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Protein Processing and Inflammatory Signaling in Cystic Fibrosis: Challenges and Therapeutic Strategies

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Abstract

Cystic Fibrosis (CF) is an autosomal recessive disorder caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) that regulates epithelial surface fluid secretion in respiratory and gastrointestinal tracts. The deletion of phenylalanine at position 508 ($\Delta F508$) in CFTR is the most common mutation that results in a temperature sensitive folding defect, retention of the protein in the endoplasmic reticulum (ER), and subsequent degradation by the proteasome. ER associated degradation (ERAD) is a major quality control pathway of the cell. The majority (99%) of the protein folding, $\Delta F508$ -, mutant of CFTR is known to be degraded by this pathway to cause CF. Recent studies have revealed that inhibition of $\Delta F508$ -CFTR ubiquitination and proteasomal degradation can increase its cell surface expression and may provide an approach to treat CF. The finely tuned balance of ER membrane interactions determine the cytosolic fate of newly synthesized CFTR. These ER membrane interactions induce ubiquitination and proteasomal targeting of $\Delta F508$ - over wild type- CFTR. We discuss here challenges and therapeutic strategies targeting protein processing of $\Delta F508$ -CFTR with the goal of rescuing functional $\Delta F508$ -CFTR to the cell surface. It is evident from recent studies that CFTR plays a critical role in inflammatory response in addition to its well-described ion transport function. Previous studies in CF have focused only on improving chloride efflux as a marker for promising treatment. We propose that methods quantifying the therapeutic efficacy and recovery from CF should not include only changes in chloride efflux, but also recovery of the chronic inflammatory signaling, as evidenced by positive changes in inflammatory markers (*in vitro* and *ex vivo*), lung function (pulmonary function tests, PFT), and chronic lung disease (state of the art molecular imaging, *in vivo*). This will provide novel therapeutics with greater opportunities of potentially attenuating the progression of the chronic CF lung disease.

Keywords

$\Delta F508$; CFTR; ERAD; Ubiquitin; Proteasome; NF κ B; IL-8; Neutrophil; Therapeutics; Cystic fibrosis

INTRODUCTION

The most common cystic fibrosis transmembrane conductance regulator (CFTR) mutation, $\Delta F508$ -, encodes a cAMP-regulated chloride channel that is retrieved from the endoplasmic reticulum (ER) during translation and folding and is targeted to the proteasome for premature degradation [1]. CFTR cellular processing involves translation, folding at the ER, Golgi transport, post-translational modifications, apical plasma membrane targeting, and

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endosomal recycling and retrieval. Plasma membrane CFTR is internalized by endocytosis and then recycled to the plasma membrane or targeted for lysosomal degradation. $\Delta F508$ -CFTR folding is inefficient, with more than 99% of $\Delta F508$ -CFTR targeted for proteasomal degradation before reaching the Golgi apparatus. In expression systems that are permissive to biosynthesis and trafficking, the $\Delta F508$ protein is partially functional with an open time period for approximately 0.1 to 0.3 seconds [2,3]. Conditions that promote at least partial rescue of misfolded CFTR from proteasomal degradation include reduced temperature [4,5], nitrosoglutathione [6,7], SERCA calcium pump inhibition [8,9], and chemical chaperones [10]. The efficiency of *in vivo* processing of $\Delta F508$ - appears to vary between the tissues [11,12]. Transient interactions between CFTR and molecular chaperones determine the early fate of the misfolded protein [13–15]. Alteration of the intracellular fate of misfolded CFTR by intervention of protein folding, processing, and proteolytic pathways has shown promise for interrupting “downstream pathology”. The identification of specific targets to correct defective $\Delta F508$ -CFTR folding or cellular processing (correctors) and channel gating (potentiators) provide a strategy for therapy of CF that corrects the underlying defect. $\Delta F508$ -CFTR correctors have been shown to be effective in increasing the amount of properly folded CFTR on the cell surface. The presence of correctors promoted folding of the mutants from approximately 2–5% without corrector to 22–35% with corrector in a study by Loo *et al.* [16]. This and similar studies substantiate the potential of therapeutic strategies to correct the $\Delta F508$ - protein processing defect. Although it remains an open question if this strategy will be able to correct or reverse the chronic CF lung disease.

CF patients exhibit a typical phenotype that is characterized by repeated pulmonary infections, leading to eventual pulmonary failure and death. Bronchoalveolar lavage fluid (BAL) in CF patients contains increased levels of proinflammatory cytokines and neutrophils. CF cells have increased basal levels of the pro-inflammatory C-X-C chemokine, interleukin (IL)-8, attributed to activated NF κ B [17]. IL-8 is a potent neutrophil chemoattractant [18] that drives the chronic inflammatory response in CF [19] by the NF κ B dependent pathway. It has also been seen in CF that the loss of CFTR function appears to attenuate the innate immune responses, possibly by altering the expression of TLR (Toll like receptors) on the airway epithelial cells [20]. Several groups have shown that lack of CFTR on the cell surface results in defective innate immune response that explains the persistent *Pseudomonas aeruginosa* infections seen in CF patients. The relationship between mutant CFTR and the inflammatory pathway is summarized in Fig. (1). Traditional approaches to treat the inflammatory pathophysiology have focused on targeting NF κ B–dependent signaling and therapy with glucocorticoids such as ibuprofen. This glucocorticoid therapy has yet to show any real promise for long term use, and is not desirable due to adverse side effects [21]. We propose that identification of specific mechanisms leading to exaggerated NF κ B response in CF will lead to identification of molecular targets and designing of selective and disease specific therapeutic approaches, especially considering the association of NF κ B with several pulmonary conditions.

We recently described a similar approach on identification of novel mechanisms of IL-8 induction in CF. It has been demonstrated that secretion of prostaglandin E2 (PGE-2), a potent mediator of inflammation produced by cyclooxygenation of arachidonic acid, is elevated in CF airways [22,23]. $\Delta F508$ - CFTR epithelial cells have enhanced expression and activity of cyclooxygenase-2 (Cox-2), resulting in PGE-2 hypersecretion, that we demonstrated as a mechanism of elevated IL-8 levels in CF. It was shown earlier in human T lymphocytes that PGE-2 induces C/EBP homologous protein (CHOP) transcription factor that binds to the IL-8 promoter [24]. We verified that PGE-2 mediates the IL-8 inflammatory response in CF cells through the CHOP transcription factor [23]. It has also been shown that the histone acetyltransferase (HAT)/HDAC balance is sensitive to CFTR function, as cells with reduced or no CFTR function have decreased HDAC2 protein,

resulting in hyperacetylation of the IL-8 promoter and increased IL-8 transcription [25,26]. These findings opened up alternative routes to controlling inflammation in CF patients other than the traditional targeting of the NF κ B-dependent signaling pathway. The control of the hyper-inflammatory response that is characteristic of CF patients is an important aspect of a therapeutic regimen and should be an important criterion when screening potential drug therapy candidates. An ideal CF drug treatment should be capable of not only rescuing the membrane-CFTR protein but will also help in alleviating the hyper-inflammatory response.

PATHOGENESIS OF CYSTIC FIBROSIS LUNG DISEASE

CFTR Protein Processing Defect

CFTR is a polytopic integral membrane glycoprotein composed of 1,480 amino acids. Biogenesis of CFTR begins with the targeting of nascent chain-ribosome complexes to the endoplasmic reticulum (ER) membrane, followed by translocation and integration of transmembrane domains into the lipid bilayer [27]. Conformational maturation of even wild-type CFTR in the ER is an inefficient process; approximately 75% of newly synthesized wt-CFTR molecules, as shown for heterologous expression systems, is degraded by the cytoplasmic proteasomes shortly after the synthesis [28]. Maturation of CFTR to post-ER compartments can be readily detected as an approximately 20-kDa decrease in electrophoretic mobility. This decrease reflects conversion by enzymes in the Golgi apparatus of the two Asn-linked glycans in the fourth extracellular loop from immature, high-mannose forms into mature, complex oligosaccharides. Once delivered to the plasma membrane, CFTR is subject to rapid internalization to a pool of sub apical vesicles that can be recycled to the plasma membrane or delivered to the lysosomes for degradation [29]. Over 1,200 mutations and sequence variants in the *CFTR* gene have been linked to CF (Cystic Fibrosis Mutation Data Base, <http://www.genet.sickkids.on.ca/cftr/>). These mutations have been grouped into six classes: class I mutants include deletions, frameshifts and non-sense mutations that result in prematurely truncated CFTR protein products, class II mutants are defective in intracellular trafficking, class III mutants are full-length proteins with little or no ion channel activity, class IV mutants results in CFTR with only slightly reduced channel activity, and class V mutants proteins are functional but expressed at reduced levels, while class VI mutants are expressed at wild-type levels but exhibit decreased stability at the plasma membrana [30,31]. Despite this large number of *CFTR* disease alleles, the vast majority (>90%) of CF patients of Northern European origin have at least one copy of a single mutant allele, Δ F508, which encodes a CFTR molecule lacking a phenylalanine at position 508.

When expressed heterologously in cultured epithelial or non-epithelial cells, Δ F508-CFTR is present as an immature, core-glycosylated species on the ER membrane, whereas wild-type CFTR is predominantly found as a complex glycosylated species at the plasma membrane. CFTR immunoreactivity is restricted to internal membranes in sweat ducts from Δ F508-CFTR homozygotes, although later studies suggest that Δ F508-CFTR is detectable at the plasma membrane and its intracellular localization is tissue-specific [29]. These findings led to the initial assignment of Δ F508-CFTR as a class II, or trafficking, mutant. Further study of the function and fate of this mutant protein indicates that the trafficking model is incomplete. As discussed below, folding defects in Δ F508-CFTR biosynthesis alter the protein interactions with the quality control system in the early secretory pathway and directly or indirectly affect its activity as an anion channel as well as its stability at the cell surface. Thus, Δ F508 should be regarded not as a simple class II mutant, but as a mixed mutant with the properties of classes II, III, and V. This makes finding appropriate treatment options of Δ F508-CFTR more complex as the protein defect cannot be treated as only a class II mutant but a more complex therapeutic strategy must be considered or designed to correct multiple protein processing defects caused by the single Δ F508 mutation [27].

Pathogenesis of CF Lung Disease

The CF gene defect (or mutation) leads to a malfunctioning CFTR protein, as discussed above. This defect has consequences in both epithelial and inflammatory cells of the lung. Most importantly, these consequences include decreased chloride efflux and increased inflammatory response. With regards to the excessive inflammation in the CF airways, some have shown that hyperinflammatory response is a result of the chronic infection while others propose this as a primary outcome of the CFTR dysfunction. It is the belief of some that excessive inflammation in CF is not a result of the CFTR defect but of such factors as the underlying bacterial infection(s) in the lungs. Boucher *et al.* showed that the hyperinflammatory response is independent of the CFTR defect, as evidenced by the decrease in hyperinflammatory markers in long term $\Delta F508$ epithelial cell primary cultures [32]. In contrast, other groups believe that CFTR dysfunction in CF results in an inherent, exaggerated NF κ B signaling leading to the pathogenesis of chronic lung disease. The significant increase in the expression of number of inflammatory markers in the sterile environment of CF fetuses prior to any direct exposure to pathogens supports that defective inflammatory response is an outcome of CFTR dysfunction. It has also been demonstrated that there is significant increase in activation and number of NF κ B driven genes and increased neutrophilic airway inflammation in CF fetus as compared to the non-CF fetus [33–35]. Thus, in addition to its chloride efflux channel function, CFTR plays a critical role in the regulation of the inflammatory response pathways.

Repeated bacterial infections, commonly with *P. aeruginosa*, are characteristic of CF lung disease [36]. The loss of proper chloride channel function leads to decreased water transport into the epithelial layer, along with other less clear consequences [37]. This may play a role in the increased mucus load seen in CF, leading to inadequate clearance of the mucus from the airways and an increased probability of bacterial infection. The bacterial infection induces an increased inflammatory response and signaling. Thus, CF patients are caught in a cycle of mucus retention, infection, and inflammation, which is amplified further as inflammatory products released by neutrophils stimulate mucus secretion and breakdown leading to further infection. These neutrophils release proteases, including neutrophil elastase that overwhelm the antiprotease capacity of the lung and cleave important structural proteins, eventually leading to bronchiectasis [38]. This cycle must be attenuated if there is any promise in therapeutic claims of curing the chronic stages of fatal CF lung disease, as this is a characteristic disorder for the majority of $\Delta F508$ -CF patients. We predict that restoring chloride channel function alone will not be sufficient in reversing this chronic lung disease. This is substantiated by the failure in the last decade of the promising CF therapeutic compounds that were screened based on chloride function alone, but failed to be a promising drug in the clinical reversal of the lung disorder(s) or restoration of optimal lung function. Thus, finding an effective treatment for CF is more complicated than we originally anticipated. We discuss here challenges and therapeutic strategies that hold great promise for designing an effective treatment of CF.

CHALLENGES IN CORRECTING CF LUNG DISEASE

Fate of Misfolded CFTR in the Endoplasmic Reticulum

Endoplasmic Reticulum (ER)-associated protein degradation (ERAD) eliminates misfolded, damaged, or mutant proteins with abnormal conformation [39]. This protein homeostasis mechanism, or proteostasis, provides a new system for targeted therapies to treat $\Delta F508$ -CF and other protein folding and aggregation diseases. ERAD targets are selected by a quality control system within the ER lumen and ultimately destroyed by the cytoplasmic ubiquitin-proteasome system (UPS). UPS plays a pivotal role in cell homeostasis and is vital in regulating various cellular processes. In normal cells, nearly all proteins are continuously

degraded and replaced by *de novo* synthesis. The spatial separation between substrate selection and degradation in ERAD requires substrate transport from the ER to the cytoplasm or extraction from the ER membrane by a process termed dislocation; recently reviewed by Meusser *et al.* [40].

The $\Delta F508$ -CFTR is the most common disease-causing protein folding mutation which results in a temperature sensitive folding defect and premature degradation by the ERAD [1]. As discussed above, absence of CFTR at the airway epithelial cell surface disrupts luminal hydration and is associated with an exaggerated immune response [31]. Functional CFTR can be restored by growth at lower temperature (25–27°C) or incubation with chemical chaperones, which rescues $\Delta F508$ -CFTR from ERAD [4]. Although, the low temperature rescue is most effective and demonstrates the possibility of rescuing functional mutant CFTR on the cell surface, it cannot be used as a therapeutic tool. Chemical chaperones similarly have shown promising results *in vitro* but lack significant clinical efficacy. This has also led to several studies designed to target the early steps of proteasomal degradation as it is becoming clear that slowing down proteostasis may help in rescuing CFTR by inhibiting both proteasomal degradation and inducing the protein folding and trafficking to the cell surface [26,41].

Several recent studies have thus focused on understanding the proteasomal targeting mechanisms for misfolded CFTR. Post translation, cytosolic Hsc70 interacts with ER membrane localized Hdj2, CHIP, and the E2 UbcH5 to form an E3 complex that ubiquitinates cytosolic regions of $\Delta F508$ -CFTR. Inhibition of E2-E3 ubiquitin ligases can increase the accumulation of ubiquitinated CFTR, but this does not show rescue of the mutant CFTR to the cell surface. This can be explained by the presence of redundant mechanisms for CFTR ubiquitination. We recently found that molecular chaperone p97/VCP (valosin containing protein) and E3/E4 ligase- gp78/AMFR (tumor autocrine motility factor receptor) form complexes with CFTR during translocation from the ER for degradation by the cytosolic proteasome. VCP physically interacts with gp78 to couple ubiquitination, retro-translocation and proteasomal degradation of misfolded proteins [42]. Interference in the VCP-CFTR complex promotes accumulation of immature CFTR in the ER and partial rescue of functional chloride channels [26,43,44]. Moreover, under these conditions, the cytokine whose expression is regulated by the proteasome, IL-8, is reduced. Inhibition of the proteasome with bortezomib (PS-341/Velcade) also rescues CFTR, but with less efficiency, and suppresses NF κ B mediated IL-8 activation--important components in controlling the inflammatory response discussed above. Immunoprecipitation of $\Delta F508$ -CFTR from primary CF bronchial epithelial cells confirmed the interaction with VCP and associated chaperones in CF [26]. We have also found that bortezomib modulate VCP activity, while another recent report demonstrated the inhibition of VCP by another proteasome inhibitor, hemin, by acting as an ATPase inhibitor [45]. As discussed earlier, selective targeting of VCP's ERAD function helps in rescuing misfolded CFTR and I κ B, endogenous inhibitor of NF κ B, from proteasomal degradation [46]. Similar therapeutic strategies are promising as rescuing $\Delta F508$ -CFTR to the cell surface may not be sufficient to control or reverse NF κ B mediated chronic inflammation. The use of small molecule drugs that not only serve to rescue the mutant CFTR to the cell surface, but also to decrease the inflammatory response through suppression of the NF κ B mediated IL-8 provides a desirable course of treatment for CF.

We have shown previously that CFTR cell surface expression, lipid raft localization and uninhibited channel function associates inversely with NF κ B and IL-8 promoter activities along with downstream inflammatory signaling, and absence of CFTR results in an inherent defect in I κ B-NF κ B mediated innate immune response [47]. Thus, CFTR lipid raft expression has a beneficial inflammatory response function as also evidenced by a decrease

in glutathione, a major anti-inflammatory agent, in the lung fluid of CF patients [48]. The clinical implication of these findings is that treatment of CF patients with small molecule or therapeutic compounds that rescues optimal amount of mutant CFTR to the cholesterol-rich cell surface lipid rafts can inhibit the NF κ B mediated chronic inflammation and rescue the pathology induced by defective CFTR, potentially attenuating the progression of CF or related obstructive lung diseases like COPD and emphysema. This conclusion should be kept in mind when developing or screening for possible therapeutic agents — the rescue of chloride channel function may not be adequate to reverse the inflammatory pathology of the chronic lung disease. Hence, it remains an open question if we can restore enough mutant CFTR to the cell surface that can control the chronic CF lung disease. Also, it is not clear if “so-called” functional Δ F508-CFTR restored on the cell surface is capable of controlling the chronic inflammatory signaling.

Rescued Δ F508-CFTR is Less Stable than Wild-Type CFTR—Once delivered to the plasma membrane, CFTR is subject to endocytic recycling through a sub-apical vesicular compartment. The steady-state distribution of Δ F508-CFTR at the plasma membrane is determined by the relative rates of endocytosis and exocytosis. The overall half-life of CFTR molecules in this recycling pool is determined by partitioning between exocytosis and degradation by the lysosome. Estimates of the half-life of the wild-type plasma membrane-recycling pool of CFTR range from 18–48 hours in BHK cells to 24–48 hours in the epithelial line LCPK1 [49–51]. In marked contrast, the half-life of Δ F508-CFTR is only about 4 hours, as determined in cultured cells exposed to chemical chaperones or held at a reduced temperature—treatments that allow the protein to accumulate on the surface prior to the assay [49–51]. Pulse-chase labeling studies indicate that this reduced half-life is a consequence of increased lysosomal degradation and not simply redistribution between surface and sub apical recycling compartments. The finding that complex glycosylated Δ F508-CFTR molecules are 20 times less stable than wild-type CFTR at modestly elevated temperatures (37°C), but exhibit stability similar to that of wild-type CFTR at reduced temperatures, suggests that the shorter half-life of plasma membrane Δ F508-CFTR is due to structural differences between Δ F508-CFTR and wild-type CFTR. Data from proteolytic mapping studies confirm this view [52].

The unstable mutant CFTR is less efficient in not only transporting chloride, but also regulating the I κ B-NF κ B mediated innate immune response. This results in a buildup of mucus within the airways and increased inflammation throughout the lungs, explaining the pathological problems associated with the chronic lung disease in CF patients. Even though possible treatment options, such as gene therapy, have shown to increase chloride efflux by up to 25% [53] there has been no significant rescue from the characteristic CF lung disease. Although, intensive efforts have been made in the last decade to identify Δ F508-CFTR correctors and potentiators to restore its chloride efflux function, there has been no significant progress in the identification of therapeutic strategies for reversal of the lung disease from the chronic stages. The underlying problem of chronic inflammation makes this a complex goal to achieve. Thus, it is necessary that further studies be designed with the goal of discovering treatments that not only increase chloride efflux, but also decrease the hyperinflammatory response. Here we examine potential therapeutic strategies, discuss their challenges, and propose methods for the discovery of novel and promising treatments for CF that may have applications in treatment of other chronic and obstructive lung disease conditions as well.

PHARMACOLOGICAL THERAPEUTIC STRATEGIES TO CORRECT CFTR PROTEIN PROCESSING

Pharmacological Rescue of $\Delta F508$ -CFTR Trafficking

Because both the instability of the rescued $\Delta F508$ -CFTR at the plasma membrane and the protein's inefficient conformational maturation in the ER are phenotypes of Phe⁵⁰⁸ deletion, it is possible that this mutant protein is prone to adopt a structurally similar, non-native conformation in both membranes. If so, this conformation may be detected by quality control machineries in the ER for ubiquitin-dependent proteolysis and lysosome in the distal secretory pathway. This model would suggest that a small-molecule drug that facilitates $\Delta F508$ -CFTR folding in the ER might also be effective in stabilizing the protein on the plasma membrane. On the other hand, it is also possible that compounds that specifically suppress off-pathway conformers in the ER do not interact with the non-native conformation in the plasma membrane. In either case, this clearly indicates the need to consider $\Delta F508$ -CFTR stability at the plasma membrane in any pharmacological rescue therapy regimen.

Fortunately, there are several promising drug candidates capable of increasing CFTR activation kinetics. These compounds, which include the alkylxanthines, flavones, and phosphatase inhibitors, appear to stimulate CFTR by a combination of amplifying the cAMP signal transduction, phosphorylation cascade and directly binding to the CFTR. The ability of these or similar compounds to ameliorate the $\Delta F508$ -CFTR defect by increasing its delivery or stability on the plasma membrane warrants further therapeutic evaluation.

One strategy to develop a definitive corrector of CF is to screen candidate chemicals from combinatorial libraries using high-throughput assays and robotics. A recent study of 150,000 diverse chemical compounds produced several classes of candidate correctors of defective $\Delta F508$ -CFTR cellular processing [39]. The effect of these correctors on chloride secretion in human CF airway epithelial cells was small, and the correctors were not screened based on the ability to ameliorate the hyper-inflammatory response seen in CF. Based on recent work from our group and others, we anticipate correcting the chloride secretion defect of CFTR will be incapable of reversing the chronic CF lung disease, as lack of CFTR results in an inherent defect in I κ B-NF κ B mediated inflammatory response [47,54]. Future screens must take into account the anti-inflammatory enhancement properties of potential candidate small molecule compounds by screening for their ability to restore both CFTRs chloride efflux and inflammatory response functions. Moreover, further studies are also needed to evaluate numerous potential off-target specificities of small molecules identified from high-throughput screens, as typically their mechanism of action is unknown. The discovery of useful correctors is anticipated to present a greater challenge than the discovery of CFTR inhibitors or activators because protein folding and trafficking is a complex multistep process involving multiple cellular targets, some of which may be cell type specific. Recently, it was demonstrated that rescuing $\Delta F508$ -CFTR to the cell surface was not sufficient to obtain optimal $\Delta F508$ function in airway epithelial cells. These findings support the idea that correction of $\Delta F508$ -CFTR requires a chemical corrector that not only promotes folding and exit from the ER but enhances surface stability and, of course, improves channel activity [55]. We propose an alternative approach for identification of specific molecular targets to stabilize optimal functional $\Delta F508$ -CFTR by rescuing it from both peripheral and ER associated degradation machinery [26,56].

The corrector or temperature-rescued $\Delta F508$ -CFTR is functional but less stable on the cell surface as discussed above. A distinct group of small molecule drugs, known as potentiators, have been found to be effective in restoring the stability and function of the rescued $\Delta F508$ -CFTR on the cell surface. However, it has been found that these treatments are very

mutation specific. Cai *et al.* found that the CFTR potentiators phloxine B and 2'-dATP, augment G551D-CFTR, a specific mutant, in channel gating, whereas only 2'-dATP enhances G1349D-CFTR [57]. The Vertex potentiator, [VX770], is currently in Phase III clinical trial and has shown promising results for G551D subjects (http://www.cff.org/UploadedFiles/aboutCFFoundation/NewsEvents/2006NewsArchive/770_Phase1_FINAL_PR3.doc.pdf). Although for $\Delta F508$ - homozygous CF patients, potentiators alone will not be able to reverse the CF lung disease. We anticipate that utilizing potentiators along with compounds that help to rescue the CFTR protein from ERAD or modulate proteostasis may be an effective course of treatment in not only restoring chloride channel function but also alleviating the hyper-inflammatory response. Recent studies found that a small molecule compound, VRT-532 [4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol] exhibited both corrector and potentiator capabilities *in vitro*. It was shown to have an effect on stabilizing $\Delta F508$ -CFTR, leading to increased channel function [58]. Although, Talebian *et al.* have recently shown that VRT-532, and another potential corrector drug, Corr4A, do not reduce the IL-6 and IL-8 inflammatory response in CFBE41o- cells [59]. Thus, these drugs will likely have little effect on reversing the chronic lung disease. This exemplifies the reason for evaluating a drugs effect on the inflammatory response as a part of the screening process. This approach will lead to promising discoveries for treatment of the chronic lung disease seen in CF. Rescuing optimal amounts of mutant CFTR to the cell surface that is capable of restoring chloride efflux and regulating inflammation may help to reverse the chronic inflammation and lung disease seen in CF patients.

Chemical Chaperones—The term “chemical chaperone” loosely describes a family of low-molecular weight compounds including polyols (e.g., glycerol, sorbitol, and *myo*-inositol), amines (e.g., betaine and trimethylamine-*N*-oxide [TMAO]), and solvents such as DMSO and D₂O. These compounds have long been recognized to have protein-stabilizing properties *in vitro*, largely due to their ability to increase protein hydration. Endogenously produced compounds like *myo*-inositol and betaine serve as osmolytes, balancing osmotic forces in cells and organisms that are chronically exposed to osmotic stress.

Incubation of $\Delta F508$ CFTR-expressing cells in high concentrations (>1 M) of glycerol or other chemical chaperones (e.g., TMAO or D₂O) increases the steady-state level of complex-glycosylated $\Delta F508$ -CFTR and functional cAMP-activated channel activity, as seen by immunoblotting and quantification of halide efflux and whole-cell patch clamp analysis. Pulse-chase analysis shows that the increase in steady-state expression of functional cell surface $\Delta F508$ -CFTR in the presence of glycerol is due to increased maturation of core-glycosylated nascent $\Delta F508$ -CFTR to a post-ER compartment and decreased degradation, most likely as a result of enhanced folding [49]. Fischer and colleagues report significant increases in forskolin-activated intestinal Cl⁻ transepithelial transport in matched wild-type and $\Delta F508$ -CFTR (but not in CFTR-null) mice injected subcutaneously with TMAO, suggesting that a correction of $\Delta F508$ -CFTR is by rescue of the misfolded CFTR protein and should provide the impetus for drug-discovery screens for compounds that are active in rescuing $\Delta F508$ -CFTR folding at nontoxic pharmacological doses [60].

A related approach to treat CFTR misfolding involves the use of “pharmacological chaperones”, defined substrates or ligands of cell surface-borne channels and receptors. Mutant membrane proteins that have been studied in this regard include P-glycoprotein, the V2 vasopressin, and δ -opioid receptors [61–63]. The identification of pharmacological chaperones constitutes proof-of-principle demonstration that small, high-affinity compounds can influence the conformational maturation pathways of proteins and ameliorate their folding defects. We anticipate that targeting molecular chaperone machinery can rescue both

CFTR and attenuate the hyper-inflammatory. As with other approaches, any attempt to modify the chaperone capabilities in CF should be quantified by not only measuring changes in chloride efflux but also by measuring the change in the inflammatory response.

SELECTIVE TARGETING OF $\Delta F508$ PROTEIN PROCESSING

Molecular Strategies to Correct CF

Although CF is a single gene disorder, there is considerable inter-individual variability amongst people with the same genotypes of CFTR. Secondly, CF is developmentally progressive and variable in progression between organs- one example being that the pancreas is attacked in utero, but the airways disease begins after birth and is impacted by environmental factors. Third, individuals metabolize drugs differently in part due to polymorphisms in key enzymes, and this could impact the response to the drug directed at correcting mutant CFTR trafficking and function. Therefore, it would be desirable to measure the level of expression of the networks of genes and proteins that contribute to disease progression and in response to interventions.

Several high throughput approaches such as gene expression profiling, proteomics, and screening of compounds to rescue functional mutant have, so far, not resulted in a new therapeutic regiment for CF. Gene therapy using viral or non-viral carriers of the normal CFTR cDNA have shown that normal CFTR cDNA can be safely transferred into CF airways, but it is inefficient and transient. We propose that identification and selective modulation of processes involved in $\Delta F508$ -CFTR degradation and folding will lead to functional correction of epithelial ion balance and reduction in inflammatory signaling. The potential advantage to this approach is that it minimizes concerns about losing physiological CFTR regulation, as might occur with activation of alternative chloride channels or ENaC modulation.

The selective rescue of functional $\Delta F508$ -CFTR holds a promise as a treatment for reversal of the characteristic chronic lung disease seen in CF and a potential cure for this fatal obstructive lung disease. With an increase in chloride channel function and proper regulation of the inflammatory pathways, an increase in lung function should follow. This approach and many of the others discussed (particularly the use of small molecule treatments) in conjunction with the rapidly developing targeted drug delivery systems such as nanoparticles will help to make these therapeutic strategies much more target specific and thus, more effective in reaching their destination with decreased detrimental side effects [64].

Selective Targeting of Proteostatic Processes by Small Molecules or Compounds

The multiple, redundant pathways seen in the ERAD mechanism such as Hsp70-CHIP and VCP-Derlin1-gp78-Rma1 have been shown to be involved in ubiquitination and proteasomal targeting of misfolded CFTR. Sato, Ward and Kopito have shown previously *in vitro* that CFTR ubiquitination might actually begin prior to the completion of protein synthesis [65]. Younger *et al.* showed that the RMA1 E3 complex regulates the assembly status of CFTR's amino-terminal regions at a folding step that occurs before NBD II synthesis and is defective in $\Delta F508$ -CFTR. The CHIP E3 pathway occurs following NBD II synthesis and searches for folding defects that involve terminal steps in CFTR assembly [66]. We and others have shown that VCP is directly associated with CFTR and required for ERAD of CFTR [26,43,44], but it is not clear if both VCP- and CHIP-mediated pathways operate at the same time. VCP is involved in retrograde translocation of tagged protein from the ER to the proteasome, indicating that its major function in ERAD is to present ubiquitinated protein to the proteasome [26]. VCP was also detected in immuno-complexes pulled down with anti-CFTR, Hsp40, Hsp70, Hsp90, gp78, ataxin-3 (aggresome marker)

and HDAC6 antibodies indicating interactions between these proteins. These results indicate the functional relationship of VCP with protein folding (Hsp40-Hsp70 and Hsp90 complex), ERAD (gp78 complex) and aggresome formation (Ataxin3-HDAC6 complex, our unpublished data) pathways [26].

These multiple redundant ERAD pathways exhibit the difficulty in rescuing the folded and functional $\Delta F508$ -CFTR by inhibiting a single target in the degradation pathways. If one arm of the pathway is blocked, other arm(s) of the pathway allow the degradation to continue *via* a different mechanism. This demands strategic selection of molecular targets based on their ability to stabilize CFTR by trafficking mutant CFTR to the cell membrane. This also supports the notion that selective modulation of proteostatic machinery will be more helpful than inhibiting ubiquitination machinery as that may result in not only a decrease in misfolded protein degradation, but also induce chaperone and trafficking machineries to stabilize functional mutant CFTR on the cell surface [26,41,56].

VCP is a promising potential target due to its interaction with multiple arms of the ERAD pathway, but its ERAD function needs to be selectively targeted as discussed earlier [26]. Another promising target is ATF6—a protein that participates in the UPR. Decreased expression of ATF6 in $\Delta F508$ -CFTR cells restores cAMP-dependent halide efflux. It has been suggested that in CF, ATF6 may be of a great importance because its decreased expression induces an increased membrane localization of CFTR [67]. Yamazaki *et al.* recently demonstrated that genetic and pharmacological inhibition of ATF6 suppressed the ATF6 arm of the UPR and also NF κ B activation, thus decreasing degradation and the inflammatory response [68] similar to the strategy we proposed earlier for inhibiting mutant CFTR degradation and inflammatory response [28,47]. These are important molecular targets and may lead to the design of an effective therapeutics for CF and other conformational diseases. Selective targeting of retrograde translocation complex or other components of proteostatic machinery are helpful in stabilizing CFTR by inhibiting both proteasomal degradation and inducing other components of chaperone machinery that may help in folding as well as trafficking of mutant CFTR to the cell membrane.

One may predict that inhibition of $\Delta F508$ -CFTR degradation should, by mass action, increase production of folded $\Delta F508$ -CFTR molecules. However, studies have shown that acute or chronic exposure of $\Delta F508$ -CFTR-expressing cells to proteasome inhibitors leads to accumulation of detergent-insoluble, multiubiquitylated $\Delta F508$ -CFTR molecules, with no detectable increase in folded CFTR. These findings suggest that the substrate of proteasome action is not in direct equilibrium with intermediates on the folding pathway. These and subsequent studies on the degradation of misfolded $\Delta F508$ -CFTR and other proteins in the secretory pathway reveal that degradation is a multistep process that involves recognition, ubiquitin conjugation and dislocation across the ER membrane, and unfolding and degradation by the proteasome. Although, complete inhibition of CFTR degradation has been observed when an active site laboratory inhibitor (MG132) was combined with an ATPase inhibitor (hemin). Recently, it was shown that hemin inhibits the ATPase activity of VCP/p97, an AAA ATPase molecular chaperone [45,69].

The latest approval of bortezomib as a drug for refractory multiple myeloma [70–72] initiated investigations on therapeutically targeting proteolysis or protein catabolism for various diseases. Bortezomib (pyrazylcarbonyl-Phe-Leuboronate) is an extremely potent, stable, reversible and selective inhibitor of chymotryptic threonine protease activity [70]. Bortezomib has shown encouraging results when employed in hematological cancers and solid tumors. Bortezomib selectively inhibits NF κ B and induces apoptosis in cancer cells while normal cells recover from proteasome inhibition [73]. Proteasome modulators have recently been also shown to be of dual therapeutic importance in pharmacogene therapy of

the cystic fibrosis airway. The proteasome modulating agents, LLnL and doxorubicin, enhanced CFTR gene delivery and, hence, the CFTR-mediated short circuit currents. Moreover, these proteasome modulators also inhibited functional ENaC activity and currents independent of CFTR vector administration [74]. We found that bortezomib could rescue some $\Delta F508$ -CFTR from ERAD that results in the partial rescue of mature CFTR. A main concern in considering the proteasome as a therapeutic target is the theoretical risk that multiple processes may be affected by proteasome inhibitors. The risk of global suppression of proteasomal degradation may be balanced by the favorable anti-inflammatory effect that we and others have observed. We confirmed that VCP or proteasome inhibition leads to the inhibition of the $\text{I}\kappa\text{B}\alpha$ degradation pathway [75,76] and hence NF κ B-mediated, IL-8 activation. Bortezomib can enter mammalian cells and inhibit NF- κ B activation and NF- κ B-dependent gene expression. Bortezomib also inhibits TNF- α -induced gene expression of the cell-surface adhesion molecules E-selectin, ICAM-1, and VCAM-1 on primary human umbilical vein endothelial cells [77,78]. In a rat model of streptococcal cell wall-induced polyarthritis [79], bortezomib attenuated the neutrophil-predominant acute phase and markedly inhibited the progression of the T cell-dependent chronic phase of the inflammatory response [77].

Bortezomib is one of the first of its kind in proteasome modulation drug that has shown promise in rescuing NF κ B dependent chronic lung disease pathophysiology of CF. mutant CFTR, but is effective in modulating IL-8, a proinflammatory cytokine. Thus, bortezomib and future proteostatic modulator drugs utilizing similar mechanism hold promise to reverse the chronic lung disease seen in CF and may be effective also in regards to other obstructive and inflammatory airway diseases by targeted controlled delivery to airway or inflammatory cells [64]. The potential to recover functional mutant CFTR and controlling inflammation, is especially promising for CF patients. Clearly, considerable basic investigation of these questions is required before this class of compounds can be considered as potential therapeutics.

Selective Targeting of Molecular Chaperone Machinery

Molecular chaperones can bind to and stabilize the folded, functional form of a mutant protein and shift the folding equilibria away from degradation and aggregation, thereby reducing the amount of misfolded and aggregated protein [41]. Nascent CFTR molecules interact transiently with cytoplasmic members of the Hsp family and the ER luminal chaperone calnexin. These chaperones contribute to the recognition of misfolded CFTR for cytosolic proteasomal degradation. The strategies which aid in inhibiting proteasomal targeting and inducing folding machinery have been reported to rescue functional $\Delta F508$ -CFTR to the cell surface, as shown in Fig. (2) which exhibits possible promising targets. Wang *et al.* found that down regulating a co-chaperone of Hsp90, Aha1, by siRNA stabilized $\Delta F508$ -CFTR mutants in HEK293 and human bronchial epithelial (HBE) cells. It was also seen that this knockdown restored halide conductance in $\Delta F508$ CFBE41o-cells [80]. The immunosuppressant drug deoxyspergualin (DSG) binds Hsp70 and Hsp90 *in vitro* with micromolar affinity and appears to compete for binding of substrates to a subset of these chaperones [81]. Treatment of $\Delta F508$ -CFTR expressing epithelial cells with DSG increases forskolin-stimulated halide efflux and whole-cell currents to levels comparable to those achieved by low temperature incubation. This suggests that, at least at rather high concentrations (50 $\mu\text{g}/\text{ml}$), this drug can partially restore functional $\Delta F508$ -CFTR. However, there is no data yet to support the hypothesis that DSG influences the folding or delivery of mutant protein to the plasma membrane, directly or indirectly. Even though it appears to exhibit some specificity with respect to Hsp70 family members, the pleiotropic effects of interfering with both the Hsp70 and the Hsp90 chaperone systems could have indirect consequences on multiple cellular pathways, including those that influence CFTR activation

or gating mechanisms. Moreover, although it is clear that DSG interacts with Hsp70 and Hsp90, there is no evidence that any of the biological effects of this drug are mediated by the drug's interaction with these chaperones; other targets of lower abundance and higher affinity may remain to be identified. Interestingly, treatment of CFTR-expressing cells with the benzoquinone, ansamycins, geldanamycin and herbimycin A-- drugs that bind with high affinity to Hsp90-family chaperones-- appears to perturb the interaction between Hsp90 and CFTR, suppressing its maturation and accelerating its degradation. Recent studies suggest that geldanamycin activates a general heat shock response, influencing other classes of molecular chaperones and components of the degradation apparatus, perhaps as an indirect consequence of its effects on Hsp90. Based on recent work from Balch group we anticipate that small molecules interfering with the Hsp90-Aha1 interaction [81] or Hsp90 acetylation can lead to the development of selective therapeutic strategies by inducing protein folding and inhibiting proteasomal targeting.

Molecular chaperones play essential roles both in protein biogenesis, where they facilitate folding and suppress aggregation, and in protein degradation, where they contribute to recognition and destruction of misfolded molecules. The studies described here suggest that drugs that interfere with the action of broad classes of molecular chaperones can influence the functionality of $\Delta F508$ -CFTR molecules in model systems. Concerns over the utility of chaperone antagonists or agonist's center on our lack of understanding of the specific chaperone functions; 1. In the intracellular fate of $\Delta F508$ -CFTR, 2. Recognition of the pleiotropic nature of chaperone action and 3. The potential for induction of proteotoxicity through protein aggregation.

Since CF is characterized by early onset of lung disease, it is necessary that possible treatments be selected with an outcome of reversing this chronic lung disease as the ultimate goal. It has been seen that targeting the ERAD and folding pathway is effective in modifying the pathways that lead to chronic lung disease, including CFTR membrane regulation and the modulation of the inflammatory response pathway(s), as seen in Fig. (2). Therefore, therapeutic options must be searched for with the idea of reversing chronic lung disease as an ultimate goal.

CAN RESCUED $\Delta F508$ -CFTR REVERSE THE CHRONIC LUNG DISEASE?

Up to this point, the majority of studies have focused on rescuing $\Delta F508$ -CFTR to restore optimal halide efflux in the membrane channel. There have been some promising discoveries that showed a small effect on increasing the halide efflux, but there is little advancement in reversing the chronic lung disease completely. In order to do so, mutant CFTR must be folded properly and trafficked to the cell membrane lipid bilayer. It has also been seen that if enough CFTR is folded properly and returned to the lipid bilayer, a significant decrease is seen in the activity of the inflammatory response. It was found that in wild type CFTR cells where CFTR is localized in lipid rafts, IL-8 and gap junctional intercellular communication (GJIC) secretion and parts of the inflammatory response cascade, were regulated in a normal fashion in comparison to the mutant CFTR [19]. The mutant CFTR showed a greater inflammatory response that explains the pathogenesis of CF and confirmed the need to both correctly fold and deliver mutant CFTR to the lipid bilayer. Having correctors to rescue the mutant CFTR chloride efflux function would not be sufficient in reversing the chronic inflammation seen in CF patients.

We also propose that measuring only chloride efflux as a therapeutic outcome for screening of potential treatment options for CF is not sufficient. It is necessary to measure the effect that a treatment has on the chronic lung disease by quantifying markers of hyper inflammatory response such as I κ B-NF κ B, as well as recovery of lung function. Moreover,

use of state of the art real time imaging techniques to examine the inflammatory state of lung during the course of the treatment will help in the discovery and development of promising drug candidates that hold potential to rescue or reverse the chronic lung disease. Most therapeutic screenings and clinical studies in the last decade have used CFTR channel function to record the level of correction and may explain why no significant recovery of clinical pulmonary function outcome was recorded in spite of the 25% correction in chloride conductance seen with gene therapy studies [53]. The rescue of pulmonary function (FEV% predicted) is the best measure of pulmonary outcome and should be used as a standard practice for evaluating the effectiveness of CF therapeutics, as it will not only demonstrate the correction of chloride conductance but rescue of the overall lung disease. Correcting the chronic CF lung disease is an achievable goal, but the present strategies for the screening and clinical evaluation of therapeutics must be modified with the goal of quantifying the levels of correction in lung disease as the final outcome. Again, without overstating the problem, it is obvious that correcting the chloride channel function alone will not be enough to find a cure for CF, as evidenced by the failed therapeutic options of the last decade. The proposed modifications in therapeutic development strategies will lead to immediate discoveries of novel, promising drug candidates that may eventually lead to a cure for this monogenic genetic disorder with multiple defects.

PERSPECTIVE

The majority of CF treatments currently being studied fall into two major categories, correctors and potentiators or anti-inflammatories. These and other treatments along with challenges are outlined in Table 1. Each available treatment brings challenges that must be appropriately addressed. It is also important that a CF treatment regime address not only the rescue of the $\Delta F508$ -mutant, but also the major pathophysiological characteristics of CF - most importantly, the chronic inflammation as seen by over expression of the pro-inflammatory cytokines, IL-8 and the resulting lung neutrophilia. Some studies have even seen increased numbers of neutrophils and IL-8 in bronchoalveolar lavage (BAL) from CF patients with mild disease symptoms or even in the absence of bacteria or other microorganisms [82–84]. There is *in vivo* evidence that the expression of anti-inflammatory products like IL-10 and lipoxins are attenuated in CF. Thus, it is likely that the immune response defect is inherent in CF and CFTR plays a critical role in inflammatory response. As discussed above in detail, promising CF treatment involves targeting the degradation and folding pathway of mutant CFTR in order to first rescue the mutant protein and traffic this protein to the cell membrane. This approach may not only help in rescuing the chloride channel function but also help in regulating the chronic inflammatory response and hence hold a promise for the reversal of the chronic CF lung disease. We cannot emphasize more that increasing chloride channel function may not be sufficient to revert the CF lung disease from chronic stages, and goals and methodology of further research and therapeutic development must be tailored in the direction of completely reversing the chronic lung disease by rescuing both ion transport (physiology) and chronic inflammation (pathology).

The traditional approach to determine the effectiveness of possible treatments for CF involves quantification of changes in halide efflux, *in vitro* and *in vivo*. This has led to many potential treatments, but little progress toward a cure for CF. Many of the potential treatments currently under development are summarized in Table 2. Standard practice has been to correlate the increase in halide efflux, recorded in the nasal cavity (nasal potential difference, NPD) and CF cells (bioelectric measurements and dye based assays), to therapeutic efficacy of the drug. This is not a completely reliable test for patients suffering from the chronic lung disease as it records only the transient changes in CFTR channel function. Limitations to the use of this strategy also include difficulties in obtaining good recordings in patients with nasal inflammation, lack of well-described differences in

baseline and borderline readings in CF subjects [85] and little correlation to overall lung function. We propose that this clinical parameter is insufficient in quantifying the therapeutic efficacy of the drug in reference to the state of chronic lung disease. As NPDs record changes in membrane potential function, they do not specifically determine the correction of other CFTR function(s) and lung disease.

It is evident from recent studies that CFTR plays a critical role in inflammatory response in addition to its well described ion transport function [47,86,87]. Thus, an effective treatment for CF may require the identification of small molecule or therapeutic corrector compound that rescue the optimal amount of mutant CFTR to the cell surface and cholesterol rich lipid rafts. It remains an open question if we can restore enough mutant CFTR to the cell surface that can control the chronic CF lung disease. Also, it is not clear if “so-called” functional $\Delta F508$ -CFTR restored on the cell surface is capable of controlling the chronic inflammatory signaling. Although, if this can be achieved, it can lead to the promising therapeutic strategy that can correct ion transport dysfunction while also inhibiting the NF κ B mediated chronic inflammation. We propose that targeting proteostasis has a potential to achieve this therapeutic goal.

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Fig. 1. The role of CFTR in the inflammatory signaling pathway

The lack of functional CFTR on the cell surface dysregulates pro-inflammatory signaling *via* innate immune response (TNFα-IL1β-TLR) pathways resulting in NFκB mediated hyper-inflammation, and the chronic CF lung disease. Therapeutic strategies that can control the chronic inflammatory pathophysiology of CF lung disease by not only restoring the chloride efflux function but also regulating the NFκB mediated chronic inflammatory signaling hold the promise as a potential cure of CF.

Fig. 2. The major classes of CF treatments and their pathological consequences

The degradation of mutant CFTR leads to an overactive innate immune response, and increased pro-inflammatory cytokine release. Although cell surface potentiators, increased chaperone recruitment, and calcium pump inhibition may increase functional CFTR, they do little to correct the hyper-inflammatory response. The figure summarizes the selective molecular targets and indicates expected pathological outcomes: I. Modulating proteostasis not only can increase functional CFTR, but also can decrease the hyper inflammatory response, and thus, increase the lung function. II. Chaperone recruitment and calcium pump inhibition induces the protein folding machinery, leading to the increased levels of properly folded CFTR. III. Potentiators and inhibitors of endocytic recycling leads to increased function and stability of mutant CFTR, but are only useful on the cell surface. IV. Anti-inflammatories are useful in controlling the progression of the chronic lung disease, but do little to correct the CFTR defect.

Table 1**Therapeutic Challenges in Designing Novel CF Therapeutics**

Therapeutic Strategy	Challenge	Pathological Outcome	Reference
Inhibition of Degradation Pathway	Selective targeting of proteostasis	Rescue CFTR and IκB from proteasomal targeting and suppress chronic lung disease pathophysiology	[34,36,62,73,74]
Chemical and Pharmacological Chaperones	Lack of understanding of the role of specific chaperones in the intracellular fate of ΔF508 CFTR. May need to be combined with proteostasis inhibitor.	Increased stabilization of the functional CFTR protein	[49–52,68]
ER Calcium Pump Inhibition	May not be effective in chronic stages of CF lung disease	Increased chaperone recruitment and subsequent decrease of aggregated and misfolded proteins	[8,9]
CF Potentiators	Mutation specific treatments effective for cell surface function. May need to be combined with proteostasis inhibitor for ΔF508 CFTR.	Restore chloride channel function	[46–48]

Table 2

Therapeutic Strategies Currently Under Development

Therapeutic Classification [#]	Drug Name [#]	Stage of Development [#]	Therapeutic Target	Anticipated Outcome for Lung Disease
Gene Therapy	Compacted DNA	Phase I	Correction of the DNA mutation by introduction of wild type CFTR through compacted DNA nanoparticles [88]	Correction of underlying CFTR defect; potential cure for lung disease if gene expression is significant
CFTR Modulation	Potentiator VX-770	Phase III	Allows defective CFTR located at the cell surface to transport more chloride *	Increased chloride efflux, but CFTR must be at the cell surface. Not likely to regulate inflammatory response, and attenuate chronic lung disease. $\Delta F508$ subjects will benefit only if effective corrector drug like VX-809 is used in combination.
	Ataluren	Phase III	Promotes read through of premature stop codons of CFTR mRNA [89]	Increased functional CFTR; potential to ameliorate all aspects of lung disease, although more studies must examine the anti-inflammatory efficacy.
	Corrector VX-809	Phase II	Increase concentration of CFTR protein to the cell surface *	May not correct function or regulate the inflammatory response. Combination with a potentiator may provide a promising treatment.
Restore Airway Surface Liquid	Hypertonic Saline Solution; Bronchitol	To Patients; Phase III	Hydration of the mucus and epithelial linings to promote normal clearance	No effect on chronic lung disease as it does not treat defective CFTR, but does improve quality of life [90].
	Denufosol; SPI-8811; Moli 1901	Phase II; Phase III	All activate alternative ion channels rather than CFTR. Denufosol is selective P2Y(2) agonist that stimulates ciliary beat frequency and Cl(-)secretion [91]	Reduces mucus viscosity and promotes clearance, but does not correct defective CFTR or control inflammation.
Mucus Alteration	Pulmozyme (dornase alfa)	To Patients	Cleaves extracellular DNA found in mucus	Reduces viscosity of mucus, leading to decreased respiratory infections and better quality of

Therapeutic Classification [#]	Drug Name [#]	Stage of Development [#]	Therapeutic Target	Anticipated Outcome for Lung Disease
				life [92], but no effect on mutant CFTR.
Anti-Inflammatory	Ibuprofen	To patients	NSAID; inhibits cyclooxygenase and thus, NFκB	Decreases inflammation, but does not effect chloride channels [47]
	Oral N-acetylcysteine; Inhaled Glutathione	Phase II	Enhances glutathione levels in neutrophils; Increases systematically low levels of glutathione seen in CF subjects [93]	Decreases inflammatory cells in lung tissue and shows positive increase in lung function [93]. But it has no effect on mutant CFTR and chloride channel function.
	Docosahexaenoic Acid(DHA)	Phase II	Increased omega 3 fatty acid concentration	Decrease mucus secretion and hyperinflammatory response [94]. But mechanism of action is unknown and has no effect on correction of mutant CFTR.
	Sildenafil	Phase II	Selective inhibitor of cGMP specific phosphodiesterase type 5 [95]	May decrease inflammation and has even been shown to increase chloride efflux at high doses [95]. Further studies are required to evaluate therapeutic effectiveness. May not affect underlying CFTR defect.
	HE-3286	Pre-Clinical	Adrenal Steroid hormone	Reduces levels of pro-inflammatory cytokines [96], which would help regulate inflammation but does little to correct or stabilize CFTR. May not have the same effect in CF subjects and possibly not be suitable for long term use. More studies are needed.

[#] Drug Development Pipeline. **Cystic Fibrosis Foundation**. (<http://www.cff.org/research/DrugDevelopmentPipeline/>); Stage of Development as of 7/15/09

* Vertex Pharmaceuticals(<http://www.vrtx.com/current-projects/drug-candidates/>).