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REVIEW

Pharmacological treatment of the biochemical defect in cystic fibrosis airways

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ABSTRACT: The understanding of the biochemical defect in cystic fibrosis (CF) has advanced considerably since discovery of the CF gene in 1989 and characterization of its product. Studies showing that the abnormality in chloride flux could be corrected by transfection of wild-type cystic fibrosis transmembrane conductance regulator (CFTR) complimentary deoxyribonucleic acid (cDNA) have led to gene therapy trials on both sides of the Atlantic. However, gene therapy as a treatment for CF has yet to be realized.

Pharmacological manipulation of the biochemical defect may provide an alternative or complementary approach to treatment. This review will discuss pharmacological agents in development which could correct the abnormal ion movement.

The mechanisms of action of these pharmacological agents can be divided broadly into drugs which affect the most common CF mutation, ΔF508, which increase trafficking of the mutant CF protein to the apical membrane; drugs which increase chloride secretion; and drugs which reduce sodium reabsorption across the apical membrane.

Treatment options for cystic fibrosis have developed rapidly since discovery of the cystic fibrosis gene over a decade ago. The targeting of specific therapies for particular cystic fibrosis genotypes and the use of combination treatments of chloride channel openers with sodium channel blockers are likely to be key advances in the next decade.
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Cystic fibrosis (CF) is caused by mutations of the CF gene on chromosome 7, which codes for the cystic fibrosis transmembrane conductance regulator protein (CFTR). CFTR is a cyclic adenosine monophosphate (cAMP) regulated chloride channel which regulates the epithelial sodium channel (ENaC) and it may have other functions including the transport of adenosine triphosphate (ATP) [1]. More than 850 CF mutations are currently recognized. These mutations cause defects in CFTR trafficking and/or activation leading to reduced epithelial chloride secretion by CFTR and excessive sodium absorption through ENaC [2]. The mechanism by which abnormal ion transport causes CF lung disease is controversial, with two theories predominating [3] (fig. 1). The isotonic volume depletion theory (figs. 1a and c) suggests that isotonic absorption of salt and water from the apical membrane in CF airway epithelium occurs as a result of increased ENaC activity [4]. This leads to volume depletion of the airway surface liquid (ASL), dehydration of the mucus layer and formation of "mucus plaques" which adhere to the airway epithelium leading to bacterial colonization. An alternative theory, the hypotonic salt hypothesis (figs. 1b and d), suggests that the pathogenesis of CF lung disease is linked to the deactivation of cationic peptides such as β-defensins [5] which are produced by airway epithelium and function optimally in hypotonic solutions. In CF, the ASL is less hypotonic thereby impairing β-defensin function and promoting bacterial colonization. Currently there is increasing evidence to support the isotonic model [6] but both may play a role [3]; however, normalization of the ion transport defect is thought to be the key to a cure for CF lung disease.

Strategies aimed at increasing chloride secretion

A number of pharmacological agents have been studied that increase chloride secretion *in vitro*. Figure 2 and table 1 give an overview of the different agents and proposed mechanism of action as discussed later in the text.

Cystic fibrosis transmembrane conductance regulator protein trafficking

The most common CF mutation is ΔF508, caused by a deletion of a phenylalanine residue at amino acid position 508. The resulting conformational change in CFTR makes it vulnerable to degradation within the endoplasmic reticulum (ER) [7]. Nascent polypeptides fail to reach the apical membrane, resulting in the absence of a functional response. Attempts to

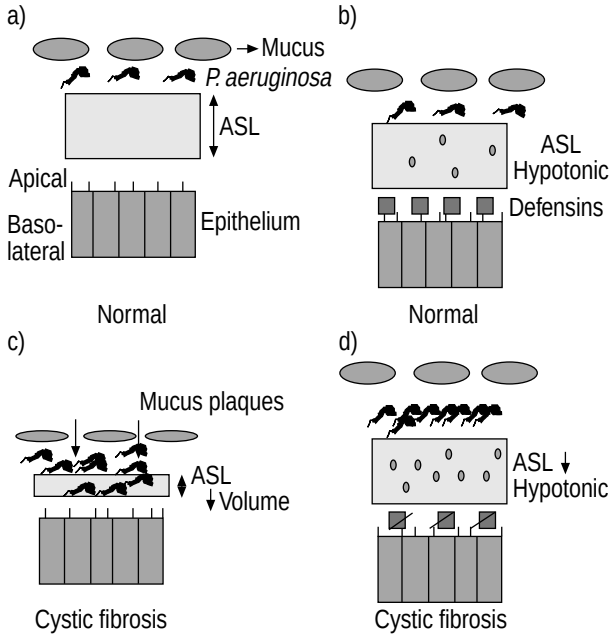

Fig. 1. – The Salt Hypotheses. ASL: airway surface liquid; *P. aeuruginosa*: *Pseudomonas aeruginosa*.

Table 1. – Proposed mechanisms for different pharmacological agents

Pharmacological agent	Proposed mechanism
4 PBA	Increase in trafficking Decrease in ubiquitination
Glycerol	Increase in trafficking
IBMX	Phosphodiesterase inhibition Direct activation CFTR
Forskolin	Adenyl cyclase activation
Milrinane	Phosphodiesterase inhibition plus adenyl cyclase activation
Amrinone	Phosphodiesterase inhibition plus adenyl cyclase activation
CPX	Phosphodiesterase inhibition Direct activation CFTR
Genistein	Tyrosine kinase activation Protein phosphatase inhibition Direct activation CFTR
CNP	cGMP regulation of CFTR
UTP, ATP	Activation of other chloride channels
Calcium ATPase inhibitors	Decrease in ubiquitination Calcium dependant involving CaMKII Activation of other chloride channels

4-PBA: sodium 4-phenylbutyrase; IBMX: 1-methyl-3-isobutylxanthine; CPX: 8-cyclo-pentyl-1,3-dipropylxanthine; CNP: C-type natriuretic peptide; UTP: uridine triphosphase; ATP: adenosine triphosphate; ATPase: adenosine triphosphatase; CFTR: cystic fibrosis transmembrane conductance regulator protein.

overcome the defect in the ΔF508 mutation involve increasing CFTR trafficking.

Chemical chaperones

The observation that low temperature induced a proportion of cultured fibroblasts to show normal maturation of ΔF508-CFTR [8, 9] lead to the search for chemical entities with similar properties. The first chemical chaperone to be studied was glycerol [9, 8], which increased expression of fully glycosylated ΔF508 protein at the plasma membrane of cultured mammary carcinoma cells [9]. However, an open parallel group study in CF subjects, using two doses of topical glycerol, showed no effect on nasal potential

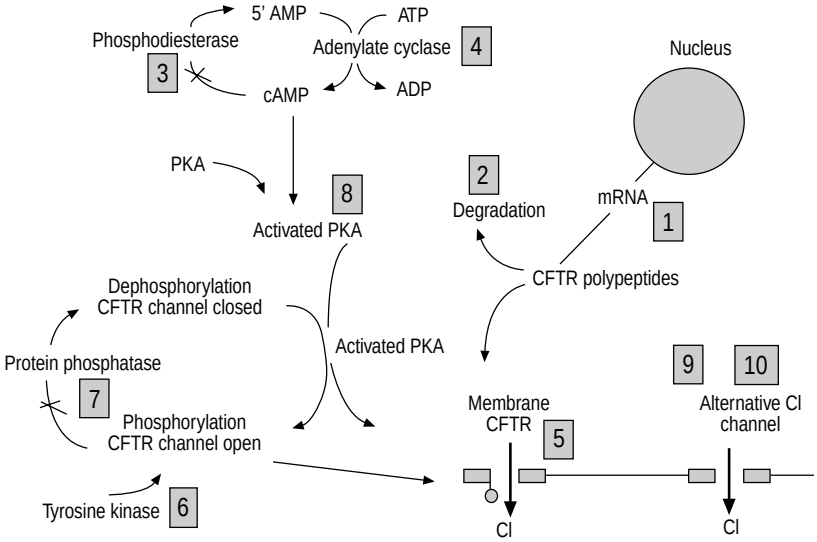

Fig. 2. – Potential targets for pharmacological manipulation of chloride channel activation. The mechanisms are indicated by the following numbers: 1: increase in trafficking; 2: decrease in ubiquitination; 3: phosphodiesterase inhibition; 4: adenyl cyclase activation; 5: direct activation of cystic fibrosis transmembrane conductance regulator (CFTR); 6: tyrosine kinase activation; 7: protein phosphatase inhibition; 8: cyclic adenosine monophosphate (cAMP) regulation of CFTR; 9: activation of other chloride channels; 10: calcium dependant involving CaMkII. AMP: adenosine monophosphate; ATP: adenosine triphosphate; ADP: adenosine diphosphate; PKA: protein kinase; mRNA: messenger ribonucleic acid.

difference (NPD) in 11 ΔF508 homozygotes [10], suggesting glycerol is unlikely to be clinically useful. Other compounds acting in a similar manner may prove more effective, such as Trimethylamine N-oxide (TMAO), which increases chloride efflux in CF trachea epithelial (CFTEo) cells, a ΔF508 expressing cell line [10]. This area is likely to expand rapidly in the future.

Sodium 4-phenylbutyrate

Butyric acid and its analogues have been shown to upregulate CFTR-messenger ribonucleic acid (mRNA) in C127 cells expressing ΔF508 [11, 12]. Butyrate itself is short-acting and toxic, but its analogue, sodium 4-phenylbutyrate (4-PBA), shows more promise. Forskolin-induced chloride secretion in IB3-1 cells expressing ΔF508/W1282X, and in primary human nasal epithelium, was increased by 4-PBA [13]. Although these agents increase CFTR-mRNA expression, they may also have other actions. The ubiquitination protein Hsc70 in IB3-1 cells and human bronchial epithelial cells was downregulated by 4-PBA. This would be expected to increase CFTR trafficking by reducing tagging of mutant protein for degradation, thereby allowing more ΔF508-CFTR protein to reach the apical membrane [14].

A pilot study of 4-PBA in cystic fibrosis patients *in vivo* showed partial restoration in nasal epithelia CFTR function in 18 ΔF508 patients treated for 1 week with oral 4-PBA or placebo (nine in each group) [15]. Longer term, larger studies of more potent 4-PBA analogues are needed.

Treatments aimed at increasing chloride currents

Phosphodiesterase inhibitors

Mutant forms of CFTR can be activated if stimulated with high levels of phosphodiesterase (PDE) inhibitors [16]. Xenopus oocytes injected with wild-type or mutant CFTR required five times as much xanthine 1-methyl-3-isobutyl-xanthine (IBMX), a nonselective PDE inhibitor, to stimulate chloride secretion, compared with wild-type. Stimulation only occurred in the presence of forskolin (an adenyl cyclase activator) [16]. However, results with IBMX have been conflicting. In normal and CF airway epithelium, high doses of IBMX and forskolin failed to increase chloride efflux, thus inhibiting chloride secretion in primary cultures from normal patients [17, 18]

Another nonselective PDE inhibitor is 8-cyclopentyl-1, 3-dipropylxanthine (CPX). CPX increased chloride channel activation of CFPAC-1 or NIH 3T3 cells expressing ΔF508 CFTR, but not in cells expressing wild-type CFTR [19]. Similar results were seen in the human airway cell line IB3 derived from a ΔF508/W1282X CF patient [20]. In contrast, CPX alone had no effect on wild-type or mutant CFTR in mouse mammary epithelial cells (Ca127 cells), although it did potentiate the forskolin response in mutant cells [21].

PDE consists of a family of enzymes which differ in their selectivity for, and activation by, cAMP and cyclic guanosine monophosphate (cGMP). Whilst IBMX and CPX are nonselective PDE inhibitors, studies with cAMP selective PDE inhibitors have been more promising. Type III inhibitors, milrinone and amrinone, are more potent activators of chloride efflux in airway epithelial cells expressing wild-type CFTR than either IBMX or type IV (rolipram) inhibitors [22]. Milrinone and amrinone stimulated chloride efflux in wild-type Calu-3 and 16 human bronchial epithelial (HBE) cells without the addition of adenyl cyclase activators [23]. However, adenyl cyclase activation, in addition to milrinone, was required for stimulation of mutant CFTR (transformed CF nasal polyp cells expressing ΔF508/ΔF508) or murine nasal epithelium *in vivo* [18, 22, 23].

The mechanism of action of PDE inhibitors is contentious, as no correlation was found between the increases in cAMP and the degree of correction of chloride efflux in some studies [18, 21, 22]. This could reflect compartmentalization of PDE activity within epithelial cells, such that total cAMP levels do not reflect membrane cAMP [22]. Alternatively, non-PDE mechanisms may be involved including adenosine antagonism or a direct effect on CFTR. Adenosine antagonism seems unlikely because the potency of these agents as adenosine antagonists correlates poorly with CFTR opening [19, 20, 24]. A more attractive suggestion is that PDE inhibitors have a direct effect on CFTR through binding to the first nucleotide binding domain (NFB-1), the site of the ΔF508 mutation [25, 26]. This hypothesis is supported by the rank order of potency for binding of different xanthines (DA-CPX > DAX > CPX > caffeine > adenosin > IBMX) to NBF-1 which parallels the action of these compounds on chloride channel opening [26].

Data on the PDE inhibitors *in vivo* are limited. Topical milrinone had no effect on NPD in the presence of amiloride, isoprenaline or ATP in low chloride solution [27].

Genistein

Genistein, a tyrosine kinase inhibitor, increased CFTR channel activity in wild-type CFTR and in Hi-5 insect cells which transiently express CFTR in the presence of forskolin [28]. The mechanism of action of genistein is unclear, but possibilities include tyrosine and protein phosphatase inhibition, direct interaction with the CFTR protein, or inhibition of topoisomerases [29, 30]. Genistein also stimulates sodium absorption in the human CF airway [31]. As genistein affects several cellular processes and its mechanism of action is unclear, it may not prove useful in CF therapy.

Cyclic guanosine-3′5′-monophosphate

cGMP is produced by guanylate cyclases (GCs) which exist in soluble and particulate forms. Soluble GCs are activated by nitric oxide and related

compounds, whereas particulate GCs are natriuretic peptide receptors. Both soluble and particulate GCs are abundant in airway epithelial cells [32]. cGMP has been shown to influence CFTR activity but the mechanism is unclear. Potential mechanisms include direct phosphorylation of CFTR by cGMP-dependant protein kinase (PKG), or by cGMP acting *via* phosphodiesterases which hydrolyse cAMP. In return, increased cAMP then activates cAMP-dependant protein kinases (PKAs) which phosphorylate CFTR.

Studies in Calu-3 cells, (which express high levels of CFTR) and CF-T43 cells (expressing ΔF508), showed that C-type natriuretic peptide (CNP), a ligand for type C particulate GC, activated wild-type and mutant CFTR through PKA [33]. Cells expressing the CF mutant only showed activation when CNP was combined with isoprenaline, and wild-type cells showed a greater response than CF cells. CNP increased CFTR-dependant chloride efflux in CF mice *in vivo* [34] suggesting that studies with CNP in humans may be warranted.

Triphosphate nucleotides

The outwardly rectifying chloride channel (ORCC) is important in airway chloride secretion and is activated by CFTR [35]. PKA activation of CFTR causes ATP movement across the cell membrane. ATP then binds to P_2 receptors, which activate ORCC. The mechanisms of ATP transport are unclear but may involve CFTR acting as a channel to directly transport ATP or by regulating ATP transport through an unidentified channel [1]. The defective regulation of ORCC in CF subjects can be overcome using topical ATP or uridine triphosphate (UTP) [36, 37]; however, longer acting P_2Y_2 receptor agonists are being evaluated. INS365 is safe in doses $\leq$100 mg in healthy volunteers or in single doses $\leq$80 mg in CF subjects, but data on efficacy are awaited [38].

Calcium adenosine triphosphatase inhibitors

A number of epithelial chloride channels are calcium dependent. Therefore, strategies which increase calcium release or reduce calcium re-uptake may be beneficial. Calcium adenosine triphosphatase (ATPase) inhibitors such as thapsigargin, cyclopiazonic acid and 2,5-di-(tert-butyl)-1,4-hydroquinone (DBHQ) inhibit Ca^{2+} re-uptake by intracellular stores and increase cytosolic free Ca^{2+}. This may increase chloride secretion *via* a calcium-regulated chloride signalling pathway [1]. Alternatively, trafficking may increase by reducing the activity of calcium dependant proteins such as calnexin and uridine diphosphate (UDP) glucose: glycoprotein glycocyl transferase (UGGT), which are involved in retaining misfolded ΔF508-CFTR in the ER. Preliminary data in CFPAC-1 cells showed that altering intraluminal ER calcium with thapsigargin allowed ΔF508-CFTR to be released from the ER while functioning at the apical membrane [39]. Although calcium ATPase inhibitors could theoretically be of use in ΔF508, their toxicity is likely to preclude use in patients.

Treatments for stop mutations

Aminoglycosides

Aminoglycosides have been used to treat stop mutations, which are class 1 mutants with their premature termination of CFTR-mRNA translation producing truncated, nonfunctional CFTR. Although these are common amongst Ashkenazi Jews [40], they affect only a small percentage of CF patients worldwide [41]. Partial functional correction of two CF stop mutations was achieved in HeLa cells using the aminoglycoside G-418 [41]. Gentamicin and tobramicin had weaker effects but did not alter chloride channel activity. IB3 cells, heterozygous for W1282X treated with G418 or gentamicin, showed increased cAMP mediated current, although possibly *via* the ORCC rather than CFTR [42]. Beneficial changes in NPD were reported with topical gentamicin (0.3% t.d.s) for 14 days in nine patients homozygous for stop mutations [43]. Larger randomized controlled trials are needed to confirm these findings.

Strategies aimed at reducing sodium absorption

An alternative or complementary approach is to target the increased sodium reabsorption. ENaC is responsible for the increased sodium absorption in CF airway epithelia as a result of abnormal regulation by defective CFTR. CFTR plays a central role in the regulation of ENaC and other ion channels. Figure 3 shows how these other ion channels are affected in CF and how they can be manipulated pharmacologically.

Amiloride and its analogues

Attempts to reduce sodium reabsorption in airway epithelium have concentrated on apical ENaC inhibitors. Amiloride inhibits several epithelial sodium transport processes including ENaC and is a potent inhibitor of sodium transport *in vitro* and *in vivo* [44, 45]. Although short-term topical amiloride administration blocks sodium transport across nasal epithelia in normal and CF subjects [46, 47], three placebo-controlled crossover studies of nebulized amiloride show conflicting results [48–50]. A 25-week North American study [48] showed a reduction in lung function decline in 18 CF subjects, whereas a 6-month UK study showed no effect [49]. Lung function was unaffected when amiloride was added to inpatient treatment of pulmonary exacerbations in 27 CF patients [50].

The lack of efficacy of amiloride *in vivo* may be due to its short duration of action [51, 52]. Amiloride is cleared rapidly from the airways and its effect on lower airway potential difference lasts for only 30 min [53]. Thus, even with repeated dosing, amiloride would only block sodium reabsorption for a short

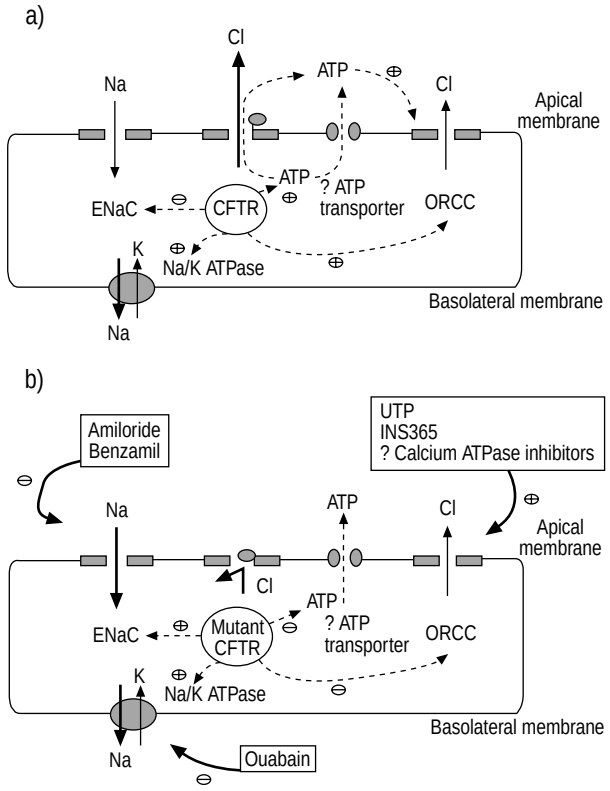

Fig. 3. – Cystic fibrosis (CF) transmembrane conductance regulator protein (CFTR) as a regulator of other channels. a) Normal airway epithelium; b) pharmacological correction in CF airway epthelium. ATP: adenosine triphosphate; ENaC: epithelial sodium channel; ORCC: outwardly rectifying chloride; ATPase: adenosine triphosphatase.

time [54]. Therefore, longer acting sodium channel blockers may prove more effective.

Benzamil, a benzyl substituted amiloride analogue, is a more potent and longer acting sodium channel inhibitor than amiloride in cultured human nasal epithelium [55]. Benzamil had a longer duration of action on NPD than amiloride in an open, parallel group study of CF subjects [56]. A randomized, placebo-controlled, crossover study in 10 CF subjects showed similar results [57] with an 8-h duration of action. Although Benzamil is promising as a long-acting sodium channel inhibitor, studies on its long-term efficacy and toxicity in the lung are required.

Sodium/potassium-adenosine triphosphatase inhibitors

Sodium absorption through the basolateral sodium-potassium-adenosine triphosphatase (Na^+K^+-ATPase) is also increased in CF [58, 59]. The Na^+/K^+-ATPase can be inhibited by cardiac glycosides such as ouabain and digoxin. Ouabain inhibits sodium reabsorption in intact epithelium strips in several species including man [60, 61] and intravenous ouabain reduces NPD in the dog *in vivo* [44]. However, two double-blind, placebo-controlled, crossover studies showed no effect on NPD for either topically applied ouabain or oral digoxin given to CF patients over 2 weeks [59]. This suggests that Na^+/K^+-ATPase inhibitors, at nontoxic doses, do not achieve sufficient inhibition of Na^+/K^+-ATPase to be of therapeutic use.

Loperamide

Loperamide, an opiod receptor agonist, inhibited sodium ion flux in the rat small bowel *in vitro* [62]. Although loperamide reduced NPD in CF mouse nasal epithelium *in vivo* [63], preliminary data in humans show that loperamide is less potent than amiloride in inhibiting NPD in CF suggesting that it is unlikely to be clinically useful [64].

Treatment combinations

Combining a sodium channel blocker with a chloride channel opener, thus mimicking normal conditions, seems an attractive option for the treatment of CF. To date, there is little data *in vivo* on this approach. A murine study showed dose-dependent NPD changes with UTP and amiloride, which suggests chloride secretion [65]. A preliminary report [66] showed an improvement in mucociliary clearance (MCC) after short-term treatment with aerosolized UTP and amiloride in 12 CF and 10 normal subjects. Future studies are likely to investigate combinations of chloride channel openers and sodium channel blockers as well as combinations of chloride channel openers which involve different pathways (*e.g.* trafficking compounds combined with drugs which directly activate CFTR).

Pharmacogenetics

Although the study of pharmacogenetics is in its infancy, it may have a role in cystic fibrosis. Genetic factors which potentially play a role in determining treatment responses in diseases such as asthma have been identified, although the clinical application of such technology has yet to be developed [67]. In CF, determination of the CF genotype has been available for many years and has mainly been used for diagnostic purposes. However, the application of genotyping in order to determine pharmacological treatment is an exciting concept. The major role of pharmacogenetics in CF is likely to be in the development of drugs targeting ΔF508, the mutation most commonly seen in the CF population. The recent development of high throughput screening provides rapid, repeatable assays capable of detecting cell chloride permeability [68]. This technology will be used to rapidly screen libraries of compounds in order to detect potential trafficking drugs of the future, and will have great impact on the efficacy of such drugs as well as their speed of development. Pharmacogenetics will also have an impact on other CF mutations such as the use of aminoglycosides for stop mutations, particularly in geographical areas where such geneotypes

are common. However the use of pharmacogenetics for other CF genotypes may be limited on pharmacoeconomic grounds.

Conclusions

There are a number of pharmacological agents in development aimed at correcting the electrophysiological defect seen in CF airways. These agents are likely to become increasingly specific, targeting patients with particular genotypes. Clinical trials have incorporated 4-PBA, probably the most advanced chloride secretagogue in development. It has the advantage of being effective as a single agent, unlike the PDE inhibitors which require concomitant treatment with adenyl cyclase activators [16, 19, 30]. However, 4-PBA and other trafficking drugs appear to increase amiloride sensitive sodium transport [11, 23]. Similarly, studies of UTP and gene therapy have shown that although these approaches produce a degree of correction of the chloride current, they do not correct sodium reabsorption [15, 36, 69]. This suggests that there may be added benefit in giving these agents in combination with sodium channel blockers. Although clinical studies with amiloride have been disappointing [48, 49, 50] the use of longer acting sodium channel blockers, such as benzamil, may prove more beneficial.

Pharmacological treatments to correct the ion transport defect in cystic fibrosis have emerged over the last decade with expanding knowledge of the structure and function of cystic fibrosis transmembrane conductance regulator. Perhaps the combination of drugs that correct different aspects of cystic fibrosis transmembrane conductance regulator function will be the way forward in the next decade.

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