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Invited Review

INVITED REVIEW

Recent advances in developing therapeutics for cystic fibrosis

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Abstract

Despite hope that a cure was imminent when the causative gene was cloned nearly 30 years ago, cystic fibrosis (CF [MIM: 219700]) remains a life-shortening disease affecting more than 70 000 individuals worldwide. However, within the last 6 years the Food and Drug Administration's approval of Ivacaftor, the first drug that corrects the defective cystic fibrosis transmembrane conductance regulator protein [CFTR (MIM: 602421)] in patients with the G551D mutation, marks a watershed in the development of novel therapeutics for this devastating disease. Here we review recent progress in diverse research areas, which all focus on curing CF at the genetic, biochemical or physiological level. In the near future it seems probable that development of mutation-specific therapies will be the focus, since it is unlikely that any one approach will be efficient in correcting the more than 2000 disease-associated variants. We discuss the new drugs and combinations of drugs that either enhance delivery of misfolded CFTR protein to the cell membrane, where it functions as an ion channel, or that activate channel opening. Next we consider approaches to correct the causative genetic lesion at the DNA or RNA level, through repressing stop mutations and nonsense-mediated decay, modulating splice mutations, fixing errors by gene editing or using novel routes to gene replacement. Finally, we explore how modifier genes, loci elsewhere in the genome that modify CF disease severity, may be used to restore a normal phenotype. Progress in all of these areas has been dramatic, generating enthusiasm that CF may soon become a broadly treatable disease.

Introduction

Cystic fibrosis [CF (MIM: 219700)] is an autosomal recessive genetic disease caused by mutations in the gene encoding the CF transmembrane conductance regulator [CFTR (MIM: 602421)] protein. The CFTR protein is found in the cell membrane of epithelial cells and acts as an ion channel. Since the initial discovery of *CFTR* and the most common mutation F508del (p.Phe508del; legacy names for mutations are used here with the variant names in parentheses) in 1989, over 2000 variants have been identified. A systematic effort to distinguish disease-causing from neutral variants (CFTR2.org) has identified 327 mutations that cause CF (1) and have varying effects on CFTR

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Table 1. Classification of CFTR mutations based on the impact of the CFTR protein

Class	How CFTR protein is affected	Examples
I	No functional CFTR protein is made	G542X, W1282X, 621 + 1G>T
II	CFTR protein is misfolded and does not reach the cell membrane	F508del, G85E
III	CFTR protein reaches the cell membrane but the channel gate does not open	G551D
IV	CFTR protein reaches the cell membrane but the channel does not move chloride efficiently	R117H, R334W, R347P
V	The CFTR protein is made and works properly but the amount is insufficient	3849 + 10kb C>T, 2789 +5 G>A
VI	The CFTR protein is made and is in the correct location but has reduced stability and accelerated turnover	rF508del, 4326del, 4279insTC

rF508 denotes the rescued protein after drug treatment.

protein function. Several mutations are now classified based on the functional abnormality seen within the protein (Table 1).

CF is a multi-organ system disease though the major causes of morbidity and mortality arise from progressive lung disease. *CFTR* mutations lead to aberrant chloride transport in epithelial tissues resulting in altered hydration and pH of airway surface fluid, and viscous mucus. Within the lungs, this leads to an increased susceptibility to infections and inflammation, further damaging the airways (Fig. 1). This cycle of infection and inflammation results in bronchiectasis and end-stage lung disease, the leading cause of premature death in patients living with this disease. Until recently, therapeutic options for CF focused on the downstream effects once damage has occurred, in an effort to delay the progression of the disease.

Over the past decade, significant therapeutic advances have been made in CF with new drugs targeting the defective protein to enhance its function. Depending on the functional abnormality, the therapeutic target may involve one or more steps. In situations where the CFTR protein is in the correct cellular location but the channel does not function optimally, so-called gating mutations, small molecule drugs have been developed to open the channel. A more complex situation exists when the CFTR protein is not properly processed and in these circumstances two drugs are necessary to enhance synthesis of functional protein and then further increase the channel opening once it is in the right location. The first drug ivacaftor (Kalydeco^{TM}) was used in a landmark trial of patients with G551D, a class III mutation. Ivacaftor therapy resulted in an average absolute increase of 10.5% in percent predicted forced expiratory volume in 1 s (FEV1pp), 55% reduction in pulmonary exacerbations, improved quality of life and a 48 mmol/L reduction in sweat chloride testing compared with placebo with relatively minimal side effects (2). (A diagnosis of CF correlates with sweat chloride levels of >60 mmol/L.) There is evidence for sustained benefits using ivacaftor in those individuals with gating mutations (3). Although there are country-specific differences in prevalence, less than 5% of the CF population worldwide carries this mutation. More recently, ivacaftor was approved for use in patients with class IV mutations that have residual CFTR function.

The most common mutation, F508del, became the focus of future clinical trials using combination therapy with both a corrector (which moves the protein to the cell surface) and a potentiator (which improves channel function, such as ivacaftor) due to the complex nature of the protein dysfunction with this mutation. The original corrector molecule, lumacaftor, in combination with ivacaftor (Orkambi^{TM}), was shown to modestly improve lung function and reduce pulmonary exacerbations, however, significant side effects were seen with worsening shortness of breath and chest tightness, particularly in those

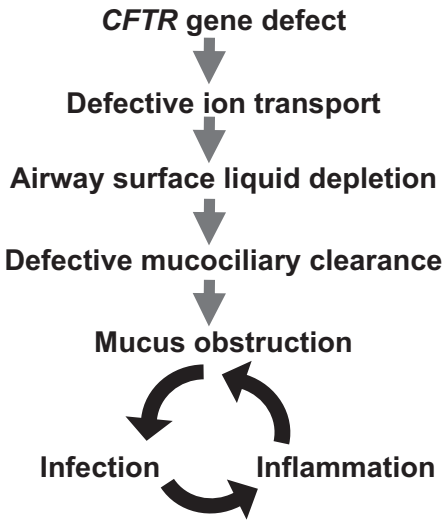

Figure 1. Pathophysiology of CF Disease. Process by which mutations in *CFTR* ultimately lead to reduced lung functional capacity. Therapeutic strategies currently target all phases of this process: Gene therapy and gene editing target the gene defect; RNA and protein therapies and modulating alternative channels aim to improve ion transport; early therapeutic paradigms targeted the downstream consequences of this process with airway clearance techniques, mucolytics, antibiotics and lung transplantation.

with low baseline lung function as well as drug–drug interactions (4). A newer corrector molecule, tezacaftor, in combination with ivacaftor (Symdeko^{TM}), appears to have a better side effect profile and showed improvements in lung function, reduction in pulmonary exacerbations in individuals who were homozygous for F508del or heterozygous with a second mutation which had residual function (5,6). Even more exciting are preliminary results from phase 1 and 2 studies using triple combination therapy for patients who are homozygous for F508del and those who carry one F508del mutation and a residual function mutation. These drugs utilize the combination therapy of ivacaftor-tezacaftor with a third molecule aimed at stabilizing the protein within the cell membrane. Phase 3 trials are ongoing with triple combination therapy, with results expected later this year (7).

Although there have been significant advances in CFTR protein modulator therapies, challenges still exist. First, not all patients with eligible mutations respond to treatment. Second, many mutations that lack treatments are rare and our understanding of their functional consequences is incomplete. Third, mutations resulting in premature termination of the protein due to stop codons, Class I mutations, are an even more

complex problem to address than Class II or III mutations since no functional protein is made. Therefore, alternative approaches to therapy are needed beyond potentiators and correctors.

The objective of this review is primarily to focus on innovative therapies for CF, many of which are still at preclinical stages. These therapies build upon advances in genetics, genomics and recent insights into CF pathology to develop strategies to make CF a treatable disease for all patients, irrespective of their specific *CFTR* mutations.

Correcting the Gene

Despite the remarkable advances in developing new drugs to circumvent the impairment of the CFTR protein and chloride ion channel, there is still a need to correct mutations associated with loss or greatly reduced amounts of mature *CFTR* transcript or protein (e.g. Classes I and V). The most frequent of these errors include 'stop/nonsense' mutations that introduce a premature termination codon (PTC) into the *CFTR* mRNA, alterations in splice sites and gross lesions deleting parts of the locus. Here we consider targeted therapeutics for each of these mechanisms and also mutation agnostic approaches that will use gene editing to correct or replace the endogenous *CFTR* locus in somatic cells.

Targeted Therapeutics

Stop mutations and "read through" drugs

About 70 'stop' mutations are recorded in CFTR2 (December 2017) (http://cftr2.org; date last accessed June 2018), the most common of these include G542X (p.Gly542X), R553X (p.Arg553X) and W1282X (p.Trp1282X) all of which have more than 1000 alleles documented in CFTR2. Therapeutic approaches targeting these mutations will likely be applicable more broadly if proven to be effective. Earlier observations showing that aminoglycoside antibiotics enhanced read through of nonsense mutations (8,9) led to hopes that these would provide valuable therapeutics, if renal and ototoxic side-effects could be overcome. This approach was evaluated for CF and also other common genetic disorders such as Duchenne Muscular Dystrophy [DMD (MIM: 310200)] and Spinal Muscular Atrophy [SMA (MIM: 253300)] among others. Ataluren, a small molecule read through therapy developed by PTC Therapeutics showed early promise, but failed to meet its endpoints of improved lung function and reduced pulmonary exacerbation in a later clinical trial (10). Several related aminoglycosides are included in pharmaceutical grade gentamycin and recent studies show that only the minor gentamycin B1 component has robust PTC read through activity, while the major gentamycins do not and further may repress B1 (11). Perhaps new aminoglycoside formulations will prove more effective. Of note, in addition to enhancing read through, therapeutics for stop mutations may also target nonsense-mediated decay, which profoundly impacts the levels of nonsense transcripts available for correction (reviewed in 12).

Splice mutations and antisense oligonucleotides

Another common class of errors in *CFTR* involves splicing of one of the 27 exons in the gene, either by reducing the efficiency of a splice donor or acceptor or through the creation of a novel cryptic splice site within an intron. Inspection of CFTR2 reveals four particularly common splice site mutations with more than 1000 alleles carrying one of the 621+1G>T (c.489+1G>T), 1717–1G>A (c.1585–1G>A), 2789+5G>A (c.2657+5G>A) and 3849+10C>T (c.3718–2477C>T) mutations. Again therapeutic approaches that build on progress in other common genetic disorders are in development for CF splicing mutations. For both SMA and DMD antisense oligonucleotides (ASOs) carefully targeted to the splice acceptor site of specific exons can induce their skipping to circumvent inclusion of an exon carrying a deleterious mutation. Chemical modification of the oligonucleotides reduces their intracellular degradation. Though the impact of ASOs in clinical trials for DMD were modest and variable (reviewed in 13), they are now an accepted therapeutic for type I SMA, where ASOs enhance inclusion of exon 7 of the Survival of Motor Neuron 2 [*SMN2* (MIM: 601627)] transcript to produce a full-length functional SMN2 protein (reviewed in 14). To date the ASO approach to restore CFTR function is less well advanced and has not yet moved beyond preclinical studies. *In vitro* proof-of-concept studies showed that ASOs could effectively restore normal splicing of the 3849+10C>T mutation in *CFTR*. This error creates a cryptic splice site resulting in the inclusion of an extra 84 bp exon that causes a premature stop codon in the transcript (15). More recently evidence for an effective ASO approach to correct the 2789+5G>A mutation *in vitro* was reported (16). Of note, though the Food and Drug Administration (FDA) in the United States approved the use of ivacaftor for the treatment of five common splice mutations in *CFTR*, based on its potency in activating the residual normally spliced mRNA, anecdotal evidence suggests that it may not always be clinically effective. More recently the combination of tezacaftor and ivacaftor was also approved for these splice mutations.

Gene Editing

Targeting specific mutations

The possibility of somatic cell editing of specific *CFTR* mutations fuelled many *in vitro* approaches to achieve this, even prior to the widespread use of CRISPR/Cas9 protocols. Earlier work with zinc finger nucleases (17,18) showed that correcting mutations in the *CFTR* locus in airway cells and induced pluripotent stem cells was efficient. Similarly CRISPR/Cas9 correction of the F508del by homology-directed repair (HDR) was achieved in intestinal organoids from a CF patient (19). Another group of disease-associated mutations in *CFTR*, which are particularly amenable to CRISPR/Cas9 editing, are those deep within introns that create cryptic splice sites. These sites are utilized instead of normal splice sites in the gene and thus incorporate additional pseudo-exons into the *CFTR* transcript, many of which contain stop codons. Examples are the 1811+1.6kbA>G (c.1679+1634A>G) error and the 3849+10kbC>T (c.3718–2477C>T) mutation mentioned above. These variants were effectively corrected by nonhomologous end joining (NHEJ) using CRISPR/Cas9 with guide RNAs flanking the mutation to delete it from the genomic sequence (20). The same approach was effective for correcting an out-of-frame 5′ extension to an existing exon 3272–26A>G (c.3140–26A>G) (20). Restoration of normal CFTR splicing was observed in targeted airway cell lines. Clearly this approach would not be applicable to the more common pathogenic splice variants in *CFTR* such as 621+1G>T, 1717–1G>A, 2789+5G>A, since these errors lie very close to intron/exon boundaries.

Fixing all CFTR mutations

In light of the more than 2000 reported *CFTR* variations, a gene editing approach that can correct all mutations is an attractive goal. Since gene therapy approaches to deliver a *CFTR* cDNA

transgene driven by a heterologous promoter to the airway have, until now, proven at best only marginally effective (with gradual loss of transgene expression being a common problem), alternative protocols are being developed. A particularly attractive approach is to use CRISPR/Cas9, zinc finger nuclease or TALEN technologies to introduce a complete *CFTR* cDNA into the endogenous *CFTR* genomic locus, within an intron close to the 5′ end of the gene. The major knowledge gap in this method is whether the regulation of the integrated transgene will be subject to the normal regulatory mechanisms of the locus. We, and others, have shown that *CFTR* has a complex tissue-specific control mechanism whereby intronic and intergenic enhancers (identified by open chromatin mapping, Fig. 2) are recruited to the gene promoter by chromatin looping to drive gene expression (21–24). This cell type-specific pattern of chromatin looping is clearly illustrated by circular chromatin conformation capture protocols, such as the 4C-seq shown in Figure 3. The *CFTR* promoter alone does not drive tissue-specific expression of the gene, so it may be essential for efficient transgene expression to not impact the recruitment of activating transcription factors, bound to distal enhancers, to the gene promoter. If the looping mechanisms are not impaired by insertion of a transgene into one of the upstream introns of the gene, then it may be possible to express a normally regulated *CFTR* transcript and protein while bypassing all other mutations located further downstream in the locus.

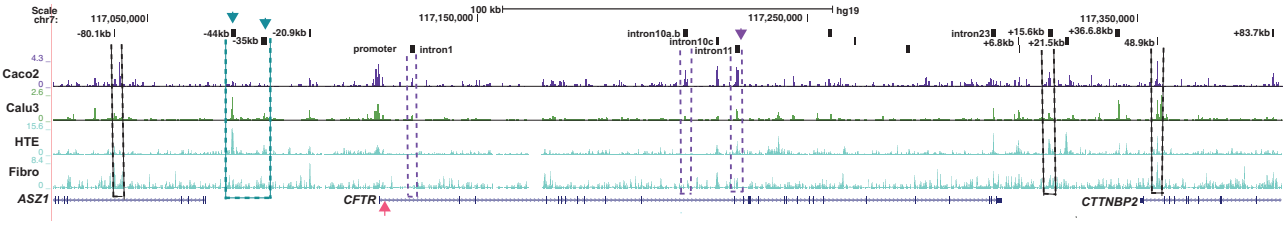

Figure 2. Open chromatin/DNase I hypersensitive sites (DHS) across the *CFTR* locus in CF-relevant cell types. DNase-seq data from Caco2 (colon carcinoma), Calu3 (lung adenocarcinoma) cell lines and HTE (primary human tracheal epithelial cells) (30,31). DHS are seen at the *CFTR* promoter and at several cell-type selective sites (23). Airway sites: teal dashed box (−44kb, −35 kb from the *CFTR* translational start site). Intestinal sites: purple boxes (introns 1, 10, 11). Ubiquitous sites; black boxes (−80.1kb, +15.6 kb, +48.9 kb 3′ to the *CFTR* last coding base). Each lane shows combined data from two biological replicas of the cell type. Pink arrow denotes a potential location of cDNA insertion in intron 1 of *CFTR*.

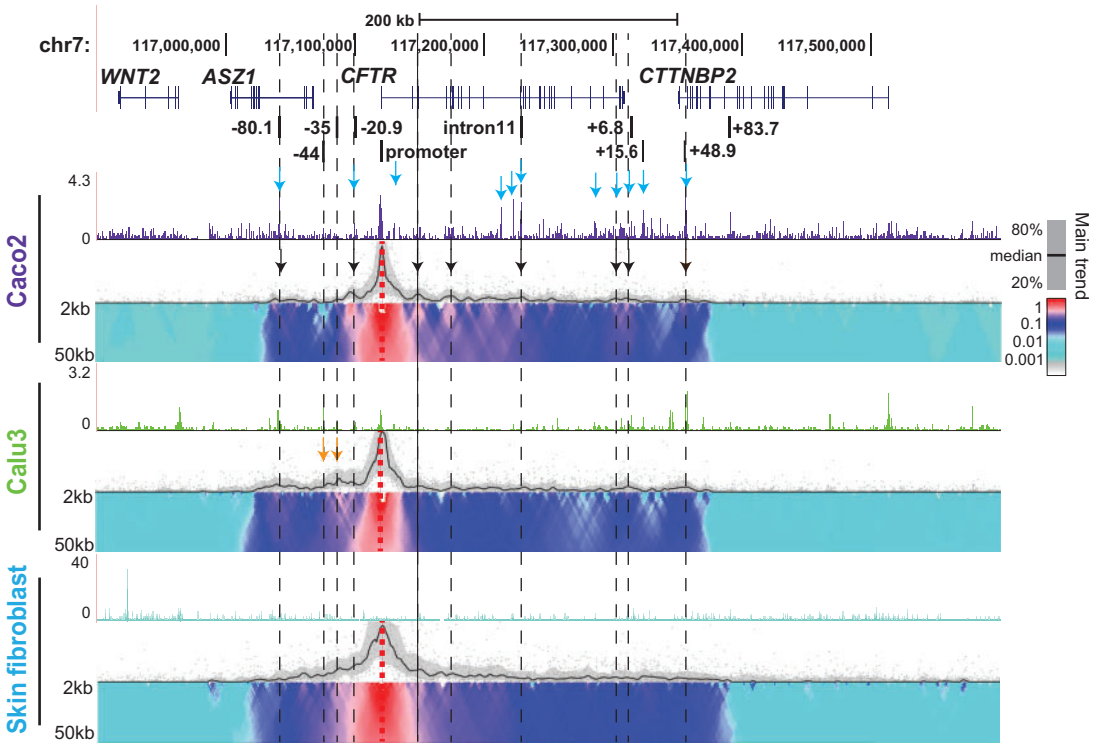

Figure 3. Cell-type-specific chromatin structure at the *CFTR* locus. 4C-seq profiles with a *CFTR* promoter viewpoint. Genomic location of *CFTR* and adjacent genes (chr 7) (top) and known *cis*-regulatory elements for the *CFTR* locus (below). The sites are named as in Figure 2. Open chromatin mapped by DNase-seq and 4C-seq data are shown for Caco2, Calu3 and skin fibroblasts. Representative data from one replica are shown. DNase-seq peaks show our data visualized on the UCSC genome browser. 4C-seq data are presented in alignment with the DNase-seq data and have two parts. The upper panel indicates the main trend of the contact profile using a 5-kb window size. Relative interactions are normalized to the strongest point (which is set to 1) within each panel. The lower panel is a domainogram with color-coded intensity values (see main text) to show relative interactions with window sizes varying from 2 to 50 kb. Arrows denote key data features: turquoise, known intestinal *cis*-regulatory elements in *CFTR*; peaks of interaction with *CFTR* promoter of known (black) *cis*-elements; orange, airway-selective regulatory elements and their interaction with the *CFTR* promoter in Calu3.

Common challenges of gene editing approaches

Significant challenges of all gene editing approaches include delivery, toxicity and potential off-target effects. These will not be considered in detail here as they are not unique to CF therapies, but deserve some comment. The development and refinement of highly efficient nanoparticle formulations (25) and adeno-associated virus (AAV) vectors (reviewed in 26) provide some optimism that even the mucus-obstructed CF lung epithelium may be accessible to these reagents. The active debate on the dangers of potential off-target gene editing by CRISPR/Cas9 is as relevant to CF as to many other genetic diseases. Perhaps more unique to CF is what fraction of cells in the airway epithelium need to be corrected in order to restore normal lung function and whether it will be possible to achieve this by focusing on stem cells in the lung. Also, it may be detrimental to induce high levels of CFTR expression in all cells in the lung epithelium, when expression of the gene is normally subject to tight cell-specific control mechanisms, including both activation (27,28) and repression (29).

Alternative Targets

An alternative strategy to repairing CFTR is to modulate other targets that can compensate for CFTR dysfunction. This mutation-agnostic approach could complement approved or advanced combination therapies that modulate F508del-CFTR (Introduction) by addressing the variation in response, and also provide a therapeutic strategy that benefits all patients.

The impact of CFTR loss on epithelial function in the airways (and several other tissues) has been reviewed elsewhere (32,33). As mentioned, abnormal CFTR function results in defective apical chloride and bicarbonate transport leading to altered airway surface liquid (ASL) that is dehydrated, reducing mucociliary clearance and innate immune defense mechanisms (34). The ASL is a fluid layer that covers the apical surface of airway epithelia and it has been proposed that modulating other ion channels, transporters and pumps that contribute to the delicate balance of ASL volume, ionic and nutrient content could compensate for loss of CFTR (33,35) (Fig. 4).

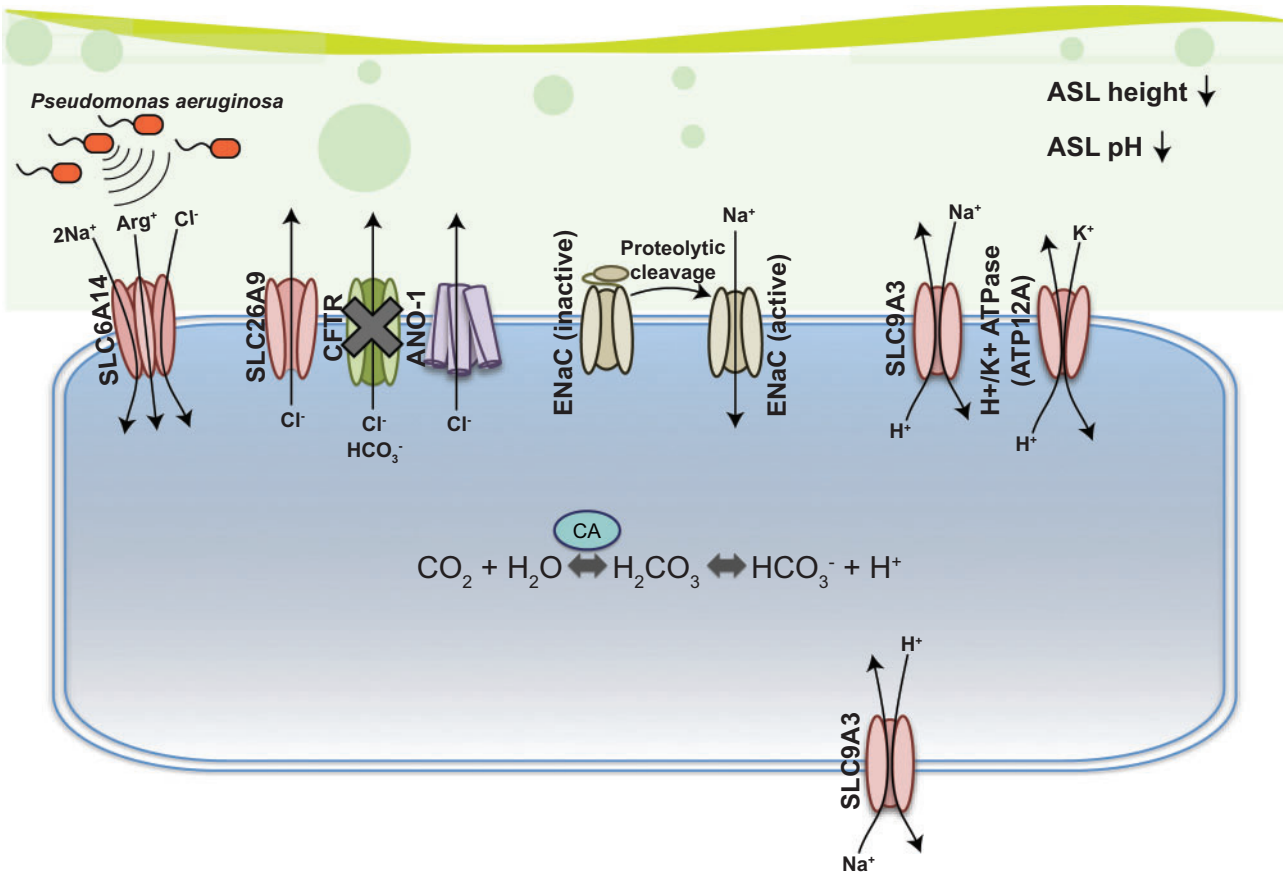

Figure 4. Hypothetical graphic of candidate therapeutic targets for CF airway disease. Figure adapted from (32,35,36). CFTR dysfunction in CF leads to decreases in chloride and bicarbonate secretion, which in turn lower ASL height due to impaired anion and fluid secretion (32). The impaired fluid secretion leads to severe mucus plugging and susceptibility to infection (37). Proteins shown in red are CF modifiers identified by Genome-Wide Association Studies (GWAS) (38–41). SLC6A14 stimulation has been shown to impact *Pseudomonas aeruginosa* infection (42). Stimulation of SLC26A9 and or ANO-1 (anoctamin-1; also known as TMEM16A) may restore chloride secretion into the lumen and thus fluidity. Inhibition of ENaC, either directly or indirectly by targeting proteolytic enzymes may also restore ASL hydration and height by preventing reabsorption of sodium ions. Lastly, ATP12A, a subunit of the nongastric H^+/K^+-ATPase, impairs the functioning of innate immune defenses, likely due to an increase in H^+ secretion, and thus, both ATP12A and SLC9A3 (aka. NHE3) could be inhibited in the apical membrane (and or stimulated in the basolateral membrane for SLC9A3) to prevent secretion of hydrogen ions into the ASL. Inhibition of SLC9A3 at the apical membrane is also beneficial due to reduced sodium reabsorption, which improves ASL fluidity. SLC, solute carrier; CA, carbonic anhydrase; ENaC, epithelial sodium channel; ASL, airway surface liquid; Arg^+, arginine; NHE3, Na^+/H^+ exchanger 3; CFTR, cystic fibrosis transmembrane conductance regulator.

Targeting other known channels

Other channels that inhibit excess sodium absorption or improve chloride transport such as the epithelial sodium channel (ENaC) and the calcium-dependent chloride channel (TMEM16A a.k.a anoctamin-1 or ANO-1, respectively) have been proposed as candidate therapeutic targets (33,35,36). Indeed, a review of the literature to catalogue ongoing clinical (or pre-clinical) trials of modulators of alternative targets identified several companies focusing on the same target, ENaC (Table 2).

ENaC is composed of three subunits (α, β and γ) (54) encoded by *SCNN1A, SCNN1B, SCNN1G*, and is regulated by SPLUNC-1 (short palate, lung and nasal epithelial clone-1), which protects ENaC from proteolytic cleavage, preventing its activation (55–57). In CF, ENaC contributes to dehydration of the ASL by mediating sodium absorption. More than 20 years ago, the ENaC blocker amiloride was tested in clinical trials by inhalation (58,59). These trials were not successful possibly due to the short half-life and potency of amiloride (60,61). More potent inhibitors such as benzamil and phenamil also proved unsuccessful (62). Later pre-clinical studies showed that early treatment (from birth) in β−ENaC-Tg mice (a CF mouse model) could prevent progression of CF lung disease (63). Direct inhibition of ENaC, however, led to hyperkalemia and sudden cardiac arrest because of effects on renal function (64). Current therapeutic paradigms aim to inhibit excessive ENaC function mainly through inhibition of channel activating proteases (CAPs) (Table 2). Broad inhibition of trypsin-like serine proteases (aprotinin and Camostat) is effective in attenuating ENaC and improving mucociliary clearance (65,66). However, more specific ENaC inhibitors are currently being evaluated (48–51). Candidate compounds that aim to inhibit ENaC-activating CAPs include QUB-TL1 (43) and NAP858 (44,67). Both compounds improve ASL height, mucociliary clearance and purport to delay CF lung disease. Another approach includes the use of a peptide mimetic of SPLUNC-1 (SPX-101), which was shown to greatly improve the survival of β-ENaC-transgenic mice (45). Other approaches targeting ENaC include nanoparticle formulations containing siRNA for ENaCα (46,68), and aerosolized ASOs (47).

TMEM16A is the channel responsible for the alternate Ca^{2+}-activated Cl^- channel (CaCC)-mediated secretion of chloride ions in the airways (69–71). Reports also show this channel is permeable to bicarbonate (72), and its stimulation may improve ASL fluidification and pH regulation (35,73,74). In the past, indirect TMEM16A stimulation using denufosol in CF patients failed to improve respiratory function in phase 3 clinical trials (75,76) likely due to the shorter half-life of the drug *in vivo* (74,77). Thus recent proposals aim to directly target the channel (78). While much progress has been made elucidating TMEM16A as an alternate chloride channel therapy in CF, more recent reports reveal an intricate connection and cross-talk of TMEM16A and CFTR. Specifically, ablation of TMEM16A in the airways (or gut) of mice obliterates CFTR-mediated chloride currents and induces a CF-like lung phenotype (79,80).

Alternative approaches such as the use of anionophores have been proposed as potential therapeutics as they confer anion channel activity (81). Cholapods (81) and decalins (e.g. bisureiododecalin) (82,83) show good promise in cell lines. *In vivo* testing, however, has not been carried out.

Targets identified through genome-wide studies

A potential new set of CF therapeutic targets arises through genome-wide association studies (GWAS) in CF population cohorts. These studies identify genetic loci that associate with the severity of disease in the presence of dysfunctional CFTR (i.e. modifier genes), irrespective of biological hypotheses. Notably, the clinical success rate of drugs in development is appreciably higher when there is human genetic evidence (from GWAS) that ties a target gene to a disease (84).

Several genome-wide studies (using exome sequencing and SNP arrays) have been carried out in CF. Modifier genes were identified that contribute to different phenotypes in the CF-affected organs and span the course of disease (Table 3). These include GWAS for intestinal obstruction at birth (meconium ileus) (39,40), infection with *Pseudomonas aeruginosa* (85,86), lung disease severity (87–89) and CF-related diabetes (38). These genome-wide studies identified common contributors [e.g. Solute Carrier Family 26 member 9 (*SLC26A9*) contributes to meconium ileus (40), CF-related diabetes (38) and CF lung disease (41)] and unique factors [e.g. EHF (ETS homologous factor)/APIP (Apoptotic peptidase activating factor 1 (APAF1) interacting protein) appears specific to lung disease severity] (Table 3). Consolidating evidence from studies of multiple CF phenotypes informs the potential broad or narrow impact should these be targeted as therapies, and provides insights into the differential relationships of modifiers with CFTR over developmental time and tissue (41).

Interestingly, 6 of the 10 loci identified through GWAS contained genes suggested to contribute to ASL volume, pH or bacterial defence, with several having already been investigated functionally as candidates contributing to CF including SLC26A9, Solute Carrier Family 6 Member 14 (SLC6A14), Solute

Table 2. Currently known active pharmacological agents targeting alternatives to CFTR and undergoing testing in patients with CF

Agent	Class	Company	References	Status
QUB-TL1	Inhibition of ENaC-activating CAPs	–	(43)	Pre-clinical
NAP858	Inhibition of ENaC-activating CAPs	–	(44)	Pre-clinical
SPX-101	SPLUNC-1 peptide mimetic (stimulates ENaC)	Spyryx Biosciences	(45)	Phase 2
GSK2225745	ENaCα siRNA	GlaxoSmithKline Plc	(46)	Pre-clinical
IONIS-ENAC-2.5_{Rx}	Antisense oligonucleotides (ASOs) against ENaCα	Ionis Pharmaceuticals	(47)	Pre-clinical
NVP-QBE170	ENaC inhibitor	Novartis	(48)	Pre-clinical
AZD5634	ENaC inhibitor	AstraZeneca	(49–51)	Phase 1b
ETD-001	Long-acting ENaC inhibitor	Enterprise Therapeutics	(52)	Pre-clinical
QBW276	ENaC inhibitor	Novartis	NA	Phase 2
ET000516-A-2	TMEM16A activator	Enterprise Therapeutics	(53)	Pre-clinical

Table was compiled in May 2018 from the review of ClinicalTrials.gov and the US Cystic Fibrosis Foundation website (https://www.cff.org/Trials/Pipeline/) of ongoing clinical trials as well as industry-released public announcements and a PubMed search. CAP, channel activating protease. NA, not available.

Table 3. Loci identified through genome-wide studies to contribute to CF co-morbidities and their functional characterization in CF

Posited gene at associated locus[a]	GWAS/Exome-associated phenotypes	GWAS/Exome reference	Functional characterisation in CF
MUC4/20	Lung function	(89)	• Encode cell surface-associated airway cell mucin 4 and 20, respectively • Important for creating an osmotic barrier at the periciliary layer, and ciliary motility • Mucins are over-abundant in patients with CF, leading to osmotic compression of the periciliary layer, and consequently mucociliary stasis and susceptibility to infection (reviewed in 106)
SLC9A3 (a.k.a. NHE3)	Lung function, Meconium ileus	(40,89)	• Major absorptive Na^+/H^+ exchanger, and mice lacking *Slc9a3* have impaired acid-base balance and Na^+-fluid volume homeostasis (107) • Deletion of *Slc9a3* rescues the intestinal phenotype in *Cftr*$^{-/-}$ mice and significantly improves their survival (104) • Regulation of SLC9A3 and CFTR by SLC9A3R1, SLC9A3R2 and ezrin (EZR), which bind via PDZ domains, has been extensively reviewed in (108) • Gene variation affecting the SLC9A3/SLC9A3R2/EZR complex may influence lung disease severity in CF (88)
HLA-DRA	Lung function	(89)	• Class II HLA (human leukocyte antigen) regulates immune response via antigen presentation to T lymphocytes • Expression variation of HLA genes and HLA immune response pathways associate with lung disease severity in patients with CF (109,110)
EHF	Lung function	(89)	• Ets family transcription factor expressed in epithelial cells • GWAS locus lies in an intergenic region between *EHF* and *APIP* genes, but physical interactions of regulatory elements confirm enhancer–promoter interactions of the GWAS locus with the *EHF* promoter (111) • EHF allele variation alters regulation of genes responsible for protein glycosylation and trafficking, processes that may be important for the folding and trafficking of F508del-CFTR, in rectal biopsies of F508del patients (112) • EHF expression variation regulates genes involved in wound closure, transepithelial resistance, inflammation and goblet cell hyperplasia (113,114) • EHF represses *CFTR* expression, with EHF knockdown improving CFTR production and function in cultured Calu-3 airway epithelial cells (29)
AGTR2	Lung function	(89)	• Encodes angiotensin II receptor type 2 • GWAS locus lies in between *AGTR2* and *SLC6A14*, and GTEx data (100) suggest associated variants are eQTLs for both genes • Deletion of *Agtr2* in *Cftr*$^{-/-}$ mice restores airway compliance, elastance, and airway damping (115,116)
SLC6A14 (a.k.a. $ATB^{0,+}$)	Lung function, Meconium ileus	(40,89)	• Sodium- and chloride-dependent neutral and cationic amino acid transporter shown to have a role in maintaining low luminal amino acid levels • Regulator of *Pseudomonas aeruginosa* (PsA) attachment to bronchial epithelial cells (42,117)
SLC26A9	Meconium ileus, CF-related diabetes	(38,40)	• Anion channel in epithelial cells (118,119), with STAS domain that interacts with the CFTR R domain (120) • Interaction enhances the functional expression of CFTR (90,121,122) • *Slc26a9*$^{-/-}$/*CFTR*$^{-/-}$ DKO mice have a much lower rate of survival post-birth than *Cftr*$^{-/-}$ or *Slc26a9*$^{-/-}$ mice (93)
DCTN4	*PsA* infection	(85)	• Encodes dynactin 4 (p62), a subunit of the 20S dynactin complex, involved in microtubule organization through processing of dynein, acting as an adapter between dynein and the cargo (123) • Has a role in ciliary beat via dynein processing (124,125)
CAV2	*PsA* infection	(86,126)	• Important component for caveolae formation and *Pseudomonas aeruginosa* infiltration (127)
TMC6	*PsA* infection	(86,126)	• Integral transmembrane protein that indirectly influences intracellular zinc concentrations, and mutations in this gene are characterized by an increased sensitivity to human papilloma virus (HPV) (128) • Unknown role in CF

(continued)

Table 3. (Continued)

Posited gene at associated locus[a]	GWAS/Exome-associated phenotypes	GWAS/Exome reference	Functional characterisation in CF
ATP12A	Meconium ileus	(39)	• The α subunit of the nongastric H^+/K^+ adenosine triphosphate (ATPase) • Responsible for the acidification of ASL pH in pigs and humans, resulting in an increased susceptibility to bacterial infections and compromised host defenses (105) • Chronic inflammation in the lungs (via IL-13) increases ATP12A expression and function, which leads to increased ASL viscosity and impaired mucociliary clearance (129) • In the pancreatic ducts, ATP12A is co-expressed with CFTR, carbonic anhydrase, and aquaporins where it may regulate the HCO_3^-/H^+ transport for the alkalinization of pancreatic fluid (130)

PsA, *Pseudomonas aeruginosa*; DKO, double knockout; GWAS, genome-wide association study; GTEx, genotype-tissue expression project.
[a]Based on evidence from published functional studies or associated protein-coding variation from exome analysis.

Carrier Family 9 Member 3 (SLC9A3) and ATPase H+/K+ Transporting Non-Gastric Alpha2 Subunit (ATP12A) (Table 3). Of note, GWAS did not provide association evidence for the ENaC subunits (*SCNN1A*, *SCNN1B* and *SCNN1G*) or TMEM16A.

SLC26A9 is an anion chloride channel that contributes to constitutive apical chloride conductance and enhances cAMP-regulated CFTR currents (90–92). It has been suggested to partially compensate for loss of CFTR channel function in the intestine in *in vivo* studies of *Cftr*$^{-/-}$ mice (93). In genome-wide studies of CF patients with severe CF-causing *CFTR* genotypes including F508del homozygosity, the same noncoding variation in the *SLC26A9* gene is associated with meconium ileus (40), CF-related diabetes (38) and early exocrine pancreatic disease (94). The role of SLC26A9 in these early-onset phenotypes is presumed to be mediated through exocrine pancreatic damage *in utero* (39,94–96). *SLC26A9* was not a modifier of CF lung disease when the majority of patients included were homozygous for F508del [(89); Fig. 5]. However, in untreated and Kalydeco-treated patients with CFTR gating mutations, and in CF F508del/F508del primary bronchial cultures treated with the CFTR corrector component of ORKAMBI™ (lumacaftor or VX-809), there was a dose–response relationship with the improvement in CFTR function dependent on *SLC26A9* SNP genotype (41,91). Bertrand *et al.* (91) show that *SLC26A9* does not confer chloride currents in epithelial cells expressing F508del. Taken together, the working hypothesis is that in the CFTR-F508del lungs, the SLC26A9 benefit to CFTR function is only achieved alongside correction of CFTR-F508del (91,97). The interplay between SLC26A9 and other CFTR mutations requires investigation.

Further supporting the complex relationship between CFTR and SLC26A9 in the lungs are the GWAS results for the airway obstruction lung function measure of peak expiratory flow (PEF) in individuals from the UK Biobank (98) (Fig. 5). The UK Biobank is a study following more than 500 000 volunteers (http://www.ukbiobank.ac.uk/; date last accessed June 2018). These individuals were sampled from across the United Kingdom and were between the ages of 40 and 69 at recruitment. Many of the phenotypes collected have been analyzed genome-wide and results made publicly available by the Global Biobank Engine (131) (http://gbe.stanford.edu; date last accessed June 2018). PEF was measured in 307 638 of the more than 500 000 participants. In these 307 638 individuals presumed to have functional CFTR, variants 5′ of *SLC26A9* are associated with PEF (Fig. 5, colored dots; min p=1.27e-28 for rs1342062), and the association pattern mirrors the GWAS results for meconium ileus in CF (Fig. 5, solid green line). This is in stark contrast to the lack of association

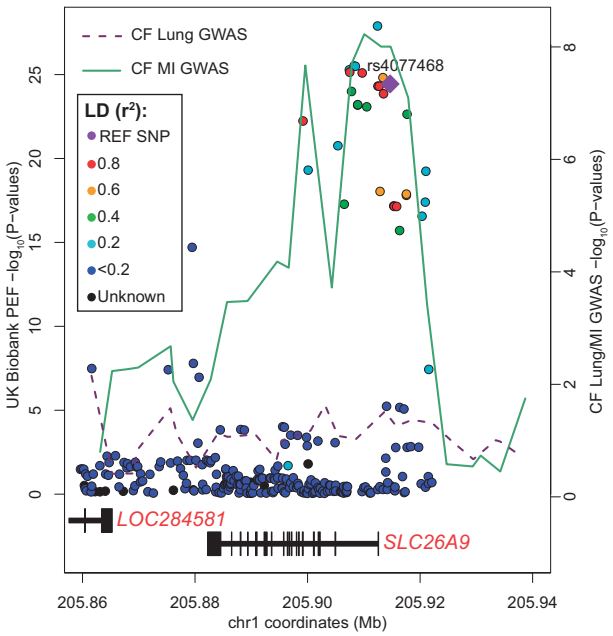

Figure 5. Genetic association of Peak Expiratory Flow (PEF) and Meconium Ileus (MI) co-localize at the *SLC26A9* locus. The lines connect the lowest *P*-value at 3.2 kb windows across the genomic region from the GWAS of the International CF Gene Modifier Consortium for MI (green line) (40) and lung function (magenta line) (89). Dots in the figure represent the genetic association with PEF in 307 638 unrelated individuals from the UK Biobank resource [analyzed and available in the Global Biobank Engine, Stanford, CA (http://gbe.stanford.edu) accessed January 2018]. The marked SNP (purple diamond shaped) was the top SNP in the MI GWAS (40). These individuals are sampled across the United Kingdom and are aged between 40 and 69 at recruitment (98). PEF is measured in L/min and measures the maximal exhalation rate during an expiratory effort. The MI genetic association at the *SLC26A9* locus mirrors the association pattern observed for PEF in the UK population, whereas there is not evidence of genetic association for lung function in patients with CF. The CF MI and lung GWAS *P*-values may be accessed from http://lab.research.sickkids.ca/strug/publications-software/; date last accessed June 2018.

demonstrated for CF lung disease in individuals predominantly homozygous for F508del (Fig. 5, magenta dotted line) and supports the functional studies showing differential SLC26A9 interaction with wild-type CFTR and with F508del-CFTR in human bronchial epithelia (41,91). Furthermore, the *SLC26A9* relationship with pulmonary function in the general population highlights the potential of SLC26A9 as a therapeutic target to

enhance lung function in other pulmonary obstructive diseases; indeed in asthma SLC26A9 has demonstrated prevention of airway obstruction and mucus plugging (99). There is no association evidence for the ENaC subunits (*SCNN1A*, *SCNN1B* and *SCNN1G*) or for TMEM16A with PEF in the UK Biobank (http://gbe.stanford.edu).

The mechanism of action and direction that SLC6A14 should be modulated is less clear. The *SLC6A14* locus was associated with both meconium ileus and CF lung disease (Table 3) in GWAS. Expression quantitative trait locus (eQTL) analysis in lung tissue provided by the Genotype Tissue Expression Consortium [GTEx; version v6p (100)] show the risk allele for both phenotypes to be associated with increased *SLC6A14* messenger RNA (39). This appears inconsistent with results in primary bronchial epithelial cell cultures from patients with CF and *ex vivo* lungs and trachea explants of *Slc6a14*$^{-/y}$ mice showing greater attachment of *Pseudomonas aeruginosa* in the presence of SLC6A14 inhibitor α-methyl-L-tryptophan (α-MT), or greater bacterial burden in the absence of the transporter (42). SLC6A14 expression and amino acid uptake activity are upregulated via inflammatory cytokines (101–103), suggesting a role in reducing the viability of pathogens (42,102).

SLC9A3 and *ATP12A* inhibition may also improve ASL height and pH in the airways. It has been proposed that SLC9A3 inhibition at the apical membrane and/or stimulation at the basolateral membrane would help with ASL pH, and inhibition of sodium absorption would help with ASL height (35). Indeed, *Cftr*-null mice lacking one or more copies of *Slc9a3* have improved fluidity of intestinal contents (104). Given the role of ATP12A in secreting protons to the ASL (105), drugs that aim to inhibit the nongastric H+/K+ ATPase are suggested (35).

Although the list of ongoing clinical (or pre-clinical) trials (Table 2) is certainly not exhaustive, efforts targeting the CF modifier loci (Table 3) were not identified, possibly due to early stages of investigation.

Concluding Remarks

There is significant allelic heterogeneity in the causal CF gene, *CFTR*. The different mutations pose a variety of challenges for restoring protein function, which cannot be addressed by a single therapeutic strategy. We reviewed the therapeutic paradigm for the F508del mutation, which now combines several molecules to address the folding, stability and gating limitations of the protein. Phase 3 trials show significant promise for these combination therapies that may enhance the average effect size of the approved drug ORKAMBI™, but many mutations remain for which there are no treatment options. Alternative strategies are required to ensure effective treatments are available for all patients. These alternatives include correcting, editing or replacing CFTR, or modulating alternative channels, transporters or pumps to compensate for dysfunctional CFTR. Though many elegant approaches to editing or circumventing *CFTR* mutations at the DNA or RNA level are currently being developed, some may be dependent on passing necessary ethical and regulatory hurdles before they become a reality. To our knowledge, therapies targeting alternatives to CFTR have largely focused on ENaC and TMEM16A, while several other relevant targets have been identified through genome-wide studies in CF. Genome-wide data from CF and population-based studies can be leveraged to guide future efforts to prioritize alternative therapeutic targets.

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