


Pharmacological Modulation of Ion Channels for the Treatment of Cystic Fibrosis

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Abstract: Cystic fibrosis (CF) is a life-shortening monogenic disease caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, an anion channel that transports chloride and bicarbonate across epithelia. Despite clinical progress in delaying disease progression with symptomatic therapies, these individuals still develop various chronic complications in lungs and other organs, which significantly restricts their life expectancy and quality of life. The development of high-throughput assays to screen drug-like compound libraries have enabled the discovery of highly effective CFTR modulator therapies. These novel therapies target the primary defect underlying CF and are now approved for clinical use for individuals with specific CF genotypes. However, the clinically approved modulators only partially reverse CFTR dysfunction and there is still a considerable number of individuals with CF carrying rare CFTR mutations who remain without any effective CFTR modulator therapy. Accordingly, additional efforts have been pursued to identify novel and more potent CFTR modulators that may benefit a larger CF population. The use of ex vivo individual-derived specimens has also become a powerful tool to evaluate novel drugs and predict their effectiveness in a personalized medicine approach. In addition to CFTR modulators, pro-drugs aiming at modulating alternative ion channels/transporters are under development to compensate for the lack of CFTR function. These therapies may restore normal mucociliary clearance through a mutation-agnostic approach (ie, independent of CFTR mutation) and include inhibitors of the epithelial sodium channel (ENaC), modulators of the calcium-activated channel transmembrane 16A (TMEM16, or anoctamin 1) or of the solute carrier family 26A member 9 (SLC26A9), and anionophores. The present review focuses on recent progress and challenges for the development of ion channel/transporter-modulating drugs for the treatment of CF.

Keywords: anionophores, CFTR modulators, drug development, ENaC, precision medicine, SLC26A9, TMEM16A

Introduction

Mutations in the gene encoding the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein cause CF – one of the most common life-shortening autosomal recessive diseases.^{1–3} CFTR is a member of the ATP-binding cassette (ABC) transporter family and functions as a chloride (Cl⁻) and bicarbonate (HCO₃⁻) channel expressed at the apical plasma membrane (PM) of epithelial cells in the airways, intestine, pancreas, sweat glands and other organs.^{4,5} This protein is composed of 1480 amino acid residues that are organized into five domains (Figure 1):^{6,7} two transmembrane domains (TMD1 and TMD2), two nucleotide binding-domains (NBD1 and NBD2) and an intrinsically disordered

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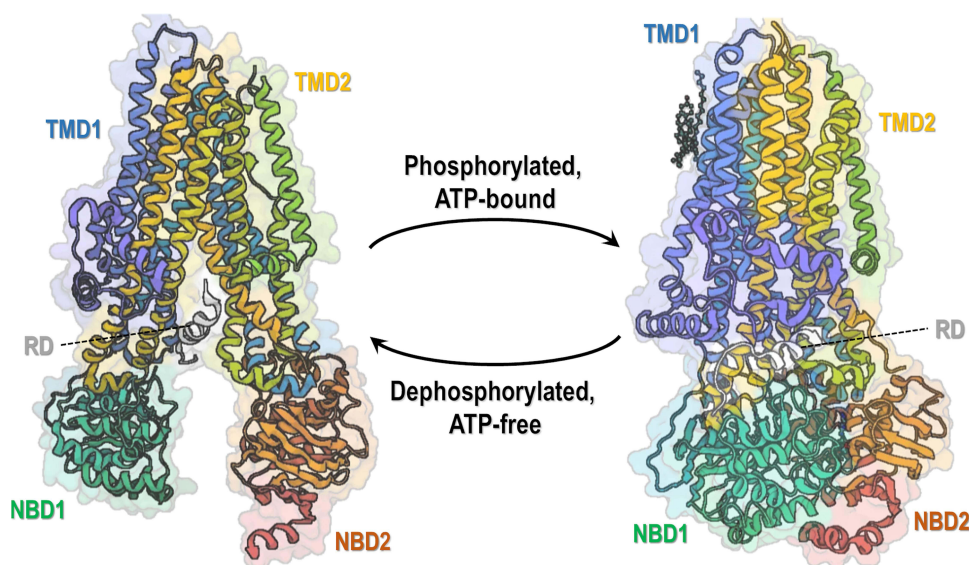


Figure 1 Overall structure of CFTR protein. CFTR structure is composed of five functional domains: two transmembrane domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2) and an intrinsically disordered regulatory domain (RD). Ribbon diagram of two conformations of human CFTR: dephosphorylation, ATP-free conformation (left, PDB: 5UAK) (data from Liu et al)⁶ and phosphorylated, ATP-bound conformation (right, PDB: 6MSM) (data from Zhang et al).⁷ Notably, only a small portion of RD is depicted as most of its structure remains undetermined due to being intrinsically unstructured.

regulatory domain (RD). The latter connects the two homologous halves of the protein and is unique to CFTR among ABC transporters. The TMD segments cross the phospholipid bilayer and are connected by extracellular and intracellular loops, thus forming the channel pore through which anions are conducted.^{6,7} Conformational changes in the protein occur following ATP binding and/or hydrolysis in NBDs and phosphorylation of RD by protein kinase A (PKA) and protein kinase C (PKC), leading to channel opening.^{6–8} For this complex protein to attain its native functional state, domain folding and interdomain interactions have to occur by cooperative mechanisms.^{9,10}

CF affects over 90,000 individuals worldwide who are heterogeneously distributed, but with a higher incidence among Caucasians.¹¹ Clinically, the disease has multi-organ involvement, being the respiratory disorder the major cause of morbidity and premature death.^{4,5,12,13} A cycle of airways dehydration and obstruction by a thick tenacious mucus, chronic inflammation and recurrent infections leads to epithelial injury, tissue remodeling and progressive loss of lung function, ultimately resulting in respiratory failure.^{4,5,12,13}

Over the last decades, major clinical and therapeutic advances have been achieved to delay CF progression. These include mostly time-consuming symptomatic therapies that mitigate lung function deterioration and compensate intestinal malabsorption and pancreatic

insufficiency (Table 1). Along with the implementation of newborn screening programs and specialized health-care management, CF life expectancy has significantly increased with many individuals currently living in their 40s and beyond.^{14–16} However, these individuals are still overwhelmed by considerable clinical, economic and psychosocial issues, which have a negative impact on their quality of life.¹¹ In order to further enhance life expectancy and significantly reduce therapeutic burdens, CF must be treated beyond its symptoms by addressing the primary defect associated to CFTR mutations, thus halting the detrimental effects downstream of CFTR dysfunction, as indeed has occurred over the last decade.

Numerous assays and high-throughput screens (HTS) have been developed and optimized to screen drug-like compound libraries and identify CFTR modulators.^{11,17} These specialized small molecules target the root cause of CF by rescuing the functional expression of several CFTR mutants. Significant success has been achieved in this field as a growing number of compounds are under experimental and early-stage clinical development, and four CFTR modulators are now approved for clinical use for individuals with specific CF genotypes (Table 1).^{18–23} However, the clinically available CFTR modulators, even the highly effective CFTR modulator therapies, only partially correct CFTR dysfunction,^{24–29} which suggests that there is scope for further enhancement. Moreover, a significant number of individuals with CF, who presumably

Table I Pharmacological Therapies Commonly Used in Therapeutic Regimens of Individuals with Cystic Fibrosis

Drug	Mode of Action
Antibiotics	
Aztreonam	Promotes bactericidal actions by binding to penicillin protein 3 and inhibiting bacterial cell wall synthesis.
Azithromycin	Promotes bactericidal action by binding to bacterial 50S ribosomal subunit and inhibiting translocation of peptide synthesis.
Colistin/Colomycin	Promotes bactericidal action by interacting with bacterial plasma membrane and increasing its permeability.
Tobramycin	Promotes bactericidal action by inhibiting translation initiation and elongation of proteins and ribosome recycling as well as affecting bacterial membrane permeability.
Bronchodilators and equivalents	
Formoterol	Activates β_2 -adrenergic receptors on airway smooth muscles that leads to an increase in intracellular cAMP levels in airway smooth muscles, which results in smooth muscle relaxation.
Salbutamol	Activates β_2 -adrenergic receptors on airway smooth muscles that leads to activation of PKA and inhibition of myosin phosphorylation, which results in smooth muscle relaxation.
Nonsteroidal anti-inflammatory drugs	
Ibuprofen	Promotes non-selective inhibition of cyclooxygenase activity, leading to decrease expression of inflammation-related mediators and neutrophil transmigration.
Mucolytics, hydrators and equivalents	
Dornase alpha (recombinant human DNase)	Promotes cleavage of extracellular DNA present in airway mucus, thus facilitating mucus removal by decreasing its viscoelasticity.
Mannitol	Promotes an osmotic gradient that alters properties of airway surface mucus layer, thus facilitating mucociliary clearance.
7% hypertonic saline	Promotes hydration of airway mucus, thus facilitating mucociliary clearance.
Gastrointestinal supplements	
Pancreatic enzymes	Lipases, proteases and amylases that facilitate the hydrolysis of lipids, proteins and carbohydrates to be absorbed by the organism.
Fat-soluble vitamins (A, D, E, K)	Restore the normal nutritional status.
CFTR modulators	
Ivacaftor (VX-770)	CFTR potentiator that increases channel open probability, thus allowing for CFTR-dependent anion transport.
Lumacaftor (VX-809)	CFTR corrector that rescues CFTR folding and trafficking to the plasma membrane.
Tezacaftor (VX-661)	CFTR corrector that rescues CFTR folding and trafficking to the plasma membrane. Note: VX-809 and VX-661 appear to act by a similar mechanism as no additive effects are observed when these molecules are used in combination.
Elexacaftor (VX-445)	Promotes dual activity as both CFTR corrector and potentiator. Note: VX-445 acts by a distinct mechanism compared to the aforementioned CFTR modulators as additive effects are observed when these molecules are used in combination.

carry very rare CFTR mutations (termed as “orphan mutations”), remain without any effective “on target” therapy.

More than 2,100 CFTR gene variants have been reported to date (Cystic Fibrosis Mutation Database, <http://www.genet.sickkids.on.ca/>), for which only 440 the associated disease

liability is established and being approximately 360 confirmed as disease causing (Clinical and Functional Translation of CFTR, <https://cftr2.org/>). However, one single mutation –

the deletion of a phenylalanine at position 508 (F508del) located in NBD1 – is found in at least one allele of 80–85% of individuals with CF worldwide.^{11,17} Nevertheless, such wide variety of CFTR mutations poses substantial challenges as specific drug development for every single mutation is unfeasible and most CFTR mutations are present in a very low number of individuals worldwide. Although all CF-causing mutations result in CFTR-dependent $\text{Cl}^-/\text{HCO}_3^-$ defective transport, these are due to distinct cellular/functional defects. Accordingly, CFTR mutations have been grouped into functional classes/theratypes,^{11,12,17,30} characterized by: (I) no production of full-length protein, (II) defective folding and trafficking, (III) defective gating, (IV) reduced anion conductance, (V) reduced protein production, (VI) reduced stability at the PM, and (VII) no mRNA production. Despite not all CFTR gene variants have been characterized according to their respective cellular/functional defect(s), this classification has been useful as mutations within the same group are expected to be treated by the same therapeutic strategy if not by the same drug.

In parallel to CFTR modulators, other pharmacological therapies have emerged aiming to modulate non-CFTR ion channels/transporters that may potentially compensate for CFTR dysfunction.^{16,31–33} These include strategies to inhibit the epithelial sodium (Na^+) channel (ENaC), which is upregulated in CF epithelia, or modulate alternative Cl^- channels/transporters, such as the calcium (Ca^{2+})-activated Cl^- channels (CaCCs), namely transmembrane 16 (TMEM16A, or anoctamin 1 [ANO1]), or the solute carrier family 26A member 9 (SLC26A9). Modulating the activity of these ion channels/transporters offers the advantage of functioning agnostically (ie, regardless of the CFTR mutation class) and, therefore, may benefit the entire CF population. These drugs might also be used alone or in combination with CFTR modulators for improved clinical outcomes. Here, we review the recent advances and challenges in the development of pharmacological modulators of CFTR and of other ion channels for the treatment of CF. Furthermore, we summarize advances in the development of anionophores, which are small artificial transmembrane anion transporters, as potential therapeutic strategies for CF.

CFTR Modulator Drugs and Personalized Medicine

CFTR Modulator Drugs

The CF drug development pipeline has been expanding with the discovery of novel small molecules from a

diversity of chemical series that are able to correct specific cellular/functional defect(s) generated by CF-causing mutations.^{17,30} Accordingly, CFTR modulator drugs may be grouped into five main types according to their actions on CFTR mutations: read-through agents, correctors, potentiators, amplifiers and stabilizers (Figure 2). Examples of promising CFTR modulators that are under both experimental and clinical investigation are described in the following sub-sections.

Class I Mutations: Read-Through Agents

Read-through agents are molecules that enable the incorporation of an amino acid in a site where a premature termination codon (PTC) was introduced into the CFTR mRNA (ie, class I CFTR mutation).^{34,35} These agents (also termed as PTC suppressors) may prevent protein translation from stopping at PTCs, ie, before the full-length CFTR is produced. PTC suppression is mediated by the base pairing of a near-cognate aminoacyl-tRNA to the PTC and subsequently, the respective amino acid becomes incorporated into the nascent polypeptide chain at the site of the PTC. Accordingly, the local mRNA sequence context plays a key role in near-cognate aminoacyl-tRNA selection during PTC suppression and different PTC mutations will incorporate distinct amino acids, despite treatment with the same read-through agent.³⁶ Furthermore, PTC-carrying transcripts are susceptible to nonsense-mediated decay (NMD)-related degradation, which significantly reduces the abundance of these PTC-carrying transcripts.^{34,35} Therefore, both NMD and PTC should be suppressed in order to achieve therapeutically relevant levels of CFTR rescue for class I mutants.

Ataluren (PTC124, PTC Therapeutics) was identified in a HTS of ~800,000 molecules using firefly luciferase-based read-through reporters³⁷ and was considered one of the leading compounds to rescue class I CFTR mutations. Despite its promising effects in the experimental setting, ataluren was unable to demonstrate efficacy in Phase III clinical trials involving individuals with CF and thus failed to reduce sweat Cl^- concentration and improve the percentage of predicted forced expiratory volume in 1 sec (ppFEV₁; a commonly used parameter to measure lung function).^{38,39} This lack of efficacy led to discontinuation of ataluren development for CF, albeit not for other conditions (eg, Duchenne muscular dystrophy), leaving an unmet need for novel drugs targeting CFTR PTC mutations.

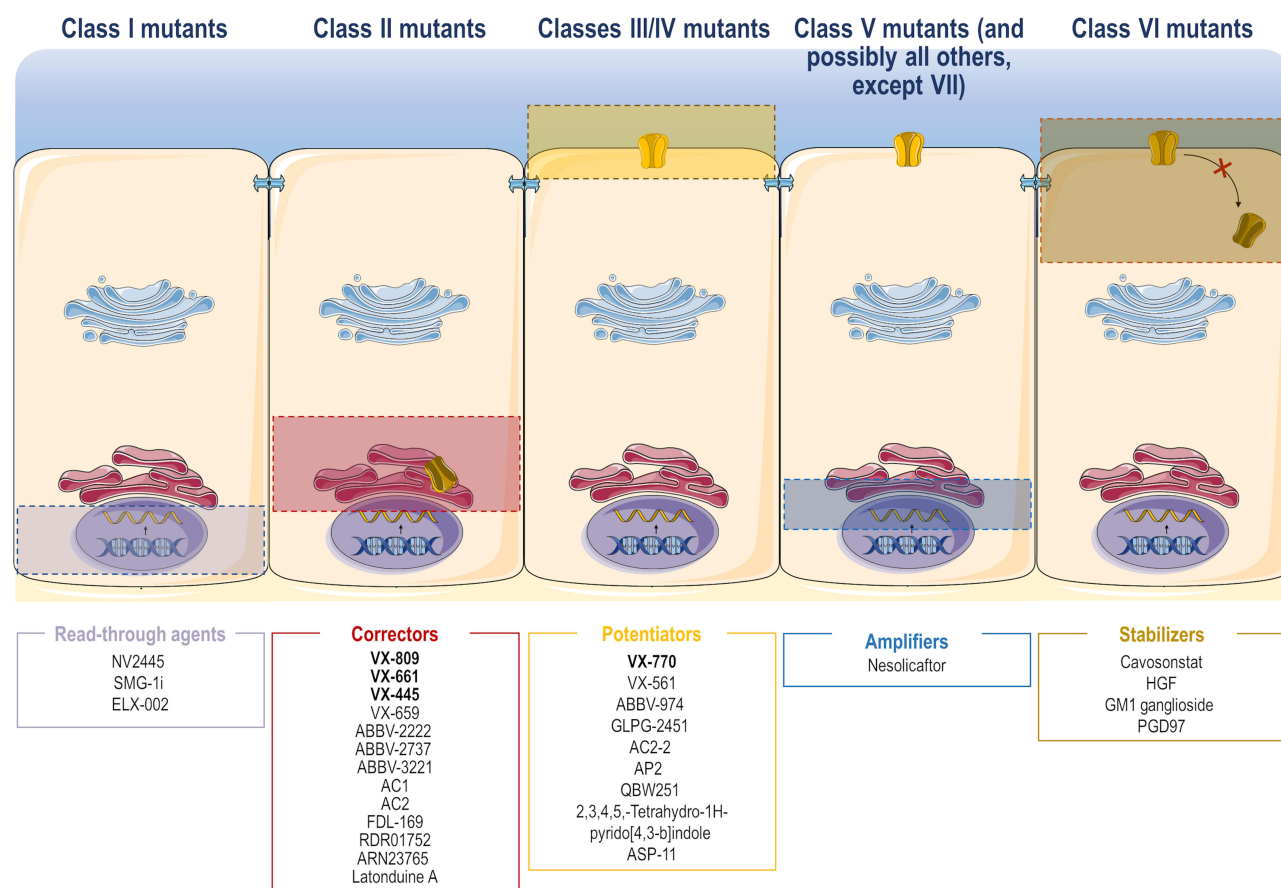


Figure 2 Site of action of the different CFTR modulator drugs. CFTR modulator drugs may be grouped into five main types according to their actions on CFTR mutations: read-through agents (for class I mutants), correctors (for class II mutants), potentiators (for classes III and IV mutants), amplifiers (for class V mutants, and possibly all others, except VII) and stabilizers (for class VI mutants). These molecules have a different putative site of action in order to correct specific defects in CFTR mutations. Some examples of promising CFTR modulators that are under experimental and clinical investigation have been provided (see text for further details).

Notes: Adapted from Lopes-Pacheco M. CFTR modulators: the changing face of cystic fibrosis in the era of precision medicine. *Front Pharmacol.* 2020;10:1662.¹¹ Copyright © 2020 Lopes-Pacheco. Creative Commons Attribution License (CC BY).

In an effort to identify more effective read-through agents, three ataluren derivative compounds were found to have a higher read-through efficacy, while maintaining low toxicity in a human bronchial epithelial cell line (W1282X/F508del CF genotype).⁴⁰ However, their efficacy in rescuing PTC CFTR function still needs further investigation. Alternatively, structural chemical replacements, such substitution of 1,2,4-oxadiazoles to 1,3,4-oxadiazoles, were demonstrated to be highly important in drug discovery and enhanced compound properties.⁴¹ A novel molecule with structural similarity to ataluren but with a 1,3,4-oxadiazole heterocycle core (termed as NV2445) revealed greater read-through activity when compared to the former molecule and rescued CFTR PM expression and function in cell models carrying different CFTR PTC mutations, including G542X and W1282X.⁴¹ NV2245 was also shown to have better

pharmacological properties than ataluren,⁴² and its effects on in vivo experimental and early-stage clinical studies are under investigation.

A molecule able to inhibit the serine/threonine-protein kinase-1 (SMG-1i) was identified as a promising NMD inhibitor.⁴³ SMG-1i was able to modestly increase CFTR mRNA abundance, protein expression and channel function in the 16HBEo⁻ cell line CRISPR-edited to express W1282X-CFTR,⁴⁴ although such effects were not observed in a following study by other group using this cell model.⁴⁵ SMG-1i also demonstrated to rescue W1282X-CFTR in primary human nasal epithelial (HNE) cells,⁴⁶ and synergistic effects were also observed when SMG-1i and other read-through agents (eg, gentamycin, G418/geneticin, paromomycin) were co-administered,⁴⁷ reinforcing the idea that combination of read-through agents and NMD inhibitors may represent a potential

therapeutic option for the treatment of individuals with CF carrying PTC mutations.

ELX-02 is a novel aminoglycoside analogue drug developed by Eloxx Pharmaceuticals that demonstrated read-through activity on CFTR PTC mutations.⁴⁸ In Phase I clinical studies (NCT02807961, NCT03292302, NCT03309605), 62 healthy volunteers were treated with ELX-02 and no serious adverse events or deaths were reported.⁴⁹ Two Phase II clinical studies have been initiated with 24 individuals with CF carrying the G542X mutation in at least one allele (NCT04126473, NCT04135495). Interestingly, ELX-02 demonstrated to rescue CFTR activity in the forskolin-induced swelling assay using intestinal organoids from individuals with CF carrying G542X-CFTR.⁵⁰ An increase in CFTR mRNA abundance and appearance of the fully-glycosylated form of CFTR were also observed in this study.⁵⁰ Such promising results have supported the ongoing clinical evaluation of ELX-02 for individuals with CF carrying the G542X mutation; however, its read-through activity for other CFTR PTC mutations needs to be further elucidated.

Several HTS have been performed over the last few years in an attempt to identify novel read-through agents for CFTR PTC mutations.^{11,30} A library containing ~85,000 molecules was screened by the CF Foundation Therapeutics laboratory and some promising hits were found to rescue the Y122X and W1282X mutations, including CFFT-0182812 and CFFT-0176974, which were able to increase both CFTR PM expression and transepithelial conductance.⁵¹ Notably, the five leading hits of W1282X increased CFTR PM expression to more than 20% of the WT-CFTR levels, which makes it encouraging to conclude that these hit compounds may yield full-length functional CFTR protein.⁵¹ Moreover, this study demonstrated that certain CF-causing mutations may benefit from development of mutation-specific modulators but efficacy ranking may differ significantly among different mutations in the same functional class. A more recent study screening over 660,000 molecules (Scripps Drug Discovery Library) for their ability to rescue G542X identified 188 compounds that rescue this PTC mutation when in combination with other modulators.⁵² These compounds are now being evaluated in primary cells from individuals with CF in order to validate the previous results and to analyze the translational read-through in a more physiologically relevant context. If successful, translational read-through would be an exciting approach to restore the expression of CFTR carrying PTC mutations

to levels approaching those of WT-CFTR. However, as shown by previous studies,^{35,36,53} the combination of a read-through agent with other modulators that have complementary mechanisms may be required to efficiently rescue CFTR PTC mutations, as the incorporation of a random amino acid may produce a full-length protein which is still misfolded or dysfunctional. Accordingly, in the pursuit of therapeutic options for individuals with CF carrying PTC mutations, and the lack of any clinically approved therapy for this group of individuals, it will be of the outmost importance to exploit the possibility of developing an effective combination of modulators.

Class II Mutations: Correctors

Correctors are small molecules that rescue CFTR mutants with a traffic defect (ie, class II CFTR mutation) to the PM.^{4,12,54} Defective traffic occurs as a result of CFTR mutations that cause protein misfolding, thus being recognized by the endoplasmic reticulum quality control and targeted to be prematurely degraded in the proteasome.^{55,56} As the F508del mutation belongs to this class, a considerable number of CF drug programs has been focused on the development of small-molecule correctors that rescue its PM traffic.

Lumacaftor (VX-809) and tezacaftor (VX-661) are two correctors (first- and second-generation, respectively) developed by Vertex Pharmaceuticals that were already approved some years ago by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).^{19,20,57,58} However, despite their promising effects in rescuing F508del-CFTR *in vitro*,^{59,60} their effects on improving lung function in clinical studies was somewhat more modest than anticipated.^{19,20} Indeed, either VX-809 or VX-661 in combination with the potentiator VX-770 (see next sub-section) only led to a fairly small improvement in lung function of individuals with CF homozygous for F508del (~4 and ~7 ppFEV₁, respectively).^{19,20} More recently, a next-generation corrector – elxacaftor (VX-445) – was approved in combination with VX-661/VX-770 and this triple combination led to greater therapeutic benefits in individuals with CF carrying one or two copies of F508del-CFTR,^{22,23} although this combination of two correctors still promotes only a partial rescue of F508del-CFTR traffic defect.⁶¹ A label extension of these drugs has been granted by the FDA to other CF-causing mutations based on *in vitro* data in cell lines,¹⁷ thus significantly increasing the number of individuals with CF who may benefit from these causative therapies.

However, there are several class II CFTR mutations unresponsive or only modestly responsive (ie, below therapeutically relevant levels) to experimental and clinically approved correctors.^{62–68} The real-life and long-term effects of clinically available correctors are also being further investigated, namely regarding liver and kidney function and overall tolerability. Another major limitation of these drugs is their very high costs, which makes them inaccessible for many individuals with CF, particularly for those living in low- and middle-income countries.^{11,69} In parallel, the same company (Vertex Pharmaceuticals) is pursuing the development of novel correctors, such as VX-121, which is currently being evaluated in combination with VX-661/VX-561 in phase II clinical studies (NCT03912233).

Many other studies have been performed by several academic laboratories and pharmaceutical companies in order to identify more potent correctors that could rescue class II CFTR mutations alone or in combination with the clinically approved ones. Some promising drugs developed by Abbvie/Galapagos include ABBV-2222, ABBV-2737 and ABBV-3221. ABBV-2222 is a first-generation corrector that demonstrated greater efficacy in rescuing CFTR function in F508del homozygous primary human bronchial epithelial (HBE) cells, being 25% more potent than VX-809 or VX-661.^{70,71} However, ABBV-2222, VX-809 and VX-661 seem to share a similar mechanism in rescuing F508del-CFTR, as these molecules were not additive when tested together.⁶⁸ Although ABBV-2222 was safe and reduced sweat Cl^- concentration in individuals with CF homozygous for F508del, it showed no improvements in ppFEV₁ in a phase IIa clinical trial,⁷² and therefore, another clinical study is currently ongoing (NCT03969888). ABBV-2737 is a second-generation corrector that was well-tolerated in phase I studies and demonstrated to rescue CFTR function in F508del/F508del HBE cells synergistically with VX-809, suggesting that these two molecules act by distinct mechanisms.^{73,74} In a phase IIa study, ABBV-2737 led to a reduction in sweat Cl^- concentration and improved ppFEV₁, albeit modestly, in individuals with CF homozygous for F508del.⁷⁵ ABBV-3221 is another second-generation corrector that demonstrated rescue of F508del-CFTR function with greater effects when in combination with corrector ABBV-2222 and potentiator ABBV-974 (formerly GLPG1837).⁷⁶ Abbvie also has two additional investigational correctors (AC1 and AC2) that were shown to rescue processing and trafficking of other

class II CFTR mutations, including G85E, M1101K and N1303K.^{46,77}

Other investigational correctors include FDL-169 (Flatley Discovery)⁶⁸ and RDR01752,⁷⁸ which also appear to share the rescue mechanism with VX-809 and VX-661. In addition, three small-molecule series were identified in a HTS of ~600,000 drug-like molecules: 6258, 3151 and 4172, which target defects at NBD1, NBD2 and TMD interfaces, respectively.⁷⁹ Although their individual use demonstrated a modest rescue of F508del-CFTR, the combination of these three compounds demonstrated much greater effects and were also able to rescue other class II mutations located at different CFTR domains.⁷⁹ ARN23765 is another novel corrector that demonstrated to be synergistic with other type of correctors in rescuing F508del-CFTR with the advantage of having picomolar potency.⁸⁰ Synthetic analogues of the marine natural product latonduine A also demonstrated to rescue F508del-CFTR traffic by inhibiting function of Poly(ADP-ribose) polymerase 3 and 16 (PARP3 and PARP16).⁸¹ Novel pyrrolothiazole derivative compounds were recently synthesized and their ability to rescue F508del-CFTR was evaluated in a small-scale screen.⁸² Among these, compound 44 rescued F508del-CFTR processing and function being additive to VX-809 but not to VX-661.⁸² Proteostasis Therapeutics also developed a potent corrector – posenacافت (PTI-801) – that was described as having a similar efficacy to VX-661 in rescuing F508del-CFTR traffic defect. Its safety, tolerability, and pharmacokinetics was evaluated in combination with other modulators from this company, namely a potentiator and an amplifier (see next sub-sections), in individuals with CF in a clinical trial (NCT03500263) with promising clinical outcomes. However, the clinical development of these compounds was discontinued after Proteostasis Therapeutics merged with another pharmaceutical company, Yumanity Therapeutics. Overall, all these compounds provide a good resource to further explore their mechanisms of action and pharmacophore structural space by medicinal chemistry to identify novel and more potent correctors.^{11,30}

Classes III and IV Mutations: Potentiators

Potentiators are compounds that increase CFTR channel open probability, thus allowing for anion transport through CFTR.^{11,83} These compounds are useful for CFTR mutations in which channel gating or conductance is impaired (ie, classes III and IV, respectively), or for those in which

protein translation or traffic are deficient and after rescue by another modulator type, the channel is still not functioning properly.

Ivacaftor (VX-770, Vertex Pharmaceuticals) was the first potentiator approved for clinical use.^{18,84} It was initially approved by both FDA and EMA for individuals with CF carrying G551D-CFTR in at least one allele after remarkable improvement in lung function (~10 ppFEV₁) and significant reduction in sweat Cl⁻ concentration.^{18,85} Thereafter, its approval was extended for other eight gating mutations.⁸⁶ More recently, the FDA has extended the approval of VX-770 for 96 residual function mutations (among gating, conductance and splicing mutations) based on in vitro data in cell lines,¹⁷ with subsequent clinical studies also confirming therapeutic benefit in some of these mutations.⁸⁷ As F508del-CFTR still exhibits a gating defect when the protein is rescued to the PM, VX-770 was also tested and approved in combination with VX-809,¹⁹ VX-661²⁰ and VX-445/VX-661^{22,23} as described above (see previous sub-section). Nevertheless, VX-770 only partially reverses G551D-CFTR dysfunction^{24,84} and individuals with CF taking this modulator may benefit from a combination of potentiators with complementary mechanisms (termed as co-potentiators).^{28,88,89} Surprisingly, VX-445 was recently demonstrated to have dual activity as both corrector and potentiator,⁹⁰ being its potentiator activity additive to VX-770 in rescuing F508del- and G551D-CFTR gating defect.²⁹ Furthermore, a deuterated form of ivacaftor (VX-561, deuticaftor) is currently under clinical investigation (NCT03911713). Since the replacement of hydrogen by the heavier deuterium atoms, renders the molecule more stable, as a drug it could be taken once daily instead of twice as it is the case of VX-770.⁹¹

ABBV-974 and GLPG-2451 are two novel potentiators developed by Abbvie/Galapagos. These molecules were demonstrated to rescue mutant CFTR carrying G551D, G178R, R334W, S549N or F508del – the latter in combination with either VX-809 or ABBV-2222 – in both cell lines and CF HBE.^{73,92,93} Patch-clamp functional studies showed that ABBV-974 and GLPG-2451 reduce the closed time and increase the open time of CFTR channels by a similar mechanism to that of VX-770, as no additive effects were observed when these molecules were tested together.^{73,92,93} ABBV-3067 is another potentiator developed by Abbvie that was demonstrated to rescue F508del-CFTR in combination with the corrector ABBV-2222,⁷⁴ and this combination is currently under clinical

investigation (NCT03969888). Abbvie also has two other investigational potentiators that are being pre-clinically tested: AC2-2 and AP2. The former was found to have dual activity as both corrector and potentiator in rescuing G85E- and M1101K-CFTR, but it only functions as a potentiator for N1303K-CFTR.⁷⁷ The combination of these two potentiators with the AC1 corrector efficiently rescued the functional expression of the ultra-rare I1234-R1239del-CFTR in HNE cells.⁹⁴

Novartis has developed icenticaftor (QBW251), a novel potentiator that showed to improve lung function (6.5 ppFEV₁) and reduce sweat Cl⁻ concentration in individuals with CF carrying a class III or IV CFTR mutation in at least one allele.⁹⁵ In parallel, QBW251 was evaluated in individuals with chronic obstructive pulmonary disease in a phase II trial and demonstrated to improve systemic inflammation and sputum bacterial colonization,⁹⁶ indicating that CFTR potentiators may benefit individuals with other lung diseases. Proteostasis Therapeutics also developed a potentiator termed as Dirocaftor (PTI-808) that was shown to have a similar efficacy to VX-770, and its effect was also assessed in an aforementioned clinical trial (NCT03500263) in individuals with the F508del mutation in at least one allele. Despite promising outcomes, its clinical development was discontinued.

A considerable number of pre-drugs has been identified by HTS programs and are under further development by academic research groups. For instance, some thiazole derivatives were found to act as both correctors and potentiators of F508del-CFTR, although the potentiator effect was lower in comparison to VX-770.^{97,98} Compounds containing a 2,3,4,5-Tetrahydro-1H-pyrido [4,3-b]indole core were able to efficiently rescue the function of F508del- and G551D-CFTR.⁹⁹ A spiro[piperidine-4,1-pyrido[3,4-b]indole] compound demonstrated potentiation activity for N1303K and I1234-R1239del CFTR with additive effects when in combination with VX-770.¹⁰⁰ ASP-11 is an arylsulfonamide-pyrrolopyridine co-potentiator that acts synergistically with VX-770 in the rescue of F508del-, G551D-, N1303K- and W1282X-CFTR mutations in cell lines.¹⁰¹ The effects of ASP-11 were confirmed for N1303K in HBE cells, but not for W1282X.^{28,101} This considerably high number of compounds with distinct chemical structures provide a great source for the development of novel potentiator drugs.

Class V Mutations: Amplifiers

In a HTS of a library containing ~54,000 drug-like compounds, a novel type of CFTR modulator was identified: the amplifiers.¹⁰² These molecules act on CFTR gene expression and, consequently, increase the total amount of protein produced.¹⁰³ Such compounds may be beneficial not only for class V mutations that result in a reduction of protein production, but also as adjuvants for all other CFTR mutation classes (except for class VII mutations that do not produce CFTR mRNA), as amplifiers may increase the protein abundance to be rescued by other CFTR modulators.

Nesolicaftor (PTI-428) is an amplifier developed by Proteostasis Therapeutics and the first-in-class evaluated in clinical studies.¹⁰² In the experimental setting, this molecule was able to selectively increase CFTR expression with no alterations on cytosolic stress or endoplasmic reticulum-associated unfolded protein response pathways. PTI-428 also augmented the effects of VX-809 and VX-770 in rescuing F508del- and I1234-R1239del-CFTR.^{102,104} A more recent study demonstrated that PTI-428 enhances the stability of CFTR mRNA by directly enhancing the binding of poly(rC)-binding protein 1 to a consensus sequence present in the open reading frame of CFTR mRNA.¹⁰³ This finding provides novel insights into the cellular regulation of CFTR biosynthesis along with mechanistic information on how amplifiers may be used to enhance the therapeutic benefits of other types of effective CFTR modulators.

Class VI Mutations: Stabilizers

Stabilizers are molecules that enhance the anchoring of CFTR channel to the PM, being thus able to significantly reduce its internalization rate. Even when CFTR mutants reach the PM, they may present intrinsic protein instability (ie, class VI CFTR mutations) that result in increased CFTR endocytosis¹⁰⁵ and/or decreased recycling back to the PM.¹⁰⁶ For instance, F508del-CFTR rescued either by low-temperature incubation¹⁰⁷ or by VX-809,^{25,108} VX-661 and/or VX-445⁶¹ exhibits much lower PM stability in comparison to WT-CFTR, thus being rapidly removed from the PM by peripheral quality controls mechanisms.¹⁰⁹

Cavosonstat (N91115) is a stabilizer developed by Nivalis Therapeutics and the first-in-class evaluated in clinical studies.¹¹⁰ It was demonstrated to enhance CFTR maturation and PM stability by inhibiting S-nitrosogluthathione reductase.¹¹¹ Hepatocyte growth

factor (HGF) was also demonstrated to facilitate the PM anchoring of F508del-CFTR by activating endogenous Rac1 signaling that promotes the interaction of CFTR with the Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1).¹¹² Furthermore, HGF treatment had a more significant effect in rescuing F508del-CFTR processing and PM stability when combined with VX-809.^{113,114}

Chemical inhibition of calpain 1 was also found to rescue F508del-CFTR expression and function as this interactor prevents ezrin recruitment, a key player in facilitating PM anchoring of F508del-CFTR.¹¹⁵ GM1 ganglioside is a bioactive lipid that also demonstrated to increase PM stability of F508del-CFTR.¹¹⁶ In cells chronically treated with VX-809 and VX-770, GM1 increased total levels of NHERF1 and ezrin, as well as levels of the mature form of F508del-CFTR, which was accompanied by an augment in CFTR-dependent Cl⁻ transport in cell lines and HBE cells.¹¹⁶

CFTR PM expression is negatively regulated by the CFTR-associated ligand (CAL)^{117,118} and PGD97 is a cyclic peptidyl inhibitor of the interaction between CAL and CFTR, leading to an increase in F508del-CFTR PM stability and function in cell lines and HBE cells with a greater effect when combined with VX-661.¹¹⁹ Such findings provide evidence for the interest in further developing PGD97 and other CAL inhibitors to rescue F508del-CFTR PM stability.

Personalized Medicine: Therotyping and Theranostics

The responsiveness of CFTR mutations to current available modulator drugs has been assessed in a process termed as therotyping, which consists in grouping mutations into classes to be treated by the same strategy (see above section and Figure 2).^{17,30,120} Although still incomplete, this process has been useful for the approval of label extension to less common CF mutations, thus clinically benefiting a larger population of individuals with CF.¹⁷ However, variability in clinical improvement was observed in clinical studies evaluating CFTR modulators even in individuals carrying the same CF genotype.^{18,57,58,85} While it is assumed that such differences may in part account for the structural tissue injury occurred over disease progression in each individual, it is currently known that responsiveness to a certain drug depends not only on the CF genotype but also on a number of modifier genes and epigenetic factors.^{11,30,121–123} Accordingly, it is generally accepted that every individual

with CF is unique, not only in terms of prognosis for the same CFTR genotype but also regarding response (and benefit) from CFTR modulators. As it is also expected that more CFTR modulator drugs will become available for clinical use, there is a need to advance in the development of tools that enable predicting the clinical effectiveness of drugs by ex vivo assays performed in individual-derived specimens so as to select the best therapeutic option(s) for that individual – a process termed theranostics, the principle underlying precision medicine.^{30,124} Such fact becomes even more relevant when treating individuals who have CF genotypes combining two different rare CFTR mutations (one in each allele) or even a complex allele.

In order to assess individual responses to CFTR modulator drugs (approved and under development), several assays have been developed and optimized using specimens from individuals with CF.^{59,125–130} Primary human airway epithelial cells are considered the gold standard since they have been successfully used as pre-clinical data for the CFTR modulators currently approved for clinical use. Indeed, this ex vivo cell model provides responsiveness in a physiologically relevant tissue and allows for the measurement of CFTR-dependent Cl^- transport.^{59,60,79} Primary HNE cells have emerged as a surrogate for bronchial epithelial cells as they share many phenotypic features^{59,126,130} and may be obtained by less invasive procedures (nasal scrapping instead of bronchoscopy or explanted lungs).

Intestinal organoids grown from stem cells obtained from biopsies have served as another robust and feasible model to evaluate the efficacy of CFTR modulators at an individual level, using the forskolin-induced swelling (FIS) assay.^{128,129} Using similar technology, airway (bronchial or nasal) organoids/spheroids have been developed based on the same FIS assay.^{127,131}

Induced pluripotent stem cells (iPSCs) might also be a useful model since they can be differentiated in several cell types and recapitulate the variable responses of individuals with the same CF genotype to modulator drugs.^{132–134} Several studies have been performed to establish correlations between responses in ex vivo individual-derived specimens and clinical parameters to identify which are the most precise biomarkers.^{59,62,135–137} The possibility of evaluating drug effectiveness at an individual level using ex vivo individual-derived specimens enables precision medicine and therapeutic counseling – ie, theranostics. More importantly, it can reduce the prescription of drugs that would create high expectation to the individual and family

members but which do not necessarily provide significant clinical benefits.

Modulation of Alternative (Non-CFTR) Channels/Transporters

Despite remarkable progress in developing highly effective CFTR modulator therapies that target the cellular/functional defects for different CFTR mutations, several issues still need to be resolved: 1) 10–15% of individuals with CF do not benefit from any clinically available CFTR modulator; 2) those who benefit, do so with different levels of responsiveness, which never reach levels of individuals without CF (or carriers); 3) some individuals cannot tolerate or respond poorly to available modulators; 4) there is no equitable access to these drugs due to their very high costs and lack of international regulatory issues; 5) real-world and long-term benefits and sequelae are yet to be demonstrated. Accordingly, an alternative therapeutic option that has gained increasing attention over the last few years consists in the modulation of other (non-CFTR) ion channels/transporters expressed at the PM of secretory epithelia.^{16,31–33} The main allure of this approach is that it may possibly be used for all individuals with CF, regardless of their CF genotypes, thus being a “mutation-agnostic” approach. Notably, CFTR modulators and drugs targeting alternative channels/transporters may also be combined for additive effects in improving fluid secretion in CF. Here, we have discussed three of these alternative targets: ENaC, TMEM16A and SLC26A9 (Figure 3).

Epithelial Na^+ Channel (ENaC)

The idea of targeting ENaC as a therapy for CF came from studies of its role in both healthy and CF airways. However, there is still a debate that encompasses two different hypotheses (not mutually exclusive) regarding the underlying cause of CF pathogenesis. The first one proposes that there is altered pH of air surface liquid (ASL) in CF resulting from absence of CFTR-dependent HCO_3^- transport, which leads to inactivation of natural antimicrobial peptides in the ASL.^{138,139} The second model is based on the idea that loss of CFTR function leads to Na^+ hyperabsorption by ENaC, leading to subsequent higher absorption of water and consequent ASL dehydration that causes impaired mucociliary clearance (MCC) in CF.^{140,141} As research data continue to support that a hyperactivation of ENaC occurs in CF cells, specific inhibition of ENaC function may represent a pathway to

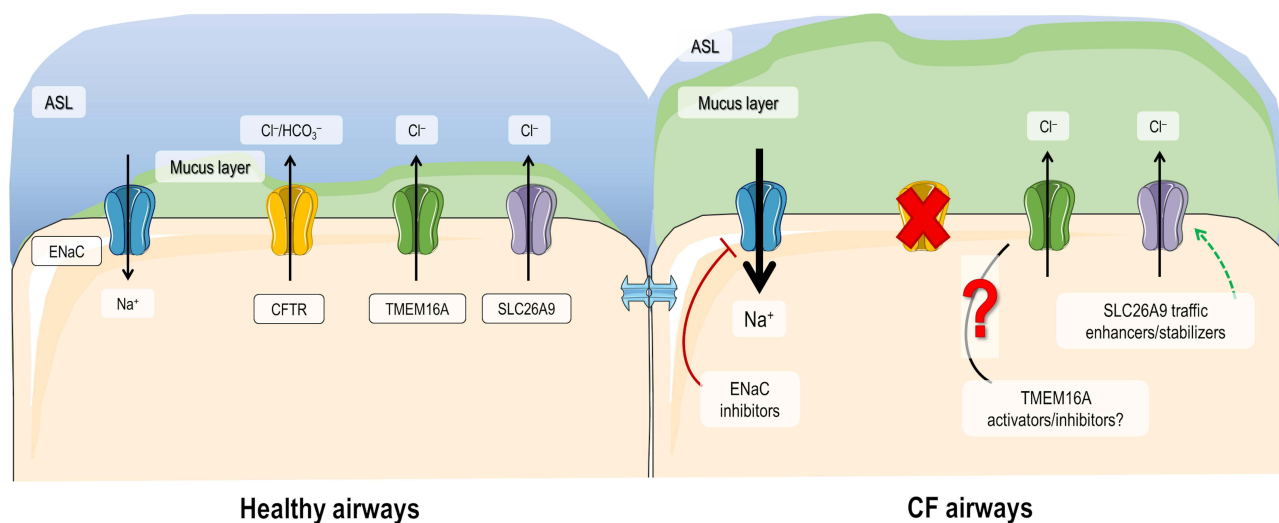


Figure 3 Modulation of ion channels/transporters as alternative therapies for CF. In healthy airways, CFTR, ENaC, TMEM16A and SLC26A9 are expressed at the plasma membrane (PM) of epithelial cells where they contribute to ion and water homeostasis. In CF airways, due to the absence of functional CFTR, Cl^- secretion is compromised and Na^+ absorption is upregulated, leading to a dehydrated air surface liquid (ASL) and impaired mucociliary clearance (MCC). Expression of TMEM16A and SLC26A9 at the PM are also diminished in CF ciliated cells, although the role of TMEM16A overexpression in secretory cells and its role in mucus secretion are still controversial. Alternative therapies for CF thus include blocking ENaC, enhancing SLC26A9 expression at the PM, and modulating TMEM16A. Although for the latter is still not clear whether activators or inhibitors are beneficial, a TMEM16A potentiator is currently under clinical investigation.

partially reverse the disruptive downstream effects of CF pathophysiology.

Numerous ENaC inhibitors have been developed with promising effects on the experimental setting but still failed to demonstrate clinical improvements in individuals with CF, including amiloride and SPX-101.^{32,141} There are several challenges that need to be overcome to successfully develop ENaC inhibitor compounds. These include the ability to penetrate the mucus barrier, the potency, the correct dose without off-target effects and a long half-life to achieve the best therapeutic effects. Accordingly, different strategies are under development to directly or indirectly inhibit ENaC function (Figure 4) and several novel molecules are advancing in the experimental pipeline, such as BI 1265162 and ARO-ENaC.^{142–144}

Amiloride was the first ENaC inhibitor developed in the 1960s by Merck and it is still commonly used in the experimental setting. Furthermore, its structural motif is present in several novel ENaC inhibitors under development. Amiloride directly binds to ENaC and promotes a decrease in the channel open probability.³² This molecule was initially used as a diuretic due to its action on blocking Na^+ absorption on the distal convoluted tubule of the kidney and in the fine tuning of Na^+ and water levels in the human body. In order to avoid any kidney side-effects or in other ENaC-expressing organs, amiloride was evaluated as an inhaled therapy in CF clinical trials.¹⁴¹ However,

results were modest and showed only short-term effects on MCC, which was mainly attributed to the short half-life of amiloride.

Due to clinical failure of amiloride and other first-generation ENaC inhibitor drugs, different strategies were pursued resulting in the discovery of SPX-101, a compound indirectly inhibiting ENaC by mimicking a protein named short palate lung and nasal epithelial clone 1 (SPLUNC1).^{145,146} Experimental data demonstrated that SPX-101 was able to significantly increase ASL height in primary HBE cultures and recover MCC in CF animal models^{145,146} and a phase I trial validated the safety of SPX-101 for clinical use.¹⁴² However, SPX-101 failed to promote any therapeutic benefit in individuals with CF in a phase II clinical trial (NCT03229252), resulting in the discontinuation of its clinical development. Other SPLUNC1-like compounds may become promising drugs to target ENaC for the treatment of CF.

BI 1265162 is a pre-drug developed by Boehringer Ingelheim that directly inhibits ENaC. It demonstrated a higher potency than amiloride with promising effects in experimental and phase I clinical studies.¹⁴⁴ BI 1265162 dose was selected based on data from a CF rat model in which this compound was administered intratracheally and then the fluid absorption net was calculated. BI 1265162 was also studied in a CF sheep model and the deposition of this drug in human airways was estimated by using a

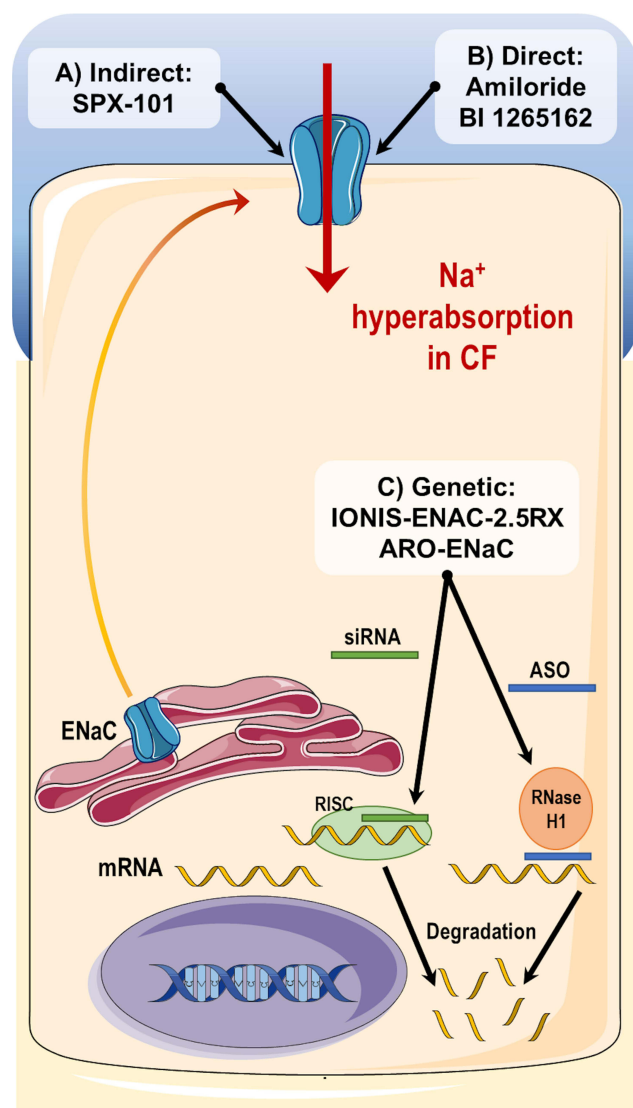


Figure 4 Summary of different strategies to inhibit ENaC. (A) Indirect inhibition: SPX-101 is a peptide analogue mimicking the inhibitory actions of SPLUNC1; (B) Direct inhibition: both amiloride and BI 1265162 promote direct inhibition of ENaC by binding to channel and decreasing its open probability; (C) Genetic inhibition: IONIS-ENAC-2.5Rx is an antisense oligonucleotide that recruits RNase H to degrade ENaC mRNA and consequently decrease ENaC protein synthesis and channel function; ARO-ENaC is a small interfering RNA (siRNA) that also promotes degradation of ENaC mRNA transcripts by the RNA-induced silencing complex (RISC) mechanism.

“soft mist aerosol” (Respimat[®]) inhaler.¹⁴³ The safety and efficacy of BI 1265162 are under investigation in a phase II clinical trial (NCT04059094).

IONIS-ENAC-2.5Rx is a novel genetic strategy developed by Ionis Pharmaceuticals to reduce ENaC function. It consists of an antisense oligonucleotide (ASO) sequence that targets and degrades the pre-mRNA α -subunit of ENaC, critical for channel formation. This degradation induced the RNase H1 activity that subsequently lead to a reduction in ENaC expression. In a phase I trial, IONIS-

ENAC-2.5Rx demonstrated to be safe and reduced ENaC mRNA expression on airways of healthy volunteers, after product inhalation via a nebulizer. Subsequent phase II clinical studies were performed to evaluate the safety and efficacy of IONIS-ENAC-2.5Rx in individuals with CF. However, due to recent pre-clinical toxicological data, the company discontinued the development of this drug.

The small interfering RNAs (siRNAs) consist of important alternatives to ASOs. These are composed of 21–23 nucleotides containing a mRNA sequence capable of degrading the mRNA of a specific target gene. The target mRNA degradation occurs with the association between the developed siRNAs with the RNA-induced silencing complex (RISC).^{32,147} ARO-ENaC (from Arrowhead Pharmaceuticals) is the key siRNA under development to target ENaC.^{32,147} ARO-ENaC was recently shown to significantly reduce ENaC mRNA and protein levels in lung tissue of rats. Although further studies are necessary to evaluate and optimize the efficacy of this therapy, its safety is currently under investigation on a phase I clinical study (NCT04375514).

Despite the several challenges, ENaC inhibitors are still a promising strategy to treat prominent abnormalities in CF lungs: the CF dehydrated mucus and its downstream detrimental consequences in CF airways. In addition, as mucus hyperproduction has been implicated in the pathogenesis of a variety of lung diseases, ENaC inhibitors may be beneficial therapeutics far beyond CF. Notwithstanding, it has been argued that an intensive ENaC inhibition should not be pursued as this may lead to excessive liquid in the lungs, ie, pulmonary edema, an equally pathological condition. Therefore, modulators that normalize Na^+ homeostasis and, consequently, water, are likely to constitute a more appropriate approach.¹⁴⁸

Transmembrane Protein 16A (TMEM16A) or Anoctamin 1 (ANO1)

TMEM16A/ANO1 is a Ca^{2+} -activated Cl^- channel (CaCC) expressed in various epithelia, including the airways, large intestine, salivary glands, pancreas, kidney and liver.^{33,149} It is also expressed in the nervous system,^{150,151} smooth muscles¹⁵² and tumor cells.¹⁵³ Such a broad tissue expression justifies its multiple physiological roles, which include airway and exocrine gland secretion, smooth muscle contraction, neuronal signaling control and peristalsis regulation of gastrointestinal system.¹⁵⁴ Furthermore, TMEM16A upregulation has been described in various

types of cancer, such as gastrointestinal squamous cancer,¹⁵⁵ head and neck squamous cell carcinoma,¹⁵⁶ breast cancer,¹⁵⁷ and lung cancer,¹⁵⁸ and it is generally associated with a poor prognosis.¹⁵⁹

Targeting TMEM16A for the treatment of CF has been a matter of debate. While some research groups support the need to activate TMEM16A in the context of CF in order to compensate for the absence of CFTR-mediated $\text{Cl}^-/\text{HCO}_3^-$ transport and thus increase airway hydration and MCC, others consider the opposite and suggest that inhibition of TMEM16A might decrease mucus secretion and bronchoconstriction. Efforts have been performed in both directions to discover TMEM16A modulators (activators and inhibitors) and to better understand the role of TMEM16A in CF.

TMEM16A Activators

The rationale for the activation of TMEM16A as a therapeutic strategy for CF was based on its role on fluid secretion in order to increase the hydration of the ASL, thus ameliorating MCC and recovering antimicrobial activities. In support of these arguments, reports demonstrated that TMEM16A knockout (KO) mice have low Cl^- secretion and mucus accumulation in the airways, which are features in common with CF lung disease.^{160–162} TMEM16A also plays a role in intestinal fluid secretion and in protecting the intestinal epithelium from colitis.¹⁶³ Furthermore, TMEM16A KO mice showed early signs of inflammation, and their airway cellular landscape is altered, lacking epithelial cell progenitors. Interestingly, in human cell models, inhibition of TMEM16A decreased ASL height in vitro, leading to airway dehydration, similarly to dysfunctional CFTR.¹⁶⁴ A strong functional relationship between TMEM16A and CFTR has also been proposed. Namely, in the absence of TMEM16A, both CaCC- and CFTR-mediated currents are reduced in mouse intestine and airways.¹⁶⁵ In human cells, genetic inactivation of TMEM16A led to dramatically reduction of WT-CFTR PM expression and function.¹⁶⁵ Along these lines, inhibiting TMEM16A would also cause CFTR inhibition, an important fact if we consider that some people with CF have mutations still associated with residual function of CFTR.

Different approaches can be used to activate TMEM16A in CF.¹⁶⁶ The most obvious would be to increase channel opening probability, enabling TMEM16A-dependent Cl^- secretion, but in a Ca^{2+} -independent way, as an increase in Ca^{2+} concentration may lead to activation of multiple

signaling pathways. To date, several TMEM16A modulator drugs have been identified, although their specificity and mechanism of action remain poorly elucidated (Table 2). An alternative strategy would be to stabilize the channel in the open state or to prevent its desensitization. TMEM16A channels are characterized by a time-dependent current decay after prolonged Ca^{2+} -dependent activation. This process is thought to be regulated by phosphatidylinositol 4,5-bisphosphate (PIP_2).¹⁶⁷ Indeed, PIP_2 has been reported to bind to TMEM16A and promote its stabilization in the open pore conformation.^{167,168} Inositol phosphates, such as $\text{Ins}(3,4,5,6)\text{P}_4$, have demonstrated to regulate Cl^- channels in multiple epithelia. Furthermore, INO-4995 (a cell permeant InsP_4 derivative compound) directly activates TMEM16A in overexpressing cells.¹⁶⁹ Another approach to stimulate TMEM16A-dependent Cl^- secretion consists in increasing its expression at the PM, either by increasing its anterograde traffic¹⁷⁰ or by blocking its endocytosis. Examples of relevant TMEM16A activators are further described below.

Some drugs able to indirectly modulate CaCCs in airway epithelia have already been investigated in clinical trials. Denufosal is a selective P2Y_2 receptor agonist that was evaluated as an inhaled therapy for CF. Experimental and clinical data demonstrated promising effects by increasing both Cl^- and water secretion, enhancing ciliary beat frequency and stimulating mucus release. Despite such effects, denufosal failed to demonstrate improvement in lung function in individuals with CF in phase III trials.^{171,172} The lack of clinical efficacy may be related to its limited time of action (shorter than its expected half-life in the airways) and receptor desensitization.¹⁷³ Furthermore, purinergic stimulation induces a transient increase in Ca^{2+} concentration that leads to a short-term activation of CaCCs, which might be insufficient to compensate for the lack of CFTR-mediated anion secretion.¹⁷⁴ An increase in intracellular Ca^{2+} concentration may also lead to undesired side effects, such as increased mucus release from airway secretory cells.¹⁷³ Duramycin (Moli1901/lancovutide) is an antibiotic that indirectly promotes CaCC activation by interacting with phosphatidylethanolamine at the PM¹⁷⁵ and raising intracellular Ca^{2+} concentration.¹⁷⁶ Although it was demonstrated to be safe and to improve lung function in individuals with CF in a phase II clinical study,^{177–179} no further studies have evaluated the utility of duramycin for the treatment of CF.

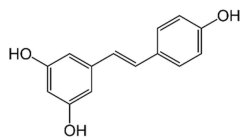
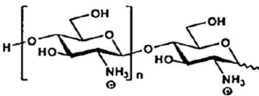
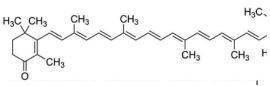
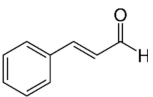
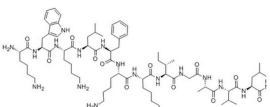
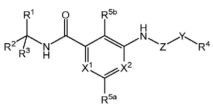
Silurian Pharmaceuticals has developed brevenal, a brevetoxin antagonist and candidate drug for CF and other respiratory diseases. Brevenal demonstrated to bind to the voltage sensitive Na^+ channel¹⁸⁰ and mobilize ATP-

Table 2 TMEM16A Activators

Molecule	Structure	IC ₅₀ (μM)	References	Clinical Stage	Putative Mode of Action/Targets
INO-4995		~5	[169]	Preclinical	Endogenous TMEM16A potentiation and activation of overexpressed TMEM16A
Denufosol		10	[171,172]	Phase III	P2Y ₂ agonist
Duramycin (Moli 901)		–	[177–179]	Phase II	Interacts with phosphatidylethanolamine in cell membranes and increases intracellular Ca ²⁺
Brevenal		–	https://www.silurianpharma.com/	Preclinical	Activation of voltage sensitive Na ⁺ channel and CaCCs
E _{act}		3	[183]	Preclinical	Intracellular Ca ²⁺ elevation by TRPV1/TRPV4 stimulation
F _{act}		6	[183]	Preclinical	Potentiator of TMEM16A
Ginsenoside Rb1		38.4	[254]	Preclinical	Activation of voltage-gated and ligand-gated ion channels and CaCCs

(Continued)

Table 2 (Continued).

Molecule	Structure	IC ₅₀ (μM)	References	Clinical Stage	Putative Mode of Action/Targets
Resveratrol		47.92	[188]	Preclinical	Activation of CaCCs in a Ca ²⁺ -independent way
Chitosan Oligosaccharide		74.5 μg/ mL	[186]	Preclinical	TMEM16A activation
Canthaxanthin		5.7	[189]	Preclinical	Activation of TMEM16A through direct binding
Cinnamaldehyde		9.73	[190]	Preclinical	Intracellular Ca ²⁺ elevation
Melittin		–	[192–194]	Preclinical	Activation of phospholipase A2
ETX001/ ETD002		0.116	[195]	Preclinical/ Phase I	Potentiator of TMEM16A

dependent intracellular Ca²⁺ that results in the TMEM16A activation. In an animal model, brevenal was able to block brevetoxin-induced bronchoconstriction, increase mucus secretion¹⁸¹ and reduce lung inflammation.¹⁸² Silurian Pharmaceuticals has attempted to initiate a phase I clinical study with brevenal for the treatment of CF; however, there are no further reports so far.

E_{act} and F_{act} are two small molecules identified by HTS that were shown to activate TMEM16A.¹⁸³ E_{act} promotes a strong increase in Cl[−] currents in the

absence of Ca²⁺. On the other hand, F_{act} potentiates TMEM16A by reducing the EC₅₀ for Ca²⁺-dependent activation of TMEM16A.¹⁸³ Although E_{act} was initially described as a direct activator of TMEM16A, subsequent studies demonstrated that its targets are indeed transient receptor potential channels, namely TRPV1¹⁸⁴ and TRPV4,¹⁸⁵ which lead to increase in intracellular Ca²⁺ concentration and, consequently, indirect activation of TMEM16A and CFTR (via Ca²⁺-dependent adenylyl cyclases).

Some natural and safe compounds have also been reported as TMEM16A activators with no apparent effects on intracellular Ca^{2+} concentration, which constitutes an advantage. These compounds include ginsenoside Rb1,^{186,187} resveratrol,¹⁸⁸ chitooligosaccharide¹⁸⁶ and canthaxanthin.¹⁸⁹ Cinnamaldehyde is another natural compound that activates TMEM16A, but it does so by increasing Ca^{2+} concentration.¹⁹⁰ These molecules may have therapeutic value for gastrointestinal disorders, such as hypomotility and constipation, as they demonstrated to increase ileum contractions in guinea pigs. Melittin, a major component of bee venom,¹⁹¹ is also a potent activator of TMEM16A and of other TMEM16 family members.¹⁹² It acts by phospholipase A2 stimulation and has anti-inflammatory properties, being widely used as an anti-cancer therapy.^{193,194} Notably, most of these compounds are not specific to TMEM16A and they actually have demonstrated a range of effects in different organs and modulate a number of other ion channels. Nevertheless, their chemical structures may serve as sources for medicinal chemistry in order to identify specific TMEM16A activators.

Enterprise Therapeutics identified a novel TMEM16A potentiator – ETX001 – that was able to enhance TMEM16A-dependent anion secretion in a Ca^{2+} -independent way, ASL height, and MCC both in vitro (in CF primary HBE cells) and in vivo (in a CF sheep model).¹⁹⁵ ETX001 demonstrated to potentiate TMEM16A-mediated Cl^- secretion without negatively affecting CFTR or ENaC.^{195,196} This portfolio has been recently acquired by Roche, including ETD002, a novel molecule that is currently in phase I clinical trials. Results are expected to demonstrate the ability of this TMEM16A potentiator in reducing mucus congestion, improving lung function and reducing frequency of lung infections. Overall, activating TMEM16A can be of interest not only for the treatment of CF, but also for other obstructive respiratory diseases and motility disorders in the gastrointestinal tract. However, it is clear that most currently known TMEM16A activators act via non-specific pathways – some of them by increasing intracellular Ca^{2+} concentration – rather than by directly binding and activating TMEM16A. Accordingly, one cannot safely assume that cellular effects promoted by these compounds occur by TMEM16A-specific mechanisms.

TMEM16A Inhibitors

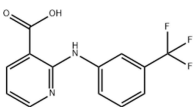
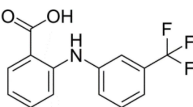
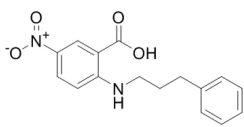
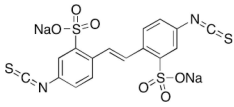
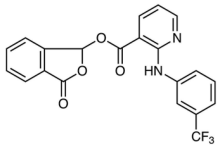
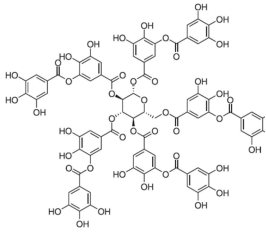
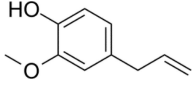
Some studies have suggested that, in the airways, TMEM16A is more abundant in mucus producing cells

rather than in ciliated cells.³³ TMEM16A expression and Ca^{2+} -dependent Cl^- secretion are also increased under inflammatory conditions, namely in asthmatic patients or in cells stimulated with Th2 interleukins (IL-4 or IL-13). Furthermore, as its expression is upregulated concomitantly with mucus hypersecretion, TMEM16A has been associated with goblet cell metaplasia.^{197,198} Following studies have demonstrated that inhibition of TMEM16A leads to a reduction in mucus secretion in airways¹⁵² and intestine,¹⁹⁸ proposing a causal relationship between these two events. It is therefore tempting to conclude that inhibition of TMEM16A (rather than activation) might be beneficial in CF.

In addition to the possible effects of TMEM16A on mucus hypersecretion, activation of TMEM16A in airway smooth muscles was shown to result in membrane depolarization, causing muscular contraction and potentially bronchoconstriction.¹⁵² Accordingly, inhibition of TMEM16A may induce bronchodilation, which could be advantageous in airway inflammatory disease.¹⁹⁹ Attenuation of intestinal contraction and diarrhea^{200,201} as well as nociception, itching and heat perception¹⁵⁰ may also be achieved by inhibiting TMEM16A. Finally, it has been argued that inhibition of TMEM16A might induce anti-cancer effects by blocking proliferation, migration and invasion of cancer cells, while also increasing the sensitivity to chemotherapies.¹⁵³ Nevertheless, the role of TMEM16A in cell proliferation is also controversial, with a recent report suggesting that TMEM16A is upregulated by cell proliferation and not the opposite.¹⁶⁴ Indeed, the data showed that, during wound, expression levels of TMEM16A rise concomitantly with the proliferation marker Ki-67, indicating that it is proliferation that triggers TMEM16A upregulation.

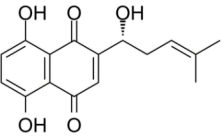
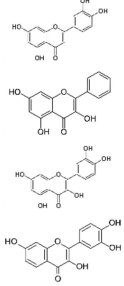
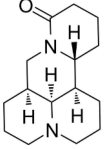
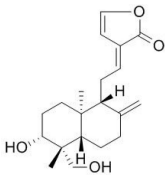
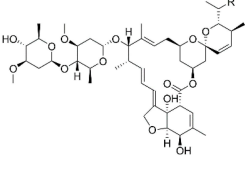
A growing number of molecules able to inhibit TMEM16A has been identified (Table 3). These include niflumic acid (NFA),²⁰² flufenamic acid (FFA),²⁰³ 5-nitro-(3-phenylpropylamino)-benzoic acid (NPPB)²⁰⁴ and 4–4'-diisothiocyanatostilbene-2–2'-disulfonic acid (DIDS),^{205,206} which are broad spectrum Cl^- channel blockers widely used for long to inhibit CaCCs and more recently demonstrated to act also on TMEM16A. However, they are non-selective and may elicit several other cellular responses. Talmiflumate, a pro-drug of NFA, is a potent analgesic and anti-inflammatory that has been used for the treatment of rheumatoid disease. Experimental studies have demonstrated that talmiflumate increases the survival rate of CF mice, which is a model of

Table 3 TMEM16A Inhibitors

Molecule	Structure	IC ₅₀ (μ M)	Reference	Clinical Stage	Putative Mode of Action/Targets
Niflumic Acid (NFA)		~10	[202]	Preclinical	Non-selective inhibition of Cl ⁻ channels, including TMEM16A and CFTR. Inhibition of phospholipase A2 and COX-2
Flufenamic Acid (FFA)		28–35	[203]	Preclinical	Inhibition of non-selective cation channels and Cl ⁻ channels. Modulation of K ⁺ , Ca ²⁺ , and Na ⁺ channels
NPPB		22–68	[204,206]	Preclinical	Non-selective inhibition of Cl ⁻ channels
DIDS		10–100	[205,206]	Preclinical	Non-selective inhibition of Cl ⁻ channels
Talniflumate		-	[207]	Phase II	Prodrug of niflumic acid
Tannic Acid		6	[208]	Preclinical	Inhibition of CaCCs
Eugenol		150	[209]	Preclinical	Inhibition of cyclooxygenase and biosynthesis of prostanoids. Cl ⁻ channel inhibition

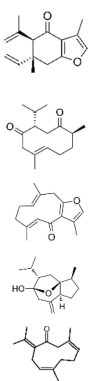
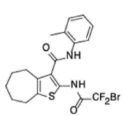
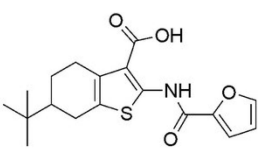
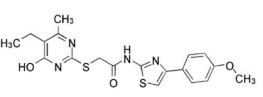
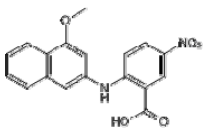
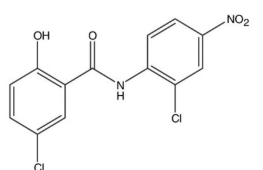
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Table 3 (Continued).

Molecule	Structure	IC ₅₀ (μ M)	Reference	Clinical Stage	Putative Mode of Action/Targets
Shikonin		6.5	[201]	Preclinical	Inhibition of CaCCs and Ca ²⁺ -activated basolateral K ⁺ channel
Natural flavonoids (1 – Luteolin, 2 – Galangin, 3 – Quercetin and 4 – Fisetin)		4.5–15	[210]	Preclinical	Modulation of several ion channels. Inhibition of TMEM16A
Matrine		27.94	[211]	Preclinical	Inhibition of TMEM16A
Dehydroandrographolide		~20	[212]	Preclinical	Inhibition of TMEM16A. Anticancer activity
Avermectins		0.15–1.32	[213]	Preclinical	Anthelmintic agents. Modulation of ligand-gated chloride channels. Inhibition of CaCCs

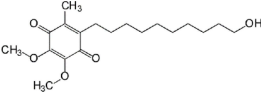
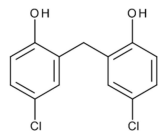
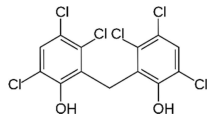
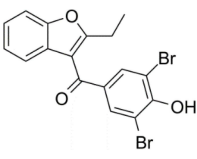
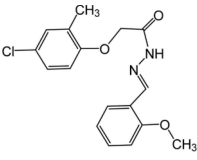
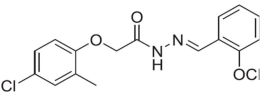
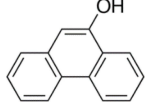
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Table 3 (Continued).

Molecule	Structure	IC ₅₀ (μ M)	Reference	Clinical Stage	Putative Mode of Action/Targets
Sesquiterpenoids (1 – Curzerenone, 2 – Curdione, 3 – Furanodienone, 4 – Curcumol, 5 – Germacrone)		13.55– 62.42	[214]	Preclinical	Inhibition of intracellular Ca ²⁺ concentration and K ⁺ channel activity. Inhibition of TMEM16A and CFTR
I0bm		0.03	[215]	Preclinical	Inhibition of TMEM16A
CaCCinh-A01		10	[216]	Preclinical	Inhibition of CaCCs
TI6A-A01		1	[217]	Preclinical	Inhibition of CaCCs
MONNA		0.08	[230]	Preclinical	Inhibition of CaCCs
Nicosamide		0.132	[199]	Preclinical	Anthelmintic. Hydrogen ionophore. Inhibition of CaCCs

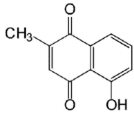
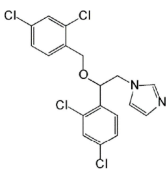
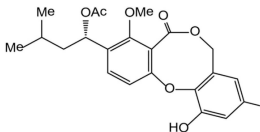
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Table 3 (Continued).

Molecule	Structure	IC ₅₀ (μ M)	Reference	Clinical Stage	Putative Mode of Action/Targets
Idebenone		9.2	[218]	Preclinical	Inhibition of CaCCs
Dichlorophen		5.49	[152]	Preclinical	Inhibition of CaCCs
Hexachlorophene		10	[152]	Preclinical	Inhibition of CaCCs
Benzbromarone		9.97	[152]	Preclinical	Inhibition of CaCCs and CFTR
Ani9		0.077	[231]	Preclinical	Inhibition of TMEM16A
5f (Ani9 derivative)		0.022	[232]	Preclinical	Inhibition of TMEM16A
9-Phenantrol		11.4	[255]	Preclinical	Inhibition of TRPM4 and TMEM16A

(Continued)

Table 3 (Continued).

Molecule	Structure	IC ₅₀ (μM)	Reference	Clinical Stage	Putative Mode of Action/Targets
Plumbagin		3–10	[218]	Preclinical	Inhibition of TMEM16A
Miconazole		10–20	[218]	Preclinical	Inhibition of TMEM16A
Purpactin A		2	[256]	Preclinical	Inhibition of TMEM16A

distal intestinal obstructive syndrome.²⁰⁷ Talniflumate has also been investigated as a mucoregulator for the treatment of various respiratory diseases,²⁰⁷ namely CF, chronic obstructive pulmonary disease and asthma; however, its clinical development has been discontinued.

Some natural products have also been identified as TMEM16A inhibitors, including tannic acid and other gallotannins, which are known to inhibit smooth muscle contraction and intestinal Cl[−] secretion.²⁰⁸ Other natural compounds like eugenol²⁰⁹ and shikonin²¹⁰ have anti-diarrheal effects, and flavonoids (galangin, luteolin, quercetin and fisetin),²¹⁰ matrine,²¹¹ dehydroandrographolide,²¹² avermectins²¹³ and sesquiterpenoids (curzerenone, curdione, curcumol, furanodienone and germacrine)²¹⁴ demonstrated anti-proliferative and anti-cancer effects.

Several TMEM16A inhibitors have been identified by HTS, such as cycloalkylthiophene 10bm,²¹⁵ CaCCinh-A01,²¹⁶ T16inh-A01,²¹⁷ MONNA,¹⁹⁹ niclosamide,²¹⁸ idebenone, dichlorophem, hexachlorophenom and benzbromarone.¹⁵² The latter is a uricosuric agent that was initially approved for the treatment of gout. Since the discovery of its role on TMEM16A inhibition, it has been suggested that it might be

repurposed for TMEM16A-related disorders. A good example has been the repurposing of benzbromarone for the treatment of idiopathic pulmonary arterial hypertension, a disease in which TMEM16A was found to be upregulated.^{219,220} However, it is important to note that benzbromarone is not a TMEM16A-specific inhibitor, thus also inhibiting other channels, such as CFTR.²²¹ Some safety concerns have also been raised for the long-term use of benzbromarone due to reports of hepatotoxicity.²²²

Niclosamide is an antihelminthic drug that has also demonstrated anti-cancer effects on several types of human cancer cells.^{223–225} It has been more extensively investigated in experimental and clinical studies for individuals suffering from prostate and colorectal cancer.^{226,227} Apart from its anti-neoplastic effects, niclosamide also demonstrated to inhibit TMEM16A and other members of the TMEM16 family.²²⁸ Other studies also suggested that niclosamide reduces bronchoconstriction¹⁹⁹ by relaxing airway smooth muscles and mitigates mucus hypersecretion.²²⁹ Interestingly, the potential antiviral efficacy of niclosamide is under clinical investigation in combination with an established therapeutic regimen for SARS-CoV-2 infection (NCT04558021).

T16inh-A01,²¹⁷ MONNA,²³⁰ and Ani9²³¹ were initially characterized as the most potent and selective TMEM16A inhibitors. However, T16inh-A01 and MONNA were found to also inhibit other members of this family, such as TMEM16B, in a dose-dependent manner.²³¹ Ani9 and its derivative 5f²³² are, therefore, presented as the most powerful and apparently selective TMEM16A inhibitors to date. Both Ani9 and 5f demonstrated high selectivity for TMEM16A without affecting intracellular Ca^{2+} concentration or other channels, such as CFTR. Nevertheless, recent studies demonstrated that Ani9 may affect TMEM16F whole-cell currents, indicating that this inhibitor is not completely selective for TMEM16A.²²⁸ Overall, despite the existence of various TMEM16A inhibitors, the potency and selectivity of these compounds are a considerable limitation. The development of potent and more selective molecules that directly target TMEM16A may provide invaluable information on the role of this channel in CF and TMEM16A-related disorders.

Solute Carrier Family 26 Member 9 (SLC26A9)

SLC26A9 has been proposed to function as a constitutively active Cl^- transporter,^{233,234} predominantly expressed in epithelial cells of the respiratory tract, stomach, duodenum, ileum and pancreas.^{31,235} In human airways, SLC26A9 contributes to constitutive Cl^- secretion and MCC,²³⁶ and, in contrast to CFTR and TMEM16A, it is described as being spontaneously active once inserted into the PM of airway epithelial cells.²³⁷

SLC26A9 was found to be mostly cytoplasmic or to localize closer to tight junctions.²³⁸ Nevertheless, it was proposed to functionally interact with CFTR, which positively regulates its transport, function and targeting to the PM.³¹ In this context, the presence of F508del-CFTR reduced SLC26A9 expression by promoting its retention in the endoplasmic reticulum and premature degradation by the proteasome, similar to F508del-CFTR itself.²³⁸

The importance of investigating SLC26A9 in the context of CF has been highlighted due to its striking effects on the pathogenesis of organs affected in CF, as well as other disorders. SLC26A9 has been described as a modifier gene of lung function and of responses to CFTR modulator drugs.^{121,122} SLC26A9 polymorphisms have been associated with the risk of developing meconium ileus and early exocrine pancreatic disease in

individuals with CF.^{239–241} Interestingly, SLC26A9 polymorphisms have also been linked to asthma in children, namely due to a reduction in SLC26A9 protein expression.²³⁶ As SLC26A9 contributes to epithelial Cl^- secretion and prevents mucus obstruction under inflammatory conditions,²³⁶ it possibly also plays a role in the regulation of the ASL, similar to CFTR. Furthermore, a recent study demonstrated that increased SLC26A9 expression in pancreatic ductal cells delays the age of onset of CF-related diabetes.²⁴²

Taken together, these data support a clinically relevant role of SLC26A9 as both a CF disease modifier and a promising therapeutic target to circumvent deficient Cl^- secretion in CF. In contrast to TMEM16A, SLC26A9 modulation as a therapeutic target for CF is unanimous – ie, all data point toward the need to increase SLC26A9 expression and function. Nevertheless, the study of SLC26A9 has been limited by the lack of sensitive and selective pharmacological modulators. Some compounds have demonstrated to inhibit SLC26A9, namely non-selective Cl^- channel inhibitors NFA, NPPB, DIDS and GlyH101²⁴³ with different degrees of potency. The use of inhibitors that are virtually specific for other channels, such as Ani9 for TMEM16A and CFTRinh172 for CFTR, for which SLC26A9 is probably not sensitive,²⁴⁴ allows only for the indirect study of SLC26A9-mediated Cl^- currents. Accordingly, identification of selective activators of SLC26A9 is necessary to further understand whether and how it can be used as a therapeutic target. Due to its spontaneous activation once inserted into the PM,²³⁷ molecules that increase or stabilize SLC26A9 PM expression may contribute to increase epithelial Cl^- secretion.

Anionophores

Besides the aforementioned channels/transporters, small artificial transmembrane ion transporters (termed as ionophores) have emerged as therapeutic candidates for CF.²⁴⁵ Among these, anionophores, which selectively facilitate the transport of anions, have the potential to compensate for the faulty CFTR transport activity in CF.²⁴⁶

Studies have identified promising molecules, including derivatives of natural products, such as prodiginines^{247,248} and tambjamins,²⁴⁹ capable of facilitating Cl^- and HCO_3^- transport in mammalian cells. More recently, these compounds were also reported to effectively correct abnormal ASL parameters in primary cultures from individuals with CF.²⁵⁰ Interestingly, the activity of some anionophores is additive to F508del-CFTR rescue by the modulators VX-809 and VX-770, while the activity of others is additive to

TMEM16A activation.²⁵¹ These data reinforce the idea that anionophores, either by themselves or in combination with other modulator drugs, may offer promising therapeutic strategies for CF.

Amphotericin B (AmB) is a channel-forming molecule capable of creating non-selective ion channels. Recent studies with AmB showed that it can increase Cl^- secretion, ASL height and pH, reduce ASL viscosity, and restore antibacterial activity in primary cultures of airway epithelia from individuals with CF caused by different mutations, including the ones that produce little to no CFTR.^{252,253} Additionally, a small clinical study involving people with CF not treated with CFTR modulators showed that intranasal application of AmB caused a significant change in nasal potential difference, a commonly used biomarker to assess experimental therapies in people with CF.²⁵³ These data combined with the fact that AmB is an already clinically approved drug used to treat fungal infections, further emphasize the need to carry out additional studies to assess if inhaled AmB can in fact benefit people with CF, regardless of their CFTR mutations.

Similar to the use of alternative channels/transporters mentioned here, taking advantage of ionophores to replace defective CFTR has the potential to provide an alternative therapy for all individuals with CF, that would work independently of their CF genotype. Nevertheless, further studies are needed to determine the efficacy and safety profiles of these molecules to enable their clinical use.

Outlook and Conclusions

Numerous milestones have been achieved in CF research since the very first pathological description of this disease in 1938. The development of novel therapies for CF has been a success story with a real transformation in the disease prognosis, quality of life and life expectancy. In fact, in the 1960s, CF was a deadly and untreatable disease in early childhood and nowadays many individuals with CF achieve the adulthood, although they still face a high therapeutic burden and several disease-related complications. While the use of symptomatic therapies remains of great importance, the introduction of CFTR modulator drugs in the clinic, namely highly effective CFTR modulator therapies, has demonstrated significant benefit by targeting the root cause of CF and thus prevent several pathological consequences downstream of CFTR dysfunction. Accordingly, it is expected that these drugs may induce a profound alteration on the course of disease, especially for individuals who initiate their use in the early disease onset. Despite such progress, not all

individuals with CF are eligible for the currently available CFTR modulator drugs, and novel and more potent CFTR modulators are still under development. Novel cell models and assays using ex vivo individual-derived specimens have also been optimized to comparatively evaluate drug efficacies and determine which may provide the greatest therapeutic benefits in a precision medicine approach, ie, theranostics. Furthermore, a better understanding of the complexity of CF epithelial cell physiology and ion transport has enabled the pursue for alternative targets to compensate for the absence of CFTR function. Modulation of alternative ion channels/transporters, such as ENaC, TMEM16A or SLC26A9, as therapeutic targets in CF is promising but still challenging. It is still critical to develop selective and potent modulators of these targets and understand how (and whether) they may be used in the clinical scenario. Nevertheless, such approaches, if successful, may be beneficial for all individuals with CF, regardless of their CFTR genotypes.

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Disclosure

The authors report no conflicts of interest in this work.

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