Early View

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Novel CFTR modulator combinations maximize rescue of G85E and N1303K in rectal organoids

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Key words: CFTR modulators; elexacaftor; G85E; N1303K; rectal organoids; label-free FIS assay

Abstract

Introduction: Cystic fibrosis (CF) is a severe monogenic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Several types of CFTR modulators (correctors/potentiators) have been developed to overcome protein dysfunction associated with these mutations. CFTR modulator therapy is now available for the major CF-causing mutations, however 10% of people with CF remain without causal treatments. By combining investigational and market approved CFTR modulators, we aimed to maximize functional rescue of *iva*-, *luma*- and *tezacaftor* refractory mutants G85E and N1303K.

Methods: We used the well-established forskolin induced swelling (FIS) in primary rectal organoids to assess responses to different CFTR corrector and potentiator types. The FIS analysis was performed with brightfield microscopy, allowing both 1h and 24h follow-up. Corrector and potentiator activity of elexacaftor was investigated.

Results: For G85E, maximal rescue was observed by a combination of elexacaftor and *corr4a*. For N1303K, the quadruple combination *teza*-*elixa*-*ivacaftor* with apigenin was required to obtain a rescue similar to that of *luma*-*ivacaftor* rescued F508del. Elexacaftor rescued G85E and N1303K by different mechanisms, with chronic corrector effects on G85E and acute potentiation of N1303K only in the presence of *ivacaftor*. Synergy in N1303K rescue for *iva*-*elexacaftor* and apigenin suggests at least three potentiator mechanisms for this mutant. 24h FIS identified *ivacaftor* as the main CFTR modulator for N1303K and elexacaftor and apigenin as co-potentiators.

Conclusions: Novel combinations of CFTR modulators can further improve functional rescue of G85E and N1303K in rectal organoids, although for N1303K more effective CFTR modulators are still needed.

Introduction

Cystic fibrosis (CF) affects over 85,000 people worldwide and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [1-3]. *CFTR* regulates chloride, bicarbonate and fluid transport across secretory epithelia in many organs. Currently, over 2,100 mutations have been described (www.genet.sickkids.on.ca) of which at least 382 are confirmed disease-causing (www.CFTR2.org). These mutations disturb the protein synthesis (Class I/II/V/VII), function (Class III/IV) or stability (Class VI) at the plasma membrane (PM) of *CFTR*, or a combination of those [4, 5].

Small molecules have been developed to improve *CFTR* processing and trafficking (“correctors”) or its gating (“potentiators”). Corrector and potentiator types have been proposed to reflect the distinct mechanisms of action of different *CFTR* modulators (Suppl. Table 1 and [6-9]). To date, four molecules have received market approval either alone or in combination: (1) potentiator ivacaftor (VX-770; type I potentiator), (2) correctors lumacaftor (VX-809; type I corrector) and its derivative (3) tezacaftor (VX-661; type I corrector), and (4) corrector elexacaftor (VX-445; type III corrector). Highly effective *CFTR* modulator treatment is now approved for the majority of people with CF (PwCF); either ivacaftor monotherapy (Kalydeco™) [10] or teza-elexa-ivacaftor triple-combination therapy (Trikafta™, Kaftrio™ in Europe) [11, 12]. Prior to the approval of Trikafta™, two other modulator combinations had been approved for PwCF homozygous for the most common mutation, F508del (c.1521_1523delCTT): Orkambi™ (luma-ivacaftor) and Symdeko™ (Symkevi™ in Europe, teza-ivacaftor), [13, 14]. In December 2020, the FDA announced a label extension for Kalydeco™, Symdeko™ and Trikafta™ to qualify over 100 additional rare mutations for these therapies [15].

CF cell models have been instrumental in guiding treatment decisions in PwCF, a strategy called theratyping [16]. While the main goal is to identify PwCF whom could benefit from a specific treatment by assessing the responsiveness of a given *CFTR* genotype to modulator therapy, it also allows to gain insights into the molecular defects of the mutation(s) studied [5]. Importantly, theratyping allows comparison of modulator responses, in either a mutation- or patient-specific approach within a given preclinical model. In particular the many different, rare mutations do not support standard clinical trial design and therefore depend on theratyping. In that light, the development of organoids as a biomarker of *CFTR* function and the possibility to quantify drug responsiveness was a major breakthrough [17, 18]. *CFTR* modulator responses, quantified by forskolin induced swelling (FIS), correlate well with non-gastro-intestinal clinical endpoints in PwCF, including lung function (FEV₁; forced expiratory volume in one second) and sweat chloride concentration [17, 19]. To date, evidence supports that organoids can be used to guide personalized medicine in CF [20]. Their most prominent advantages include the ease and minimal discomfort of obtaining rectum biopsies, which have led to the rapid growth of different biobanks around the world, including the UZ Leuven biobank used here. In particular, many rare genotypes have been included. Further, the rapid *in vitro* expansion of stem-cell derived organoids speeds up preclinical assessment, allowing to obtain functional data within a month after biopsy collection. Based on this favorable profile, we opted to use primary rectal organoids for our study.

One mutation for which Trikafta™ was recently approved by FDA label extension is G85E (c.254G>A; 0.4% of alleles, *CFTR2* database). G85E, located in the first membrane spanning domain (MSD1), causes severely impaired processing and trafficking, completely abolishing *CFTR* mediated ion transport [21]. We and others have previously shown that this mutant is drug-refractory to

potentiators and type I correctors alone or in combination [8, 21]. Recently, elixa-teza-ivacaftor has been shown to partially rescue G85E function [8, 22]. The exact mode of action of elixaftor has not been fully uncovered, but recent reports show that besides its known corrector function, it also acts as a potentiator, with mutation-specific activity [9, 22]. Therefore, we aimed to study elixaftor both as corrector (24h incubation) and potentiator (added acutely). We further sought to investigate whether the severe trafficking defect of G85E was maximally corrected by the corrector activity of elixaftor, which has been shown to stabilize nucleotide binding domain 1 (NBD1) [8], or whether correctors with different mechanisms could further improve correction. We thus tested tezacaftor (correcting the MSD-NBD interface) and a type II corrector (stabilizing NBD2/MSD2), *i.e.* the investigational corrector 4a (corr4a; C4) [7, 23].

Many studies have tried to rescue the severe NBD2 mutant, N1303K (c.3909C>G), the 4th most common mutation (1.6% of alleles in CFTR2). N1303K has been shown to be an atypical CFTR mutant in many ways, by both its trafficking and functional defects [24]. In fact, to date there is no consensus which of these two defects is the major cause of N1303K dysfunction and hence, should be the main focus of therapeutic targeting [25, 26]. N1303K is completely refractory to iva-, luma- and tezacaftor in patient-derived organoids and airway cultures but recent reports show a modest functional rescue in cell lines and primary human bronchial epithelial cells (HBE) with elixa-teza-ivacaftor [8, 22], which precluded N1303K from inclusion in the Trikafta™ FDA label extension [27]. We therefore investigated whether a rational choice of modulators, *i.e.* NBD2/MSD2 corrector corr4a and co-potentiator apigenin (type II potentiator), would augment the limited but hopeful functional correction induced by Trikafta™ [28].

Methods

Human rectal organoid generation and biobanking

Informed consent was obtained prior to biopsy collection, in accordance with the ethical committee of UZ Leuven (S56329). Human rectal organoids were generated from rectum biopsies of PwCF as previously described, and biobanked after successful culture [19, 29]. Organoids from the biobank were thawed and cultured for evaluation in this study.

Forskolin induced swelling assay in human rectal organoids

Responses to CFTR modulator combinations were determined in the forskolin induced swelling (FIS) assay, which was performed as described previously [18, 21, 29], with modifications. Briefly, organoids with F508del, G85E or N1303K genotypes were mechanically disrupted and plated in Matrigel® Matrix (Corning; #356231) in 96-well plates. Corrector compounds (lumacaftor/tezacaftor/elixaftor (all Selleckchem; S1565/S7059/S8851; all 3 µM) and corr4a (Merck; #219673; 5 µM) or DMSO controls were added for 24h prior to FIS. At the time of analysis 0.8 µM of forskolin (Selleckchem; S2449) was added together with either potentiator compounds ((ivacaftor (Selleckchem; S1144; 3 µM) and/or apigenin (Selleckchem; S2262; 50 µM)) or DMSO controls. When the potentiator activity of elixaftor was investigated, it was added at the time of analysis, conform the other potentiators. In selected experiments, calcein green AM live dye (Invitrogen; C34852; 5 µM) was added to fluorescently label organoids. In most experiments however, organoids were imaged label-free using brightfield microscopy. Fluorescent and brightfield images were taken every 10 minutes for one hour or every 30 minutes for 24h (for brightfield only) on the ZEISS LSM880 system at 5X magnification. Tiff files were analyzed in ImageJ. Customized macros were developed to determine total organoid area in each image, by removing the background and enhancing the contrast of each image. Color thresholds recognizing organoid structures were set and holes in the mask were filled after which it was despeckled to remove noise. The area of the resulting mask was determined for each timepoint and organoid swelling was

calculated as the relative increase in this area over time, which was analyzed in Microsoft Excel. Compound responses between t=0 and t=60min or t=24h were calculated as the area under the curve (AUC) of the organoid swelling in GraphPad Prism 9.

Flow cytometry PM density in CFTR overexpressing HEK293T cells

HEK293T cells overexpressing CFTR variants with an extracellular triple hemagglutinin (3HA) tag [30], were plated in 96-well plates and 24h prior to analysis cells were treated either with corrector combinations of tezacaftor (3 μ M), elexacaftor (3 μ M), and corr4a (5 μ M), or DMSO controls. PM density was then determined as described previously [21], and corrected for differences in mRNA expression levels measured by RT-PCR. For RT-PCR, RNA was isolated using Aurum Total RNA mini kit (Bio-Rad, #7326820) and converted to cDNA with the High Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific, #4368813). Samples contained LightCycler 480 SYBR Green I Mastermix (Roche; #4707516001) and CFTR (Fw:GCAGTTGATGTGCTTGGCTA; Rev:ACTGCCGCACTTTGTTCTCT) or β -actin (Fw:TCACCCACACTGTGCCCATCTACGA; Rev: CAGCGGAACCGCTCATTGCCAATGG) primers and were run and analyzed in LightCycler 480 (Roche). *CFTR* mRNA expression levels were normalized to β -actin levels.

Statistics

At least three independent repeats of each experiment were performed, and are shown as dots in the diagrams (mean $\pm$ SEM). Each experiment was performed in triplicate/quadruplicate. All statistical analysis was performed in Graphpad Prism 9. Pearson correlation r^2 was determined between fluorescent and brightfield FIS analysis. Different treatments were analyzed with ANOVA with multiple comparisons, unless specified otherwise in the text.

Results

Label-free analysis of forskolin induced swelling using brightfield microscopy

We set out to optimize a label-free version of the forskolin induced swelling (FIS) assay which would allow long-term monitoring of organoid swelling. Calcein, the most commonly used fluorescent dye to label organoids in FIS, diffuses out of cells over time (Suppl. Figure 1A). Re-application of calcein at later timepoints was not feasible due to further loss of the signal-to-noise ratio and toxicity (Suppl. Figure 1B,C). In brightfield microscopy by contrast, no labels are needed for organoid detection. An example of a brightfield image converted to a binary mask representing the total organoid surface area is shown in figure 1A. In order to validate our optimized analysis, market approved CFTR modulators were tested alone and in existing and novel combinations on F508del/F508del organoids (Figure 1B). The two analysis methods, brightfield and fluorescence microscopy (calcein labeling), were performed in parallel and showed an excellent correlation ($r^2=0.98$, $p<0.0001$) (Figure 1C). Therefore, all subsequent FIS assays were analyzed using brightfield microscopy.

Limited effect of type I corrector on top of elixa-ivacaftor in F508del rectal organoids

We first quantified rescue of F508del function by combinations of the currently approved CFTR modulators (Figure 1B) as a reference for further comparison with G85E and N1303K responses. As a proxy for a relevant response to modulator treatment, the published average response of F508del/F508del organoids to luma-ivacaftor (Orkambi™; 1600 AUC after 60 minutes, dotted line, [19]) was set as the arbitrary threshold in all experiments. A clear additive effect was observed when elexacaftor was added to luma-ivacaftor or teza-ivacaftor in line with published data for elixa-teza-ivacaftor [8, 31]. Intriguingly, adding a second corrector (luma- or tezacaftor) to elixa-ivacaftor did not seem to further improve CFTR function in human rectal organoids with F508del ($p=0.99$ and $p=0.15$ for homozygous F508del and F508del/G542X(c.1624G>T) (Figure 1D) organoids, respectively; CFTR modulator responses of G85E/F508del and N1303K/F508del organoids in Suppl. Figure 2).

Functional rescue of G85E by elxacaftor alone

We next evaluated drug responsiveness in two G85E genotypes, both compound heterozygous, one with F508del on the other allele and the other with the W1282X (c.3846G>A) mutation for which Trikafta/Kaftrio™ is not approved. Indeed, in contrast to the previously market-approved CFTR modulator combinations, elxa-teza-ivacaftor could functionally rescue G85E in both organoid samples well above the threshold considered relevant (Figure 2A; Suppl. Figure 2). Interestingly, we observed that elxacaftor by itself resulted in a similar response compared to elxa-teza-ivacaftor in G85E/W1282X organoids (ANOVA, multiple comparisons, $p=0.17$).

Corrector combinations synergistically improve G85E PM density and function

We previously reported that G85E suffers from a severe trafficking defect, which reduces PM expression below 1% of WT-CFTR ([21], Figure 2B). To address if the elxacaftor-induced functional rescue we observed in organoids originated from a rescue in trafficking, in line with elxacaftor's corrector activity, we determined the PM density of G85E (Figure 2B). As hypothesized, elxacaftor increased PM density to ~6% of WT-CFTR (13-fold increase over DMSO; $p=0.0032$), with no additive benefit of teza- and elxacaftor ($p=0.56$). We set out to further enhance G85E trafficking by evaluating different corrector combinations. Adding corr4a onto elxacaftor led to a synergistic increase in PM density to ~26% of WT-CFTR (62-fold increase over DMSO; 4-fold increase over elxacaftor alone, $p<0.001$). Rescue by corr4a in the absence of elxacaftor did not reach significance. We next evaluated corr4a-mediated rescue in G85E/W1282X organoids, to verify whether it would enhance function of (elxacaftor-corrected) G85E (Figure 2C). Combined elxacaftor and corr4a synergistically rescued G85E function compared to elxacaftor only ($p<0.0001$). In elxacaftor-corr4a and in particular the triple corrector (teza-elxacaftor-corr4a) treated organoids, CFTR activity was observed even in the absence of forskolin stimulation. These organoids showed a visible lumen (Figure 2D), the phenotype of rectal organoids from non-CF subjects [18] and F508del/F508del organoids rescued with teza-elxacaftor (Suppl. Figure 3), this in contrast to the dense lumen-less phenotype of CF organoids. The effect of ivacaftor on top of corrector-mediated rescue was minor (Figure 2C).

Functional rescue of N1303K minimally requires elxa-ivacaftor combinations

We used a rational choice of CFTR modulators to investigate whether NBD2 corrector corr4a and co-potentiator apigenin would further augment the modest functional improvement by elxa-teza-ivacaftor [8, 22, 28, 32]. First, we reproduced this functional correction, now in the rectal organoid model (Figure 3A). Although significantly higher than previously approved modulator therapies (ivacaftor, luma-ivacaftor, teza-lumacaftor), CFTR function did not surpass the threshold for proposed relevance (dotted line, Figure 3A) in either homozygous N1303K or N1303K/3121-1A>G compound heterozygous organoids, the latter carrying a class I mutation on the second allele. The minimally effective combination necessary to rescue N1303K-CFTR function above baseline was ivaplus elxacaftor ($p<0.0001$, both genotypes), although adding tezacaftor provided significant benefit ($p<0.0001$, both genotypes). To next investigate whether we could further increase N1303K function, we combined the above correctors and potentiators with apigenin or corr4a. Apigenin synergistically enhanced elxa-ivacaftor rescued N1303K (Figure 3B). Compared to elxa-teza-ivacaftor, adding apigenin doubled CFTR function, thereby reaching levels with predicted clinical benefit. Corr4a however could not functionally rescue N1303K alone or in combination with ivacaftor or ivacaftor plus apigenin (Figure 3B).

Elxacaftor rescues N1303K and G85E by different mechanisms

Based on recent reports stating that elxacaftor acts both as a corrector and potentiator [8, 9, 22, 33], we took a closer look at the mechanism(s) by which elxacaftor restores function of G85E and

N1303K. 24h incubation and acute addition of elexacaftor resulted in similar levels of N1303K functional rescue for all compound combinations tested (Figure 4A; 2-way ANOVA, $p=0.52$). Here, acute addition represents the potentiator activity of elexacaftor, while 24h incubation shows the combined effect of potentiator and corrector activities. Since the FIS assay does not allow discrimination of corrector and potentiator activities with 24h incubation, we investigated the corrector activity via PM density in HEK293T cells (Figure 4B). Elxacaftor improved PM expression of N1303K a modest but significant ~ 1.3 -fold over DMSO ($p=0.03$), compared to ~ 1.5 -fold for tezacaftor ($p=0.001$). Corr4a alone could not significantly augment N1303K PM expression ($p=0.21$). The triple corrector teza-elxacaftor-corr4a yielded a ~ 2 -fold increase in PM N1303K. Comparing 24h incubation and acute elxacaftor treatment in the FIS assay confirmed that, in contrast to N1303K, pre-incubation with elxacaftor was necessary to achieve relevant levels of G85E rescue (Figure 4C).

Long-term FIS reveals ivacaftor as the major potentiator for N1303K

Finally, we aimed to unravel which CFTR modulator is the major contributor to the functional rescue of N1303K. To this end, we optimized an extended version of FIS more sensitive for detecting small improvements in CFTR function as accumulated swelling is analyzed over a 24-hour rather than 1-hour period (Figure 5A). This allowed detection of near-linear swelling for CFTR modulators and combinations undetectable at 1 hour (Figure 5B-D). Since the combination of ivacaftor and elxacaftor was the minimal treatment needed to moderately rescue N1303K function in the 1h FIS (Figure 3), we now investigated within the 24-hour detection window, how each modulator on its own affected N1303K function. We identified that only ivacaftor mono-treatment resulted in a functional correction, in contrast to mono-treatment with tezacaftor, elxacaftor or apigenin, suggesting a major role of ivacaftor to improve the gating defect of N1303K (Figure 5C). Ivacaftor was effectively co-potentiated though by apigenin and elxacaftor (Figure 5D, E). The Venn diagrams summarize the contribution of each modulator tested for N1303K in the 1h and 24h detection window, respectively (Fig. 5F,G).

Discussion

Over the past decade, four CFTR modulator therapies have reached the market and have provided a causal therapy for the majority of PwCF. For the last 10% of PwCF however, no causal treatment is available. The remaining untreatable mutations are mainly non-sense, frameshift and deletion mutations, but also several missense mutations. N1303K is the most common missense mutation without approved modulators. With over 2,100 PwCF registered in the CFTR2 database, an effective treatment for this mutation remains an important need for the CF community. G85E is the 13th most common *CFTR* mutation, also refractory to iva-, luma- and tezacaftor. Elxacaftor-teza-ivacaftor was approved for this mutation through label extension of Trikafta™ by the FDA but not EMA. Nevertheless, in light of its previous modulator refractoriness, questions about G85E's underlying molecular defects remain. The FIS assay performed in patient-derived rectal organoids is a robust and well-validated preclinical model for phenotyping and drug testing [17, 19, 34], a procedure called theratyping [16]. We have used patient-derived rectal organoids to test the latest approved CFTR modulator, elxacaftor, as well as combinations of known types of correctors (I/II/III) and potentiators (I/II) with distinct mechanisms of action for previously drug-refractory mutants G85E and N1303K. By combining approved and investigational types of CFTR modulators we found novel combinations that enhanced G85E and N1303K function beyond that of the Trikafta/Kaftrio™ triple (teza-elxacaftor-ivacaftor), which is currently the best combination with market approval. Our study also showed for both F508del and G85E that not all compounds of the triple combination contributed to CFTR functional rescue in rectal organoids, suggesting a remaining need to optimize combination therapies for in-patient use. Finally, we identified a distinct mode of action of CFTR modulator elxacaftor for both CFTR mutants studied. For G85E, it predominantly restored its folding defect and

coupled to that, CFTR function, whereas for N1303K, it largely improved its gating defect when combined minimally with ivacaftor.

Indeed, as elexacaftor is the latest CFTR modulator to reach market approval, the full scale of its effects is still under investigation, especially for the many rare mutations that cause CF. To date, several studies have investigated the effect of elexacaftor in cell lines and primary HBE both for the most common mutation F508del as well as for several gating and processing mutations, including G85E and N1303K [8, 9, 22, 35]. However, in rectal organoids, only the added effect of elexacaftor on top of teza-ivacaftor for F508del was recently reported [31]. In our study, we set out to investigate novel combinations of CFTR modulators beyond the market approved combinations, aiming to achieve maximized functional rescue for these specific mutations, as well as better understand the underlying molecular defects. For determining the minimal level of organoid responsivity correlating with reported clinical benefit, we set the published average response of F508del/F508del organoids to luma-ivacaftor treatment (Orkambi™) as cut-off [19]. Interestingly, we observed that adding tezacaftor did not further enhance elexa-ivacaftor rescue of F508del much. The same limited benefit of tezacaftor on top of elexacaftor was observed for G85E. For N1303K, a clear benefit of tezacaftor on top of elexa-ivacaftor was observed in the 1h FIS. Our study thus suggests that the contribution of each of the three individual components of Trikafta/Kaftrio™ should be investigated case by case.

For G85E, mono-treatment with elexacaftor was sufficient to restore CFTR function to clinically relevant levels and adding extra CFTR modulators (iva-, luma- or tezacaftor) provided only limited benefit (Figure 2A). Currently, only the triple teza-elexa-ivacaftor (Trikafta™) is FDA-approved for PwCF carrying the G85E mutation, but an elexacaftor monotherapy might be considered in the future. A synergistic effect on top of elexacaftor was observed for type II corrector corr4a (Figure 2C). This investigational compound was previously described as a corrector of F508del by stabilizing MSD2/NBD2 [7, 23, 36]. For G85E it appears to act as a co-corrector, dependent on simultaneous elexacaftor rescue. Notably, G85E organoids pre-incubated with elexacaftor-corr4a and even more so, teza-elexacaftor-corr4a showed CFTR functionality even in the absence of forskolin stimulation. This is indicative of potent rescue, which is sufficient to rescue both trafficking and ion channel function of G85E.

On the other hand, effective rescue of N1303K remains more elusive. In the 1h FIS, we identified elexa-ivacaftor as the minimal treatment necessary to restore detectable CFTR function in organoids, although below the arbitrary threshold for effectivity (Figure 3A). Interestingly, co-potentiator apigenin approximately doubled CFTR function of elexa-ivacaftor and teza-elexa-ivacaftor rescued N1303K organoids (Figure 3C). The exact mechanism of apigenin and other co-potentiators has not yet been elucidated, but in line with previous cell line data [6], our study shows that ivacaftor is necessary for apigenin-mediated rescue of N1303K function. Pre-incubation with elexacaftor was not necessary to restore N1303K function in the presence of ivacaftor, suggesting N1303K rescue is mainly achieved by (co-)potentiation of its gating defect (Figure 4A). The potentiator activity of elexacaftor has previously been described for other CFTR mutants, in particular gating mutants in need of a potentiator [9, 22, 33], but we report for the first time that elexacaftor's potentiator activity also is the main mode of action for N1303K. Indeed, elexacaftor only moderately increased N1303K PM density (Figure 4B), in contrast to G85E (Figure 2B). Laselva and colleagues investigated the potentiator effect of elexacaftor and ivacaftor on N1303K in cell lines, reporting that both showed some minimal potentiator activity by itself [22]. Our study by contrast shows that even when CFTR functional responses were magnified by long-term FIS (24h), no potentiator or corrector activity of elexacaftor was detectable in the absence of ivacaftor in organoids, which is a primary cell model expressing physiological levels of CFTR. Only ivacaftor alone allowed some functional rescue of

N1303K in the 24h FIS, in contrast to mono-treatment of tezacaftor, elexacaftor or apigenin, suggesting a major role of ivacaftor to improve N1303K gating (Figure 5B). Our study shows that at least for N1303K, elexacaftor acts as a co-potentiator of ivacaftor (Figure 5C). The synergy of apigenin on top of elexa-ivacaftor (Figure 3C) suggests there are at least three different mechanisms for CFTR potentiation, some of which are dependent on each other (summarized in Figure 5F,G; Suppl. Table 1). In follow-up studies, the co-potentiators elexacaftor and apigenin should be evaluated in more mechanistic assays, such as by patch clamp, to allow precise elucidation of the different co-potentiator mechanisms. Our study thus underscores that N1303K requires more effective CFTR modulators, as even the six proposed modulator types (*i.e.* three corrector and three potentiator types, Suppl. Table 1) only moderately restore CFTR function in organoids. In particular, CFTR potentiation, with or without supporting correction, appears necessary to fully restore N1303K function.

In conclusion, while some of the tested modulators used in this study are investigational, these data may serve to further optimize drug discovery initiatives for these mutants, in particular N1303K, to reach further functional rescue, and coupled to that, clinical benefit in PwCF. In that light, the label-free 24h FIS assay we developed may accelerate identifying drug responses for difficult to correct mutations, due to its increased sensitivity and potential to be implemented in labs without the need for large, expensive confocal microscopy equipment.

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Author contributions

M. Ensink, M. Carlon, A. Ramalho and F. Vermeulen conceptualized the research questions. M. Ensink and L. De Keersmaecker performed all experiments. A. Ramalho, S. Cuyx, S. Van Biervliet, F. Vermeulen assisted with or collected organoid samples. M. Ensink and M. Carlon analyzed the data and prepared figures. Data were discussed with all authors. M. Ensink and M. Carlon wrote the original draft, which was reviewed and edited by all.

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Figure 1. Label-free analysis of forskolin induced swelling (FIS) by brightfield microscopy allows evaluation of CFTR modulator responses. Brightfield and fluorescence microscopy read outs for FIS of rectal organoids were compared for several market approved CFTR modulators. (A) Detection of organoid area from brightfield images. Left: brightfield image of rectal organoids at 5x magnification. Right: Mask of total organoid area after analysis. Scale bar: 500 μ m. (B) F508del/F508del organoids were treated with combinations of CFTR correctors (lumacaftor, tezacaftor, elexacaftor; all at 3 μ M) or DMSO 24h before analysis. At the start of the assay forskolin (0.8 μ M) with or without the CFTR potentiator ivacaftor (3 μ M) was added and organoid swelling was assessed over the next 60 minutes using either brightfield or fluorescence microscopy. Area-under-the-curve (AUC) of the relative increase in organoid area over time is shown. (C) Correlation between brightfield and fluorescence read outs for each modulator combination in B. (D) FIS analysis as described in B of F508del/G542X organoids treated with the same modulator combinations. Abbreviations: AUC – area-under-the-curve; Iva – ivacaftor; Luma – lumacaftor; Teza – tezacaftor; Elexa – elexacaftor. Each dot in B,D represents the result of an independent experiment, each performed in quadruplicate. Bars show mean+SEM. Dotted line represents the arbitrary threshold for relevant functional rescue (average response of F508del/F508del organoids to luma-ivacaftor).

Figure 2: Elexacaftor alone restores G85E function. CFTR modulators were added at a concentration of 3 μ M, except for corr4a (5 μ M) and organoids were analyzed by brightfield microscopy. (A) FIS analysis of G85E/W1282X organoids treated with CFTR modulators or DMSO controls after stimulation with 0.8 μ M forskolin for one hour. (B) Plasma membrane (PM) density of HEK293T cells overexpressing 3HA-G85E-CFTR treated with CFTR correctors or DMSO controls, measured by flow cytometry. PM density is determined as the percentage of positive cells multiplied with the median fluorescence intensity of positive cells, relative to 3HA-CFTR PM density after correction for mRNA expression, determined by RT-PCR. 10,000 events were analyzed per well. (C) FIS analysis of G85E/W1282X organoids treated with CFTR modulators or DMSO controls after stimulation with 0.8 μ M forskolin for one hour. (D) Brightfield images of G85E/W1282X organoids after 24 h incubation with of mentioned CFTR modulators or DMSO controls. Scale bar: 200 μ m. Abbreviations: FIS: forskolin induced swelling; AUC – area-under-the-curve; PM – plasma membrane; fsk – forskolin; Iva – ivacaftor; Luma – lumacaftor; Teza – tezacaftor; Elexa – elexacaftor. Each dot represents the result of an independent, each performed in triplicate or quadruplicate. Bars show mean+SEM. Dotted line represents the arbitrary threshold for relevant functional rescue (average response of F508del/F508del organoids to luma-ivacaftor).

Figure 3: Elexa-ivacaftor combinations restore some CFTR function in N1303K rectal organoids. CFTR modulators were added at a concentration of 3 μ M, except for apigenin (50 μ M) and corr4a (5 μ M) and rectal organoids analyzed by brightfield microscopy. (A) FIS analysis of N1303K/N1303K and N1303K/3121- 1A>G organoids treated with approved CFTR modulators or DMSO controls after stimulation with 0.8 μ M forskolin for one hour. Dots/triangles represent data from organoids from two different donors with the same genotype. (B) FIS analysis of N1303K/N1303K organoids treated with CFTR modulators or DMSO controls after stimulation with 0.8 μ M forskolin for one hour. Abbreviations: AUC – area-under-the-curve; fsk – forskolin; Iva – ivacaftor; Luma – lumacaftor; Teza – tezacaftor; Elexa – elexacaftor. Each dot/triangle represents the result of an independent experiment, each performed in triplicate/quadruplicate. Bars show mean+SEM. Dotted line represents the arbitrary threshold for relevant functional rescue (average response of F508del/F508del organoids to luma-ivacaftor).

Figure 4: Elexacaftor mediates rescue of N1303K and G85E by different mechanisms. CFTR modulators were added at 3 μ M except for corr4a (5 μ M) and apigenin (50 μ M). FIS analysis of N1303K/N1303K (A) and G85E/W1282X (C) organoids treated with CFTR modulators and activated with 0.8 μ M forskolin. Elexacaftor was added 24h before analysis together with the CFTR correctors (24h) or at the start of the assay together with CFTR potentiators (acute). Organoid swelling was evaluated by brightfield microscopy for the next hour. (B) PM density of HEK293T cells overexpressing 3HA-N1303K-CFTR treated with different CFTR correctors/corrector combinations or DMSO controls, measured by flow cytometry. PM density is determined as the percentage of positive cells multiplied with the median fluorescence of positive cells, put relative to 3HA-CFTR PM density after correction for mRNA expression, determined by RT-PCR. 10,000 events were analyzed per well. Abbreviations: AUC – area-under-the-curve; PM – plasma membrane; fsk – forskolin; Iva – ivacaftor; Luma – lumacaftor; Teza – tezacaftor; Elexa – elexacaftor. Each dot represents the result of an independent experiment, each based on a triplicate. Bars shows mean+SEM.

Figure 5: Elexacaftor and apigenin co-potentiate ivacaftor rescue of N1303K in rectal organoids. CFTR modulators were added at a concentration of 3 μ M, except for apigenin (50 μ M) to N1303K/N1303K organoids. (A) Scheme of 24h FIS. Tezacaftor and elexacaftor were added 24 h before the start of analysis. Forskolin (0.8 μ M), ivacaftor and apigenin were added and organoid swelling was analyzed every 30 minutes for 24h. (B-D) Time course of 1h (B) and 24h FIS (C, D) in N1303K/N1303K organoids with mentioned compounds. Time course shows mean+SD of representative experiments. (E) Brightfield images of organoids in (D) at time points 0h, 1h, and 24h. Scale bar: 200 μ m. (F, G) Venn diagram summarizing responses of CFTR modulators or combinations in N1303K/N1303K organoids in 1h FIS (F), based on data from Figure 3D, or 24 h FIS (G), based on data in Suppl. Figure 4.

Supplementary table 1:

Modulator	Type	Compound	Mechanism of action	Ref
Corrector	I	VX-809 (lumacaftor), VX-661 (tezacaftor)	Stabilization of MSD-NBD interface	[1]
	II	Corr4a	Stabilization of MSD2/NBD2	[1]
	III	VX-445 (elexacaftor)	Stabilization of NBD1	[2]
Potentiator	I	VX-770 (ivacaftor)	Phosphorylation-dependent, ATP-independent increase in P_o	[3]
	II	Apigenin	Co-potentiator, specific for NBD2 mutants, mechanism unknown	[3]
	III	VX-445 (elexacaftor)	Co-potentiator, mechanism unknown	[4]

Overview of the different CFTR modulator types with distinct mechanisms of action, used in this publication. CFTR modulators are grouped into different types based on distinct mechanisms of action and additive rescue effects when combined. In the current study, we conclude there are at least three different potentiator mechanisms, due to the clear additive effect of ivacaftor, elexacaftor and apigenin, in this manuscript studied on the class II/III/VI mutant N1303K [5, 6]. This is in line with the recent finding of the co-potentiator activity of elexacaftor on top of ivacaftor for F508del and other gating mutants [4], which we here extended to the triple potentiator combination for N1303K. Abbreviations: MSD: membrane spanning domain; NBD: nucleotide binding domain; P_o : open probability.

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Supplementary figures

Suppl. Figure 1: Calcein-labeled organoids do not allow for long term follow-up. Calcein AM (5 μ M) was added to rectal organoids which were then imaged for up to 24h. **(A)** Fluorescent images (4x) were taken at the mentioned time points, showing a decrease in organoid fluorescence compared to background fluorescence over time. **(B)** Calcein AM was re-added after 4 and 8 hours, but resulted in loss of a preferential signal-to-noise ratio. **(C)** Brightfield images (10x) showing rectal organoids 24h after receiving calcein AM (5 μ M) for the specified number of times. Increased organoid toxicity is apparent with increasing repeats of calcein addition.

Suppl. Figure 2: CFTR modulator responses of G85E/F508del and N1303K/F508del organoids. (A) G85E/F508del or (B) N1303K/F508del rectal organoids were treated with combinations of CFTR correctors (lumacaftor, tezacaftor, elxacaftor; all at 3 μ M) or DMSO 24h before analysis. At the start of the assay, forskolin (0.8 μ M) with or without the CFTR potentiator ivacaftor (3 μ M) was added and organoid swelling was assessed over the next 60 minutes using brightfield microscopy. Area-under-the-curve (AUC) of the relative increase in organoid area over time is shown. Abbreviations: FIS: forskolin induced swelling; AUC – area-under-the-curve; PM – plasma membrane; fsk – forskolin; Iva – ivacaftor; Luma – lumacaftor; Teza – tezacaftor; Elexa – elxacaftor. Each dot represents the result of an independent repeat, each performed in quadruplicate. Bars show mean+SEM. Dotted line represents the arbitrary threshold for relevant functional rescue (average response of F508del/F508del organoids to luma-ivacaftor).

Suppl. Figure 3: Evidence of a functional correction of F508del-CFTR in rectal organoids by specific CFTR corrector combinations in the absence of forskolin stimulation. F508del/F508del organoids were treated with the mentioned CFTR modulators (teza/elexacaftor 3 μ M; corr4a 5 μ M). Brightfield images were taken after 24h incubation with compounds or DMSO controls. The appearance of an organoid lumen for teza+elexacaftor and teza+elexacaftor+corr4a treated organoids in the absence of forskolin stimulation suggests a substantial functional correction of CFTR, resembling that of non-CF organoids (for example, see Dekkers et al., Nat. Med. 2013).

N1303K/N1303K

Suppl. Figure 4: CFTR modulator responses determined by long term (24h) FIS in N1303K/N1303K organoids. This graph provides the corresponding data on which the Venn diagram shown in Figure 5H is based. N1303K/N1303K organoids were treated with teza/elexacaftor (3 μ M) or DMSO controls for 24h before analysis. At the start of FIS, forskolin (0.8 μ M) and ivacaftor (3 μ M)/apigenin (50 μ M) were added and organoids were followed up every 30 minutes for the next 24h. Increases in organoid area over time were calculated. AUC: area-under-the-curve; teza: tezacaftor; elexa: elexacaftor; fsk: forskolin; iva: ivacaftor. Each dot represents the result of an independent experiment, each performed in triplicate. Bars show mean+SEM.