Review

CFTR Modulator Therapy for Rare CFTR Mutants

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Abstract: Cystic fibrosis (CF), the most common genetic disease among the Caucasian population, is caused by mutations in the gene encoding for the CF transmembrane conductance regulator (CFTR), a chloride epithelial channel whose dysfunction results in severe airway obstruction and inflammation, eventually leading to respiratory failure. The discovery of the CFTR gene in 1989 provided new insights into the basic genetic defect of CF and allowed the study of potential therapies targeting the aberrant protein. In recent years, the approval of “CFTR modulators”, the first molecules designed to selectively target the underlying molecular defects caused by specific CF-causing mutations, marked the beginning of a new era in CF treatment. These drugs have been demonstrated to significantly improve lung function and ameliorate the quality of life of many patients, especially those bearing the most common CFTR mutant F508del. However, a substantial portion of CF subjects, accounting for ~20% of the European CF population, carry rare CFTR mutations and are still not eligible for CFTR modulator therapy, partly due to our limited understanding of the molecular defects associated with these genetic alterations. Thus, the implementation of models to study the phenotype of these rare CFTR mutations and their response to currently approved drugs, as well as to compounds under research and clinical development, is of key importance. The purpose of this review is to summarize the current knowledge on the potential of CFTR modulators in rescuing the function of rare CF-causing CFTR variants, focusing on both investigational and clinically approved molecules.

Keywords: cystic fibrosis; CFTR; rare CFTR mutations; CFTR modulator; drug screening; personalized medicine; approved CFTR modulators; CFTR modulators under development

1. Introduction

Cystic fibrosis (CF) is the most common life-threatening inherited disorder among the Caucasian population, affecting more than 100,000 people worldwide. It is caused by mutations in the gene encoding for the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride (Cl⁻) channel expressed in all epithelial tissues [1]. As a consequence, CF affects the function of multiple organs, although the major manifestations of the disease occur in the digestive tract and respiratory system. One of the typical signs of gastrointestinal disease is pancreatic insufficiency, which can be detected by greasy stools and poor weight gain, resulting in fat-soluble-vitamin deficiency and malnutrition. Of note, this latter symptom had diagnostic value until the 1950s [2]. While pancreatic enzyme replacement therapy can limit gastrointestinal symptoms, lung dysfunction remains the most difficult CF manifestation to treat. CF respiratory pathology is characterized by chronic airway obstruction that evolves from early onset mucus plugging in the small airways to chronic neutrophilic airway inflammation and infections. These features lead to a progressive decline in airway function and eventually to respiratory failure, which is the main cause of death among CF patients.
The significant advances made towards the comprehension of the pathophysiology and the underlying causes of the disease since the discovery of the CFTR gene in 1989 have dramatically improved the symptomatic treatment of CF, transforming this genetic disorder from a fatal disease of infancy into a pathology of the adult [2]. This milestone was achieved thanks to a holistic approach adopted in specialized CF centers, which exploits the aforementioned pancreatic enzymes to target pancreatic insufficiency, mucolytics and chest physical therapy (CPT) to drain mucus from the lungs, and antibiotics and anti-inflammatory drugs to treat chronic infection and inflammation. Nevertheless, the breakthrough era in CF therapy started 10 years ago with the approval of the first molecular treatments, named CFTR modulators, directly targeting the basic genetic defects of the pathology. Since then, four CFTR modulators have reached the market for the treatment of patients with specific CF-causing variants and several molecules are currently under preclinical and clinical development [3]. In 2019, the Food and Drug Administration (FDA) approved Ivacaftor-Tezacaftor-Elexacaftor (ETI), a triple combination therapy that is expected to be a gamechanger in the treatment of CF, especially of patients carrying at least one F508del allele, accounting for 90% of the total U.S. CF population [4]. Although this revolutionary triad of modulators has been found to be highly effective in clinics, ameliorating lung function up to 14%, the activity of the mutant channel is only partially rescued to up to 60% of physiological values [5], indicating that there is still room for improvement. Furthermore, patients carrying some rare CFTR mutations, which represent around 20% of the total European CF population, are not currently eligible for treatment with these modulators [6].

This review highlights the therapeutic potential of CFTR modulators, either under development or approved for clinical use, in rescuing the function of rare CF-causing CFTR variants, with a brief excursus on preclinical models that are currently available to study the phenotype of these rare mutations and their response to drugs.

2. The CFTR: A Unique Channel with Multiple Facets

The CFTR is a cyclic adenosine monophosphate (cAMP)-dependent, phosphorylation-activated anion channel which functions as a Cl\(^{-}\) and bicarbonate transporter across the apical membrane of epithelial cells of different anatomical districts, such as airways and alveoli, pancreatic duct, gastrointestinal tract, sperm canal, and bile duct [7]. In the lungs, by regulating the ionic composition of the extracellular space, the CFTR plays a major role in maintaining both the optimal luminal pH and the airway surface liquid hydration (ASL), thus being crucial for the mucociliary clearance of pathogens and debris in the respiratory tract. Therefore, loss of CFTR function results in impaired ASL and thicker mucus, which in turn trigger a series of events, including accumulation of pathogens, leading to recurrent inflammation and, eventually, respiratory failure [8].

Concerning the structural and mechanical features, the CFTR belongs to the ABC transporter superfamily, whose peculiarity relies on the utilization of energy derived from the binding and hydrolysis of ATP to drive the active unilateral transport of a variety of substrates across the plasma membrane [9,10]. These transporters typically contain two structures composed of a tandem of a nucleotide-binding domain (NBD), responsible for intracellular ATP binding and hydrolysis, and a transmembrane domain (TMD), encompassing several membrane-spanning alpha-helices [11]. However, differently from other members of the ABC transporter superfamily, the CFTR also carries a unique regulatory (R) domain, linking the two NBD-TMD tandems, which is phosphorylated by protein kinase A (PKA), making the ATP-dependent Cl\(^{-}\) active transport strictly controlled by the cyclic AMP (cAMP)-mediated phosphorylation of the channel [11]. In particular, Cl\(^{-}\) secretion across the channel is activated by the PKA-mediated phosphorylation of the R domain, followed by the binding of cytoplasmic ATP to the NBD domain and its consequent hydrolysis and release, that ultimately closes the CFTR [11,12]. Moreover, aside from the activity of each individual channel, total CFTR-dependent Cl\(^{-}\) secretion depends on channel density at the apical plasma membrane of epithelial cells, that is dynamically
regulated by a complex proteostatic network of proteins, also referred to as the CFTR Functional Landscape, which affects different processes, from protein synthesis and folding to stability and function at the plasma membrane [11,13,14]. Some of the interactors of this network, including the Na\(^+\)/H\(^+\) exchanger regulatory factor isoform-1 (NHERF1), guanine nucleotide exchange factor EPAC-1, receptor for activated C-kinase-1 (RACK1), protein kinase C (PKC), ERM and ezrin, have been demonstrated to control the CFTR membrane abundance through different mechanisms, while also favoring the physical association between PKA and the R domain, thus impacting channel opening [11,14–16]. Although the protein interactome of both the normal (wild-type) and the most common mutant (F508del) channels have been characterized, little is known about how this network is modified in the case of other and rare CFTR mutations.

Since the discovery of the CFTR gene, more than 2000 variants have been identified. However, based on research studies and clinical evidence, only ~300 CFTR mutations have been classified as CF-causing variants and subdivided into six different classes according to their functional repercussion [17]. These comprise mutations that lead to no protein formation (class I), defective channel folding and trafficking (class II), including the most prevalent F508del, completely absent (class III) or partially defective (class IV) function and regulation, decreased protein production (class V) and impaired stability (class VI). Notably, certain mutations have been found to co-exist in patients, resulting in both strong allelic heterogeneity and high variability in terms of phenotype, ranging from absent or milder to more severe symptoms [8]. Unfortunately, the majority of CFTR variants remain largely unexplored from a molecular standpoint and a major challenge in the field is to establish a genotype-phenotype correlation for these rare mutants since at present their biological outcome can only be presumed from clinical and epidemiological data. More importantly, the efficacy of approved CFTR modulators in subjects bearing rare CFTR mutants is still to be defined.

3. The Breakthrough Era of CFTR Modulators

For patients carrying selected CFTR mutations, the last decade has marked a significant change in treatment opportunities through the approval of the first molecular treatments, named CFTR modulators. This breakthrough era started in 2012, when the U.S. Food and Drug Administration (FDA) approved the use of the CFTR modulator Ivacaftor (IVA), targeting the basic molecular defect of the pathology and not only the underlying symptoms for the first time in CF history. This compound, also called “potentiator”, increases the amount of time that the CFTR is open, improving Cl\(^-\) transport through the channel [18]. Therefore, only patients carrying mutations affecting CFTR opening and Cl\(^-\) secretion, but not channel trafficking (i.e., G551D), could benefit from this treatment. Since its approval, the label of Ivacaftor has been extended to 38 other mutations [19], which collectively represent ~4% of CF patients worldwide. From a clinical perspective, this drug is highly effective as it leads to an improvement in lung function up to 10% and a 50% reduction in pulmonary exacerbations [20].

For patients carrying the most prevalent mutation, namely F508del, two double combinations of CFTR modulators, called Ivacaftor-Lumacaftor (IVA-LUMA) and Ivacaftor-Tezacaftor (IVA-TEZA), have been authorized in 2015 and 2018, respectively, in which the potentiator is associated with one so-called “corrector”, a drug that rescues the trafficking defect of the CFTR channel and promotes its translocation to the cell surface [4]. Despite having a similar molecular mechanism, IVA-TEZA shows a more favorable outcome in terms of pulmonary adverse events and drug interaction profile compared to IVA-LUMA, with an improvement in lung function of up to 5%. However, the radical change in the field of CFTR modulators and CF treatment occurred in 2019 with the FDA approval of ETI, a triple therapy combining IVA with two different correctors, for patients carrying at least one F508del allele, representing 90% of the total CF population in the U.S. [4]. This revolutionary combination of modulators has been found to be highly effective in clinics, with lung function improvements up to 14%, and rescue of CFTR activity up to
60% of physiological values [5]. Notably, ETI proved to be beneficial even in patients with advanced lung tissue damage, suggesting that early treatment with this triple combination could prevent airway remodeling and lung function decline.

Currently, ETI is not available for patients devoid of one F508del allele, leaving around 20% of CF patients, excluding those eligible for IVA, without an effective modulating therapy in Europe [6]. Among these patients, some bear rare mutations that might be amenable to CFTR modulators or approved for ETI in the U.S. based on in vitro data, while others carry mutations that alter the production of any CFTR protein, underlying the need for molecules with a completely different mechanism of action compared to existing modulators and/or gene therapy approaches. Another critical issue is the fact that only 0.8% of the reported CFTR variants have an allele frequency of 0.01%, whereas the others are extremely rare, with largely unknown functional consequences [6], thus urging the need for new ways of evaluating drug efficacy in these limited populations. In this regard, the imperative is to find and optimize suitable models to investigate the functional repercussions of a given mutation, as well as to perform drug screenings to target those patients with rare CFTR variants.

3.1. Modeling CF for the Screening of CFTR Modulators

During the last decades, animal models have remarkably contributed not only to the elucidation of the pathophysiological mechanisms behind CF but also to designing new therapies to rescue channel functionality [21–23]. However, there is no single animal model available yet that completely recapitulates the complexity of the disease observed in humans [21,23,24], and this is of particular relevance given the intricacy of CF lung disease. Consequently, in vitro and ex-vivo cell-based models derived from patients have acquired increasing importance in the preclinical research scenario.

Several immortalized cell lines (i.e., 16HBE14o-, CFBE41o-) have been implemented in the field [25,26], albeit both physiological relevance and drug screening predictivity of these cell cultures remain limited due to the lack of structural complexity, compared to that observed in vivo in the airway epithelium [22,25].

Nowadays, patient-derived three-dimensional (3-D) cell-based models, including organoids and spheroids, represent the most used model in the field of basic and translational CF research, as they have been shown to better resemble the in vivo tissue architecture compared to other systems [27,28]. Intestinal organoids derived from patients expressing a broad range of CFTR variants have been previously optimized to study pathogenic mechanisms and to perform screening of CFTR modulators [29,30], thus potentially helping individuals carrying CFTR rare mutants to benefit from already approved therapies [31,32]. Moreover, induced pluripotent stem cells (iPSCs) can be generated from CF patients and can be differentiated into airway epithelial progenitor cells, which display self-renew and self-assemble capacities, allowing the formation of iPSC-derived 3D airway organoids suitable for studying CFTR modulation [33–36]. Similarly, nasal brushing-derived spheroids have been successfully validated as a promising tool for individualized CFTR studies of channel functionality and modulator response [37,38].

Another cell-based system that has been implemented to closely recapitulate the low respiratory tract is the two-dimensional (2-D), air-liquid interface (ALI) cell culture of airway epithelial cells (AEC). This model is based on the use of permeable supports that allow cell polarization and differentiation, which in turn enable epithelial pseudostratification and air exposure of the differentiated epithelium. ALI culture of transplant-derived human bronchial epithelial cells (HBE) is currently considered the “gold-standard” for measurement of channel functionality during the preclinical screening of CFTR modulators by Ussing chamber and patch clamping experiments [28,31].

Nevertheless, primary HBE cultures are extremely invasive to obtain since they require lung explant or bronchial brushing/biopsies [28]. Throughout the years, in search of easy-to-obtain and non-invasive sources of respiratory tissues, researchers have examined alternative CF patient-specific cell-based models for preclinical drug screening, such as
primary human nasal epithelial cells (HNEs) suitable for ALI culture, harvested by nasal epithelial brushings [28,39]. A study from Brewington and colleagues [40] revealed that nasal and bronchial cultures display a remarkable similarity in terms of CFTR function and regulation. In particular, comparable results have been obtained in terms of changes in CFTR currents elicited by CFTR modulators in HNE and HBE specimens from the same brushed subject. Moreover, recent work from Amaral’s group demonstrated a correlation between the CFTR rescue achieved by CFTR modulators in HNE and that observed in rectal organoids from the same individual, thus confirming the validity of this cell-based model to perform drug screening [41].

Altogether, these studies demonstrate that patient-specific cell-based models can be a key tool to predict CFTR modulator response of rare CFTR variants and expand patient eligibility to already approved CFTR modulators. In this view, the next paragraphs will focus on the most recent and significant studies wherein CFTR modulating agents, both approved and under development, have been found to rescue the function of rare CFTR mutants.

3.2. Rare CFTR Variants Potentially Eligible for Approved CFTR Modulators

While the clinical relevance of ETI has been well assessed for patients carrying the F508del mutation [8,42,43], numerous rare pathogenic genotypes are not included in the list of those eligible for ETI treatment. Albeit patients affected by these mutations represent a minority, there is great hope that these highly effective drugs could, at least partially, be used to rescue the function of rare CFTR variants. For instance, it is plausible that CFTR correctors which are part of the approved combinations could be exploited to rescue the trafficking defect of other class II variants besides the F508del mutant.

Currently, available CFTR correctors have been subgrouped into three classes depending on the specific molecular defect that they can rescue in the mutant protein [44]. In particular, type I CFTR correctors, including Lumacaftor/VX-809 and Tezacaftor/VX-661, have been shown to revert the folding defect of F508del-CFTR by inserting a hydrophobic pocket in the TMB1, favoring the binding of four thermodynamically unstable helices and the consequent correction of channel folding during its early biogenesis [45]. Potentially, these drugs could allosterically stabilize the processing of many other class II CFTR variants, preventing premature degradation of the mutant channel. This could be the case of rare class II mutations, such as c.170C > T (S13F), c.91C > T (R31C), c.274G > A (E92K), c.1558G > T (V520F), c.3302T > A (M1101K), c.254G > A (G85E), c.1570T > G (Y569D) and c.3909C > G (N1303K), displaying defects in channel processing and functionality similar to those of F508del-CFTR [46–48]. As a matter of fact, a study from Lukacs group demonstrated substantial rescue of many rare misprocessing mutations by the triple combination ETI, including S13F, R31C, G85E, E92K, V520F, M1101K, and N1303K [48]. In further support of these observations, Laselva et al. confirmed that some of these rare CFTR variants (i.e., M1101K, G85E) can be functionally rescued by ETI, as demonstrated by Ussing chamber experiments in HNE cell cultures. Likewise, other rare class II mutations associated with severe lung disease, including c.1826A > G (H609R) and c.3067_3072delATAGTG (I1023_V1024del), have been shown to respond to ETI in vitro [49]. The c.3700A > G mutation, another rare CF-causing variant whose incidence is relatively high in the Middle East, produces a full-length transcript encoding a missense mutation (I1234V-CFTR) and a cryptic splice site that deletes six amino acids in the NBD2 (I1234del-CFTR) [50,51], resulting in defective protein folding and impaired channel activity. Of note, it has been shown that CFTR modulators, including ETI, are able to restore the function of I1234del-CFTR to wild-type levels observed in both human embryonal kidney 293T (HEK293T) cells and gene-edited I1234-CFTR-expressing 16HBE14o- cells. Moreover, the rescue of CFTR function in primary HNE cells from two CF patients heterozygous for I1234-R1239del/W1282X was observed in this work [52]. Taken together, these studies encourage a possible application of the triple combination for patients homozygous for rare misfolding CFTR mutants, supporting
a precision medicine approach aimed at optimizing modulator therapy for these subjects and extending patient eligibility for this type of treatment.

However, rare class II CFTR mutants do not represent the only variants that can be addressed by combinations of CFTR modulators. The FDA recently agreed to expand the list of CF-causing mutations for which ETI treatment could be clinically beneficial, comprising subjects heterozygous for 177 additional mutations [53]. Moreover, class III and class IV CFTR variants, characterized by defective gating and decreased conductance respectively, are only partially normalized by Ivacaftor/VX-770, underlying the need for alternative therapeutic approaches [54,55]. In this regard, there is increasing evidence that the CFTR corrector Elexacaftor/VX-445 can also serve as a potentiator [46,56,57], indicating its possible implementation to fulfill the need for a complete rescue of these CFTR variants. As demonstrated by Veit et al. [56], Elexacaftor/VX-445 significantly potentiates the activity of F508del and other gating mutants, including G551D and G1244E. Shaughnessy and colleagues reported that Elexacaftor/VX-445 exhibits multiplicative synergy with VX-770 in potentiating class III and IV CFTR mutations (represented by G551D and R117H mutations, respectively) through distinct mechanisms of action [57]. Given that CFTR potentiator activity also depends on CFTR density at the plasma membrane, and patients homozygous for G551D CFTR still experience progressive loss of lung function [54,58], combination therapy could provide clinical benefit to these individuals.

The effects of approved CFTR modulators on rare CFTR variants are summarized in Table 1.

| CFTR Variants | Experimental Models | CFTR Modulators | Results | Reference |
|---------------|---------------------|-----------------|---------|-----------|
| S13F, R31C, G85E, E92K, V520F, M110K, N1303K | Primary HNE cells | VX-661, VX-445, VX-770 (ETI, Vertex Pharmaceuticals) | Rescue of CFTR functionality to therapeutically significant levels (>20% of WT-CFTR) | [48] |
| M1101K, G85E, N1303K | HEK293T cells, primary HNE | VX-661, VX-445, VX-770 (ETI, Vertex Pharmaceuticals) | Responses comparable to, or inferior to, those observed for F508del-CFTR | [46] |
| H609R, I1023_V1024del | HEK293T cells, | VX-661, VX-445, VX-770 (ETI, Vertex Pharmaceuticals) | Rescue of channel misprocessing and functionality | [49] |
| I1234del, W1282X | HEK293T cells, 16HBE14o-, primary HNE | VX-661, VX-445, VX-770 (ETI, Vertex Pharmaceuticals) | Rescue of I1234del-CFTR to WT activity in HEK293T cells; rescue in gene-edited I1234-CFTR-expressing 16HBE14o- cells and in primary HNE cells from two CF patients heterozygous for I1234-R1239del/W1282X; No rescue in primary HNE cells homozygous for I1234-R1239del | [52] |
| G551D, G1244E, Y1092X | CFBE41o-, primary HNE | VX-661, VX-445, VX-770 (ETI, Vertex Pharmaceuticals) | Acute VX-445 addition increased VX-770-potentiated CFTR current by ~70–85% in HNE cells homozygous for G551D-CFTR and heterozygous for G551D/Y1092X | [56] |
| G551D, R117H | FRT cells | VX-661, VX-445, VX-770 (ETI, Vertex Pharmaceuticals) | VX-445 synergizes with VX-770 in potentiating CFTR activity in FRT cells heterologously expressing both G551D- and R117H-CFTR | [57] |
3.3. Investigational CFTR Modulators to Target Rare CFTR Mutations

3.3.1. CFTR Modulators by AbbVie/Galapagos

Both type 1 correctors, targeting the NBD1 membrane spanning domains (MSDs) interface, and type 2 ones, instead of targeting the NBD2, are under development by AbbVie and Galapagos. Among these is the type 2 corrector ABBV/GLPG2737 that has been found to rescue the V232D-CFTR, a rare mutant characterized by protein misfolding and, consequently, impaired channel maturation. As for the F508del variant, ABBV/GLPG2737 is able to rescue V232D-CFTR when overexpressed in human HEK293T cells with exquisite potency marked by an EC50 of 161 nM, which is significantly higher than that shown by the same compound towards the F508del mutant, being 0.2–2.2 mM [59]. The other AbbVie/Galapagos corrector under evaluation is the type 1 ABBV/GLPG2222, also known as Galicaftor, which is able to rescue several rare CFTR mutants. This compound was tested in HEK293T cells overexpressing the E92K-CFTR, a poorly characterized transmembrane-domain mutant, as well as the P67L-CFTR, characterized by a maturational processing defect, and the already mentioned V232D-CFTR. Towards these variants, ABBV/GLPG2222 exerts the same function as the Vertex compound Lumacaftor/VX-809, albeit with a reduced potency compared to that observed with F508del-CFTR [59]. Similar to the modulators developed by Vertex, these correctors by Galapagos/AbbVie have improved activity towards CFTR maturation and activation when used in combination. GLPG2737, in combination with GLPG2222, is about 25-fold more potent than the compound alone in F508del/F508del human bronchial epithelial (HBE) cells, suggesting that GLPG2222 brings the mutant CFTR into a conformation that allows GLPG2737 to better exert its biological action [59]. The same combinatorial effect has been observed with another AbbVie/Galapagos CFTR modulator, namely GLPG3067, which acts as a potentiator. In primary HBE cells, the triple combination composed by GLPG2737, GLPG2222 and GLPG3067 substantially increases CFTR-mediated Cl\(^{-}\) transport compared to the combination of the two AbbVie/Galapagos correctors alone, suggesting a possible higher clinical benefit for CF patients carrying the F508del mutation as well as other CFTR variants that have been proven responsive to these new compounds [59].

This is also corroborated by the fact that GLPG2737 has already reached the clinical trial phase for safety assessment in healthy volunteers, in which the pharmacokinetic properties of the compound allow for a single daily dosing regimen in patients [60]. The drug was also evaluated in F508del homozygous patients, for which it is currently investigated as an add-on treatment with LUMA/IVA and as a triple combination with GLPG2222 and GLPG2451, another experimental AbbVie/Galapagos potentiator that has not been tested for the rescue of rare CFTR mutants yet [61]. These promising results with GLPG2222 in 2016 led to the first human study focused on safety and pharmacokinetic features of the compound in healthy adult subjects, and subsequently to two placebo-controlled phase 2 studies, namely FLAMINGO [62] and ALBATROSS [63], aimed at assessing the therapeutic effect of GLPG2222 in CF patients. In particular, 59 subjects were homozygous for the F508del mutation and 37 patients heterozygous for F508del and a gating mutation, who were receiving IVA, as well as the GLPG compound, were enrolled in the FLAMINGO and ALBASTROSS trials, respectively. Of note, Galicaftor was well tolerated in patients, as already seen in the first human study, but its efficacy was demonstrated to be quite low, with a slight decrease in sweat Cl\(^{-}\) concentration but no significant changes in pulmonary function or respiratory symptoms [64].

A recent study by Laselva and colleagues highlighted the therapeutic potential of other AbbVie/Galapagos compounds targeting rare mutations, with a particular focus on AC1, and two type 2 correctors, namely AC2-1, which belongs to the ABBV/GLPG2737 series, and AC2-2 [65]. In HEK293T cells expressing the I1234_R1239del mutation, pre-treatment with these three novel correctors individually resulted in significant improvements in channel activity [52]. However, the greatest effect was seen when AC1 and AC2-2 were combined, with the AC1 and AC2-1 combination exerting similar effects to AC2-1 alone [52]. This evidence was further corroborated in nasal epithelial cultures derived from two patients homozygous for the I1234_R1239del-CFTR mutation, in which AC1-AC2-2 rescues
the function of the CFTR variant up to ~130% of the mean forskolin response in non-CF cultures [52]. Noteworthy, in nasal epithelial cells from two I1234_R1239del/W1282X siblings, the improvement induced by AC1-AC2-2 treatment was only ~50% of the mean forskolin response observed in non-CF cultures [52], which was somewhat unexpected considering the study from Laselva and colleagues in which AC1/AC2-2 was used to target the nonsense W1282X-CFTR mutation. In HEK293T cells expressing this latter mutant CFTR, residual cAMP-dependent channel activity is augmented by pre-treatment with AC1 in combination with AC2-2 and the AbbVie potentiator AP2, a close analog of GLPG2451, suggesting that the AC corrector combination may be very effective in enhancing the abundance and/or functional competency of the truncated protein [66]. To better study the effect of nonsense mediated decay (NMD), the process responsible for the elimination of mRNA transcripts that contain premature stop codons, on W1282X-CFTR expression, 16HBE14o-cells were genetically modified using the CRISPR/Cas9 technology to express the variant in the context of the complete CFTR gene. In these cells, the AC1-AC2-2-AP2 combination still induced a modest activation of W1282X-CFTR [66]. To further increase the response of W1282X-CFTR, a promising strategy was found by combining the AbbVie/Galapagos drugs with SMG1i, an inhibitor of the SMG-1 kinase recently identified as an NMD effector protein, which is able to induce a robust 50% restoration of wt-CFTR channel activity [66]. Unfortunately, the AC1-AC2–2–AP2 combination only modestly improves maturation of the missense N1303K-CFTR, but rescues channel function to a higher extent compared to AC1 and AP2 alone, suggesting an unexpected potentiator activity of AC2–2 [67].

The therapeutic potential of another combination of AbbVie/Galapagos modulators, namely the AC1 and AC2–1 compounds, together with the potentiator AP2, was also recently demonstrated towards the CFTR variants M1101K and G85E, characterized by mis-processing and reduced protein function [67]. The combination of these AbbVie/Galapagos modulating agents is indeed effective in rescuing both defects of the M1101K- and G85E-CFTR proteins in patient-derived nasal cells. Intriguingly, in cells expressing M1101K-CFTR, the response to the novel combination was substantial, ranging between 100 and 280% of the mean forskolin response in non-CF cultures [67].

3.3.2. CFTR Modulators by Proteostasis

Another company developing new modulators targeting the CFTR channel is Proteostasis Therapeutics Inc. The most interesting product of this company is the triple combination, which includes a potentiator, namely Dirocaftor (PTI-808), the corrector Pose-nacaftor (PTI-801), and Nesolicaftor (PTI-428), a molecule classified as a “CFTR amplifier”. These latter compounds, capable of promoting CFTR protein synthesis, are defined as “agnostic” because any CF-causing mutation can potentially benefit from their action [68,69]. In CRISPR/Cas9-engineered HBE cells expressing the I1234_R1239del variant, PTI-428 is able to increase CFTR mRNA levels and, when combined with Lumacaftor/VX-809, significantly increases the expression of the channel, both in its core-glycosylated and complex-glycosylated forms [51]. Furthermore, the PTI-428-Lumacaftor/VX-809 combination significantly enhances the peak activity of the mutant channel above that achieved with Lumacaftor/VX-809 treatment alone. In agreement with these data, nasal cultures from I1234_R1239del patients treated with Lumacaftor/VX-809-PTI-428 show increased CFTR function compared to Lumacaftor/VX-809 alone [51]. Interestingly, PTI-428 is not able to increase mRNA levels of the nonsense mutation G542X-CFTR, and a slight induction can be detected only upon the addition of genetin (G418), a read-through compound that cannot be used in vivo because of its severe toxicity [68]. However, Nesolifactor has already been tested in F508del CF patients in phase 1–2 clinical trial [70], showing an improvement of 8% in percent predicted forced expiratory volume in 1 s (ppFEV1) and a decrease of 29 mmol/L in sweat Cl− after 4 weeks of treatment compared to the placebo. These results underly the possibility of using this compound in patients expressing responsive rare mutations, such as the I1234_R1239del, through an accelerated pathway, in virtue of the already completed regulatory procedures, such as certified safety and toxicology studies [71].
3.3.3. Ataluren (PTC124) from PTC Therapeutics

Nonsense mutations, which account for 10% of CF cases worldwide, can be targeted using either NMD inhibitors, as already seen with SMG1i, or read-through agents, promoting the production of a full-length CFTR protein. One of the best studied molecules of this latter category, beyond the CF research field, is Ataluren, also called PTC124, developed by PTC Therapeutics. This drug is already used in clinical practice to target nonsense mutations found in patients affected by Duchenne muscular dystrophy and it is currently under clinical evaluation for CF [72]. Two phase 2 clinical trials have been conducted in patients carrying at least one class I nonsense mutation, with W1282X and G542X being the two prevalent ones. In the first study [73], an improvement in total Cl⁻ transport was seen in the majority of patients, and this was accompanied by a slight increase in lung functionality measured as FEV1, although this was evident only in the first phase of the treatment. A second phase-2 clinical trial [74] has also been performed in children carrying the nonsense mutations Q493X, G542X, R553X, W846X, W882X, E1104X, R1162X, W1282 and Q1313X, in which half of the patients showed a normal range of total Cl⁻ transport. Finally, a phase 3 clinical trial [75] has been conducted to evaluate the long-term clinical efficacy and safety of a 48-week Ataluren therapy. Specifically, the most common nonsense mutations, R553X (18 patients), R1162X (22 patients), G542X (83 patients), and W1282X (86 patients) were present in one or both alleles. Ataluren resulted in a smaller decrease in FEV1 compared to the control group, although the difference between treatment and placebo was not statistically significant, and the same held true for exacerbation frequency. Intriguingly, relevant differences in both clinical parameters were observed when patients using chronic inhaled tobramycin were excluded from analyses. This discrepancy can be explained by the fact that the two drugs have similar mechanisms of action, both targeting the activity of ribosomes. However, in a second phase 3 trial [75] conducted in patients mostly carrying G542X, W1282X, R553X and R1162X mutants and not subjected to tobramycin, ataluren therapy did not reach any statistical significance in terms of FEV1 absolute changes compared to placebo. Overall, the failure of ataluren monotherapy to produce clinical benefits in CF compared to Duchenne muscular dystrophy may be explained by poor availability of the compound in the lungs and by low levels of CFTR mRNA accessible to readthrough.

3.3.4. ELX-02 by Eloxx Pharmaceuticals

A chemically-engineered aminoglycoside derivative, termed ELX-02 (NB124), is currently under development by Eloxx Pharmaceuticals. This molecule functions as a read-through agent that, by interacting with ribosomes, allows for the production of a full-length CFTR in cells carrying a nonsense mutation [76]. Several proofs of the efficacy of ELX-02 have been generated in different biological systems. ELX-02 is able to restore CFTR function in 16HBE14o- bronchial epithelial cells, whose CFTR gene has been edited to introduce the CF-causing mutations PTCs R553X, R1162X and Y122X [68]. Moreover, Ivacaftor/VX-770 is able to enhance CFTR function above the levels promoted by the readthrough drug alone in CFBE41o− cells expressing either R1162X- or W1282X-CFTR [77]. Another piece of evidence for ELX-02 efficacy in CF was obtained in intestinal patient-derived organoids (PDO) with G542X/G542X, G542X/W1282X, or G542X/minimal function (MF) genotypes, in which the compound shows a significant restoration of CFTR-dependent swelling upon forskolin treatment [78]. Further studies regarding ELX-02 activity on the G542X variant have been carried out by Xue and colleagues demonstrating that the molecule restores full-length CFTR expression and Cl⁻ transport in Fischer rat thyroid (FRT) cells stably transduced with a CFTR-G542XcDNA transgene, with a 2.5-fold increase in Cl⁻ conductance [77]. In primary HBE cells derived from G542X/F508del patients, ELX-02 induces a 2.5-fold increase in short circuit current (I_SC), as seen in FRT monolayers, rescuing CFTR function to roughly 7% of wild-type activity. Moreover, the combination of ELX-02 with the correctors Lumacaftor/VX-809 and Elexacaftor/VX-445 was shown to raise CFTR-mediated currents compared to the readthrough agent alone in G542X-16HBE14o- bronchial epithelial
cells [68]. Interestingly, ELX-02 also restores CFTR activity, indicated by cAMP–activated transepithelial currents, in a CF mouse model expressing a human CFTR-G542X transgene, with favorable pharmacokinetic properties, suggesting a possible clinical benefit in patients [77]. In this regard, a phase 1 multiple-ascending-dose trial has been already performed in healthy subjects in which ELX-02 appeared to be well tolerated with no severe adverse events reported [79]. A phase 2 trial is ongoing in CF patients with a G542X/MF genotype to assess ELX-02 safety, tolerability, pharmacokinetics and pharmacodynamics as a stand-alone drug or in combination with Ivacaftor/VX-770 [80], and top-line study results are expected by mid-2022.

3.3.5. Icenticaftor by Novartis

Icenticaftor (QBW251) is a new Novartis medicinal compound that has been discovered as a potentiator via a high-throughput screening in primary human bronchial epithelial cells of both healthy subjects and F508del CF patients [81]. The first phase 1/2 clinical study assessed safety, pharmacodynamics and pharmacokinetics of Icenticaftor in a randomized, double-blind, placebo-controlled study in healthy volunteers, as well as its efficacy as a CFTR potentiator in adult CF patients with one pre-specified CFTR class III or IV mutation or homozygous for the F508del-CFTR variant [82]. The compound was well-tolerated in all subjects, and in patients with class III and IV mutations was able to improve FEV1 by 6.46%, compared to placebo, and to decrease lung clearance index variation (LCI2,5) and sweat Cl− by 1.13 points and 8.36 mmol/L, respectively [83]. Importantly, in patients affected by another obstructive airway disease with similar features to CF, namely chronic obstructive pulmonary disease (COPD), a 28-day treatment with Icenticaftor improved systemic inflammation and sputum bacterial colonization [84], suggesting that this medicinal product may have the same therapeutical effects in subjects affected by CF.

3.3.6. Ensifentrine by Verona Pharma

Ensifentrine (also known as RPL554) is an inhibitor of both phosphodiesterase 3 (PDE3) and 4 (PDE4), the enzymes hydrolyzing the second messenger cAMP. cAMP elevating agents are indeed potent inducers of the CFTR protein function, stimulating PKA-mediated channel phosphorylation, and are known to regulate airway constriction and inflammation, two of the main pathological features of CF [85]. By virtue of its unique mechanism of action, RPL554 augments forskolin-stimulated Cl− currents in FRT cells expressing R334W- and T338I-CFTR, two class IV CFTR mutants [86]. Moreover, FRT cells expressing G551D- and S549R-CFTR variants are responsive to RPL554 and forskolin stimulation after chronic exposure to IVA/VX-770, with an even higher response when treated with the VX-770-VX-809/IVA-LUMA combination [86]. Of note, RPL554 stimulates CFTR-dependent ion secretion across bronchial epithelial cells isolated from patients carrying the R117H/F508del CF genotype, with an increased response upon Ivacaftor/VX-770 treatment, while also elevating cilia beat frequency. Thus, the drug could potentially promote mucociliary clearance in CF through two different mechanisms, i.e., increased CFTR opening and enhanced ciliary movement [87]. In addition to several clinical trials in COPD [88–90] and asthmatic patients [91], which showed the potential of this medicinal product to reduce respiratory phlegm and airway obstruction, as well as to inhibit inflammation, Ensifentrine has been under clinical evaluation also in CF patients. Specifically, a phase 2a clinical trial [92] demonstrated that a single administration of both high and low doses of RPL554 significantly increases FEV1 in CF subjects, with a sustained effect for at least eight hours. Furthermore, RPL554 displays favorable stability and distribution profiles and is well tolerated by the patients, further underlying the potential of Ensifentrine as a medicinal product targeting CF.

The effects of CFTR modulators under development on rare CFTR mutants are summarized in Table 2.
Table 2. Rare CFTR variants rescued by CFTR modulators under preclinical and clinical development.

| CFTR Variants | Experimental Models | CFTR Modulators | Results | Reference |
|---------------|---------------------|----------------|---------|-----------|
| V232D         | HEK293T cells       | ABBV/GLPG2737 (AbbVie/Galapagos) | CFTR rescue with higher potency than against the F508del-CFTR (EC50 of 161 nM) | [59] |
| E92K, P67L, V232D | HEK293T cells       | ABBV/GLPG2222 (AbbVie/Galapagos) | CFTR rescue with lower potency than against the F508del-CFTR (EC50 of 161 nM) | [59] |
| I1234_R1239del | HEK293T cells, Primary HNE cells | AC1, AC2-2 (AbbVie/Galapagos) | CFTR rescue up to ~130% of the mean forskolin response in non-CF cultures | [52] |
| I1234_R1239del/W1282X | Primary HNE cells | AC1, AC2-2 (AbbVie/Galapagos) | CFTR rescue up to ~50% of the mean Fsk response observed in non-CF cultures | [52] |
| W1282X         | HEK293T cells       | AC1, AC2-2, AP2 (AbbVie/Galapagos) | Augmented residual cAMP-dependent channel activity | [66] |
| W1282X         | 16HBE14o-           | AC1, AC2-2, AP2 (AbbVie/Galapagos) | Modest CFTR activation 50% rescue of wt-CFTR channel activity in combination with SMG1i | [66] |
| M1101K, G85E   | Primary HNE cells   | AC1, AC2–1 (AbbVie/Galapagos) | 100–280% rescue of the mean Fsk response in non-CF cultures | [67] |
| N1303K         | Primary HNE cells   | AC1, AC2–2, AP2 | Modest improvement in maturation; concomitant channel function rescue | [67] |
| I1234_R1239del | HBE                 | PTI-428 (Proteostasis) | Increased CFTR mRNA; induction of CFTR expression in combination with VX-809; enhanced CFTR activity in combination with VX-809 compared to VX-809 alone | [51] |
| I1234_R1239del | Primary HNE cells   | PTI-428 (Proteostasis) | Increased CFTR channel upon Fsk and VX-770 stimulation in combination with VX-809 compared to VX-809 alone | [51] |
| Heterozygous for class I nonsense mutation (W1282X and G542X more prevalent variants) | Phase 2 clinical trial | PTC124 (PTC Therapeutics) | Slight increase in FEV1; improvements in total Cl− transport | [73] |
| Q493X, G542X, R553X, W846X, W882X, E1104X, R1162X, W1282, Q1313X | Phase 2 clinical trial in children | PTC124 (PTC Therapeutics) | Normal range of total Cl− transport in half of the patients | [74] |
| R553X, R1162X, G542X, W1282X | Phase 3 clinical trial | PTC124 (PTC Therapeutics) | Smaller decrease in FEV1 compared to placebo | [75] |
| R553X, R1162X, Y122X | 16HBE14o-          | NB124 (Eloxx Pharmaceuticals) | Restored CFTR function | [68] |
| R1162X, W1282X | CFBE41o−           | NB124 (Eloxx Pharmaceuticals) | Increased CFTR function with VX-770 compared to NB124 alone | [77] |
Table 2. Cont.

| CFTR Variants | Experimental Models | CFTR Modulators | Results | Reference |
|---------------|---------------------|-----------------|---------|-----------|
| G542X/G542X, G542X/W1282X G542X/MF | PDO | NB124 (Eloxx Pharmaceuticals) | Significant restoration of CFTR-dependent swelling upon Fsk | [78] |
| G542X | FRT | NB124 (Eloxx Pharmaceuticals) | Restored full-length CFTR expression 2.5-fold increase in Cl⁻ conductance | [77] |
| G542X/delF508 | Primary HBE | NB124 (Eloxx Pharmaceuticals) | 2.5-fold increase in Cl⁻ conductance CFTR rescue up to 7% of wt-CFTR | [68] |
| G542X | CF mouse model | NB124 (Eloxx Pharmaceuticals) | Rescue of cAMP-activated transepithelial currents | [77] |
| G542X/MF | Phase 2 clinical trial | NB124 (Eloxx Pharmaceuticals) Alone or in combination with VX-770 | Ongoing | [80] |
| Class III or IV mutation | Phase 1/2 clinical trial | QBW251 (Novartis) | FEV1 improved by 6.46% compared to placebo Decrease in lung clearance index variation (LCI₂₋₅) by 1.13 points Decrease in sweat Cl⁻ by 8.36 mmol/L | [82] |
| R334W, T338I FRT | RPL554 (Verona Pharma) | Increased forskolin-stimulated Cl⁻ currents | | [86] |
| G551D, S549R FRT | RPL554 (Verona Pharma) | CFTR rescue in combination with VX-770 alone or together with VX-809 | | [86] |
| R117H/F508del Primary HBE | RPL554 (Verona Pharma) | Increased CFTR-dependent ion secretion in response to VX-770 Increased cilia beat frequency | | [87] |

4. Conclusions

Although tremendous steps forward have been made in CF patient care by transforming a devastating childhood disease into a chronic condition with better life expectancy, the efficacy of currently available therapeutic strategies is still amenable to improvement [8,93,94]. The breakthrough of CFTR modulators from Vertex Pharmaceuticals has dramatically ameliorated clinical outcomes in CF patients, but these life-changing drugs only partially restore the function of the channel, reaching 60% of physiological values [48]. More importantly, despite the clinical relevance of some CFTR-targeting agents being well-assessed for common CFTR variants, the applicability of these therapeutic approaches to rare mutations is still undefined.

Data summarized herein encourage the use of cell-based, patient-specific models as a key tool for personalized medicine approaches. These can be used to predict the response of rare CFTR genotypes to available drugs and, potentially, to extend the use of CFTR modulators to patients with rare CFTR mutations. On these grounds, these models could be exploited not only to identify mutants responsive to current modulators but also to design mutation-specific combinations of modulators, in a precision-based fashion [95,96]. Future studies are needed to conclusively define which cell-based model is preferable for such approaches, in terms of practicability and predictive capacity [28,97]. ALI cultures of brushed HNE set out to be a viable surrogate of gold-standard HBE to
perform CFTR phenotyping and drug screening, as well as a promising tool in the era of precision medicine, especially considering the low invasiveness of sample acquisition and accuracy in phenocopying the CF respiratory epithelium [28].

On the basis of preclinical investigations carried out in these cellular systems, it is becoming clear that patients bearing rare CFTR mutations could benefit from currently approved CFTR modulator therapy. Several class II CFTR variants, displaying processing and functional defects similar to F508del-CFTR, can be rescued by ETI [48,49,52,98]. Moreover, the dual activity of Elexacaftor/VX-445 as both corrector and potentiator could be potentially exploited to increase Ivacaftor/VX-770 activity and fully restore the function of class III and IV CFTR mutants [56,57] (Table 1). In this view, the FDA extended ETI eligibility to subjects heterozygous for 177 additional mutations in the past year [53].

However, individuals bearing selected mutations on both CFTR alleles still remain without a CFTR modulator therapy, and albeit they represent a minority, further studies in this direction would be of vital importance to ensure efficacious treatments for all CF patients. Indeed, while the majority of CFTR variants can be globally considered rare, some of them are more prevalent or represent a relevant fraction among groups or specific regions [99]. This is the case of the W1282X mutation in the Jewish community, the M1101K variant among Hutterite colonies, the c.3700G > A genotype which is common in individuals descended from Bedouin tribes and represents the second most frequent CF mutation in the Middle East, and the Y122X substitution, mainly occurring in the Reunion Island population, a French overseas department [52,99,100].

The interest of the pharmaceutical industry in the development of novel CF therapeutics is growing, as demonstrated by the fact that many pharmaceutical companies are focusing their effort not only on designing new CFTR modulators but also on finding alternative molecules that could indirectly rescue CFTR function, such as PDE inhibitors and read-through agents, including RPL554 (Verona Pharma) and NB124 (Eloxx Pharmaceuticals), respectively. Some of these compounds have shown promising efficacy in restoring the function of a wide spectrum of rare CFTR mutants and have successfully reached the late stages of the drug development process, being tested in phase 1–2 clinical trials (Table 2). Finally, it is noteworthy to emphasize that the CFTR channel does not represent the only molecular target for therapies aimed at restoring epithelial homeostasis in CF. Small molecules targeting non-CFTR channels, such as the epithelial Na\(^+\) channel (ENaC) or the transmembrane protein 16 A (TMEM16A), are in the pipeline to be advanced to the clinical scenario. In particular, ENaC inhibition is a promising strategy for patients partially or not responding to already approved CF therapeutics, regardless of the CFTR class mutation. The ENaC inhibitor BI 1265162 has been shown to optimize outcomes in CF patients either eligible or ineligible for CFTR modulator therapy, and it is currently in the phase 2 clinical stage [101].

Further investigations in this direction are expected to bring new CF therapeutics that could rescue the function of rare CFTR mutants, either as single agents or as combination therapies with already approved modulators, for currently ineligible patients.

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