116-118). In addition to VX-809 and VX-661, several other correctors discovered by Vertex Pharmaceuticals (VX-152, VX-440, VX-445, VX-659) and by other companies, such as Genzyme/Sanofi, Pfizer and Reata (FDL169, GLPG2222, PTI-428, PTI-801), entered in phase 1/2 clinical trials (https://www.cff.org/Research/Developing-New-Treatments/).

Molecules for class III "No function" defects

Treatment of F508del *CFTR* homozygous patients requires both correctors and potentiators to rescue the gating defect also present in F508del CFTR protein (119).

Approval of VX-770 for CF patients with G551D mutation (56), is one of the major breakthroughs for CF cure (66-68). Nevertheless, negative interactions between VX-770 and VX-809 (77,78) prompted academic groups to search novel potentiators that do not present these limitations (120). In parallel, other companies launched phase 2 and phase 1 clinical trials on new potentiators (https://www.cff.org/Research/Developing-New-Treatments/). Very interestingly, dual-acting compounds, i.e., corrector and potentiator activity, may be a very appealing therapeutic perspective for CF treatment (see below).

Molecules for class IV "Less function" defects

This mutated CFTR protein displays low ion conductance that could be repaired by increasing protein expression at the plasma membrane or potentiating its open state period. In this regard, clinical trials with VX-770 (Ivacaftor, Kalydeco) in CF patients carrying the R117H mutation showed some benefit in lung function of adults with stable disease (121). This evidence supports further testing of potentiators in patients with *CFTR* class IV mutations.

Molecules for class V "Less protein" defects

As detailed above, class V mutations reduce the expression of functional CFTR. As a consequence of abnormal splicing both aberrant and normal transcripts are produced. To repair this defect, increase CF gene transcription as well as CFTR correctors and potentiators could represent useful remedies (122).

Molecules for class VI "Less stable" defects

Less stable CFTR protein needs to strengthen its anchoring at the plasma membrane. Importantly, rescued F508del CFTR protein by correctors displays increased turnover due to its removal by the peripheral quality control machinery and Disabled-2 (Dab2)-dependent ubiquitination (123-126), further worsened by *P. aeruginosa* chronic infection that decreases, the expression of critical proteins, such as Na^+/H^+ exchanger regulatory factor 1 (NHERF1) (127-129). Therefore, treatment of F508del CFTR homozygous patients should be addressed not only with correctors and potentiators but also with compounds stabilizing the rescued CFTR, by targeting both the CFTR anchoring proteins and the peripheral quality control machinery.

Dual-acting CFTR corrector and potentiator compounds

Consensus was reached that multiple defects of F508del CFTR protein should be addressed by combination of correctors and potentiators [for review see (130)]. In order to avoid negative side effects due to multiple drug interactions, compounds able to act at the same time both as correctors and potentiators, i.e., dual-acting compounds, have been proposed (131,132). Several dual-acting compounds have been identified so far (99,103,104, 133-135). In this regard, an interesting example has been given by 4,6,4'-trimethylangelicin (TMA) which besides correcting and potentiating CFTR activity displays anti-inflammatory properties (99,134). TMA exerts its dual action by interacting directly with the MSD1 on F508del CFTR protein (136).

The CFTR "amplifiers"

Beside the above mentioned molecular defects, F508del mutation produces a somewhat low amount of non-glycosylated immature Band B CFTR (44). Transcriptional inducers such as 4-phenylbutirrate were found to repair CFTR function by increasing band B CFTR protein that could escape at least in part the quality control systems (137,138). Therefore, forcing the production of band B CFTR protein in association with CFTR correctors could improve the overall efficacy of treatment (139). In this respect, new class of compounds called "CFTR amplifiers" seem to provide promising results *in vitro* (140). In particular, PTI-428 has been tested in a phase 1 clinical trial in CF patients under sponsorship of Proteostasis Therapeutics (https://www.cff.org/Research/Developing-New-Treatments/). An alternative approach is to inhibit the degradative pathway of CFTR mRNA intervening on the epigenetic down regulation of *CFTR* expression, e.g., by microRNA miR-145, which inhibits CFTR translation by degrading CFTR mRNA and blocking CFTR protein translation. MiR-145-specific cell permeable peptide-nucleic acid chimera relevantly increased CFTR protein (141).

Effects of repairing mutated CFTR on lung infection and inflammation

It has been suggested that repairing of the ion transport defect by CFTR correctors and potentiators can by itself solve CF chronic lung infection and inflammation. In vitro evidence supports this idea as VX-809 abolished the exaggerated inflammatory pathways in F508del *CFTR* bronchial epithelial cells (142,143). A different F508del CFTR corrector, miglustat, was also found to have anti-inflammatory effects in CF bronchial epithelial cells, although not directly related to correction of mutated *CFTR* (144). On the contrary, derivatives of the angular furocoumarin angelicin already proved as correctors of F508del CFTR protein (TMA analogues) showed that rescue and anti-inflammatory activity can coexist or be separated in the same molecule as a function of structural changes (145). These findings provide evidence that CFTR rescue *per se* is not enough to reduce excessive inflammation. Despite different results *in vitro* indicate that F508del CFTR rescue could *per se* repairs excessive inflammation (142,144), no evidence of reduced lung inflammation after VX-809 has been presented so far in CF patients. Moreover, CFTR restoration for all individuals with CF is challenging because approximately 2000 CFTR variants have been reported, most of them are rare (see the Cystic Fibrosis Mutation Database of the Cystic Fibrosis Gene Analysis Consortium, www.genet.sickkids.on.ca/cftr) and

personalized medicine approaches based on each individual's genetic profile may not be sufficiently efficacious in patients with irreversible lung damage. Thus, it appears that both combinations of novel CFTR potentiators and correctors as well as newer compounds for conventional therapies, such as inhaled antibiotics and anti-inflammatory agents, remain a cornerstone of treatment for CF lung disease [for review see (146-149)].

Conclusions and open issues

The ability to repair CF defect by using personalized medicine based on each patient's genetic profile represents a new challenge for CF research community. Despite exciting advances, several issues still remain open:

(I) As not all *CFTR* gene mutations have been classified within the six classes and many defects lack repairing molecules, the right drug for each CF patient is not available yet;

(II) In order to increase the amount of rescued F508del CFTR, more effective correctors are still needed as well as clear-cut biomarkers to evaluate their efficacy;

(III) Considering that CF patients should be treated by combination of more drugs, the interaction between these drugs needs to be investigated in depth;

(IV) Different therapeutic response along different patients is emerging, therefore, clinical efficacy of a specific treatment in every patient should be predicted by novel tools;

(V) Long-term safety of new drugs is still unknown;

(VI) It is still a matter of debate whether rescuing CFTR defect avoids infection and exaggerated inflammation occurring in CF patients, thus newer compounds for conventional therapies, such as antibiotics and anti-inflammatories will likely remain a cornerstone of treatment for CF lung disease;

(VII) Many questions are still open on the role of other genes, besides the CFTR one, in modulating pulmonary phenotype.

All this considered, a good therapeutic strategy should be based on more than one option.

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Footnote

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