Therapeutic Options for Hydrating Airway Mucus in Cystic Fibrosis

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Key Words

Mucus · Lung · Cystic fibrosis · Cystic fibrosis transmembrane conductance regulator gene · Hypertonic saline · Ivacaftor · Bronchitol · Mannitol · Mucus hydration · Moli1901

Abstract

Background: In cystic fibrosis (CF), genetic mutations in the CF transmembrane conductance regulator (CFTR) gene cause reduced chloride efflux from ciliated airway epithelial cells. This results in a reduction in periciliary liquid (PCL) depth of the airway surface liquid due to associated reduced water efflux. PCL layer dehydration reduces mucociliary clearance (MCC), leading to airway obstruction (reduced airflow and inflammation due to pathogen invasion) with mucus plug formation. **Summary:** Rehydrating mucus increases MCC. Mucus hydration can be achieved by direct hydration (administering osmotic agents to set up an osmotic gradient), using CFTR modulators to correct dysfunctional CFTR, or it can be achieved pharmacologically (targeting other ion channels on airway epithelial cells). **Key Messages:** The molecular mechanisms of several therapies are discussed in the context of pre-clinical and clinical trial studies. Currently, only the osmotic agent 7% hypertonic saline and the CFTR

'potentiator' VX-770 (ivacaftor) are used clinically to hydrate mucus. Emerging therapies include the osmotic agent mannitol (Bronchitol), the intracellular Ca^{2+} -raising agent Moli1901/lancovutide, the CFTR potentiator sildenafil [phosphodiesterase type 5 (PDE5) inhibitor] and the CFTR 'corrector' VX-809 (lumacaftor). Other CFTR correctors (e.g. 'chemical chaperones') are also showing pre-clinical promise.

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1 Introduction

Airway mucus secretion is a normal physiological process involved with homeostasis and is a component of the innate immune response in the respiratory tract [1] (table 1). However, mucus hypersecretion, with abnormal mucus consistency, causes airway obstruction by reducing mucociliary clearance (MCC) in respiratory diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease and asthma [1].

Airway mucus, also termed airway surface liquid (ASL), comprises a gel-like layer above the periciliary liquid (PCL) layer which surrounds the cilia of ciliated airway epithelial cells [2] (fig. 1). Of the three diseases of the respiratory tract mentioned, it is CF where there is the

Table 1. Functions of ASL ('mucus')

Provision of a physical barrier to inhaled airborne irritants, particles and micro-organisms as well as aspirated food and liquid
Trapping of irritants, particles, micro-organisms and food
Formation of a transportable 'vehicle' upon which irritants are removed from the airways by mucociliary clearance
Provision of a 'waterproof' layer to limit desiccation (e.g. of the epithelium)
'Humidification' of inspired air
pH buffering capacity
Neutralisation of inhaled noxious gases
Provision of a selective macromolecular 'sieve'
Provision of a 'store' of immunoglobulins and enzymes (e.g. antibacterial – lysozyme) and provision of an extracellular surface for their activity

most intense interest in identifying targets and drugs to alleviate the mucus problems associated with airway pathophysiology [1].

2 CF: Genetics and Pathophysiology

CF is an autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) gene [3]. The frequency of CF occurrence varies worldwide, but is approximately 1 in 2,500 live births. Nearly 2,000 mutations have been identified, although ~70% of CF patients are homozygous for the F508del mutation [4]. Other CFTR mutations are rare, with just four (G542X, N1303K, G551D and W1282X) having a worldwide prevalence of ~1–3% each; the remaining proportion of CF cases are unique to individuals/families (frequency <0.1%) [5]. Mutations have variable effects on disease severity, with some (e.g. N1303K) not being correlated with lung disease severity [6].

The predominant function of CFTR is a cAMP-dependent transmembrane Cl^- channel at epithelial surfaces [7]. When intracellular cAMP-dependent protein kinases A and C phosphorylate the CFTR regulatory domain (R-domain), the ATP-binding affinity of the nucleotide-binding domains (NBDs) of the CFTR is increased. When ATP hydrolyses the ATP-binding sites of the NBDs, the two NBDs dimerise and the resulting conformational change opens the channel [8] (fig. 2).

Fig. 1. Relative locations of the gel-like mucus layer and PCL components of ASL. Mutated CFTR leads to dehydrated PCL/ASL, impaired MCC and the pathophysiological sequelae of CF. Methods to achieve mucus hydration are targeted at the ion channels (of ciliated airway epithelial cells?), which are adjacent to the mucus-secreting goblet cells in the respiratory tract.

In CF, there is a reduction in PCL depth linked to reduced water efflux due to reduced Cl^- efflux caused by malfunctioning CFTR [9]. PCL layer dehydration reduces ciliary beat frequency, which slows the MCC rate [10], allowing mucus plug formation, resulting in the following symptoms of the disease: reduced airflow and inflammation due to pathogen invasion [11].

One therapeutic approach against mucus accumulation is mucus hydration, because this increases MCC. Mucus hydration can be achieved by three main methods:

- Direct hydration
- CFTR modulators
- Pharmacologically – using drugs which target other ion channels on airway epithelial cells

An extensive portfolio of drug compounds is being developed to aid the above approaches to airway mucus hydration (table 2; fig. 3) and is described in detail below.

Fig. 2. CFTR. It is an ATP-binding cassette transporter, consisting of two main MSDs (either side of the dashed line), two NBDs, and a regulatory domain (R-domain). **a** In the 'resting state', the NBDs are disassociated from each other, do not interact with the R-domain and the channel is 'closed' (reduced open probability). **b** Upon stimulation by intracellular cAMP-dependent protein kinases A and C (PKA/PKC) the R-domain phosphorylates, thereby increasing ATP-binding affinity. ATP binds to the NBDs, causing dimerisation of NBD1 and NBD2. This results in a conformational change, leading to an opening of the ion channel (increased open probability).

3 Direct Hydration

Administering aerosolised hypertonic saline (HS) or dry powder mannitol (Bronchitol) into the airways sets up an osmotic gradient which draws water through epithelial cells' aquaporins, rehydrating the PCL [12, 13] (fig. 4).

Table 2. Drug compounds intended to aid hydration of ASL (see text for details): classification for hydrating airway mucus in CF

<i>Osmolytics</i>
HS
Mannitol (Bronchitol)
<i>Pharmacological compounds</i>
Amiloride
Amiloride analogues: 552-02, GS-9411
Camostat
'Compound 12g' (α -branched quaternary amine)
Denufosol
INO-4995
Moli1901/lancovutide (duramycin)
Small peptide RFSHRIPLLIF
<i>CFTR modulators</i>
3-Isobutyl-1-methylxanthine (IBMX)
Betaine
Bortezomib/Velcade/PS-341
Corrector 'Corr-4a' (bisaminomethylbithiazole)
Curcumin
G418 (Geneticin)
Genistein
Gentamicin
Glycerol
Matrine
Miglustat
Myoinositol
PCT124 (ataluren)
Sildenafil
Sildenafil analogues: ardenafil, tadalafil, KM11060
Sodium 4-phenylbutyrate (Buphenyl)
Suberoylanilide hydroxamic acid (SAHA)
Thapsigargin
Tobramycin
Trimethylamine N-oxide
VRT-325
VRT-532
VX-770 (ivacaftor)
VX-809 (lumacaftor)

3.1 Hypertonic Saline

HS is used for experimental sputum induction in respiratory diseases such as chronic obstructive pulmonary disease and asthma, but can also be used therapeutically, usually as a 7% solution [14].

Therapeutic efficacy can be measured by the MCC rate, for example as the amount of sulphur colloid in expectorated sputum [15]. However, using the sulphur colloid content as a marker for clearance does not differentiate between mucus composition (e.g. hydration) and ciliary beating, or both in combination [16]. Consequently,

Osmolytic

Mannitol (Bronchitol)

Pharmacological compounds

Amiloride

INO-4995

Camostat

Denufosol

Moli1901/lancovutide (duramycin)

3

(For legend see page 122.)

whether or not increased mucus clearance by HS is due to hydration is unclear. Inhalation of HS for 90 min improved MCC by 12%, although concomitant amiloride (Na^+ channel blocker) did not significantly further increase clearance [14] (due to a possible negative feedback effect on epithelial Na^+ channel (ENaC) triggered by increased intracellular Na^+ concentration as a result of water efflux [17]). The clinical significance of a 12% increase in MCC was not defined. HS changes the rheology of expectorated mucus in vitro (reducing viscoelasticity by disrupting ionic bonds) [18], induces inflammatory mediator release such as prostaglandin E_2 [19] (which increases ciliary beat frequency) [20] and induces coughing [14], which increases total MCC [17].

Nevertheless, although the exact mechanism of action of HS is unknown, HS is an effective treatment. In clinical trials, the effect of HS was measured focusing on lung function, rate of pulmonary exacerbations and mucus clearance. HS significantly increased forced vital capacity compared to baseline values, and HS on its own produced a sustained increase (≥ 8 h) in mucus clearance [21]. Accompanying in vitro experiments (cultures of patients' bronchial epithelium) demonstrated changed ASL volume after HS application [21]. HS has long-term positive effects. The absolute lung function increase was significantly different between the control and the HS group, and the '48-week exacerbation-free survival' rate was 14% significantly higher in the HS group in comparison to the control group [22]. Recently, HS has shown short-term

CF modulators

(CF modulators continued)

Fig. 3. Chemical structures of drug compounds intended to aid hydration of ASL (see text for details).

efficacy and a lack of adverse effects in infants [23]. An ISIS trial with 321 <6-year-old participants found no significant difference in the rate of pulmonary exacerbations during the 48-week treatment period between HS and isotonic saline groups [24].

When adjusted for height, the ‘lung clearance index’ as an endpoint showed a significant difference between HS and isotonic saline groups in children aged <6 years, albeit with a small sample size ($n = 25$), after 48 weeks’ treatment [25].

In summary, HS is relatively inexpensive and well-tolerated by adults and infants. However, although there is a dose-dependent relationship between the HS concentration and MCC, most efficacious with 7% HS, clinical trials show mixed results for HS improving pulmonary function and reducing exacerbation rates [12]. However, there may be an issue with the endpoints against which the efficacy of CF drugs is currently measured [26], because trial results do not translate into clinical outcomes. This issue is worsened by problems of an attempted com-

Fig. 4. Effect of HS on hydration of ASL in CF. **a** In non-CF people, the ASL comprises a PCL layer, in which cilia beat, and an upper mucus layer (which contributes to MCC). CFTR acts as a Cl^- channel, permitting Cl^- efflux into the airway lumen, with some concomitant Na^+ absorption via ENaC, thereby establishing an osmotic gradient to drive water efflux into the PCL. **b** In CF, mu-

tated CFTR results in reduced Cl^- efflux and, thereby, reduced water secretion. Continued Na^+ absorption leads to water absorption with associated dehydration of the PCL and defective MCC. **c** In CF patients, inhaled 7% HS sets up an osmotic gradient, causing water efflux into the PCL, resulting in ASL rehydration and restored MCC.

parison of CF in infants with CF in adults, where baseline pulmonary function values for very young CF children reflect non-CF values. These endpoints make it difficult to find treatments with best efficacy when administered before CF symptoms are present.

3.2 Mannitol

Mannitol is a non-ionic osmotic agent [27]. Inhaled dry mannitol causes mucus hydration by the same mechanism as HS: increasing the osmotic gradient causing water efflux into the lung lumen [28]. The disadvantage of mannitol powder over HS is its relatively large size, so deposition at the intended site, the small airways and the lung periphery [29], is hard to achieve [30]. Similar to HS, mannitol has rheologic effects on mucus; disruption of hydrogen bonds in the mucus reduces viscoelasticity, which increases mucus clearance, and induces cough [31].

Phase 3 studies (in ≥ 6 -year-old CF children) show improved FEV_1 and $\sim 35\%$ reduction in pulmonary exacerbation occurrence [32] and improved lung function [33] after 26 weeks. It should be noted that the control group for these studies received 50 mg mannitol (a dose determined as subtherapeutic in a previous dose-escalation study [34]). However, the 2012 study ($n = 3,138$) [33] questioned this 50-mg dose, where, although there was $\sim 8\%$ improvement in FEV_1 from baseline in the mannitol

group, statistically significant results were not obtained between the 400-mg mannitol and the control group, suggesting that the 50-mg dose may have had some efficacy. Another reason for statistically insignificant results may be that only one data point was collected for baseline FEV_1 .

In summary, mannitol's easier administration via inhaler (compared to HS nebuliser), similar adverse event rates, more consistent clinical efficacy and longer epithelial retention time may make mannitol the leading treatment in the future. Mannitol may cause bronchoconstriction in patients with hyperresponsive airways, but this is not proven [32]. Although a crossover trial in 6- to 17-year-olds [35] is currently underway, additional studies are needed to assess mannitol in patients < 6 years old as well as its inflammation-inducing effects.

4 CFTR Modulators

Another way to increase mucus hydration in CF is to correct dysfunctional CFTR.

4.1 Mutation Classes

Different CFTR mutations can cause problems with synthesis, gating, conductance or stability of the channel (fig. 5).

Fig. 5. Summary of the effects on Cl^- efflux of the different CFTR mutation classes. WT-CFTR are abundant at the apical surface and permit effective Cl^- efflux into the airway lumen. Class I mutations produce truncated CFTR which are degraded before reaching the apical surface. Class II mutations produce misfolded CFTR which are degraded before reaching the apical surface. Class III muta-

tions produce CFTR with defective activation (do not permit Cl^- efflux). Class IV mutations produce CFTR with low ion channel conductance (suboptimal Cl^- efflux). Class V mutations produce suboptimal amounts of functional CFTR. Class VI mutations produce unstable CFTR which are quickly degraded.

4.1.1 Class I: Reduced Synthesis of CFTR

These mutations synthesise unstable or truncated CFTRs due to prematurely coding a termination codon into the amino acid sequence. The most common class I mutation is G542X [6] and W1282X in the Ashkenazi Jewish population [36]. If a splicing error occurs instead of a nonsense or a frameshift mutation, some functional CFTRs are synthesised, but total CFTR expression is suboptimal.

4.1.2 Class II: Defective Processing of CFTR

The most common mutation (F508del) results in misfolded CFTR synthesis due to deletion of the phenylalanine codon at the 508th amino acid position [4]. Usually, dysfunctional CFTR is degraded before localisation to the apical membrane, but when F508del-CFTR is 'rescued' (trafficked and inserted into the membrane) its gating is defective, classifying it simultaneously as a class III mutation [37].

4.1.3 Class III: Defective Gating of CFTR

Amino acid codons are substituted for other amino acids. While transcription and processing events are successful, these channels have defective regulation of ion transfer upon insertion into the membrane (e.g. G551D, glycine-to-aspartate missense mutation) [5].

Class III mutations confer a conformational change to the NBD, negatively affecting ATP-binding capacity, failing to be stimulated by ATP [38].

4.1.4 Class IV: Reduced Cl^- Conductance of CFTR

CFTR is successfully inserted into the cell membrane but the channel's Cl^- conductance is reduced by decreased open state frequency, linked to reduced phosphorylation capacity of cAMP-dependent protein kinases, due to membrane-spanning domain (MSD) mutations [4].

4.1.5 Class V: Reduced CFTR Synthesis

Defective CFTR transcription forms both correctly and incorrectly spliced mRNA, resulting in suboptimal amounts of functional CFTRs [5].

4.1.6 Class VI: Decreased Stability of CFTR at the Apical Membrane

CFTRs which are functional but have a high turnover at the cell surface are produced; they are degraded 5–6 times faster than normal due to deletion of the C-terminal tail which stabilises folded CFTR [39].

4.2 Therapy

Correction of the underlying defect of CFTR mutations by gene replacement therapy is progressing slowly and has significant efficacy and safety problems [40]. Instead, mutation-class-specific drugs aim to correct the molecular mechanism of CFTR dysfunction to regain CFTR function [40].

4.2.1 Class I

Drugs which correct class I CFTR mutations are termed 'premature stop codon suppressors' or 'read-through agents' [41].

Aminoglycoside antibiotics disrupt translation by binding to rRNA, thereby inhibiting premature termination by inserting a different amino acid into the sequence [40]. G418 (Geneticin) increased expression of full-length CFTR to 25% of wild-type CFTR (WT-CFTR) levels in vitro in cells containing R553X-mutated CFTR [42]. Treatment with gentamicin or tobramycin (separately) in vivo in intestinal tissues of mice caused full-length hCFTR localisation to the apical surface [43]. However, long-term use of aminoglycoside antibiotics can cause nephro- and ototoxicity [40], and gentamicin and tobramycin are risk factors for acute renal failure in patients with renal/hydration problems [44].

PCT124 (ataluren) allows 'read-through' of stop codons, allowing transcription to continue, with synthesis of full-length CFTR [45]. Nasal potential difference (NPD) was the biomarker of Cl^- transport and drug activity/efficacy. PCT124 induced a ≥ 5 mV change in NPD in children [46] and adults [47] with few/mild adverse effects. PCT124 is undergoing a phase 3 extension study to observe long-term effects [48].

4.2.2 Class II

Due to the close interaction between CFTR channel gating and cellular processing, and uncertainties of drug mechanisms, the distinction between CFTR correctors (targeted at cellular processing and trafficking defects [49]) and CFTR potentiators (which correct Cl^- channel activity in mutant CFTRs at the cell surface [49]) is ambiguous [50].

4.2.2.1 CFTR Correctors

CFTR synthesis, co-translational folding and interaction with chaperone proteins occur in the endoplasmic reticulum (ER). If CFTR folding time is increased and CFTR is retained in the ER, molecular chaperones transport improperly folded CFTRs to the cytoplasm, where the 26S proteasome degrades them. This process is termed ER-associated degradation. WT-CFTR is transported from the ER to the Golgi where it undergoes N-glycosylation before transportation to the apical membrane [51, 52].

The mechanism underlying the primary therapeutic method against F508del was to reduce CFTR retention in the ER. In vitro, low temperature [53], glycerol [54] and trimethylamine N-oxide [55] correct the processing defect of F508del-CFTR but had cytotoxic effects.

Organic solutes (myoinositol alone/with taurine and betaine) stabilised and promoted glycosylation of F508del-CFTR by increasing hydration of the peptide in IB3 (bronchial) and NIH 3T3 (fibroblast) cells [56]. However, merely releasing F508del-CFTR from the ER was not sufficient to treat CF; protein generation and maintenance by interaction with chaperone proteins are important. CF may be due to incorrect recognition of F508del-CFTR by the 'protein homeostasis network' [57]. However, mutated CFTR interaction with certain chaperone proteins (expressed during cell stress response [58]) promotes degradation [44].

The following chaperone proteins have been investigated:

- Hsc70, which associates more strongly with F508del-CFTR than with WT-CFTR and promotes degradation [59].
- Hsp70, which enhances F508del-CFTR maturation [60].
- Hsp90, which provides ATPase activity for the proteasome [61], integral to its function, thereby contributing to mutated CFTR degradation.

Chemical and pharmacological chaperones have been investigated. Chemical chaperones are defined as non-specific small molecules which stabilise cellular proteins by improving the rate or fidelity of protein folding, by acting on chaperone proteins or the proteasome rather than directly on CFTR [62]. Pharmacological chaperones act directly on mutated CFTR.

4.2.2.1.1 Chemical Chaperones. Sodium 4-phenylbutyrate (Buphenyl) with genistein were used in a phase 1/2 clinical trial in 2001 [63]. A similar study in heterozygous F508del patients was conducted in 2007 [64] but was terminated in 2008; the data were not analyzed due to the identification of more clinically efficacious compounds. This may seem reasonable, because Buphenyl only showed statistically significant improvement in NPD (e.g. sweat Cl^- concentration was not significantly reduced) in its pilot study [65], although it seemed promising, since in vitro it trafficked mutated CFTR to the apical membrane [66] and downregulated Hsc70 [67].

The sarco-/ER calcium (SERCA) pump inhibitors curcumin and thapsigargin interfere with chaperone proteins by decreasing the ER Ca^{2+} concentration, allowing F508del-CFTR release from the ER and restoring CFTR's function [68, 69]. Conflicting results [70–73] suggest that curcumin does enhance CFTR expression but by down-regulating another ER chaperone protein, calreticulin [74].

Bortezomib/Velcade/PS-341 is used clinically to treat multiple myeloma by increasing the expression of certain stress response proteins by inhibiting specific proteasomal subunits [75]. Bortezomib increases intracellular Hsp70 [76], thereby rescuing F508del-CFTR from ER-associated degradation [77].

Miglustat inhibits deglycosylation of F508del-CFTR in the ER by inhibiting α -1,2-glucosidase in vitro in human nasal, tracheal and pancreatic cells, thereby inhibiting F508del-CFTR interaction with calnexin (another ER-located chaperone protein), preventing CFTR's transport into the cytoplasm and subsequent degradation [78]. Miglustat increased Cl^- efflux and reduced Na^+ influx when administered to mice either intratracheally or orally [79]. However, using RNAi [80] or knock-out mice [81], inhibiting F508del-CFTR-calnexin interaction does not prevent degradation of mutated CFTR. Despite these conflicting pre-clinical data, a phase 2 clinical trial of miglustat [82] ($n = 11$) went ahead, but produced no significantly different NPD results [83].

Ruling the drug as inefficacious was disputed, suggesting that NPD is not the best measure of drug efficacy, and measurements were prone to error [84]. Ultimately, it was agreed that the effect size was correctly determined (accounting for the small sample size) and the results could not be classed as statistically significant [85].

In vitro in human bronchial cells, inhibiting histone deacetylase 7 (HDAC7) using suberoylanilide hydroxamic acid (SAHA) restores F508del-CFTR function by increasing CFTR expression (due to altering histone acetylation states) and producing a protective and corrective post-translational environment (by modulating protein activity, e.g. downregulating Hsp90) [86].

In vitro in HEK293 cells, downregulating a significant co-chaperone of Hsp90, Aha1, decreases F508del-CFTR degradation [87]. Matrine decreases Hsc70 levels in human alveolar epithelial cells in vitro, promoting F508del-CFTR binding to chaperone, BAG3, which promotes translocation to the membrane [88].

4.2.2.1.2 Pharmacological Chaperones. High-throughput screening yielded compounds VRT-325 and VRT-532 [89]. VRT-325 binding to CFTR is not specific to F508del-CFTR. VRT-325 binds to F508del-CFTR allosterically, indirectly affecting the NBD [90].

Corrector 'Corr-4a' (bisaminomethylbithiazole) has binding specificity for F508del-CFTR over other mutants and increased folding efficiency (by correcting NDB2 misassembly [91] or interacting with transmembrane domains [92]), preventing degradation by the proteasome via reducing ubiquitination susceptibility, re-

sulting in 8% normal CFTR activity (deemed clinically significant to reduce pathology) [93]. Corr-4a also increases F508del-CFTR residence time at the apical membrane [93].

VX-809 (lumacaftor) binds to mutated CFTR's MSD1, inducing a protein-stabilising conformational change, protecting it from degradation [94]. VX-809's specificity depends on the MSD structure but was shown to rescue CFTR in F508del and other mutations [94]. VX-809 produced a significant change in NPD in a phase 2 clinical trial [95] and is investigated with concomitant VX-770 (CFTR potentiator) and other CF drugs which prevent infection [96].

4.2.2.2 CFTR Potentiators

CFTR potentiators are effective for (class III/IV) mutations where the CFTR has been trafficked to the apical membrane but has a defective ion channel function.

VRT-532 corrects the gating defect of several CFTR mutants, with the highest affinity for F508del-CFTR [89]. It increases ATP-binding affinity of mutated CFTR after protein kinase A activation [97]. VRT-532 corrects G551D-CFTR by increasing ATP binding and hydrolysis, with no effect on WT-CFTR where ATPase activity is already maximal [98].

VX-770 (ivacaftor) has efficacy in both WT-CFTR and mutated CFTR [99], including G511D-CFTR [100]. Although its binding site is unknown, VX-770 mediates channel opening ATP-independently, being able to potentiate gating in G511D mutants, where the ATP-binding site is mutated [100]. Current clinical trials for VX-770 include non-G551D mutations [101] and VX-770 in combination with other CF drugs against infection [102] and also with the CFTR corrector VX-809, which improves FEV_1 in patients homozygous for F508del-CFTR [103].

The CFTR corrector Buphenyl has been investigated in conjunction with genistein because genistein alone was shown to be a CFTR potentiator [104]. Genistein acts at the NBD, showing 5-fold higher affinity for F508del-CFTR than G551D-CFTR [89], increasing ATP binding capacity and so increasing channel open probability [105]. Capsaicin (pungent essence of hot chillies) was shown to have a similar mechanism [106]. Genistein in conjunction with curcumin demonstrated a significant synergistic effect [107]. It also increases ciliary beat frequency [108].

Increasing intracellular cAMP activates CFTR. The phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX), in conjunction with adenylylate cy-

clase activators [109], produces significant Cl^- efflux, albeit at high concentrations and lowered temperature [110, 111].

The inhaled PDE5 inhibitor, sildenafil, inhibits cGMP breakdown in vivo in mice [112]. The relevance of cGMP to cAMP was addressed: there is 'crosstalk' between signalling pathways, with increased cGMP inhibiting PDE3 (PDE3 degrades cAMP) [113], suggesting sildenafil increases intracellular cAMP. Sildenafil's potent analogue, KM11060, is effective, but its safety in vivo is unknown [114]. Sildenafil, vardenafil and tadalafil each corrected Cl^- conductance in mice orally [115] and when inhaled [112]. Sildenafil had both exocytotic effects on F508del-CFTR and ion channel potentiating function, making it both a CFTR corrector and potentiator. However, sildenafil's efficacy is only achieved at high doses [116], but a current phase 2 clinical trial [117], following a phase 1/2 safety trial in 2008 [118], suggests a potentially positive clinical future.

5 Pharmacological Approaches

Mucus hydration can be achieved pharmacologically, by targeting either the apical ENaC, the P2Y_2 receptor or the Ca^{2+} -activated Cl^- channel (CaCC) (fig. 6).

5.1 ENaC Inhibitors

Although the existence of dysfunctional CFTR-mediated hyperabsorption via ENaC is disputed [119–121], Na^+ absorption via ENaC controls fluid absorption [122]; therefore, ENaC activity contributes to CF pathophysiology by reducing PCL depth. Mucus hydration can be achieved using direct and indirect ENaC inhibitors [123].

5.1.1 Direct ENaC Inhibitors

These compounds block open ENaC directly.

5.1.1.1 Amiloride and Amiloride Derivatives

Amiloride and early amiloride derivatives (phenamil and benzamil) had limited therapeutic usefulness due to rapid absorption/clearance from the lungs [124]. A third-generation amiloride derivative, 552-02, which in vitro in human bronchial cells was 60-fold more potent and 2 times less reversible than amiloride, showed a significant positive synergistic effect with HS (unlike amiloride-HS treatment, see section on Hypertonic Saline above) and in vivo in sheep increased MCC for >5 h [125]. Since a 2006

Fig. 6. Summary of pharmacological approaches to hydrating ASL in CF. As well as CFTR modulation, pharmacological approaches targeting the P2Y_2 receptor, the ENaC and the CaCC are being examined in attempts to achieve mucus hydration in CF. ATP activates P2Y_2 purinoceptors, causing PIP_2 hydrolysis to IP_3 by phospholipase C, and Ca^{2+} release from intracellular stores, increasing intracellular Ca^{2+} concentration. ATP degradation to adenosine (via ADP and AMP) in the lumen results in the activation of adenosine A_{2b} receptors, leading to the increase of intracellular cAMP concentration, which activates WT-CFTR. Na^+ absorption via ENaC contributes greatly to fluid absorption, resulting in PCL depth reduction in the lumen. Direct and indirect ENaC inhibitors can be used to downregulate ENaC activity. Since P2Y_2 purinoceptor activation results in the decrease of intracellular PIP_2 , ENaC activity can be decreased using P2Y_2 purinoceptor agonists. The activation of CaCC results in Cl^- efflux from the cell, increasing mucus hydration. CaCC activation can be achieved by intracellular agonists or by increasing intracellular Ca^{2+} concentration. One of the pharmacological methods to increase intracellular Ca^{2+} concentration involves again using a P2Y_2 agonist.

phase 1/2 clinical trial [126], development has not advanced, except for a 2009 trial in xerostomia [127].

Another amiloride analogue, GS-9411, had greater solubility in HS, was 100-fold more potent in vitro, maintained 75% more ENaC block following apical surface washes than amiloride, and had less permeability and increased metabolism in airway epithelium than amiloride, suggesting a better safety profile [128]. However, in phase 1 ascending-dose trials in healthy people [129, 130], GS-9411 also blocked renal ENaCs, causing clinically significant hyperkalaemia [131]. Hence, the phase 1 trial in CF patients was withdrawn in 2009 [132].

5.1.1.2 Pyrazinoyl Quaternary Amines

Pyrazinoyl quaternary amines are structurally different from pyrazinoyl guanidine amiloride. Some show efficacy *in vitro* in human bronchial cells and *in vivo* in guinea pigs, having decreased cellular permeability and increased solubility (significant for administration methods, i.e. dry powder) [133]. 'Compound 12g' (α -branched quaternary amine) blocked ENaC and produced a guinea-pig tracheal potential difference 16 times greater than that induced by amiloride [134].

5.1.2 Indirect ENaC Inhibitors

5.1.2.1 Channel-Activating Protease Inhibitors

Channel-activating protease (CAP), prostasin, is a trypsin-like serine protease which cleaves ENaC channel subunits at specific sites, increasing ENaC activity [123].

Camostat is a low-molecular-weight CAP inhibitor which inhibits prostasin *in vitro* [135]. By inhibiting prostasin, camostat inhibits ENaC for 6 h after washing *in vitro* in human bronchial cells and increases MCC *in vivo* in sheep for 5 h after dosing [136]. In a phase 2 clinical trial in CF patients ($n = 9$) [137], camostat (nasal spray) reduced Na^+ efflux, causing a change in NPD of +13.1 mV from baseline, but 3 patients experienced serious adverse effects [138]. Concerns exist about camostat's specificity for certain CAPs and there is uncertainty regarding tolerance/safety with administration to lower airways by nebuliser [138].

5.1.2.2 Small Peptide Inhibitors

The small peptide RFSHRIPLLIF inhibits endogenous ENaC *in vitro* in human airway epithelia by blocking an ENaC γ -subunit domain [139].

5.1.3 Purinergic Receptor Agonists

ENaC is held open by PIP_2 binding to the β -subunit of ENaC [140]. Agonising P2Y_2 purinergic receptors decreases PIP_2 levels by allowing hydrolysis of PIP_2 to IP_3 by phospholipase C, thereby decreasing ENaC activation and increasing mucus hydration [140] (fig. 6). P2Y_2 agonists also increase CFTR-independent Cl^- efflux by activating CaCC by calcium-mediated signalling [141, 142] (fig. 6).

Previously, UTP (INS316) was used as a P2Y_2 agonist because the endogenous agonist (ATP) caused adverse effects (bronchoconstriction and pulmonary vasodilation) [143]. Synthesis of diadenosine 5'-polyphosphates yielded denufosal [144], which inhibited Na^+ influx via ENaC [145], and increased Cl^- secretion via CaCC activation (by increasing intracellular Ca^{2+}) [145], resulting

in mucus hydration and improved MCC. This caused prolonged (8 h) increased tracheal mucus velocity *in vivo* in sheep [146]. In a phase 3 trial [147] (TIGER-1; $n = 352$), denufosal was well-tolerated and increased FEV_1 from baseline to 0.048 litre (at week 24; placebo was 0.003 litre). Lung function change by denufosal, relative to placebo, was therefore small but significant (45 ml) [148].

In a second phase 3 trial [146] (TIGER-2; $n = 466$), denufosal caused a mean change in FEV_1 from baseline to 0.040 litre (at week 48; placebo was 0.032 litre): lung function change by denufosal, relative to placebo, was now just 8 ml. The statistically insignificant difference may be attributed to less patient adherence in TIGER-2 than TIGER-1 or to the fact that denufosal fails to prevent long-term lung function decline (TIGER-2 lasted twice as long as TIGER-1) [149].

A phase 2 trial of denufosal [150] was initiated in 2010 in 2- to 4-year-old CF patients based on the concept that drugs with 'disease-modifying capability' be trialled in young children with low disease severity, where small short-term effects should be expected, but which have possible long-term efficacy [151].

5.1.4 CaCC Agonists

Several compounds activate CaCC (by increasing intracellular Ca^{2+} , by binding near the Ca^{2+} binding site or allosterically – the latter two having increased target specificity, producing a sustained response, thereby increasing efficacy) [141]. Activation of CaCC results in Cl^- efflux, which increases mucus hydration [152]. INO-4995 [1-O-octyl-2-O-butyryl-myo-inositol 3,4,5,6-tetrakisphosphate octakis(propionoxymethyl)ester] inhibits ENaC *in vitro* [153] and enhances CaCC action, showing increased Cl^- efflux in human airway epithelial cells with enhanced effect in CF compared to WT [154, 155].

The bacterial polycyclic peptide duramycin (Moli1901/lancovutide) activates CaCC in airway epithelium by causing Ca^{2+} influx via Ca^{2+} -permeable pores and releases Ca^{2+} from intracellular stores [156]. However, *in vitro* in human colonic epithelial cells, duramycin disrupted cell membrane integrity by forming non-specific ion channels [157]. In a phase 2 trial ($n = 24$), nebulised duramycin was well-tolerated, had a long half-life, and did not enter the systemic circulation. FEV_1 was increased (on day 26) by 8% from baseline, with the highest concentration of duramycin administered (2.5 mg/day) [158]. Another phase 2 (estimated $n = 160$) dosage-finding study was conducted in 2008 [159], the increased sample size showing promise.

6 Conclusions

Properly hydrated mucus is a critical component of airway homeostasis. Failure to maintain adequate mucus hydration results in reduced MCC, which leads to mucus accumulation in the airways, causing impaired gas exchange and inflammation due to pathogen and proteolytic enzyme accumulation [11].

A 2013 meta-analysis of randomised clinical trials of CFTR gene replacement therapy concluded that transferring the correct CFTR gene via topical administration fails to produce clinical benefit [160]. Other ways to correct mutated-CFTR-induced abnormal ion concentration include correction of the processing of mutated CFTR using CFTR modulators, directly applying osmotic agents onto the ASL, or targeting other ion channels on the ciliated airway epithelial cell surface.

Of the therapies discussed herein, only 7% HS and CFTR potentiator VX-770 (ivacaftor) are used clinically to hydrate mucus. Ivacaftor is currently only used with G551D-CFTR patients (clinical trials are underway testing its efficacy with other mutations). Therapies which may also succeed clinically are the osmolytic agent, mannitol, which shows more consistent efficacy than HS in clinical trials, the intracellular Ca^{2+} -raising agent Moli1901/lancovutide, the PDE5 inhibitor sildenafil and the pharmacological chaperone VX-809 (lumacaftor), which is in clinical trials in combination with other CF drugs. Also, research into some chemical chaperones is relatively new, with SAHA, Aha1 inhibitors and matrine showing positive results in vitro. Nevertheless, HS surprisingly remains the best treatment today, despite the CFTR gene mutation being first described in 1989 [3].

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