Mutation-specific Therapy in Cystic Fibrosis

a report by

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The cystic fibrosis transmembrane conductance regulator (CFTR) chloride (Cl-) channel comprises five domains: two membrane-spanning domains (MSDs) (MSD1 and MSD2), each composed of six transmembrane segments (TMSs) (TM1 to TM12) that form the channel; two nucleotide-binding domains (NBDs) (NBD1 and NBD2), capable of adenine triphosphate (ATP) hydrolysis; and a regulatory (R) domain, which contains numerous phosphorylation sites.1,2 The phosphorylation of sites in the R domain by protein kinase A (PKA), regulated by cyclic adenosine monophosphate (cAMP), and the hydrolysis of ATP by NBDs are essential for activating the chloride channel.2 This complex multidomain assembly is thought to be responsible for the inefficient conformational maturation process of the CFTR (only 20–50%) in the endoplasmic reticulum (ER).3

Over 1,400 sequence variations (mutations that are involved in disease expression and polymorphisms, with no effect on the phenotype) have been identified so far on the entire CFTR gene.4 ΔF508 (the most common mutation) is found in ~70% of patients worldwide; however, its frequency varies greatly among different ethnic groups, i.e. from 18% in Tunisia to 100% in the isolated Faroe Islands of Denmark. In Europe, a clear decreasing gradient in the frequency of ΔF508 is observed from north-east to south-west. All other CFTR mutations are rare. However, several of them appear with a high incidence in isolated populations.4

The location of a mutation on the CFTR gene has no direct effect on the severity of cystic fibrosis (CF) disease, and neither does the type of mutation (deletion, insertion, non-sense, etc.) directly affect disease severity. CFTR mutations can be either classical or atypical, spanning all known mutation types. The increased understanding of the genotype-phenotype relationship and the molecular mechanisms by which mutations cause CF led to the classification of the different CFTR mutations into five major classes according to their effect on CFTR production and function (see Table 1 and Figure 1). This classification led to the development of mutation-specific therapies aimed at correcting or bypassing the molecular defect created by each group of mutations.

Class I Mutations – Defective Protein Synthesis

Class I includes mutations creating premature termination codons (PTCs), which lead to the disruption of CFTR protein synthesis. These mutations are also called stop mutations or non-sense mutations. They are known to result in truncated proteins; however, it is now apparent that they have additional effects on the resulting transcripts. PTCs can dramatically decrease the half-lives of mutant messenger RNA (mRNAs) by the non-sense-mediated mRNA decay (NMD) pathway, as well as altering the pattern of pre-mRNA splicing. Therefore, these mutations are expected to result in little or no protein. Indeed, genotype-phenotype studies have revealed that CFTR non-sense mutations are associated with a severe form of the disease.5

A specific therapy for PTCs has been proposed that aims to read through the non-sense codon, thereby allowing synthesis of full-length proteins. It has been shown that aminoglycoside antibiotics, in addition to their antimicrobial activity, can inefficiently interact with the aminoacyl (A) site of eukaryotic ribosomal ribonucleic acids (tRNA), leading to alteration in RNA conformation, which reduces the accuracy between codon–anticodon pairings. This can lead to read-through of the PTCs by binding of any transfer RNA (tRNA) to the non-sense codon, thereby permitting protein translation to continue to the normal end of the transcript.6 Usually, as termination of eukaryote genes is coded for by different contexts of termination codons, the aminoglycosides are not expected to affect the normal termination processes. In addition, in cases where even low levels of physiologically functional proteins are sufficient to restore function, aminoglycosides may be suitable for treatment.

Several in vitro studies have demonstrated that aminoglycosides can read through PTCs in the CFTR gene, producing functional full-length CFTR proteins.7 Ex vivo exposure of airway cells from CF patients carrying non-sense mutations led to the identification of surface-localised CFTR in a dose-dependent fashion.7 Clinical studies provided evidence that the aminoglycoside gentamicin can read through PTCs in vivo.8 An additional clinical study in which systemic gentamicin was administrated also showed correction of the CFTR abnormalities.9

A double-blind, placebo-controlled, cross-over study demonstrated expression of full-length CFTR proteins and restoration of CFTR function following topical application of gentamicin to the nasal epithelium of 19 CF patients carrying the W1282X mutation.10 Complete normalisation of electrophysiological abnormalities was found in 21% of the patients. In 68%, restoration of either chloride or sodium transport was observed. Furthermore, a significant increase in peripheral and surface staining for full-length CFTR proteins was detected in the nasal epithelial cells of the patients following treatment.10 Together, these studies imply that aminoglycosides can induce read-through of PTCs. This has also been shown in other genetic diseases caused by PTCs.5

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Cystic Fibrosis

Table 1: Classification of Different Cystic Fibrosis Transmembrane Regulator Defects and Potential Therapies

<table>
<thead>
<tr>
<th>Class</th>
<th>Effect on CFTR</th>
<th>Functional CFTR</th>
<th>Presence of CFTR on Cell Membrane</th>
<th>Potential Treatments*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Unstable RNA Production of truncated protein</td>
<td>No</td>
<td>No</td>
<td>Aminoglycosides, PTC124</td>
</tr>
<tr>
<td>II</td>
<td>Impaired protein processing in the Golgi</td>
<td>No</td>
<td>No</td>
<td>Stabilising chaperones – Sodium-4-phenylbutyrate, ER Ca++ pump inhibitor – thapsigargin</td>
</tr>
<tr>
<td>III</td>
<td>Defective regulation</td>
<td>No</td>
<td>Yes</td>
<td>Alkylxanthines (e.g. 8-cyclopentyl-1,3-dipropylxanthine (CPX), flavonoids – genistein</td>
</tr>
<tr>
<td>IV</td>
<td>Impaired function causing reduced chloride transport</td>
<td>Yes, but reduced</td>
<td>Yes</td>
<td>CFTR activators: Alkylxanthines (e.g. 8-cyclopentyl-1,3-dipropylxanthine (CPX), flavonoids – genistein, NS-004, milrinone, modulators of CFTR protein-protein interactions – with PDZ-binding proteins and syntaxins</td>
</tr>
<tr>
<td>V</td>
<td>Reduced synthesis of normal functioning of functional CFTR</td>
<td>Depending on number of functional CFTR</td>
<td>Splicing factors that promote exon inclusion or factors that promote skipping of CFTR mRNA, induce mRNA alternative splicing</td>
<td></td>
</tr>
</tbody>
</table>

*CFTR activators may be beneficial additives for all classes of CFTR mutations.

A new drug, PTC124 (3-{5-[2-fluorophenyl]-[1,2,4]oxadiazol-3-yl}-benzoic acid), is a 284Da orally bioavailable, non-aminoglycoside compound specifically developed to induce ribosomes to read through premature stop codons but not normal stop codons. When tested in a stop-mutation-mediated mouse model of CF, PTC124 generated production of full-length, functional CFTR protein. Phase I studies in healthy volunteers established the initial PTC124 safety profile and defined dosing regimens for achieving target trough plasma concentrations known to be active in pre-clinical models.

In a phase II study performed in Israel, PTC124 induced improvements in CFTR-mediated chloride transport in all non-sense mutation genotypes tested, shifting values into the normal range in many patients. Small but statistically significant improvements in basal nasal potential difference (NPD) were also noted, suggesting that partial restoration of CFTR function may have had benefits in terms of ENaC-related sodium transport. Changes in clinical parameters, including increases in forced expiratory volume in one second (FEV1) and forced vital capacity (FVC), improvements in bodyweight and reductions in neutrophilia, were also observed in this study. A parallel phase II study performed in five centres in the US using the same protocol as the study in Israel did not show significant improvement in NPD and in the clinical parameters. Another phase II study in Europe was performed in children who received two 14-day treatment courses of oral PTC124 therapy at two different dose levels. Patients ranged from six to 18 years of age. All had non-sense-mutation-mediated disease, pathological lung infection and pancreatic insufficiency. Statistically significant (p<0.05) increases in the proportion of epithelial cells showing surface staining with the CFTR protein were observed. In addition, statistically significant (p<0.05) improvements in CFTR-mediated chloride conductance as measured by NPD were evident. PTC124 was generally well tolerated in paediatric patients and mean compliance with treatment was excellent (>95%). It is unclear why not all patients carrying non-sense mutations respond to PTC124 therapy. Our data suggest that the efficiency of the non-sense-mediated decay (NMD) machinery regulates the level of mRNA. PTC124 works on mRNA transcripts and the level of mRNA determines the response. Thus, patients with low mRNA levels will not respond to PTC124 therapy.

A further phase II extension study was performed in Israel and assessed three months of oral PTC124 therapy in adult patients with non-sense-mutation-mediated CF who had participated in a prior short-term PTC124 phase II study. Results from the study showed that treatment with PTC124 resulted in statistically significant improvements in CFTR function as measured by NPD in both dose groups. The proportion of patients showing improvement in NPD chloride secretion increased over time in the extension study. Trends towards improvements in mean FEV1 and FVC values were observed. Patients receiving PTC124 experienced a decrease in cough frequency by the end of three months of therapy. In addition, the level of CFTR transcripts carrying non-sense mutations is a limiting factor in the response to read-through treatment. Following two weeks of treatment with gentamicin or PTC124, improvement in the CFTR electrophysiological abnormalities was found only in patients with relatively high transcript levels. However, following the three-month treatment with PTC124, response was found also in patients with low levels of CFTR non-sense transcripts.

Class II Mutations – Defective Protein Processing

Class II mutations are known to cause defective protein processing. On completion of the CFTR protein translation, the normal protein undergoes glycosylation and folding in the ER and the Golgi apparatus that enables protein trafficking to the apical membrane. During its folding, CFTR binds to molecular chaperons, such as the heat shock proteins (HSPs) Hsc70, Hsp40 and Hsp90, and calnexin, a calcium-binding transmembrane protein that assists newly synthesised proteins to fold into a normal structure in the ER (see Figure 1). Complex formation with these chaperons is involved not only in facilitating the folding, but also in the degradation of non-native conformers. The latter are targeted for proteolysis by the ER-associated degradation (ERAD) to prevent accumulation of toxic polypeptides in the cell. After the CFTR protein reaches the plasma membrane it undergoes rapid endocytosis and efficient recycling into the cell surface. Class II mutations cause impairment of this process, leading to early degradation of the abnormally processed protein. In vitro studies of the ΔF508-CFTR...
proteins demonstrated that this mutant polypeptide can function as a cAMP-dependent chloride channel once it reaches the cell membrane. It is clear, therefore, that the most efficient treatment would be to enhance the folding of the variant protein, thus increasing the amount of active protein.

Chemicals or molecules that could correct this early degradation problem and restore CFTR to the cell surface are called ‘correctors’. One important goal of corrector therapy is the restoration of sufficient levels of CFTR activity at the cell surface to achieve chloride secretion and downregulation of sodium absorption. This may require much higher densities of ∆F508 than of wild type due to the reduction in open time for the chloride pore. Chemical chaperones such as glycerol have been shown to stabilise protein structure and to augment the stability and kinetics of oligomeric structures, often through the processes of ubiquitination and proteolysis. A number of stabilising chaperones that are found within the lumen of the ER and in the cytosol, such as the 70-kDa HSPs, including Hsp70 and Hsc70, can stabilise these misfolded structures. Hsc70 binds to the N-terminus of the NBF-1 peptide during the intermediate stages of folding and stabilises the protein, thereby inhibiting aggregation. Another molecular chaperone is calnexin, a calcium-binding transmembrane protein chaperone in the ER that assists newly synthesised proteins to fold into a normal structure. Mutant CFTR undergoes prolonged specific association with both calnexin and Hsp70, which may promote ubiquitination and degradation. Sodium-4-phenylbutyrate (4-PBA), a drug developed to treat elevated blood ammonia levels in urea cycle disorders, is a histone deacetylase inhibitor that promotes ∆F508 CFTR trafficking. 4-PBA allows mutant CFTR to escape the ER, thereby permitting its glycosylation in the Golgi apparatus, transportation to the plasma membrane and subsequent display of residual intrinsic channel activity. 4-PBA has already been tested in phase I clinical trials and in a randomised, double-blind, placebo-controlled study of patients homozygous for the ∆F508 mutation, yet no significant change in sweat chloride concentration was observed. Evidence of functional ∆F508 CFTR was detected in nasal epithelia. 18

Once ∆F508 CFTR escapes the ER and passes through the Golgi apparatus, additional barriers have to be crossed to reach the plasma membrane. Estimates of the in vitro half-life of ∆F508 CFTR on the cell surface suggest that this residence time is shortened compared with wild-type CFTR. 19 Differences in the relative rates of recycling and degradation in lysosomes are likely to account for the substantial differences in half-lives between wild-type and ∆F508 CFTR.

For molecular chaperones to function optimally they require calcium concentrations in the millimolar range, mobilisation of sequestered ER calcium (Ca++) stores with agents such as the ER Ca++ pump inhibitor thapsigargin substantially reduces the ER luminal calcium concentration. Thapsigargin treatment released ER-retained ∆F508-CFTR to the plasma membrane, where it functioned effectively as a Cl- channel. Treatment with aerosolised calcium-pump inhibitors reversed the NPD defect observed in a murine model of ∆F508-CFTR expression. 20 Likewise, curcumin, a non-toxic Ca–adenosine triphosphatase pump, administered to homozygous ∆F508 CFTR mice corrected the characteristic nasal potential difference defect; however, it is unclear whether this can be adapted to humans.

Class III Mutations – Defective Cystic Fibrosis Transmembrane Conductance Regulator Activators

Phosphorylation of CFTR by protein kinases (PK) and dephosphorylation by protein phosphatases (PP) is considered the major pathway by which CFTR chloride channel activity is physiologically regulated. Phosphorylation of the regulatory domain causes binding of ATP to the NBD, resulting in induction of chloride transport. Class III includes mutations that lead to the production of proteins that reach the plasma membrane; however, their regulation is defective and thus they cannot be activated by ATP or cAMP. G551D is an example of a class III mutation. It is the third most common mutation, with a worldwide frequency of 3.1% among CF patients, although populations of Celtic descent display frequencies as high as 8%.

Cystic Fibrosis Transmembrane Conductance Regulator Activators

Activator compounds are designed to increase the probability that the CFTR channel is open, which could result in an increase in chloride transport across the cell surface in some patients. A prerequisite for an activator to be effective is a mutant CFTR at the cell surface. Several compounds have been found to directly activate both wild-type and mutant CFTR, such as the alkylxanthines and genistein. 21 The flavonoid genistein is a potent activator of CFTR and can overcome the affected ATP binding to G551D. More recent data have indicated that the most likely explanation for the stimulatory effect of genistein is through direct binding to an NBD of phosphorylated CFTR. Genistein did not directly open CFTR channels, and required both ATP and prior phosphorylation by PKA; it also restored cAMP-dependent G551D-CFTR activity in vitro and in vivo. 22-24 CF patients with at least one G551D allele who underwent nasal PD testing in which genistein was superfused following isoproterenol showed modest activation of CFTR. 27 The overall degree of chloride transport (17% of that observed in normal subjects) might be augmented by increasing the level of G551D expression in nasal mucosa. Perfusion of the nasal mucosa with genistein significantly hyperpolarised nasal PD in both G551D CF patients and healthy subjects, indicative of a genistein-induced Cl- conductance in both groups. Furthermore, genistein stimulated ∆F508-CFTR if available in the cell membrane, for example after treatment with 4-PB. 23,24 Thus, genistein is considered to be a CFTR activator and can be used to augment the function of chloride conductance of available mutated CFTR located in the cell membrane.

The substituted benzimidazolone NS-004 has quite similar effects to those exhibited by genistein. NS-004 activated CFTR and ∆F508-CFTR without raising cAMP concentration in the presence of PKA inhibitors. NS-004 may use a mechanism similar to that of genistein to increase open probabilities of phosphorylated active channels without affecting either PK or PP. The cardiotoxic drug milrinone is an inhibitor of cGMP-inhibited phosphodiesterase (PDE), and was selected from several drugs inhibiting PDE isozymes. 28 Because PDE inhibitors raise cellular cAMP concentration and CFTR is activated by increases in cAMP concentration, it was assumed that milrinone (and also IBMX, see below) stimulates CFTR by raising cAMP levels. 29,30
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Class IV Mutations – Defective Cystic Fibrosis Transmembrane Regulator Channel Conduction

In this class of mutations CFTR can be produced, processed in the cytoplasm, transported and inserted into the apical membrane. CF patients with one or two of these mutations tend to have a milder CF phenotype.31,32 R117H, D1152H, R334W and R347P are class IV mutants with reduced single-channel conductance.32 Augmentation of CFTR function by CFTR activators in this setting may restore sufficient function to ameliorate the disease. Alternatively, restoring native chloride pore characteristics pharmacologically might be effective. Adenosine and its nucleotides have been shown to activate wild-type and R117H forms of CFTR in cell cultures through the A2B receptor, which is present in human bronchial epithelium.33 Activators of CFTR at the plasma membrane may function by elevating cytosolic cAMP (promoting CFTR phosphorylation), by inhibiting phosphatase activity (blocking CFTR dephosphorylation) and/or by interacting directly with CFTR.44 Modulation of CFTR protein–protein interactions, such as with PDZ-binding proteins36,37 and syntaxins,38 may also affect CFTR function. Several chemical classes of CFTR activators exist that might function by direct interaction with CFTR, including flavones/soflavones (e.g. genistein and apigenin,33 benzoc[4]quinoliziniums,40 xanthines,41 benzimidazolones42 and fluorescein derivatives.43

Recently, a novel approach to identifying new classes of CFTR activators and potentiators was used that involved high-throughput screening of diverse drug-like compound collections.44 The primary screen was designed to identify CFTR activators that were effective both without and in synergy with cAMP activators, the latter mimicking the normal physiological response.45 Vertex Pharmaceuticals and Negelescu recently described a portfolio of small molecules that rescue either the defective trafficking or gating (potentiators) of ∆F508-CFTR in both recombinant cells and primary CF human bronchial epithelia cells.46 The corrector is not yet in clinical trials; however, a potentiatior, VX-770, is in early-phase clinical trials in healthy volunteers and R117H forms of CFTR in cell cultures through the A2B receptor, which is present in human bronchial epithelium.33 Activators of CFTR at the plasma membrane may function by elevating cytosolic cAMP (promoting CFTR phosphorylation), by inhibiting phosphatase activity (blocking CFTR dephosphorylation) and/or by interacting directly with CFTR.44 Modulation of CFTR protein–protein interactions, such as with PDZ-binding proteins36,37 and syntaxins,38 may also affect CFTR function. Several chemical classes of CFTR activators exist that might function by direct interaction with CFTR, including flavones/soflavones (e.g. genistein and apigenin,33 benzoc[4]quinoliziniums,40 xanthines,41 benzimidazolones42 and fluorescein derivatives.43

Future Prospects

Additional studies aiming to better understand the effect of different CFTR gene mutations on CFTR function will broaden our understanding of the different functions of the CFTR protein as well as the function of each CFTR domain. Further studies of the potential modulation of the CF phenotype by polymorphisms in the CFTR sequence and/or by other genes will enable us to better understand the genetic complexity of the disease. The development of mutation-specific therapies using high-throughput screening for small molecules will lead to pharmacotherapy targeting the basic CF defect. Since mutation-specific therapy will result in the expression of partially functioning mutated CFTR on the apical membrane, it is expected that combined therapy with CFTR potentiators will be needed in order to augment CFTR function.45

References