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TARGETING EARLY EVENTS IN CYSTIC FIBROSIS DISEASE PATHOGENESIS

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SUMMARY

Cystic fibrosis (CF) is a chronic and debilitating genetic disease that occurs in over 70,000 people worldwide. Advances in treatment have led to significant improvements in longevity since the disorder was first described over half a century ago, and standardized care at CF care centers is central to these achievements. Traditional therapies for CF have focused on disease symptoms, including pancreatic enzyme replacement, mucus clearance from the lungs, treatment of lung infections, use of antiinflammatory agents and management of acute processes that periodically occur on a background of chronic therapies. Based on our understanding of early steps in the cascade leading to clinical CF, new therapeutic strategies are entering clinical trials that target upstream processes. These new therapies have the potential to dramatically change the face of CF, moving care from a symptom-based approach to a preventive, presymptomatic paradigm. This review will summarize recent progress in studies of airways hydrators and cystic fibrosis transmembrane conductance regulator (CFTR) modulators, focusing on medium and later phase clinical trials that target primary events in CF pathogenesis.

CYSTIC FIBROSIS AND ION TRANSPORT DEFECTS

Cystic fibrosis (CF) is a serious, life-threatening and -shortening disease that affects over 70,000 people worldwide (CFF registry statistics, 2008; 1, 2). It results from autosomal recessive mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator protein (CFTR), which is a plasma membrane localized traffic ATPase. CFTR is regulated by ATP binding and hydrolysis during heterodimerization of nucleotide binding domains 1 and 2, and protein kinase A (PKA)-dependent phosphorylation of the regulatory (R) domain, and it functions as a chloride channel (3). CFTR also regulates additional ion transporters and pathways, such as other chloride channels, bicarbonate (HCO_3^-) transport, the epithelial sodium channel ENaC, thiocyanate (SCN^-), and other small molecules (4-14), and serves a primary role in regulating fluid and ion transport in the tissues where it is expressed. In the airways, high CFTR expression is found in the submucosal gland ducts, with moderate expression seen in the apical cell membrane of pseudostratified epithelium (medium and large airways), and in distal small airways epithelial cells (15, 16).

Mutations in the *CFTR* gene result in a variety of molecular mechanisms that lead to impaired production or function of CFTR (Fig. 1). The disease-causing mutations can generally be grouped into common class defects that logically follow the various steps between the gene and the integral membrane protein. While these class distinctions are not absolute, they do serve as a starting point to understand how mutations cause disease, and strategies to restore their function. The CFTR mutation classes include: impaired biosynthesis (class I); defective protein maturation and accelerated degradation (class II); defective regulation of CFTR at the plasma membrane (class III); defective chloride conductance (class IV); diminished CFTR transcription (class V); and accelerated turnover at the cell surface (class VI).

CF airways pathology is believed to be directly related to the absence or reduction of functional CFTR present at the airways surface to regulate airways surface liquid (ASL) derived from both the submucosal glands and the surface epithelia (7, 8, 17-19). The volume produced by submucosal glands that is released onto the airways surface is believed to be significant, containing high levels of diverse innate defense molecules. This fluid is then adjusted by the surface airways epithelial cells, which are normally bathed by a critical fluid compartment known as the periciliary liquid layer (PCL; a subcomponent of the ASL). Surface epithelial cell ion transporters thus optimize conditions for mucociliary clearance (MCC) (7, 9, 20).

Cilia beat in a coordinated manner, moving the overlying gel layer from the lung periphery to the large airways for clearance. The gel layer includes high levels of hydrated mucins (MUC-5AC and -5B), and recent data suggest that it may also serve as a volume reservoir for the ASL. Under healthy conditions, this coordinated glandular and surface epithelial system rapidly clears inhaled particulates and pathogens, and this is accomplished without awakening other secondary host defense mechanisms.

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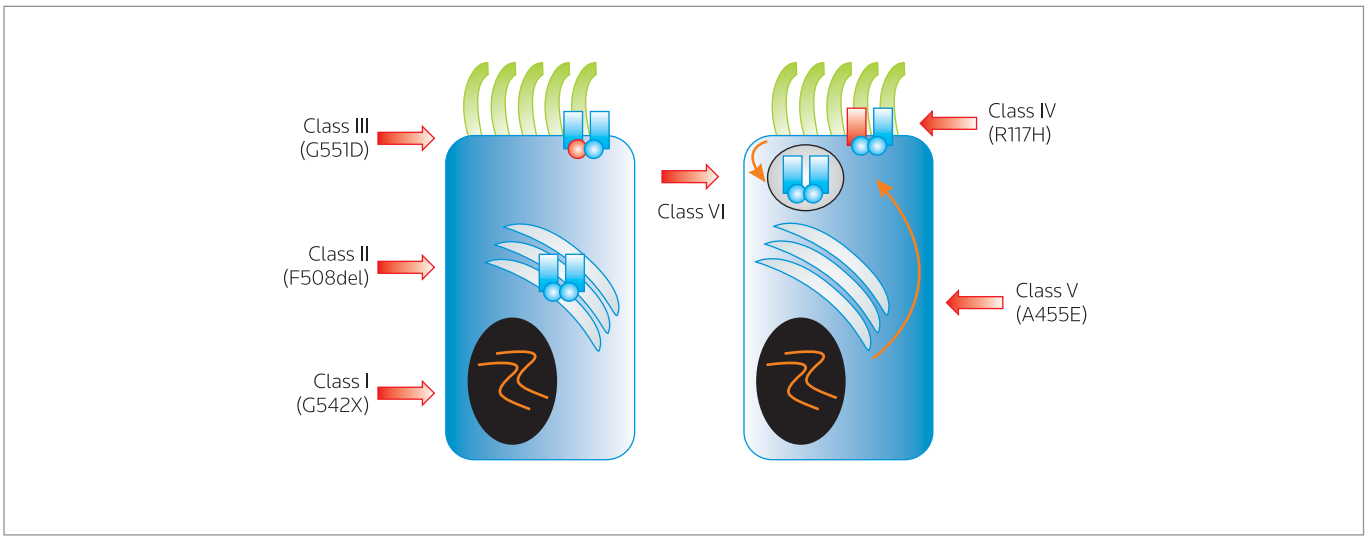

Figure 1. Classes of cystic fibrosis transmembrane conductance regulator protein (CFTR) mutations. The approximately 1,800 disease-causing CFTR mutations can be classified by their mechanism of defect, including class I (biosynthetic defects), class II (folding and maturation defects), class III (regulatory defects), class IV (chloride conductive defects), class V (reduced numbers of normal transcripts) and class VI (shortened half-life at the plasma membrane). Examples of "severe" mutations are shown on the left (with minimal if any function), while mutations with partial activity are shown on the right.

Under high ASL volume conditions, ENaC absorbs sodium across the airways epithelium, with passive chloride flow through CFTR (and other chloride transport pathways) to promote a "dry" mucosal surface. The ASL volume is reduced until the PCL is approximately 7 μm (which is similar to the length of extended cilia). Chloride secretion is believed to then balance with sodium absorption such that the PCL and effective MCC are maintained (9, 20, 21). Evidence supports the concept of tight coordination between sodium and chloride transport to maintain (and when necessary to increase) MCC. H_2O transport through the paracellular pathway and through aquaporins follows ionic forces, and is driven by the dominant transcellular and paracellular ion flux (8, 19, 22, 23). As CFTR has been shown to transport additional anions (HCO_3^-, SCN^-), defects in their transport have also been hypothesized to be major contributors to CF pathology, including disrupted mucin unpackaging during its release onto the epithelial surface, and defects in innate bacterial killing (10-12).

In addition to CFTR, the airways epithelium also has a separate chloride transport pathway that is regulated by cellular calcium. This calcium-responsive chloride channel was formerly known as the calcium-activated chloride channel regulator (CaCC), but recent studies have identified it as a member of the anoctamin-1 channel family (24-30). The CFTR and anoctamin-1 chloride channels and their associated signaling pathways are well understood in the context of CF lung disease, and each pathway serves as a target for drug development. The calcium-regulated chloride transport pathway functions normally (or above normal) in the CF airways and is activated following stimulation of G protein-coupled $P2Y_2$ receptors. It has been postulated that the retention of calcium-regulated chloride transport may substitute for CFTR dysfunction, and upregulation of CaCC has been hypothesized to minimize the CF lung phenotype in *Cftr* knockout mice (31, 32). Activation of calcium-dependent chloride transport produces a rapid but somewhat short-lived increase in chloride conductance in vitro due in part to the short half-life of ATP in the airways. Chloride transport through this pathway is enhanced in CF, which may reflect endoplasmic reticulum expansion (with increased calcium storage produced by chronic inflammation and infection) (33-38). The calcium-regulated chloride transport pathway appears to be vulnerable to insults such as upper and lower respiratory tract viral infections (21, 22, 38, 39). In the absence of these chloride transport pathways, the ASL and PCL volume is reduced, leading to impaired MCC, endobronchial infection by opportunistic bacteria and an exuberant inflammatory host response that results in CF lung disease (7, 9, 20, 38, 39).

TARGETING EARLY EVENTS IN CF PATHOGENESIS

The current treatments for CF lung disease focus on treating the downstream pathological processes that occur in the absence of normal ASL regulation and MCC. Mucus obstruction, infection and excessive inflammation produce chronic airways obstruction that is managed by physical clearance, anti-infective and antiinflammatory agents. This includes enhanced clearance of mucus from the airways with bronchodilators, inhaled recombinant human DNase, and more recently, the use of inhaled hypertonic saline (further discussed in the "Airways Hydrators in CF" section). These agents improve mucus rheology by decreasing mucus viscosity (rhDNase) and promoting osmotic hydration (hypertonic saline). Chronic suppression or eradication of pathological bacteria with inhaled and systemic antibiotics helps to temper the impact of infection, while the use of antiinflammatory agents can improve lung function, and may slow airways damage and disease progression (40, 41). All of these aspects of CF lung disease are at last somewhat reversible, and thus therapies tar-

geting mucus obstruction, infection and inflammation typically assess efficacy through analysis of lung function, often coupled with the need for rescue therapies or increased treatments for new pulmonary symptoms (42). During periods of increased symptoms (i.e., pulmonary exacerbations), therapies are intensified, with the addition of prolonged courses of i.v. antibiotics with and without inpatient hospitalization. While effective, these treatments are challenging for CF patients to adhere to for optimal effectiveness. Over time, these processes lead to damage of the airways structure, producing irreversible lung damage and airways obstruction through initially regional and ultimately panbronchiectasis.

New strategies (Fig. 2) that are in clinical trials to treat underlying CF pathology and target early events in disease pathogenesis include inhaled airways hydrators, which aim to increase the amount of fluid in the PCL and ASL compartment (including denufosol and activators of other alternative chloride transport pathways, osmotic agents and ENaC inhibitors), and CFTR modulators, which are systemically available small molecules that target disease-causing CFTR protein defects (and thus increase the amount and/or activity of CFTR at the plasma membrane). One major difference between these strategies relates to whether airways pathology in CF requires restoration of CFTR activity (including chloride transport, regulation of sodium transport and transport of other anions, such as HCO_3^- and SCN^-), or whether restoration of airways hydration alone can substitute for CFTR deficiency. Airways hydrators have been studied in phase III trials to treat CF since 2006, and CFTR modulators have more recently entered phase III trials (VX-770 and ataluren [PTC-124]). While there is some philosophical overlap among these approaches (as they target early steps in CF pathogenesis rather than downstream consequences), they are each focused on different aspects of CF airways disease, and thus may have the potential for complementary effects.

AIRWAYS HYDRATORS IN CF

Two airways hydrators have advanced to phase III clinical trials in CF, i.e., denufosol and mannitol. Both agents are topically delivered to the airways, but they differ in terms of mechanism of action (activation of CFTR-independent chloride transport pathway for denufosol, osmotic stimulus for mannitol), delivery mechanisms and dosing (nebulized solution three times a day for denufosol, dry powder inhaled twice a day for mannitol), and population under evaluation (patients with FEV_1 > 75% for denufosol and > 40% for mannitol).

Denufosol (INS-37217; Inspire Pharmaceuticals) is a $P2Y_2$ receptor agonist that stimulates calcium-activated chloride transport and

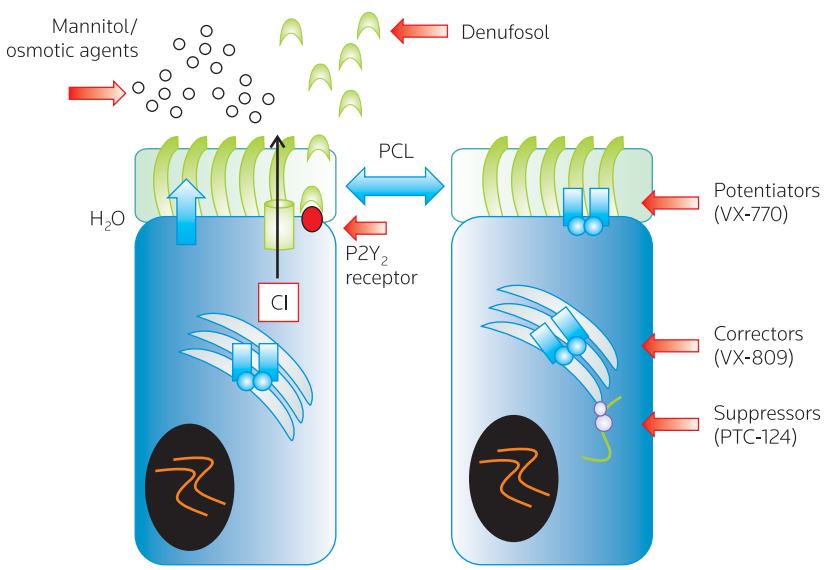

Figure 2. Early events in cystic fibrosis – targets for new therapies. The figure on the left identifies sites of action of airways hydrators. Mannitol is an osmotic agent that draws fluid into the airways, expanding the periciliary liquid layer (PCL) and airways surface liquid (ASL) and promoting mucociliary clearance. Denufosol binds to $P2Y_2$ receptors, activating IP3 signaling and elevating cell calcium. This activates the calcium-activated chloride channel regulator (CaCC, TMEM16A) and apical chloride transport, contributing to airways hydration. The figure on the right summarizes targets of cystic fibrosis transmembrane conductance regulator protein (CFTR) modulators. Suppressors of premature termination codon (PTC) interact with the ribosome during translation, altering proofreading function at PTCs and promoting readthrough and full-length protein production. Correctors aim to increase the amount of F508del CFTR at the plasma membrane, presumably through promotion of correct folding during protein processing and maturation in the endoplasmic reticulum. Potentiators aim to increase the gating of CFTR at the cell membrane, enhancing chloride flow through CFTR.

improves hydration of the airways epithelial surface (43, 44). It has also been shown to reduce sodium absorption in vitro, and has a prolonged airways surface half-life relative to other nucleotides that normally regulate airways ion transport. The results of a two-part, multicenter phase I/II clinical trial of denufosol were published in 2005, demonstrating safety and tolerability in pediatric and adult CF patients (45). Subsequently, 89 individuals with mild CF across 12 U.S. centers were randomized to a double-blind study investigating the safely of 28 days of denufosol versus placebo (isotonic saline) (46). Subjects enrolled had an age range of 8-50 years with an FEV_1 > 75% predicted. Subjects were randomized 1:1:1:1 to receive either 28 days of inhaled denufosol (20, 40 or 60 mg in 3 of the cohorts) or placebo. A total of 94% of enrolled patients completed the study and demonstrated similar rates of compliance by treatment diary review. The tolerability of denufosol was similar to that of placebo across all dose cohorts. Five patients discontinued the study due to adverse events (two on placebo and three on denufosol). Two serious adverse events considered unrelated to study drug occurred in randomized patients (development of Hodgkin's lymphoma and a CF pulmonary exacerbation). Denufosol achieved the primary endpoint of safety and tolerability, while demonstrating evidence of biological activity based on analysis of some secondary endpoints. Spirometry demonstrated small but statistically significant improvements in FEV_1, forced expiratory flow ($FEF_{25-75\%}$) and forced vital capacity (FVC) in the denufosol treatment groups compared to placebo, despite a relatively low power to detect changes in lung function. This improvement occurred over a short duration and approached that reported in previous investigations of azithromycin and dornase alfa (47-49). An additional randomized, double-blind, placebo-controlled phase II trial in 72 CF patients evaluated 2 doses of denufosol (20 and 60 mg) or placebo 3 times a day across 17 U.S. centers. Overall there was no difference in reported respiratory symptoms between the denufosol and placebo groups. The study concluded that 20 and 60 mg of denufosol dosed 3 times a day for 28 days was generally well tolerated in subjects with milder CF lung disease. Patients with more severe lung disease reported more adverse events and greater lung function decline, which in some cases led to withdrawals from the trial (43).

Phase III studies of denufosol in CF began in July 2006 with the initiation of the multicenter, randomized, double-blind TIGER-1 (Transport of Ions to Generate Epithelial Rehydration) trial. TIGER-1 examined the efficacy of 60 mg of denufosol nebulized 3 times daily over a 24-week treatment period, as determined by change in FEV_1 compared to placebo. The double-blind portion was followed by a 24-week open-label safety extension phase. Secondary endpoints included other lung function parameters, pulmonary exacerbations, use of CF-related medications and a patient-reported outcome measure (the CFQ-R, a validated quality-of-life instrument) (Quittner AL, et al. Cystic fibrosis questionnaire: a health-related quality of life measure. © 2000: Version 1.0). TIGER-1 enrolled a total of 352 patients, limiting enrollment to subjects with mild CF lung disease (FEV_1 > 75% predicted) (ClinicalTrials.gov NCT00357279). Following 24 weeks of treatment, the primary efficacy endpoint (FEV_1) was met, with the denufosol group demonstrating a mean improvement of 45 mL compared to placebo (P = 0.047). The majority of secondary endpoints did not achieve statistical significance, but $FEF_{25-75\%}$ did show a trend towards improvement favoring denufosol over placebo (P = 0.072). The subsequent 24-week open-label extension phase included 210 subjects. Those who received denufosol in the initial 24-week study had continued improvements in FEV_1, with a total increase of 115 mL by 48 weeks. Those who received placebo during the blinded portion improved their FEV_1 by 76 mL by 48 weeks, with 75 mL of the improvement occurring in the open-label denufosol phase.

A second phase III study of denufosol in CF patients with an FEV_1 > 75% predicted (TIGER-2) began enrollment in May of 2008, and concluded enrollment in November of 2009 (> 450 patients). Recent topline results released in January 2011 reported that the study did not achieve statistical significance for the primary efficacy endpoint, which was the change from baseline in FEV_1 at week 48 (50). Ongoing analysis of this study and its impact on further development of this agent is anticipated in late 2011. These contradictory phase III results will raise a number of questions relevant to new drug development in the CF research community, including study design for airways hydrators, clinical trials in patients with mild lung disease (including choice of study endpoints, study size and duration), predictability of preclinical model systems and drug targets.

Inhaled mannitol (Bronchitol®; Pharmaxis) is the second airways hydrator that has progressed to phase III trials in CF. Intravenous mannitol has been used clinically for decades in the management of increased intracranial pressure. Its use in the airways is based on similar principles, in that osmotic pressure will draw water into the lumen of the airways, mobilizing mucus and improving its clearance. Topical mannitol can improve mucociliary clearance and lung function (51-54). In a recent phase III trial in 295 CF patients (age > 6 years; mean FEV_1 63.8 ± 15.9%), inhaled dry powder mannitol was well tolerated in patients treated for 6 months, with moderate improvements in lung function (96.2 mL over baseline) (55). A related study included 643 patients across 11 countries. Mannitol-treated subjects had a 7.3% increase in FEV_1 over their baseline and controls (P < 0.001) (56). These two means of airways hydration are novel approaches to address the underlying cause of CF lung disease based on our leading hypotheses of disease.

The principle of using osmotic stimuli to enhance airways hydration is postulated as the mechanism of action for a commonly used airways therapy in CF, i.e., hypertonic saline (57, 58), and another agent (nebulized xylitol) that is in early development to treat CF (59, 60). Preclinical studies in various model systems indicate that they are capable of increasing ASL volume, but questions remain regarding the optimal agent for use in CF. For example, while hypertonic saline has been shown to improve lung function and improve clinical stability in CF, compliance in large clinical trials has been reported to be relatively low (~60%), primarily due to respiratory symptoms (cough and wheeze). Additionally, non-saline-based agents (such as bronchitol and xylitol) are predicted to reduce the salt concentration of the ASL, which has been postulated to be abnormally elevated in CF. According to this theory, normalizing the sodium and chloride concentration of the ASL may have additional benefits, improving bacterial killing by innate antimicrobial peptides (61). Clearly, future trials will need to address these questions to help optimize hydrator therapy in CF patients.

CFTR MODULATORS

Modulators of CFTR currently in clinical trials are the product of high-throughput screening (HTS) initiatives that have identified unique compounds for subsequent CF drug development. They specifically target disease-causing *CFTR* mutations, with the aim of restoring protein function towards normal levels. There are three lines of modulators in development for CF, including suppressors of premature termination codons (PTCs; in phase III), potentiators of CFTR at the plasma membrane (in phase III), and correctors of CFTR folding defects (in phase II). These strategies encompass the majority of CF-causing mutations, and have the capacity to target dysfunctional CFTR across all organ systems (62).

PTC suppressors

Biosynthetic mutations in *CFTR* include PTCs, which are characterized by single base-pair substitutions that create a PTC (UAG, UAA, UGA) within the open reading frame. When the ribosome encounters a PTC, protein translation ceases and the result is low levels of truncated and nonfunctional protein. Small molecules that suppress PTCs bind to the eukaryotic ribosomal machinery and alter its function sufficiently to cause the occasional insertion of near cognate amino-acyl tRNAs into the ribosomal A site (63). This can produce translational readthrough and full-length protein derived from PTC-containing transcripts. Several aminoglycosides have been shown to produce this effect in preclinical model systems and in proof-of-concept trials in CF patients (64-73).

A novel oral compound, ataluren, is under development to treat CF caused by PTCs, and has been evaluated in three randomized, two-dose phase II clinical trials (open-label). In the first trial (completed in Israel), 23 adult CF subjects possessing at least one CF-causing PTC were treated with oral ataluren 3 times a day for two 2-week treatment periods. Two doses were tested in a dose-ascending, open-label format with a 2-week washout between treatment blocks. In general, ataluren was well tolerated and no subjects discontinued study drug during the trial. Patients improved CFTR-dependent chloride transport (as measured by the nasal potential difference, NPD) by –7.1 mV (standard deviation [SD]: 7.0; $P < 0.0001$ for low dose) and –3.7 mV (SD: 7.3; $P < 0.032$ for high dose), respectively. There were small improvements in FEV_1, FVC and body weight during the first treatment period relative to baseline, but no significant changes in other clinical parameters or sweat chloride values (another biomarker of CFTR).

In a parallel trial completed in the U.S. (N = 24 subjects at 6 centers), ataluren was generally well tolerated, without study drug discontinuations. There were no group changes in NPD measures of CFTR activity or other clinical parameters compared with baseline values (personal communication, Dr. S. Rowe). More recently, a third phase II trial of ataluren has been completed in France and Belgium (74). Thirty CF children (age range 6-18 years) possessing at least 1 PTC in CFTR were enrolled in this crossover trial, in which subjects were randomly treated with either a low or a high dose of ataluren (dosing as described previously) for two 2-week treatment blocks with a 2-week washout period. Approximately 50% of subjects were considered NPD responders based on meeting threshold NPD responses or changes in CFTR-dependent chloride transport. These effects were identified across a variety of CF-causing PTCs. In addition, CFTR was detectable at the plasma membrane of brushed nasal cells by immunofluorescent staining during ataluren treatment, providing evidence for appropriate localization of full-length CFTR while on study drug.

Based on these results, ataluren has advanced to a randomized, double-blind, placebo-controlled phase III trial evaluating the efficacy, safety and tolerability of oral study drug compared with placebo for 48 weeks, followed by an open-label extension (NCT00803205: A Phase 3 Efficacy and Safety Study of PTC124 as an Oral Treatment for Nonsense-Mutation-Mediated Cystic Fibrosis). This study will be the first blinded and placebo-controlled trial of ataluren in CF, and parallels evaluation of the agent in other genetic diseases caused by PTCs.

CFTR potentiators

Potentiators are CFTR modulators that aim to increase the gating of CFTR at the plasma membrane. VX-770 (Vertex Pharmaceuticals) is an orally available compound that normalizes gating of G551D CFTR, and was developed following an extensive HTS program (75). G551D CFTR is found in about 4% of CF patients, with characteristic ATP binding and hydrolysis defects that cause low open channel probability and chloride transport. G551D CFTR localizes normally to the plasma membrane, and is therefore an appropriate target for potentiator development. In a recently completed study, VX-770 was studied at four doses in a randomized, double-blind, placebo-controlled trial in CF adults possessing at least one copy of the G551D CFTR mutation (N = 39 CF subjects). Oral VX-770 (25, 75, 150 and 250 mg twice daily) was well tolerated and VX-770-treated patients demonstrated improvements in two CFTR biomarkers (sweat chloride values, NPD) and FEV_1 relative to pretreatment values and controls (76).

VX-770 has recently been studied in a phase III clinical trial in CF patients > 12 years of age with the G551D CFTR mutation, with topline results reported in early 2011 (NCT00909532: A Phase 3, Randomized, Double-Blind, Placebo-Controlled, Parallel Group Study to Evaluate the Efficacy and Safety of VX-770 in Subjects With Cystic Fibrosis and the G551D Mutation). The study met its primary efficacy endpoint, reporting mean absolute improvements in lung function (FEV_1) for VX-770-treated subjects from baseline compared to placebo of 10.6% and 10.5%, respectively, at weeks 24 and 48 of the trial ($P < 0.0001$ for both time points). In addition, statistically significant improvements in weight gain, reduced pulmonary exacerbations and improved quality of life measures were reported in the VX-770-treated group compared with the placebo group over the 24- and 48-week time points (77).

F508del CFTR correctors

Correctors of F508del CFTR aim to increase the amount of mutant protein at the cell surface. Phenylalanine deletion from position 508 of CFTR interrupts protein folding and processing, reducing F508del CFTR maturation and targeting to the plasma membrane (1). Strategies that increase the amount of F508del CFTR at the cell membrane are termed correctors, and they are typically intended to improve F508del CFTR protein folding and trafficking. F508 CFTR correction could also theoretically include strategies

that increase F508del CFTR membrane targeting independent of folding effects (i.e., release of incorrectly folded F508 CFTR to the cell membrane), or extending the plasma membrane residence time of F508del CFTR.

A recently developed agent (VX-809; Vertex Pharmaceuticals) has been evaluated in a multicenter, randomized, double-blind, placebo-controlled phase II clinical trial in CF patients homozygous for the F508del CFTR mutation. VX-809 is also the product of HTS and subsequent drug optimization, and has been demonstrated to improve F508del CFTR trafficking to the cell membrane and function in primary human bronchial epithelial cells isolated from F508del CFTR homozygous CF patients (78). Eighty-nine subjects were enrolled in this trial, which evaluated 28 days of once-daily oral VX-809 compared with placebo (25, 50, 100 and 200 mg). VX-809 was generally well tolerated across the four dose groups, with a safety profile similar to that seen in the placebo group. VX-809 treatment was associated with dose-dependent reductions in sweat chloride in treated patients compared with placebo controls. The effects on sweat chloride were less than with VX-770 in G551D CFTR CF patients, and other outcome measures (NPD, lung function, patient-reported outcomes) did not demonstrate statistically different changes compared with baseline or placebo group results. Clinical trials have been initiated examining VX-809 combined with VX-770 in F508del CFTR homozygous patients, evaluating safety, tolerability, pharmacokinetics and biomarkers of CFTR activity.

CONCLUSIONS

New CF therapies that target early events in disease pathogenesis have advanced to late-phase clinical trials in CF patients, and offer new paths of treatment that could potentially modify disease progression. It remains unclear whether restoring CFTR function or substituting for CFTR function(s) are viable treatment strategies for CF, but results from recently completed phase II and III trials provide evidence that agents that hydrate the airways and modulate CFTR improve biomarkers and clinical outcome measures in short- and medium-length trials. Results of ongoing work will help to clarify these remaining questions, and should help to define viable targets for future intervention.

DISCLOSURES

The authors state no conflicts of interest.

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