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REVIEW

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# Discovery of CFTR modulators for the treatment of cystic fibrosis

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**ABSTRACT**

**Introduction:** Cystic fibrosis (CF) is a life-threatening inherited disease caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, an anion channel expressed at the apical membrane of secretory epithelia. CF leads to multiorgan dysfunction with progressive deterioration of lung function being the major cause of untimely death. Conventional CF therapies target only symptoms and consequences downstream of the primary genetic defect and the current life expectancy and quality of life of these individuals are still very limited.

**Area covered:** CFTR modulator drugs are novel-specialized therapies that enhance or even restore functional expression of CFTR mutants and have been approved for clinical use for individuals with specific CF genotypes. This review summarizes classical approaches used for the pre-clinical development of CFTR correctors and potentiators as well as emerging strategies aiming to accelerate modulator development and expand theratyping efforts.

**Expert opinion:** Highly effective CFTR modulator drugs are expected to deeply modify the disease course for the majority of individuals with CF. A multitude of experimental approaches have been established to accelerate the development of novel modulators. CF patient-derived specimens are valuable cell models to predict therapeutic effectiveness of existing (and novel) modulators in a precision medicine approach.

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## 1. Introduction

Cystic fibrosis (CF), the most common lethal autosomal recessive inherited disease in Caucasians, is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein, a chloride ($Cl^-$) and bicarbonate ($HCO_3^-$) channel expressed at the apical plasma membrane (PM) of epithelial cells [1,2]. The premature degradation or dysfunction of CFTR protein leads to an impaired transepithelial balance of fluid and electrolyte, resulting in dehydration and alteration of physicochemical properties of mucus in the epithelial lining of several organs [1,2]. Clinically, the disease is characterized by severe sinopulmonary and gastrointestinal manifestations, pancreatic insufficiency, elevated levels of sweat chloride, and male infertility, among other symptoms and signs. Despite its multifaceted effects, the progressive deterioration of lung function depicts the major cause of morbidity and mortality in CF [1–3].

Traditionally, CF therapeutic regimens have been focused on managing symptoms and preventing complications. These include time-consuming physical and inhaled therapies, and a large number of daily medications to enhance airway clearance, alleviate inflammation, combat lung infections and supplement the missing pancreatic enzymes. Together with early diagnosis and improvements in interdisciplinary healthcare, CF life expectancy has increased from the early childhood in the 1960s to beyond the third decade nowadays [4,5]. However, these individuals are still susceptible to substantial clinical and psychosocial burdens, which negatively impact on their quality of life [3]. In order to efficiently enhance quality of life and the current life expectancy in CF, the disease must be treated beyond its symptoms by targeting the primary defect associated to CFTR mutations, thereby preventing the pathological cascade of events downstream of CFTR dysfunction.

CFTR is a member of the ATP-binding cassette (ABC) transporters superfamily and its structure is composed of two equivalent halves, each containing a transmembrane domain (TMD1 and TMD2) and a nucleotide-binding domain (NBD1 and NBD2) (Figure 1). The TMD segments form the channel pore through which anions are conducted and these segments are linked through extra– and intracellular loops (ECLs and ICLs, respectively). The NBDs regulate channel gating by binding and hydrolyzing ATP. Finally, a highly disordered regulatory domain (RD) – unique to CFTR – connects the two halves of the protein and participates in the regulation of channel activity [6,7]. Interdomain interactions occur during CFTR folding process and these are crucial for this complex protein to achieve its native conformation and channel function [8].

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**Article Highlights**

- Different CFTR modulator therapies are required for CFTR mutants with distinct primary defects.
- Unraveling the mechanisms of action of individual modulators will aid in selecting combinations with additive/synergistic actions to increase clinical outcomes.
- *In vitro* studies have been useful in extending the label of approved modulator therapies for rare and ultra-rare CFTR mutations.
- A multitude of novel approaches have been developed to circumvent barriers and accelerate the discovery of novel modulator drugs.
- Patient-derived specimens are valuable tools for precision medicine application.
- Highly effective CFTR modulator therapies are available for clinical use and are expected to deeply modify the disease course for the majority of individuals with CF.

This box summarizes key points contained in the article.

Although the deletion of a phenylalanine at position 508 (F508del in NBD1) is the most prevalent CFTR mutant and found in ~80% of individuals with CF worldwide [1,3], over 2,100 CFTR gene variants have been identified (Cystic Fibrosis Mutation Database, http://www.genet.sickkids.on.ca/Home.html), of which more than 350 have confirmed disease liability (Clinical and Functional Translation of CFTR, https://cftr2.org/). These mutants have been segregated into six main classes according to their primary cellular defects, characterized by alterations in: expression (class I), folding/traffic (class II), gating (class III), conductance (IV), abundance (class V) and PM stability (class VI) [3,9,10]. Nevertheless, several mutants possess pleiotropic defects and are associated to multiple classes, including F508del, which primarily impairs CFTR protein folding, processing and trafficking to the PM, but also exhibits defective gating and reduced stability when it achieves the PM [9–13].

Over the last decade, significant efforts into high-throughput screening (HTS) of small molecule libraries have enabled the identification of CFTR modulator drugs. These specialized types of small molecules can restore the cooperative domain folding of the CFTR protein and its trafficking to the PM (correctors), enhance the channel open probability (potentiators), or stabilize the protein when it is located at the PM (stabilizers). In addition of these modulator types, read-through agents and amplifiers are in early-stage clinical trials aiming to provide 'on target' therapies for individuals with CF carrying premature termination codon mutations, which lead to no CFTR protein synthesis or translation of shortened, truncated forms [3]. Indeed, the first-in-class amplifier was found to selectively enhance CFTR translation by stabilizing CFTR mRNA [14], which might compensate eventual failures over the various steps of CFTR gene expression and thus promoting additive actions in combination with other CFTR modulator drugs. Moreover, microRNA-based therapeutics may have a potential role as adjunct strategies to enhance CFTR modulator drugs effects and have been further discussed elsewhere [15].

The CF drug development pipeline continues to expand with the discovery of novel small molecules from different chemical series able to correct specific defects in the mutated CFTR structure. Furthermore, recent novel insights into the 3D CFTR structure provide great support for the rational design of next-generation modulator drugs [6,7]. To date, four CFTR modulator drugs have reached the market: the potentiator VX-770 (ivacaftor) and the correctors VX-809 (lumacaftor), VX-661 (tezacaftor) and VX-445 (elexacaftor). These drugs represent landmarks in CF therapeutics as they act on the root cause of the disease and have demonstrated to improve lung function and nutritional status and reduce pulmonary exacerbations and sweat chloride levels in individuals with specific CF genotypes [16–21]. Despite such progress, there is still scope for further enhancement as these drugs only partially correct CFTR dysfunction [22,23] and long-term clinical studies indicate that individuals with CF still face disease-related symptoms and complications, albeit at lower frequency [22,23]. Furthermore, the process of matching modulators with mutations, known as theratyping, remains unfinished. Indeed, some CFTR mutations are demonstrated to be unresponsive or only modestly corrected (below therapeutically relevant levels) by experimental and clinically available modulator drugs [24–32]. A considerable challenge persists for rare and ultra-rare CFTR mutations as traditional

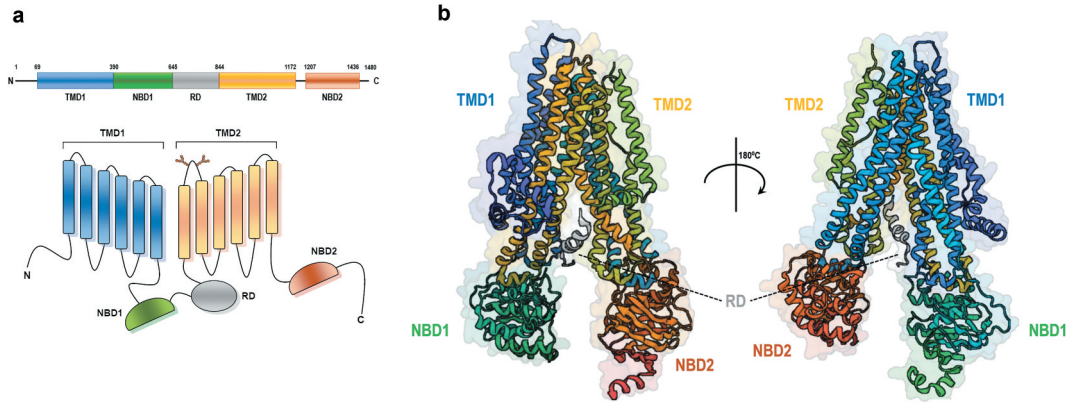


Figure 1. Overall structure of CFTR. **(a)** CFTR structure is composed of 5 domains: two transmembrane domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2) and a regulatory domain (RD). **(b)** Ribbon diagram of two views of the dephosphorylated, ATP-free conformation of human CFTR. Notably, only a small fraction of RD is represented as most of its structure remains unresolved (PDB: 5UAK) [6].

clinical trial designs may not be feasible (and underpowered) due to low number of individuals worldwide.

Various experimental protocols and assays have been developed and optimized to evaluate modulator effects in *ex vivo* individual-derived specimens, such as human nasal epithelial (HNE) cells as a surrogate for human bronchial epithelia (HBE), and intestinal/respiratory organoids [33–37]. These cell models are precision medicine tools as they recapitulate several features of parental tissue and may be used to predict therapeutic effectiveness of modulators at an individual level. Here, we have summarized classical and novel approaches for the development of CFTR modulator drugs and remaining challenges that should be addressed to expand the number of individuals with CF benefiting from highly effective modulator therapies.

## 2. Classical drug discovery

The identification of CFTR modulator drugs has been pursued primarily by screening libraries of natural or synthetic compounds, having maximal chemical diversity, using a diversity of cell models and HTS methodologies. The search for CFTR potentiators has been particularly successful since its beginning. Indeed, many active compounds were identified during the very first campaigns of HTS [38,39]. However, only one of these potentiators – the already mentioned VX-770 [40] – has been approved so far for clinical use. On the other hand, the search for CFTR correctors has been more challenging, and, at least in its early phases, less successful compared to that for potentiators. While in the case of potentiators the use of a functional assay for the primary screens was intuitive, the identification of correctors relied on HTS approaches using functional or biochemical assays, based on the assumption that improved CFTR processing could be detected by measuring CFTR activity and expression at the PM.

### 2.1. The first drug discovery projects: classical assays and cell lines

The first F508del-CFTR correctors were reported in 2005 [41]. A large library consisting of 150,000 small molecules was screened using the microfluorimetric, functional assay based on a halide-sensitive yellow fluorescent protein (HS-YFP) (Figure 2A). Fischer rat thyroid (FRT) epithelial cells co-expressing F508del-CFTR and the HS-YFP were chosen as cell model given their negligible activity of endogenous $Cl^-$ channels, and their suitability with HTS workflow [42–44]. The study led to the identification of five chemical classes of F508del-CFTR correctors, among which class 4 correctors (Corr-4) appeared as the most interesting, improving folding efficiency and thus stabilizing core-glycosylated F508del-CFTR mutant, and showing modest efficacy on primary HBE derived from individuals with CF homozygous for the F508del mutant [41].

In the same period, Vertex Pharmaceuticals was also pursuing the discovery of correctors. A library of 164,000 drug-like molecules was screened using a microfluorimetric, functional assay on NIH-3T3 cells, a mouse embryonic fibroblast cell line, stably expressing F508del-CFTR [45]. The functional assay detected mutant CFTR channel activity as changes in a fluorescence signal (resembling cellular membrane potential) caused by cAMP-stimulated depolarization in the presence of a $Cl^-$ gradient (Figure 2B). The first correctors screen resulted in the identification of 13 chemical classes and a counter screen in a second cell model (FRT cells stably expressing this CFTR mutant) confirmed the activity as correctors for 6 of these classes [45]. Subsequent round of optimization of one of the hit compounds identified in this primary screening led to corrector VX-809, particularly effective on CF primary bronchial epithelia [46]. Indeed, its *in vitro* efficacy pushed forward its advancement into clinical trials, considering also the favorable drug properties.

A third example of drug discovery project for correctors was the one performed at the McGill University, where a library of 42,000 compounds was tested by means of a biochemical high-throughput assay on baby hamster kidney (BHK) cells stably expressing F508del-CFTR bearing a triple hemagglutinin (HA) epitope tag on the fourth extracellular loop, so that expression at the PM could be detected using a fluorescent secondary antibody [47] (Figure 2C). This study identified several active compounds, in particular the approved drug sildenafil, having activity as F508del-CFTR correctors. Later, the same authors adopted a new assay, based on differential scanning fluorimetry, to identify compounds directly acting on the mutated NBD1 domain of F508del-CFTR. On this way, the authors were able to demonstrate the ability of the previously identified phenylhydrazone RDR01752 [48] to bind to and stabilize purified murine F508del-NBD1 *in vitro* [49].

Very recently, a novel assay exploiting high-content imaging methodology has been developed [50]. This assay provides simultaneous measurements of both CFTR function and CFTR membrane proximity based on the expression of two fluorescent proteins: a cytosolic mCherry protein and a HS-YFP fused to the intracellular N-terminal of CFTR [50]. The mCherry expression allows image segmentation and accurate localization of the cell membrane by marking the border of cells, but it is also useful as an internal standard for the normalization of YFP-CFTR expression [50]. The time course of YFP quenching in response to extracellular iodide addition informs on anion conductance [50]. At the same time, evaluation of fluorescent signals corresponding to total cellular YFP-CFTR and YFP-CFTR within the membrane-proximal zone provides a readout of efficiency of CFTR maturation and trafficking, as well as of the overall rates of biosynthesis and degradation of the protein [50].

### 2.2. Relevance of primary cell models in the development of CFTR modulators

In general, the development of assays for CFTR modulators based on heterologous expression systems has several advantages. First, these cells are available in large quantities, and usually their cell culture procedures are relatively easy. Second, heterologous expression systems allow to study any CFTR mutant, once the desired mutation has been introduced in the CFTR coding sequence and then, by means of an appropriate expression vector, delivered to the recipient cell.

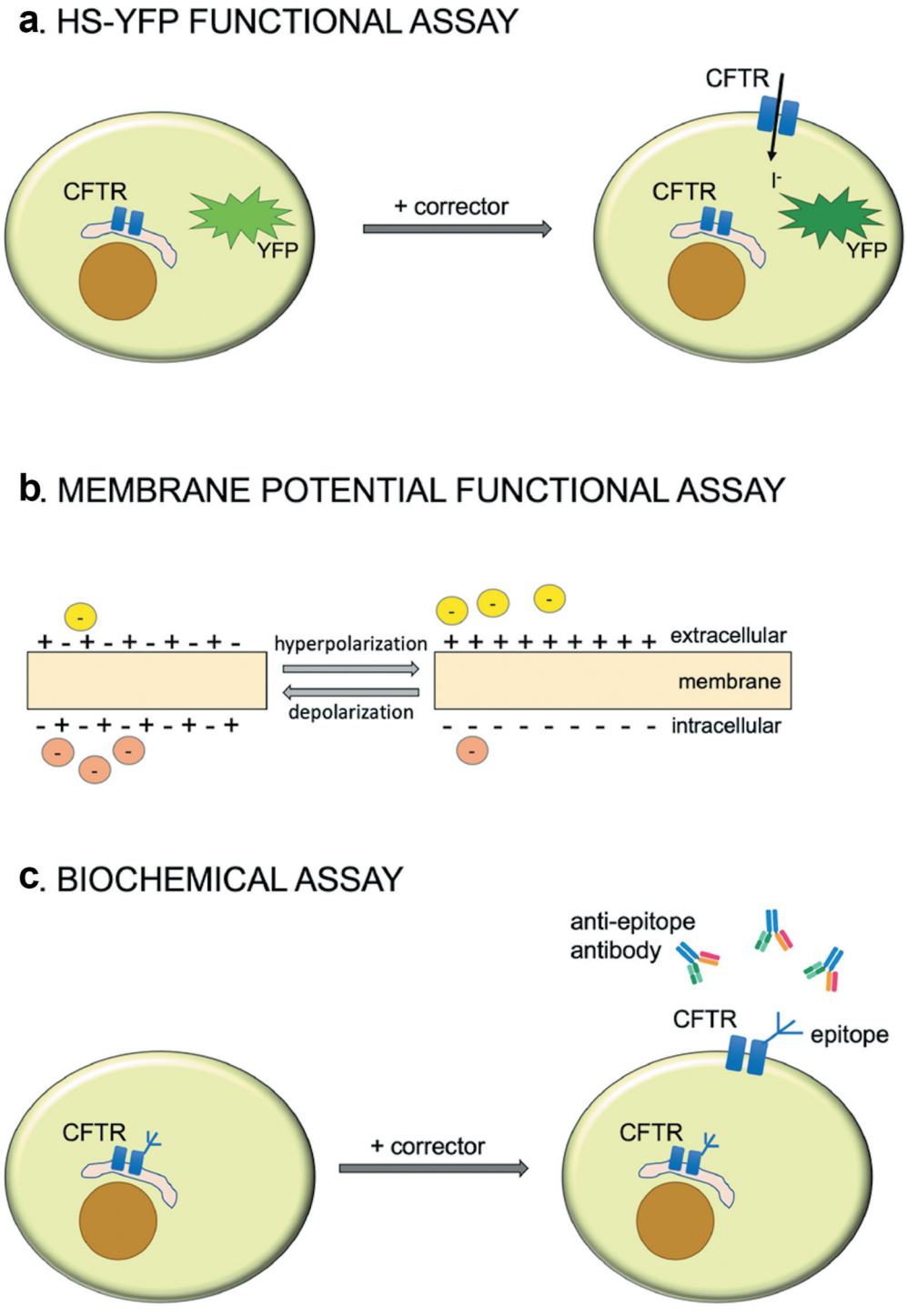


Figure 2. Classical assays used in CFTR modulator discovery process. **(a)** Halide sensitive-yellow fluorescence protein (HS-YFP) assay [41,44]. **(b)** Membrane potential functional assay [45]. **(c)** Biochemical assay [47].

On the other hand, primary HBE cells from individuals with CF are usually available in limited number and cannot be used for large-scale studies. Primary cell cultures are usually more expensive and difficult to obtain, in particular for uncommon CFTR mutations. In addition, responsiveness of a rare CF mutation in these native cells might be difficult to assess due to the presence of a different mutation in the second allele.

It should be considered that only primary airway epithelial cells derived from individuals with CF may provide the advantage to test compounds in a native system. In the case of pharmacological modulation of gating mutations, this might be not relevant, since potentiators are in general not affected by cell background [51]. However, it has been demonstrated that corrector activity is strongly influenced by cell

background, and pharmacological rescue of F508del-CFTR may differ when the same compound is evaluated in different cell models [51]. As a consequence, it is highly recommended that putative correctors identified by HTS in a heterologous expression system, are rapidly validated in primary HBE. However, validation of many putative correctors on primary cultures of bronchial cells might be impractical, depending not only on the genotype under investigation, which is particularly important when dealing with precision medicine approaches, but also on the type of assays to be performed. Indeed, the classical method for testing the efficacy of modulators on primary airway epithelial cultures consists of short-circuit current recordings, which is a low-throughput electrophysiological technique. However, a novel assay has been recently proposed, based on the use of a membrane potential sensitive dye to monitor apical chloride conductance mediated by normal and mutant CFTR in airway epithelium, in a medium-throughput manner [52].

The use of immortalized CF bronchial epithelial (CFBE41o$^-$) cells heterologously expressing a desired CFTR mutation may be a feasible option to narrow the number of hit compounds to be further validated in primary HBE cells as it is a more physiologically relevant cell model to CF compared to other cell lines. Distinct systems have been optimized to facilitate the generation of CFBE41o$^-$ cell lines expressing certain CFTR mutations, including those which allow for incorporation of multiple copies, inducible promoters or containing a single recombinant target site for integration [24,53–55].

The increasing number of CFTR modulator drugs that are in experimental development or in clinical trials has urged the need for predictive *in vitro* models for testing CF therapies in a precision approach. Several protocols have been optimized to circumvent the limitations in obtaining HBE by using other patient-derived specimens, including HNE cells [33,34] and rectal biopsy-derived intestinal organoids [35,36]. These cell models also offer the advantage of predicting modulator effectiveness at an individual level. Indeed, a variability in therapeutic responses for modulator therapy has been observed even in those individuals carrying the same CF genotypes [3,16,56]. These novel cell models are further discussed in section 3.

### 2.3. Mechanism of action of modulators

The reason why the activity of CFTR modulators can be influenced by cell background resides in their mechanism of action. Several evidences demonstrate that potentiators act by interacting directly with CFTR. This interaction can occur at different sites of CFTR domains. Several potentiators, including genistein, likely interact with the NBD1:NBD2 dimer to modify ATP-dependent channel gating [57–59]. On the other hand, in the case of VX-770 and ABBV-974, an investigational potentiator, their binding site is more likely located between the TMDs near a twist in the transmembrane segment 8 [60,61].

Small molecules that overcome protein misfolding improving mutant CFTR trafficking to the PM may have different mechanism of actions, acting as either pharmacological chaperones or as protein homeostasis (or proteostasis, i.e. dynamic balance of the proteome) regulators [62]. Proteostasis regulators lead to beneficial effects on CFTR processing by modulating the proteostasis network, which includes all the molecular components that control biogenesis, folding, trafficking and degradation of proteins [62–66]. On the opposite, pharmacological chaperones act directly on mutant CFTR by stabilizing specific CFTR domains and/or by improving CFTR interdomain interactions [55,65]. Thus, only pharmacological chaperones are indeed CFTR modulators, since the target of proteostasis regulators is not CFTR itself, but other cell proteins whose modulation eventually results in increased CFTR processing. As a consequence, activity of proteostasis regulators may be dependent on the cell model on which the compounds are being tested, whereas the activity of CFTR correctors (i.e. pharmacological chaperones) is usually conserved across different cell models [51].

Single correctors demonstrated to have limited efficacy in rescuing F508del-CFTR, and the consequences of F508del on NBD1 folding and its interdomain interactions was further investigated [2,66]. By using CFTR genetic revertants (i.e. second site mutations that are *in cis* with F508del) able to correct either NBD1 misfolding or its interaction with ICL4, Mendoza and collaborators demonstrated that a higher level of CFTR rescue could be achieved when combining revertants that act on different structural defects, thus bypassing the efficacy ceiling [66]. Thermodynamics analyses of NBD1 folding and the stability of the NBD1:ICL4 domain interface led to similar conclusions [67]. Based on these studies, the existence of three different classes of CFTR correctors was postulated, depending on their mechanism of action: type I correctors targeting the NBD-TMD interface, type II targeting NBD2 and/or its interfaces and type III facilitates NBD1 folding and/or impedes its unfolding [65]. Analysis of known CFTR correctors demonstrated that VX-809 is the prototype of the first class of molecules, while Corr-4a belongs to type II correctors [65]. A recent analysis of the mechanism of action of RDR01752 indicated that this compound fills the pocket at the NBD1:ICL4 interface in F508del-CFTR, thus acting as a type I corrector, similarly to VX-809 and VX-661 [68], and the investigational correctors ABBV-2222 and FDL-169 [26].

This increased knowledge about the mechanisms of action of correctors has allowed novel drug discovery campaigns to identify novel compounds targeting distinct structural defects of F508del-CFTR. A recent study performed by the Lukacs group reported the screening of a library of 600,000 drug-like compounds by means of a novel biochemical high-throughput assay based on CFBE41o$^-$ cells expressing F508del-CFTR containing a horseradish peroxidase isoenzyme C (HRP-C) in its fourth extracellular loop [55]. Two primary screens were performed to preferentially select type II and type III corrector hits: one in the presence of VX-809 and another using a F508del-CFTR variant that contained a revertant mutation, R1070W [67], thus stabilizing the NBD1:ICL4 interface [55]. The project led to the identification of three small-molecule series that target defects at NBD1, NBD2 and their TMD interfaces: group I consisting of the 6258 series of sulfamoyl–pyrrol compounds, group II containing the 3151 series of aminothiazole compounds (having

structural similarity with Corr-4a) and group III collecting the 4172 series of pyrazole compounds [55]. This mechanistic approach led to the first identification of small molecules endowed with profound rescue efficacy in primary HBE and HNE cells of individuals with CF, and acting synergistically to restore up to 50–100% wild-type CFTR function to F508del and rare mutations located in different domains of CFTR through structural allostery [55]. More recently, a study from the same group demonstrated that VX-445 synergistically restores F508del-CFTR processing in combination with type I or II correctors that target the NBD1-TMDs interface and NBD2, respectively, consistent with a type III corrector mechanism [26]. Other studies demonstrated that VX-445 may exert dual activity as both corrector and potentiator [69] and that its potentiator activity is additive with VX-770 for potentiation of F508del- and G551D-CFTR channels [70].

## 3. Novel approaches to modulator discovery

With the rapid adoption of highly effective modulator therapies for CF [16,20,21], the shrinking number of modulator naïve individuals with CF and the demands of regulators will make the clinical development of novel CFTR modulators increasingly challenging. This further increases the requirements for pre-clinical modulator development to focus on the most promising candidates for clinical trials, a threshold that was not always met in the past, as indicated by the poor reproducibility of some pre-clinical studies that led to poorly justified drug trials in individuals with CF [71,72]. In order to meet these challenges, a multitude of novel approaches to modulator development emerged.

### 3.1. Expanding the use of the existing modulator drugs to additional CFTR mutants

Therapy with the potentiator VX-770, which was initially developed for individuals with CF carrying the G551D mutation [16], has been FDA-approved for monotherapy of 96 mutants (and a complex allele) on the basis of results in heterologous expression systems (Table 1) [3,73–75]. While subsequent studies largely confirmed the clinical benefit of VX-770 for several of these mutants [76,77], it has been noticed that efficacy of CFTR modulator drugs can depend on the model system [51,78]. Similarly, therapy with the combination VX-661+VX-770, initially developed for the F508del mutation, was approved for additional 149 mutations (plus six complex alleles) (Table 2) [3,18,19,56,79]. The combination VX-445+VX-661+VX-770 was initially approved for CF individuals who carry the F508del mutation in at least allele [20,21]. The approval was recently expanded to 171 additional CFTR mutations (plus six complex alleles) located throughout the channel based on *in vitro* data in FRT cells, so far without formal publication of these results (Table 3) [80]. Notably, many CF individuals may benefit from more than one therapy as their mutations may be present in more than one list. However, the disease liability and response to modulators of only a fraction of the more than 2,100 mutations in the *CFTR* gene has been mapped so far in a process termed theratyping [81].

As an alternative to individual mutant testing, the concept of allosteric corrector combinations provides a more generalized approach. This concept is based on the post-translational completion of the CFTR cooperative domain-folding and the coupled domain-misfolding of F508del and other folding mutations [8,65–67,82]. It predicts that the localized stabilization of CFTR domains by correctors of distinct folding defects will be propagated to distant regions of the channel and, thereby, combinations of correctors targeting different CFTR domains may synergistically stabilize a variety of mutants [26,55]. When this concept was tested for the type I+II+III corrector combination VX-809+3151+4172 [55] or the type I+III corrector combination VX-661+VX-445 [26], the corrector combination efficacy exceeded that of single correctors in 14 out of 17 corrector responsive mutants, suggesting that the adoption of the best available corrector combination therapy for most folding mutations is a sensible approach.

Similarly, the additivity or synergy of dual potentiator combinations for gating/conductance mutants with incomplete functional correction by single potentiators was proposed [25,-,83–87]. While the mechanistic classification of VX-770 and investigational potentiators is currently only based on their pharmacological properties [25,87], combinations could provide therapeutic benefit if the potentiator activity of VX-445 is confirmed [70].

Table 1. List of CFTR mutations responsive to VX-770 (Kalydeco®) and approved by the FDA.

| | | | | | | |
|---|---|---|---|---|---|---|
| 711 + 3A→G | D1152H | G194R | I807M | Q237H | R553Q | S1159F |
| 2789 + 5 G→A | D1270N | G314E | I1027T | Q359R | R668C | S1159P |
| 3272–26A→G | E56K | G551D | I1139V | Q1291R | R792G | S1251N |
| 3849 + 10kbC→T | E193 K | G551S | K1060T | R74W | R933G | S1255P |
| A120T | E822K | G576A | L206W | R75Q | R1070Q | T338I |
| A234D | E831X | G970D | L320V | R117C | R1070W | T1053I |
| A349V | F311del | G1069R | L967S | R117G | R1162L | V232D |
| A455E | F311L | G1244E | L997F | R117H | R1283M | V562I |
| A1067T | F508C | G1249R | L1480P | R117L | S549N | V754M |
| D110E | F508C/S1251N | G1349D | M152V | R117P | S549R | V1293G |
| D110H | F1052V | H939R | M952I | R170H | S589N | W1282R |
| D192G | F1074L | H1375P | M952T | R347H | S737F | Y1014C |
| D579G | G178E | I148T | P67L | R347L | S945 L | Y1032 C |
| D924N | G178R | I175V | Q237E | R352Q | S977F | |

**Note**: Individuals with CF should have one of the mutations listed here in at least one allele. Adapted from Ref [75].

Table 2. List of CFTR mutations responsive to VX-661+VX-770 (Symdeko®) and approved by the FDA.

| 546insCTA | D1152H | G126D | I601F | P5L | R334L | S912L |
|---|---|---|---|---|---|---|
| 711 + 3A→G | D1270N | G178E | I618T | P67L | R334Q | S945 L |
| 2789 + 5 G→A | E56K | G178R | I807M | P205S | R347H | S977F |
| 3272–26A→G | E60K | G194R | I980K | Q98R | R347L | S1159F |
| 3849 + 10kbC→T | E92K | G194V | I1027T | Q237E | R347P | S1159P |
| A120T | E116K | G314E | I1139V | Q237H | R352Q | S1251N |
| A234D | E193K | G551D | I1269N | Q359R | R352W | S1255P |
| A349V | E403D | G551S | I1366N | Q1291R | R553Q | T338I |
| A455E | E588V | G576A | K1060T | R31L | R668C | T1036N |
| A554E | E822K | G576A/R668C | L15P | R74Q | R751L | T1053I |
| A1006E | E831X | G622D | L206W | R74W | R792G | V201M |
| A1067T | F191V | G970D | L320V | R74W/D1270N | R933G | V232D |
| D110E | F311del | G1069R | L346P | R74W/V201M | R1066H | V562I |
| D110H | F311L | G1244E | L967S | R74W/V201M/D1270N | R1070Q | V754M |
| D192G | F508C | G1249R | L997F | R75Q | R1070W | V1153E |
| D443Y | F508C/S1251N | G1349D | L1324P | R117C | R1162L | V1240G |
| D443Y/G576A/R668C | F508del | H939R | L1335P | R117G | R1283M | V1293G |
| D579G | F575Y | H1054D | L1480P | R117H | R1283S | W1282R |
| D614G | F1016S | H1375P | M152V | R117L | S549N | Y109N |
| D836Y | F1052V | I148T | M265R | R117P | S549R | Y161S |
| D924N | F1074L | I175V | M952I | R170H | S589N | Y1014C |
| D979V | F1099L | I336K | M952T | R258G | S737F | Y1032C |

**Note**: Individuals with CF should have the F508del mutation in one allele and one of the mutations listed here in the second allele. Adapted from Ref [79].

### 3.2. CFTR structure as basis for rational design and *in silico* optimization of modulators

The complex polytopic multidomain architecture and thermal instability have made crystal structures of CFTR so far, an unachievable goal. Early structural studies were able to solve the structures of the CFTR NBD1 that was stabilized by multiple mutations [88,89]. The first *in silico* docking experiments to predict the binding sites of potentiators used this structure and NBD2 homology modeling to create a model for the NBD1:NBD2 heterodimer [90,91]. The following iterations were full-length CFTR homology models based on the structure of bacterial ABC transporters [92–95]. These homology models were used for docking studies to predict the binding sites of established correctors, including VX-809 [65,96,97] and Corr-4a [65]. Putative binding pockets in the NBD1 structure and in homology models of full-length CFTR were also used for *in silico* screening of chemical libraries with some success [98,99]; however, none of the identified compounds seems to have moved beyond an initial characterization. A more iterative process uses molecular modeling to increase the potency and efficacy of CFTR modulators by optimizing their interaction with a putative binding pocket, which led to promising results [100,101].

Table 3. List of CFTR mutations responsive to VX-445+VX-661+VX-770 (Trikafta®) and approved by the FDA.

| 3141del9 | E193K | G551D | I980K | P574H | R352W | S1255P |
|---|---|---|---|---|---|---|
| 546insCTA | E403D | G551S | I1027T | Q98R | R553Q | T338I |
| A46D | E474K | G576A | I1139V | Q237E | R668C | T1036N |
| A120T | E588V | G576A/R668C | I1269N | Q237H | R751L | T1053I |
| A234D | E822K | G622D | I1366N | Q359R | R792G | V201M |
| A349V | F191V | G628R | K1060T | Q1291R | R933G | V232D |
| A455E | F311del | G970D | L15P | R31L | R1066H | V456A |
| A554E | F311L | G1061R | L165S | R74Q | R1070Q | V456F |
| A1006E | F508C | G1069R | L206W | R74W | R1070W | V562I |
| A1067T | F508C/S1251N | G1244E | L320V | R74W/D1270N | R1162L | V754M |
| D110E | F508del | G1249R | L346P | R74W/V201M | R1283M | V1153E |
| D110H | F575Y | G1349D | L453S | R74W/V201M/D1270N | R1283S | V1240G |
| D192G | F1016S | H139R | L967S | R75Q | S13F | V1293G |
| D443Y | F1052V | H199Y | L997F | R117C | S341P | W361R |
| D443Y/G576A/R668C | F1074L | H939R | L1077P | R117G | S364P | W1098C |
| D579G | F1099L | H1054D | L1324P | R117H | S492F | W1282R |
| D614G | G27R | H1085P | L1335P | R117L | S549N | Y109N |
| D836Y | G85E | H1085R | L1480P | R117P | S549R | Y161D |
| D924N | G126D | H1375P | M152V | R170H | S589N | Y161S |
| D979V | G178E | I148T | M265R | R258G | S737F | Y563N |
| D1152H | G178R | I175V | M952I | R334L | S912L | Y1014C |
| D1270N | G194R | I336K | M952T | R334Q | S945L | Y1032C |
| E56K | G194V | I502T | M1101K | R347H | S977F | |
| E60K | G314E | I601F | P5L | R347L | S1159F | |
| E92K | G463V | I618T | P67L | R347P | S1159P | |
| E116K | G480C | I807M | P205S | R352Q | S1251N | |

**Note**: Individuals with CF should have one of the mutations listed here in at least one allele. Adapted from Ref [80].

The recent breakthrough in cryo-electron microscopy (Cryo-EM) provided for the first time the structure of different states of CFTR with a resolution in the 3–4 Å range [6,7]. This structural information was used to dock the type I correctors VX-809, VX-661 and C18 to CFTR, which showed a multi-domain binding site at the interface of TMD1, NBD1 and TMD2 [102], in agreement with previous results [65,103]. Cryo-EM also produced the first full-length structure bound to CFTR modulators, indicating a binding site of the potentiators VX-770 and ABBV-974 in the TMDs in a cleft formed by transmembrane helices 4, 5, and 8 [60]. The hope is that the structural data will facilitate structure-based optimization of therapeutic compounds.

### 3.3. Novel translational cell models and their predictive capacity

Heterologous overexpression cell models, allowing for HTS screening of compound libraries, were a key component in the CFTR modulator discovery pipeline [40,41,45,46,48]. The high attrition rate for compounds subsequently tested in primary airway epithelia, led to the development of some screening assays in patient-derived specimens [36,104]. However, the low number of CF patients with rare mutations and the shortage of CF HBE, mostly obtained from end-stage lung disease by invasive procedures (bronchoscopy or lung transplantation), limits the accessibility of these cells and restricts the progress of mutation-specific pre-clinical drug development and optimization of precision therapy.

A major limitation in obtaining a feasible number of airway epithelial cells was overcome by adopting the method of conditional programming first introduced by Liu and collaborators [105,106], which allows primary cells to acquire stem-like characteristics while retaining their ability to differentiate into the various epithelial cell types [107]. Under those conditions, the CFTR function is maintained for at least 20 population doublings allowing the expansion of any starting material by a factor of $10^6$ [24]. The limit of rare genotypes was, at least in part, surmounted by the use of HNE cells instead of HBE, which are collected by minimal-invasive procedures such as nasal brushing or scraping of the lower turbinates [33,34,108]. However, since most CF patients with rare genotypes have compound heterozygous mutations, that is, they carry different mutations on both alleles, the attribution of phenotypes to a specific mutation remains challenging. CRISPR/Cas9-mediated gene editing to create isogenic 16HBE14o$^-$ immortalized bronchial epithelial cells expressing homozygous CFTR mutants has been exploited [109], but since these cell lines underwent at least two rounds of clonal selection, at the points of cell-line creation and gene-editing, their physiology does not necessarily reflect that of primary bronchial cells. Accordingly, HNE cells have been used more extensively to identify modulator-responsive mutants [26,110–112]. The responsiveness of F508del homozygous HNE to VX-809+VX-770 correlated with the clinical responsiveness of individual patients, suggesting that functional measurements in HNE are a predictive biomarker for CF [113,114]. To increase the throughput of CFTR functional measurements in primary airway cells, human airway spheroids and organoids were developed [37,115–117]. However, these models show limited response to CFTR modulators leading to an unfavorable response-to-background ratio.

As an alternative to airway epithelial cells, the isolation and propagation of LGR5$^+$ adult stem cells from intestinal crypts was established [118,119]. When cultured in matrigel, these cells spontaneously form fluid-filled lumen structures (termed as organoids), which allow determining the CFTR function by forskolin-induced swelling (FIS) and steady-state lumen area (SLA) assays [35,36]. Intestinal organoids were used to identify CFTR modulator-responsive mutants [36,86]. CFTR-activity measurements showed a correlation between the organoid and clinical responsiveness of a variety of different mutants to modulator drugs [120] as well as between the residual F508del-CFTR function in organoids and the lung function in individual patients [121]. However, since the organoids are derived from the intestinal epithelium, they might not predict airway responses to modulators as accurately as airway epithelia models.

Another line of research focuses on the generation of induced pluripotent stem cells (iPSCs), which overcome the availability issues in respect to both cell numbers and rare mutations and that allow the differentiation into several CF relevant cell types/tissues, including pancreatic ductal epithelial cells [122], cholangiocytes [123,124], intestinal organoids [125] and macrophages [126,127]. The differentiation of iPSCs through definitive endoderm, anterior foregut endoderm, and NKx 2.1$^+$ lung progenitors gives rise to proximal airway epithelial cells; however, the resulting cell populations contain mesenchymal cells [128–130]. A recent publication by Hawkins and collaborators reports the directed differentiation of human iPSCs into a homogeneous population of airway basal cells using a dual fluorescent reporter system (NKX2-1$^{GFP}$; TP63$^{tdTomato}$), which can subsequently form a mature airway epithelium [131]. The hope is that, similar to iPSC-derived intestinal organoids [132], these cells may be used for CFTR modulator development. Nevertheless, it is still unclear to which degree iPSC-derived airway epithelial cells exhibit the epigenetic- and modifier gene-encoded individual responsiveness to CFTR modulators, necessary for their function as a predictive biomarker for clinical responses.

Gene-editing strategies to correct CF mutations in primary airway cells, intestinal organoids and iPSC with the prospect of regenerative medicine approaches have been reported and are reviewed elsewhere [133,134].

### 3.4. Beyond measurements of CFTR function: additional readouts to determine modulator efficacy

Loss-of-CFTR-function in airway epithelia leads to mucociliary clearance (MCC) defects [135,136], reduced air surface liquid (ASL) height [137], ASL acidification [138,139], as well as imbalance between pro-inflammatory and anti-inflammatory lipid mediators [140–142]. The MCC defect is the result of increased mucus viscosity and compromised, uncoordinated ciliary beating, which can be partially rescued by CFTR modulators, as determined by multiple particle tracking mucus microrheology [143] and multiscale differential dynamic microscopy [144], respectively. CFTR modulators VX-809+VX-770 were

found to partially reverse the reduced ASL height of F508del HBE [46]. To increase the speed of ASL height acquisition, automated, or semi-automated techniques based on laser confocal microscopy [145,146] or surface laser reflectance microscopy [147] have been developed and validated by ion channel activators and inhibitors. These techniques may be used for the discovery of novel modulator compounds. Determination of the apical pH of F508del HBE by dextran coupled, pH-sensitive, ratiometric fluorophores demonstrated single or corrector combination-mediated increase in ASL pH [148]. The modulator combination VX-445+VX-661+VX-770 led to distinctive changes in the bronchial epithelial cell lipidome, including a reduction of ceramide levels [149], which are associated with apoptotic and inflammatory signaling [150]. Measurement of these factors allows determining the physiological response to CFTR modulators in pre-clinical cell models, which may facilitate the prioritization of modulator compounds for clinical development.

### 3.5. Co-culture cell models that capture the effect of complex interactions between hyper-inflammation, infection and the modulator efficacy.

Multiple lines of evidence suggest that the excessive inflammation in CF, immune cell infiltration of the airways and bacterial colonization affect the residual mutant CFTR function and potentially the modulator efficacy. Bacterial infection – particularly with *P. aeruginosa* as the most prevalent pathogen chronically infecting up to 80% of adults with CF [151] – exacerbates the inflammatory phenotype in CF by promoting activation of toll-like receptor (TLR) signaling that further increases the airway epithelia pro-inflammatory cytokine and chemokines release as well as further recruiting inflammatory cells. Furthermore, certain bacterial virulence factors are able to decrease CFTR total and membrane expression as well as anion currents, including LasB elastase [152,153] and the *P. aeruginosa* CFTR inhibitory factor (Cif), which is present in secreted outer membrane vesicles and enhances the ubiquitination and degradation of CFTR [154,155].

Neutrophils are the predominant leukocytes recruited to the lung lumen. Once recruited and activated, neutrophils release antimicrobial peptides, neutrophil extracellular traps (NETs), reactive oxygen species and proteases [156–158]. The sheer amount of neutrophil released-serine proteases (neutrophil elastase (NE), proteinase 3, and cathepsin G) overwhelms the antiprotease production resulting in a protease-antiprotease imbalance [159,160]. Excessive amounts of NE in the airway-surface liquid in turn induce interleukin (IL)-8 expression [161,162], upregulate mucin production [163,164], cleave antimicrobial peptides [165], promote the degradation of CFTR [166] further perpetuating the inflammatory response that cumulates in progressive tissue damage, and correlates to the rate of lung function decline [167,168]. Despite their excessive infiltration into the CF lung, CF neutrophils fail to eradicate bacterial infections [157,169] and a number of studies suggest an intrinsic neutrophil function defect in CF [170–172].

Innate immune responses also have the potential to downregulate CFTR expression. Transforming growth factor (TGF)-β [173–175] and interferon (IFN)-γ [176,177] by decreasing CFTR mRNA, as well as high concentrations of IL-8 via $\beta_2$-adrenergic receptor desensitization and downregulation [178] lead to a decreased functional expression of CFTR. Furthermore, although controversial, reactive oxygen radicals [179], such as those released by neutrophils, and *P. aeruginosa* virulence factor pyocyanin [180] may reduce CFTR function by inhibiting PKA and/or CFTR mRNA expression [181].

Co-cultures of CF HBE with secreted exoproducts of *P. aeruginosa* were the first *in vitro* models to capture these interactions and demonstrated an attenuation of efficacy of modulator-mediated mutant CFTR correction [182,183] (Figure 3A). Deletion of the LasR transcriptional regulator of quorum sensing [153] or inhibition of outer-membrane vesicle fusion with epithelial cells [184] prevented the deleterious effect of *P. aeruginosa* on F508del-CFTR correction, indicating that the modulator efficacy may depend on the bacterial strains present in the patient's lung. On the other hand, Gentzsch and collaborators reported that apical exposure of HBE cultures with supernatants from mucopurulent material, containing the soluble infectious and inflammatory factors present in CF airways *in vivo*, led to an increased response to CFTR

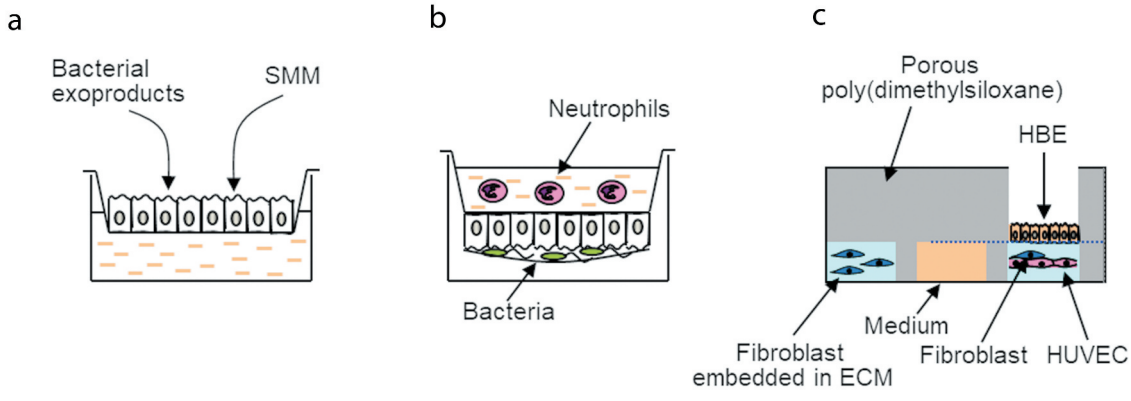


Figure 3. Schematic depictions of *in vitro* co-culture systems. **(a)** Differentiated HBE cultured at air-liquid interface (ALI) are apically exposed to bacterial exoproducts [164,165] or supernatants from mucopurulent material (SMM) [185]. **(b)** Inverted ALI model which allows studying neutrophil transmigration upon apical HBE exposure to bacteria or chemoattractants [186]. **(c)** Lung-on-a-chip model which contains channels for extracellular matrix (ECM) embedded fibroblast culture, combined human umbilical vein endothelial cell (HUVEC)/fibroblast culture and HBE culture at ALI. Cells are fed via medium channels and nutrients diffuse through porous poly(dimethylsiloxane) (PDMS). Reproduced from Ref [190] with permission from the Royal Society of Chemistry.

modulators [185], substantiating the notion that the inflammatory milieu has a major impact on the efficacy of CFTR modulators.

As a next step, *in vitro* models incorporating innate immune cells were created. The co-culture of primary human neutrophils with HBE on opposite sides of a 3 μm pore size filter support allowed measuring the transmigration of neutrophils in response to a chemoattractant gradient or apical infection with *P. aeruginosa* [186,187] (Figure 3B). A similar model, using a CF bronchial epithelial cell line, basolateral macrophages derived from the THP-1 monocyte cell line and apical infection with *P. aeruginosa* was recently reported [188]. To our knowledge, similar experiments using primary CF airway epithelia to study the effect of CFTR modulators on transmigration, cytokine secretion and bacterial eradication have not been reported, but could serve as a powerful tool to study modulator efficacy in a pre-clinical model.

To more closely mimic the lung environment *in vitro*, lung-on-a-chip devices have been constructed. First systems, combining a differentiated, mucociliary bronchiolar epithelium and an underlying microvascular endothelium that experiences fluid flow, were used to study the drug-sensitive cytokine secretion in asthma and chronic obstructive pulmonary disease [189]. Recent iterations of such systems included human lung fibroblasts embedded in extracellular matrix in a 96-well plate that could support medium throughput compound screening approaches [190] (Figure 3C). Using this lung-on-a-chip system, Mejías and collaborators showed increased neutrophil recruitment into the microvascular compartment if CF HBE were used in the epithelial layer [190]. The prospect of lung-on-a-chip systems is to replace animal models [191], which bears particular relevance for CF, since rodent models do not recapitulate all aspects of the lung disease [192,193].

## 4. Conclusion

The introduction of highly effective modulator therapies constitutes a milestone in CF therapy. The combination VX-445+VX-661+VX-770 provides unprecedented clinical benefits to individuals with CF carrying at least one allele of the F508del-CFTR mutation by promoting a gain in percentage of predicted forced expiratory volume in 1 sec ($ppFEV_1$) of 14.3% relative to placebo administration and reduction of sweat chloride by 41.8 mmol per liter in compound heterozygous patients [21]. In individuals with CF homozygous for the F508del mutation, this triple combination resulted in 10.0% gain in the $ppFEV_1$ in addition to that of only VX-661+VX-770 treatment and reduction of sweat chloride by 45.1 mmol per liter [20]. Considering the ~6.8% improvement in the $ppFEV_1$ upon VX-661+VX-770 administration [18], the estimated absolute clinical efficacy of the combination VX-445+VX-661+VX-770 in homozygous F508del-CFTR (~16.8% increase in ppFEV1% relative to placebo) exceeds that of ivacaftor in compound heterozygous G551D patients, which results in $ppFEV_1$ gain of 10.6% and sweat-chloride reduction of 48.1 mmol per liter [16]. With the discovery of novel modulators and label extension of those already approved for clinical use, the hope is that CFTR modulator therapies will become available for all patients with folding, gating or residual function mutations and CF will be transformed from a life threatening to a more manageable chronic disease.

## 5. Expert opinion

The advent of highly effective modulator therapies in the CF clinic has created excitement not only for patients and familiars but also for clinicians and researchers in the field. The hope is that these therapies will deeply modify the disease course for the majority of individuals with CF. While long-term benefits of the triple combination VX-445+VX-661+VX-770 remain to be demonstrated, long-term use of VX-770 has indeed been associated with multiple clinical benefits, including indicators of a reduced need for lung transplant [194,195]. Nevertheless, in the first phase III clinical trials of VX-770 alone or in combination VX-445+VX-661, the mean baseline $ppFEV_1$ was between 60% and 65%, implying only partial normalization of the lung function after 24 weeks treatment [16,21] or at 4 weeks [20]. Long-term studies also indicate that adult G551D patients still experience progressive loss of their lung function [22,196,197] and *P. aeruginosa* infection, albeit at reduced frequency [198]. While the incomplete lung function correction is presumably in part due to the structural damage incurred during the course of the disease, which may be prevented by early therapy onset, increasing the functional correction efficacy may provide additional benefit to patients with modulator responsive mutations.

The CFTR modulators currently available represent effective causative therapies for 85–90% of the entire CF population; however, 10–15% of CF individuals, who are likely to carry rarer CF genotypes, remain without any of these therapies. Moreover, many eligible CF patients do not have access to these drugs due to their very high costs and regulatory issues in national health systems that do not approve their reimbursement, which poses an additional challenge that should be considered and further discussed in order to find feasible solutions.

The CF therapeutic development landscape is robust with a considerable number of promising novel CFTR modulator drugs under investigation. A better understanding of the mechanism of action of these investigational molecules, rather than only knowing they exert a desired effect, may significantly increase their chances to successfully achieve clinical approval. Furthermore, despite the absence of reliable small animal models that recapitulate the complexity of CF nature [192,193], scientific community was able to establish alternative models with patient-derived specimens as a key component of many approaches. Indeed, these specimens provide a translational perspective not only to perform a comparative assessment of drug efficacies and select which may provide better therapeutic responses for each individual (as more drugs are expected to become available), but also to extend the approval of existing (and novel) modulator drugs to

a larger CF population by including rare CF genotypes that are responsive.

Individuals with CF carrying mutations that are unresponsive to currently available modulators may benefit yet from mutation-specific modulator development. A multitude of novel approaches have been developed to circumvent barriers and accelerate the discovery of novel modulator drugs. The incorporation of extensive pre-clinical development approaches as discussed in section 3, could increase the speed and cost-effectiveness of modulator development and may follow an approach as outlined in Figure 4 in order to enhance the success rate during clinical development.

Despite all the aforementioned drugs under development, it is likely that a small percentage of patients (but still significant number) may not benefit from any CFTR modulator because of unresponsive CF genotypes or adverse effects. For this CF population, there are still promising pharmacological strategies that work independently of the CFTR mutation type by targeting alternative ion channels. These include inhibitors of the epithelial sodium channel (ENaC), which is upregulated in CF, and activators of non-CFTR alternative chloride channels to compensate for the lack of CFTR activity [199–201].

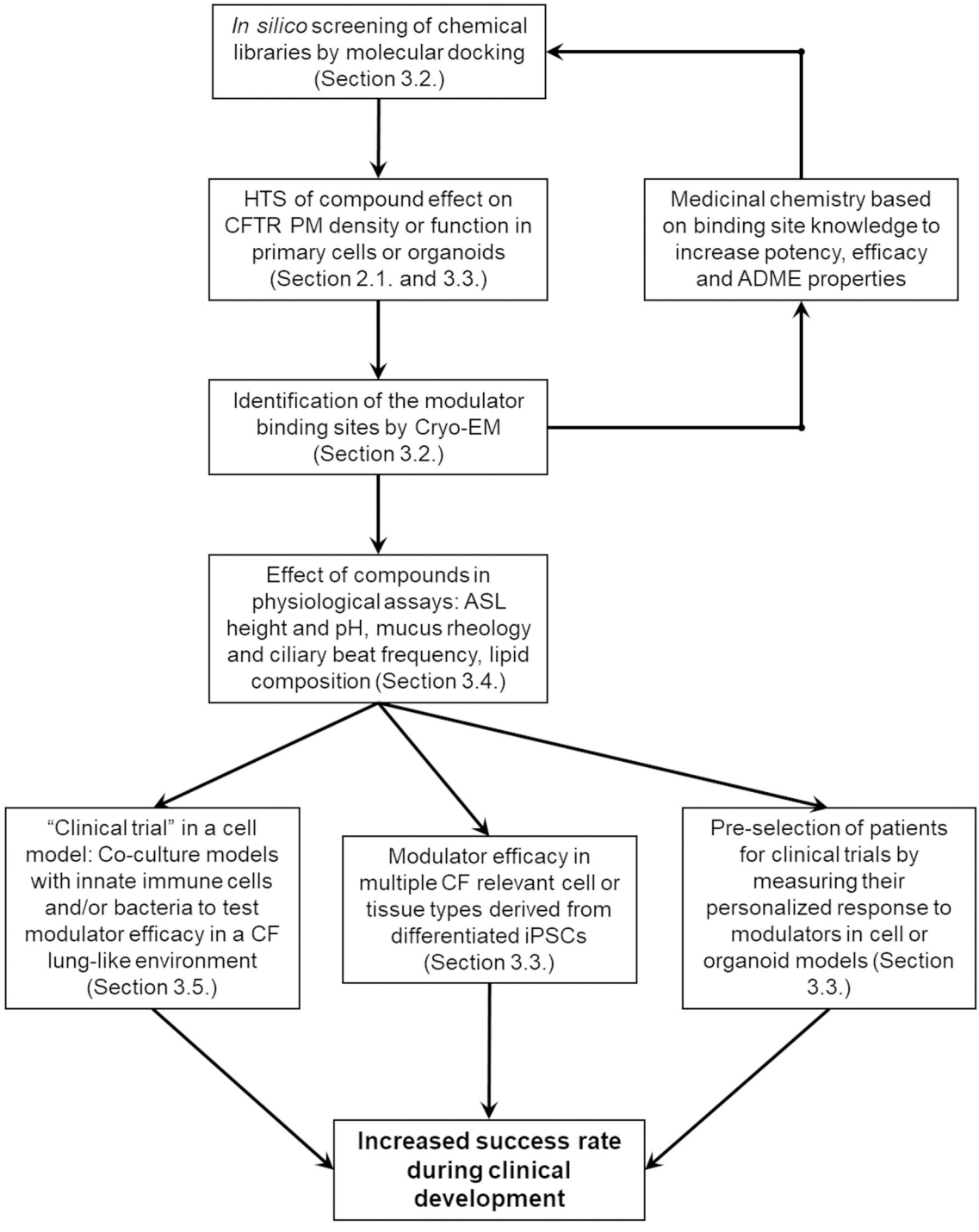


Figure 4. Flow-chart of an optimized pre-clinical CFTR modulator discovery process.

One can envision a day when newborns diagnosed with CF will be assigned to highly effective therapies soon after selecting the best therapeutic option by predicting the effectiveness in their own specimens. Such approach may provide a real transformation in the healthcare paradigm and the disease face as these individuals would be able to live much healthier and longer lives and symptomatic therapies would be included later on a patient basis if necessary.

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