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Original Article

Functional Restoration of *CFTR* Nonsense Mutations in Intestinal Organoids

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ABSTRACT

Background: Pharmacotherapies for people with cystic fibrosis (pwCF) who have premature termination codons (PTCs) in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene are under development. Thus far, clinical studies focused on compounds that induce translational readthrough (RT) at the mRNA PTC location. Recent studies using primary airway cells showed that PTC functional restoration can be achieved through combining compounds with multiple mode-of-actions. Here, we assessed induction of CFTR function in PTC-containing intestinal organoids using compounds targeting RT, nonsense mRNA mediated decay (NMD) and CFTR protein modulation. *Methods:* Rescue of PTC CFTR protein was assessed by forskolin-induced swelling of 12 intestinal organoid cultures carrying distinct PTC mutations. Effects of compounds on mRNA CFTR level was assessed by RT-qPCRs. *Results:* Whilst response varied between donors, significant rescue of CFTR function was achieved for most donors with the quintuple combination of a commercially available pharmacological equivalent of the RT compound (ELX-02-disulfate or ELX-02ds), NMD inhibitor SMG1i, correctors VX-445 and VX-661 and potentiator VX-770. The quintuple combination of pharmacotherapies reached swelling quantities higher than the mean swelling of three VX-809/VX-770-rescued F508del/F508del organoid cultures, indicating level of rescue is of clinical relevance as VX-770/VX-809-mediated F508del/F508del rescue in organoids correlate with substantial improvement of clinical outcome. *Conclusions:* Whilst variation in efficacy was observed between genotypes as well as within genotypes, the data suggests that strong pharmacological rescue of PTC requires a combination of drugs that target RT, NMD and protein function.

Introduction

Cystic fibrosis (CF) is a monogenic, autosomal-recessive disease caused by mutations in the *CFTR* gene [1]. Highly efficacious pharmacotherapy of the most prevalent F508del mutation shifts the unmet clinical need towards approximately 15% of people with CF (pwCF) who carry non- or low-responder CFTR mutations. The spectrum of mutations that are poorly responsive to clinically approved pharmacotherapies include the class I mutations that do not lead to full length protein (e.g. by nonsense mutations, frameshifts, consensus splice mutations, or larger rearrangements).

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Approximately 10% of the worldwide CF population carry mutations that result in premature termination codons (PTC) resulting in production of truncated CFTR protein. Early work demonstrates that aminoglycoside antibiotics including gentamicin and G418 enable rescue of CFTR PTC in cell lines [2]. These compounds reduce the fidelity of translation by affecting the pairing of cognate and near-cognate tRNAs with the mRNA, resulting in incorporation of non-cognate amino acids at the PTC site. This readthrough (RT) process facilitates continuation of translation, albeit at low efficacy [2]. Subsequent efforts identified PTC124 (Ataluren) as selective inducer of PTC-readthrough [3]. However, efficacy in many preclinical models was not reproduced [4,5] and clinical trials with Ataluren failed to reach their primary endpoints [6]. A recently chemically-engineered aminoglycosides derivative termed ELX-02 (NB124; Eloxx Pharmaceuticals) is currently in early clinical development [7] and showed to be effective as single treatment in intestinal organoids [8].

Whilst readthrough agents hold potential for increasing full length protein production, their efficacy is inhibited by a control system called nonsense-mediated mRNA decay (NMD) that leads to degradation of PTC-containing mRNA molecules [9,10]. By pharmacological inhibition of critical effectors of NMD such as SMG1 kinase (through SMG1i) or SMG7 (through NMDI-14), increased efficacy of readthrough agents has been observed in various preclinical models and laboratories [10–14]. A potential alternative to NMD-inhibition may be a recently identified CFTR amplifier (PTI-428 or nesolicaftor [15]) that increases CFTR mRNA quantity independent of PTC-mutations.

The reduced translational fidelity by readthrough agents induces a pool of proteins with different amino acids at the PTC site [16], underlining the potential of combining CFTR protein modulators with readthrough agents to further enhance CFTR restoration [8,10,17]. CFTR (co-)potentiators such as VX-770, ASP-11[18] and to some extend VX-445 [19], may increase the channel open probability of the readthrough-induced CFTR protein pool, whereas CFTR correctors may enhance trafficking of readthrough-CFTR protein towards the apical surface. Their combination will likely be most effective in restoring CFTR function upon readthrough.

To study the impact and repair of PTCs, we use intestinal organoids and the forskolin-induced swelling (FIS) assay as CFTR-dependent phenotypic readout that allows to quantitate individual CFTR function in response to CFTR function modulators [20,21]. CFTR function measurements in this assay model correlate with clinical disease indicators [22,23] and CFTR modulator responses [21,24]. Our previous work on readthrough demonstrated no efficacy of PTC124 in intestinal organoids [4], consistent with clinical trial data by others [6], supporting the use of this assay for preclinical drug development. The purpose of this study was to investigate the capacity of commercially-available compounds with different modes-of-action to increase ELX-02ds-induced CFTR function rescue in organoids with multiple PTCs.

Materials and methods

Collection of primary epithelial cells of CF patients (pwCF)

Informed consent for tissue collection, generation, storage, and use of the organoids was obtained from all participating patients. Biobanked intestinal organoids are stored and catalogued (https://huborganoids.nl/) at the foundation Hubrecht Organoid Technology (http://hub4organoids.eu). Collection of patient tissue and data was performed following the guidelines of the European Network of Research Ethics Committees (EUREC) following European, national, and local law, and the study was approved by the local the medical ethical committee at UMC Utrecht biobank (TcBio document 14-008), at Charite, Berlin and at The Hebrew University, Jerusalem.

Human intestinal organoid culture

Crypts were isolated from biopsies of subjects with cystic fibrosis as previously described [21]. Organoids were incubated in a humidified chamber with 5% CO2 at 37°C. Medium was refreshed every 2–3 days, and organoids were passaged 1:4 every 7–8 days. Prior to forskolin induced swelling assay measurements, organoids were grown at least 3 weeks after crypt isolation or thawing.

Functional assessment of CFTR function

Functional assessment of CFTR function was assessed with the forskolin-induced swelling assay, performed as described by Vonk et al. [25]. But instead of using recombinant Human R-Spondin 3 Protein, we used R-Spondin condition medium [21]. Details about the compound concentrations and incubation times can be found in Table 1. Organoid swelling was monitored during 60-180 minutes using a Zeiss LSM 710 confocal microscope. Total organoid surface area per well was quantified based on calcein green staining as described by Vonk et al. [25].

Quantitative real time PCR

Organoids were cultured in tissue culture plates, either in regular culture medium or culture medium supplemented with compounds described in Table 1. Organoids were collected from 24-well tissue culture plates, washed once with advanced DMEM/F12 and RNA was extracted using RNeasy Mini Kit (Qiagen, catalog no. 74104), following manufacturers protocol. cDNA was synthesized of 100 ng RNA with Iscript™ according to the supplied protocol (Biorad, catalog no. 1708891). qPCR reactions were executed in 96-well format with IQ SYBR green (Bio-Rad, catalog no. 1708880) and following primer sets: CFTR reverse: CCCAGGTAAGGGATGTATTGTG, CFTR forward: CAACATCTAGTGAGCAGTCAGG;YHWAZ reverse: AAGGGACTTCCTGTAACAATGCA, YHWAZ forward: CTGGAACGGTGAAGGTGACA. Using a Biorad CFX PCR device, samples were incubated for 3 minutes at 95°C and for 39 cycles at: 10 seconds at 95°C, 30 seconds at 62°C. Relative expression levels of the treated PTC organoids were analyzed by means of ΔΔCt calculations, for which YWHAZ served as housekeeping gene and mean expression level of two replicate experiments of 5 healthy control organoid samples was used as calibrator. YWHAZ expression was not affected by the different compound therapies. Melt peaks were analyzed to confirm specific primer binding.

Statistics

Data are represented as mean ± SD or SEM (specified in figure legends). One-way ANOVA's were performed to compare mean FIS or ΔΔCt values upon treatment with pharmacotherapies with DMSO at group level with Dunnett T-test as post-hoc analysis. No statistical testing was performed between the different pharmacotherapies on individual donor level. P values < 0.05 were considered statistically significant. Data analysis was performed in SPSS.

Results

Overview of compound mode-of-actions and patient samples used in this study

We selected a diverse set of compounds that are commercially available (Fig. 1**A**) to study their capacity to enhance functional restoration upon RT of CFTR. The incorporation of an amino acid

Table 1
Final assay conditions of pharmacotherapies included in this manuscript.

Compound	hours added prior to FIS assay or organoid collection for PCR	Final concentration (μM)	Manufacturer	Mode of action
ELX-02 disulfate	48	80	MedChemExpress	RT-agent
SMG1i	24	0.3	Cystic fibrosis foundation	NMD-inhibitor
PTI-428	24	0-20	MedChemExpress	CFTR amplifier
VX-770	0, added together with forskolin	3	selleck biochemicals	CFTR potentiator
VX-661, VX-445	24	3	selleck biochemicals	CFTR corrector
ASP-11	0, added together with forskolin	0-20	Kindly provided by UCSF	CFTR co-potentiator
NMDI-14	24 and 48	0-20	MedChemExpress	NMD-inhibitor
Vidaza	24	0-20	SelleckChem	NMD-inhibitor

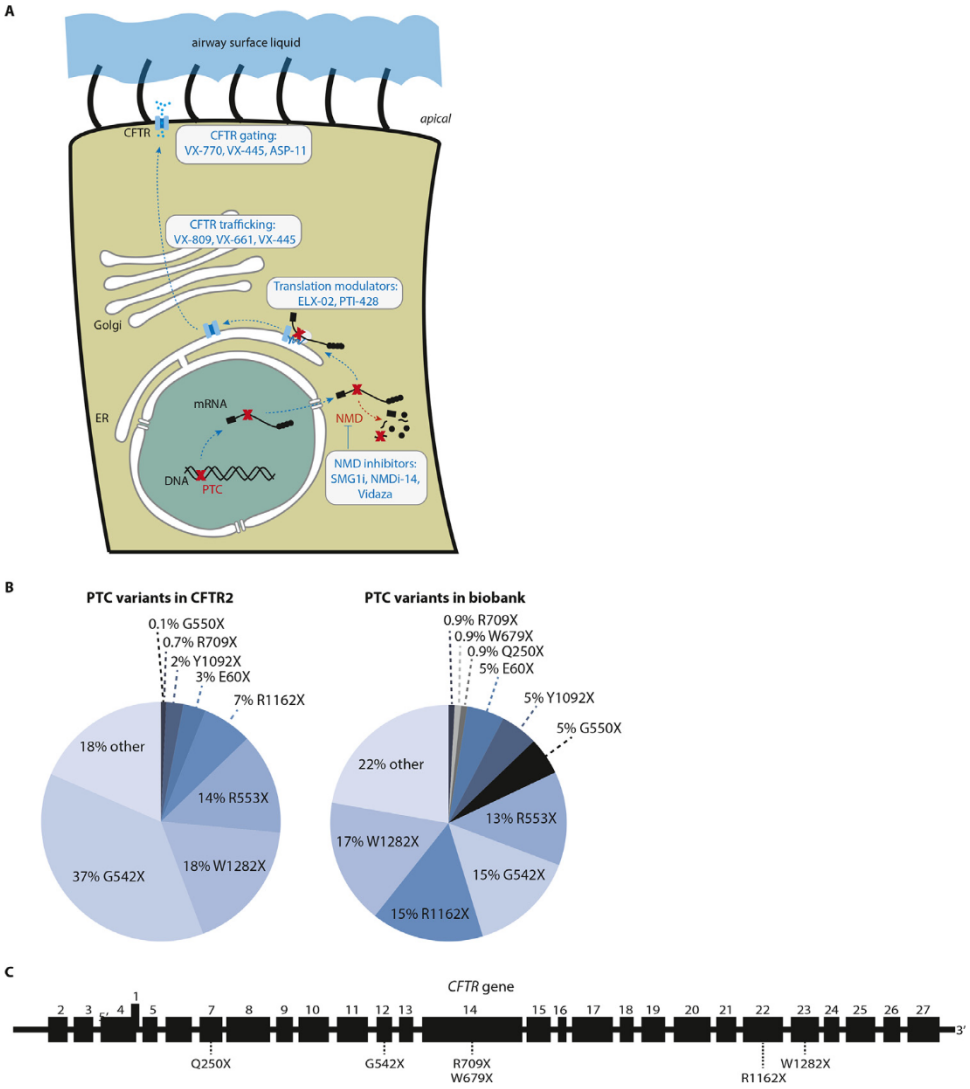

Fig. 1. Schematic presentation of impact of pharmacotherapies and demographics of CFTR mutation types and PTC allele frequencies. (**A**) Schematic presentation of intracellular trafficking of CFTR and impact of pharmacotherapies. **(B)** Relative allele frequencies of specific PTC mutations in our biobank (right) compared to world wide incidence (left). **(C)** Location in the CFTR gene of the PTC mutations included in this study.

at the place of the PTC results in a pool of full-length transcripts of which function could be enhanced with CFTR modulation therapy (VX-770, VX-661, VX-445 and ASP-11). The amplifier PTI-428, or the NMD inhibitors SMG1i, NMDi-14 and Vidaza should increase the level of mRNA transcript and thereby expand the pool of PTC mRNA prone for RT. Combining these small molecules acting on different steps along the CFTR biosynthesis pathway, channel trafficking and channel gating might collectively result in CFTR function restoration of PTCs in general, to clinically relevant levels.

Currently 10% of our biobank consists of PTC mutations in which most prevalent PTC mutations are represented (Fig. 1**B**); yet distribution is to some extent shifted towards rare PTC variants. Also, locations of PTC mutations described in this study are well distributed across the CFTR gene (Fig. 1**C**) which allows us to investigate whether the location of the PTC in the CFTR gene influences drug response.

Rescue of W1282X/W1282X function by RT, NMD-inhibition and CFTR modulation

First, we assessed the dose-dependency and incubation times required for optimal rescue of CFTR function by RT agent ELX-

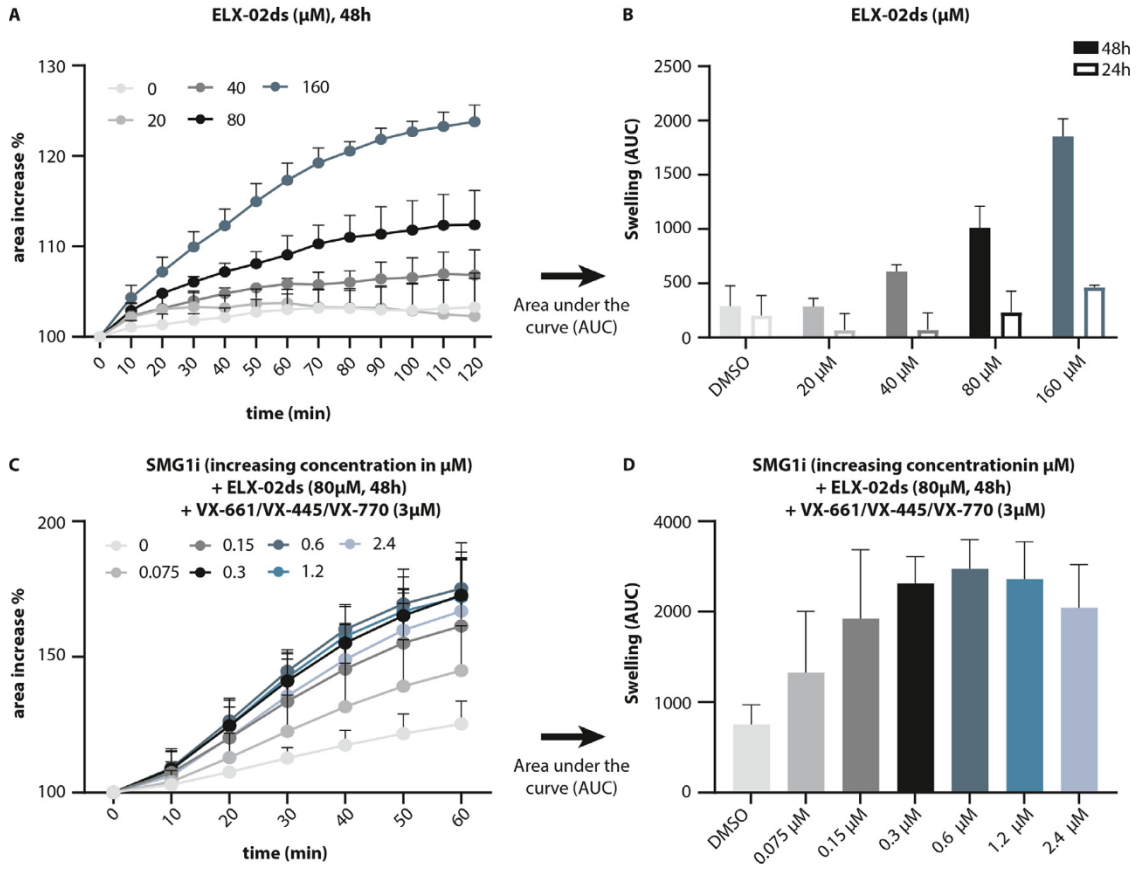

Fig. 2. Dose-response assays on a W1282X/W1282X organoid culture to define optimal pharmacotherapy conditions. **(A)** Area increase in time (in total for 120 min) of W1282X/W1282X organoids upon addition of increasing concentration of ELX-02ds for 24h or 48h prior to the FIS-assay. Organoid area increase was measured with the addition of 5 μM forskolin. Datapoints represent mean+SD, n=3. **(B)** FIS, measured as area under the curve of each condition shown in **(A). (C)** Area increase in time (in total for 60 min) of W1282X/W1282X organoids upon addition of an increasing concentration of SMG1i for 24h or 48h and ELX-02ds (80 μM) for 48h + VX-661/VX-445 (3 μM) for 24h prior to the FIS-assay. Organoid area increase was measured with the addition of 0.128 μM forskolin + 3 μM VX-770. Datapoints represent mean+SD, n=3. **(D)** FIS, measured as area under the curve of each condition shown in **(C)**.

02ds in 2-hours FIS measurements in a W1282X/W1282X organoid culture. ELX-02ds increased FIS dose-dependently and to a higher extend after 48h ELX-02ds pre-incubation when compared to 24h (Fig. 2**A-B**). We selected 80 μM of ELX-02ds and 48h pre-incubation as condition for combination studies. Based on the dose-response of SMG1i on top of ELX-02ds and VX-661/VX-445/VX-770 (Fig. 2**C-D**) and reported toxicity concerns of SMG1i [17], 0.3 μM and 24h incubation were chosen for further studies. Concentrations of VX-661, VX-445 and VX-770 were set to 3 μM based on previous work and dose-dependency was not studied in detail here. Pre-stimulation with 0.625 μM NMDI-14 for 24 or 48h slightly increased swelling when combined with ELX-02ds (**Fig. S1**), but significantly less than the combination of SMG1i and ELX-02ds. NMDI-14 concentrations >2.5 μM became toxic, shown by a decreased swelling response. We could not detect any impact of PTI-428, ASP-11 and Vidaza on rescuing W1282X/W1282X-CFTR, despite varying concentrations (0-20 μM, 1:2 diluted) and compound backgrounds (ELX-02ds + SMG1i w/w/o VX-661/VX-445/VX-770) (**Fig. S1**). For these reasons, NMDi-14, PTI-428, ASP-11 and Vidaza were excluded from further experiments.

PTC rescue with combinations of ELX-02ds, SMG1i and VX-661/VX-445/VX-770

We next set out to study rescue of CFTR function by combined use of ELX-02ds, SMG1 and VX-661/VX-445/VX-770, and compared efficacy to VX809/VX770 or VX661/VX445/VX770 treatment of F508del/F508del organoids. Organoids were stimulated with different fsk concentrations for 1h to define a fsk concentration that can quantitate PTC rescue in the dynamic range of the assay, and enable comparison with previous work. Fsk titrations demonstrated a dose-dependent relation with swelling (Fig. 3**A-B**). Maximal swelling was observed from 0.128 μM fsk and higher, and fsk dose dependency and efficacy of EST in W1282X/W1282X organoids (n=3 donors) conditions was comparable to VX770/VX809 in F508del/F508del (n=3 donors). We selected fsk 0.128 μM for 1h for comparison of RT compound efficacies between the various organoid conditions.

We next assessed the CFTR restoring capacity of ELX-02ds, SMG1i and VX-661/VX-445/VX-770 as stand-alone compounds and combinations thereof in 8 organoid cultures homozygous for distinct PTC mutations (Fig. 3**C-F**) and 3 organoid cultures compound heterozygous for PTC mutations (**Fig. S2A**). Two organoid cultures homozygous for consensus splice mutations showed no response to compound treatment indicating PTC-dependent rescue (**Fig. S2B**). No single compound restored CFTR function to such extend it could be detected with 1h 0.128 μM fsk stimulation. Swelling levels significantly increased when organoids were treated with ELX-02ds and either SMG1i (ES in Fig. 3) or VX-661/VX-445/VX-770 (ET in Fig. 3), nearly reaching AUC levels similar to VX-809/VX-770-rescued F508del/F508del organoids (Fig. 3**C-D**).The magnitude of swelling increase was donor dependent and within-genotype (W1282X/W1282X) variation was observed (Fig. 3**E-F**). As recent literature described that differences in mRNA sequence surrounding the PTC might influence RT, all four homozygous W1282X organoid cultures were sequenced. However,

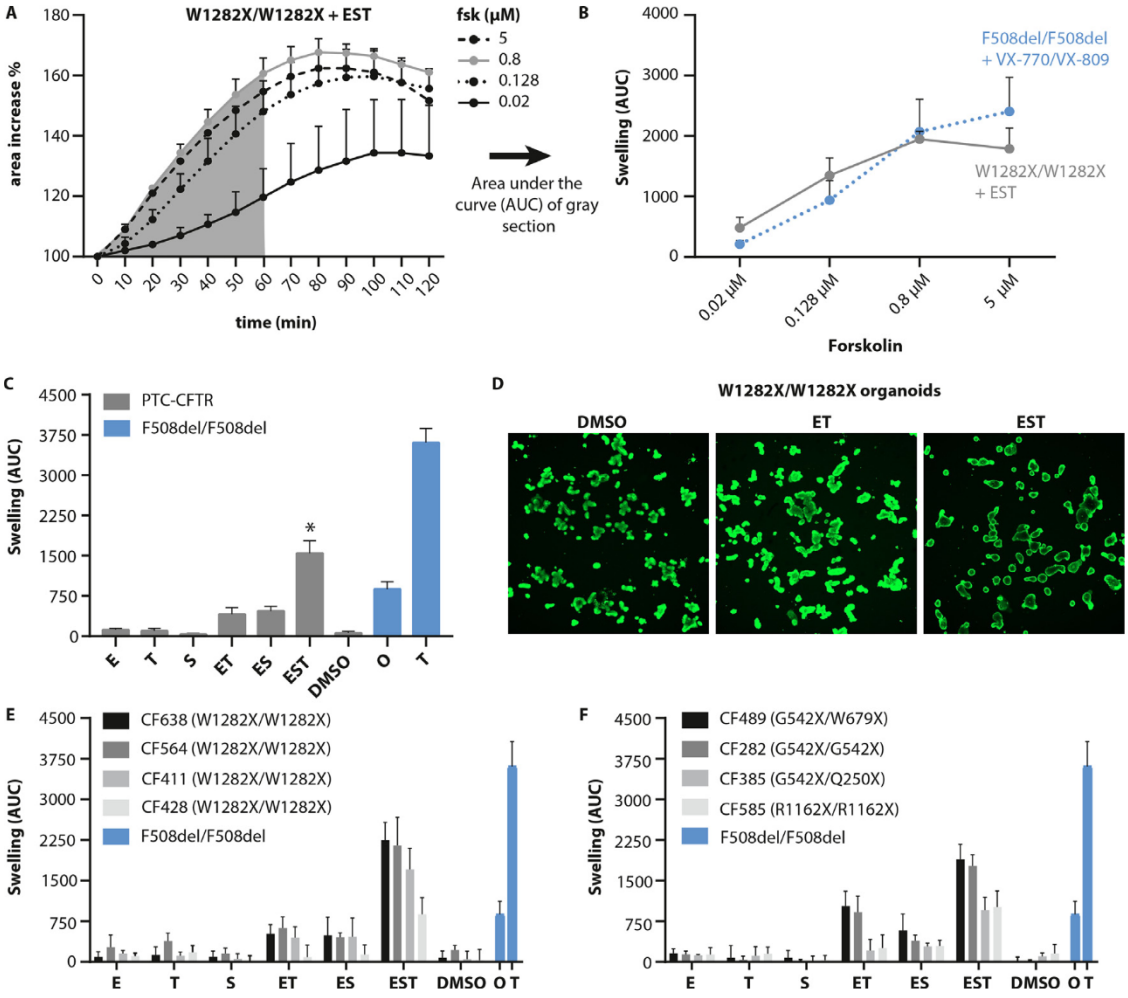

Fig. 3. FIS of various homozygous PTC expressing organoid cultures upon treatment with pharmacotherapies that (collectively) stimulate read-through, inhibit NMD and/or modulate CFTR protein trafficking or gating. **(A)** Area increase in time (min) of W1282X/W1282X organoids upon addition of an increasing concentration of fsk. Organoids were pre-stimulated with EST (bars represent mean+SD, n=3). **(B)** Swelling (area under the curve of t=0 to t=60 minutes, gray area in **A**) of W1282X/W1282X organoids pre-stimulated with EST (datapoints represent mean+SD, n=3) and mean swelling of three F508del/F508del organoid cultures pre-stimulated with VX-809 (3 μM, 24h) + VX-770 (3 μM, 0h) (datapoints represent mean+SD, n=9) upon stimulation with an increasing concentration of fsk. To determine the therapeutic value of EST with 0.128 μM fsk, the swelling levels were compared to the mean swelling levels of three organoid cultures expressing F508del/F508del-CFTR upon rescue with VX-770 (3 μM, added with fsk) + VX-809 (3 μM, 24h), datapoints represent mean+SD, n=9). Similar F508del/F508del data is shown in C-F and supplmental Fig. 2**(C)**. Mean FIS of all homozygous PTC organoid cultures, measured for 1h in presence of 0.128 μM fsk. Organoid cultures were pre-stimulated with E, S or T and all combinations thereof. Bars represent mean+SEM, n=8. *p-value<0.05, compared to DMSO. **(D)** Miscroscopic images of W1282X/W1282X organoids after 60 minutes stimulation with 0.128 μM fsk, untreated or pre-stimulated with ET or EST. **(E and F)**. FIS (1h, 0.128 μM fsk) responses of four distinct organoid cultures, carrying two copies of the W1282X mutation **(E)** or two PTC mutations other than W1282X **(F)** upon pharmacotherapy treatment. Abbreviations: fsk = forskolin; E = ELX-02ds (80 μM, 48h); S = SMG1i (0.3 μM, 24h); T = VX-661 (3 μM, 24h) + VX-445 (3 μM, 24h) + VX-770 (3 μM, added simultaneously with fsk); O = VX-809 (3 μM, 24h) + VX-770 (3 μM, added simultaneously with fsk).

no SNPs were observed in the region 600 nucleotides before and 400 nucleotides after the PTC (data not shown). Whilst the combination SMG1i and VX-661/VX-445/VX-770 (ST in **Fig. S2C**) moderately rescued W1282X-CFTR, although again with within-genotype variation, functional rescue of the PTCs R1162X, G542X or W679X require the addition of the RT-agent: ELX-02ds (**Fig. S2C**). The most effective pharmacotherapy showed to consist of the combination of ELX-02, SMG1i and VX-661/VX-445/VX-770 (EST in Fig. 3) and resulted in tripling of the mean AUC value compared to the dual compound therapies (Fig. 3**C-D**). Interestingly, in three of the W1282X/W1282X (Fig. 3**E**) and two homozygous PTC donors (Fig. 3**F**) rescue of CFTR function reached AUC levels that were in between reference AUC values of F508del/F508del organoids treated with VX-809/VX-770 (mean AUC 940 +/- 32, n=9) and VX-661/VX-445/VX-770 (mean AUC of 3327 +/-383, n=9). As can be observed in **Fig. S2A,** level of swelling was halved in organoid cultures expressing one PTC mutation, yet levels were still comparable to VX-809/VX-770-rescued F508del/F508del-CFTR. These data show that combining RT agents with NMD inhibitors and CFTR modulators represents a potential therapeutic option for treating PTC mutations.

CFTR mRNA rescue of panel of various PTC harboring organoid lines in response to combinations of ELX-02ds, SMG1i and VX-661/VX-445/VX-770

Additionally, the expression of CFTR mRNA in response to all compounds by qRT-PCR was characterized. In general, the expression level of CFTR mRNA is lower in organoid cultures having two PTC mutations compared to WT organoids, as the expression levels of the different donors in the DMSO condition shown in Fig. 4**A** and **B** are below 50% of WT expression . Whilst not significant, ELX-02 shows a minor increase in CFTR mRNA. Yet, all conditions including SMG1i resulted in a significant increase in mRNA similar

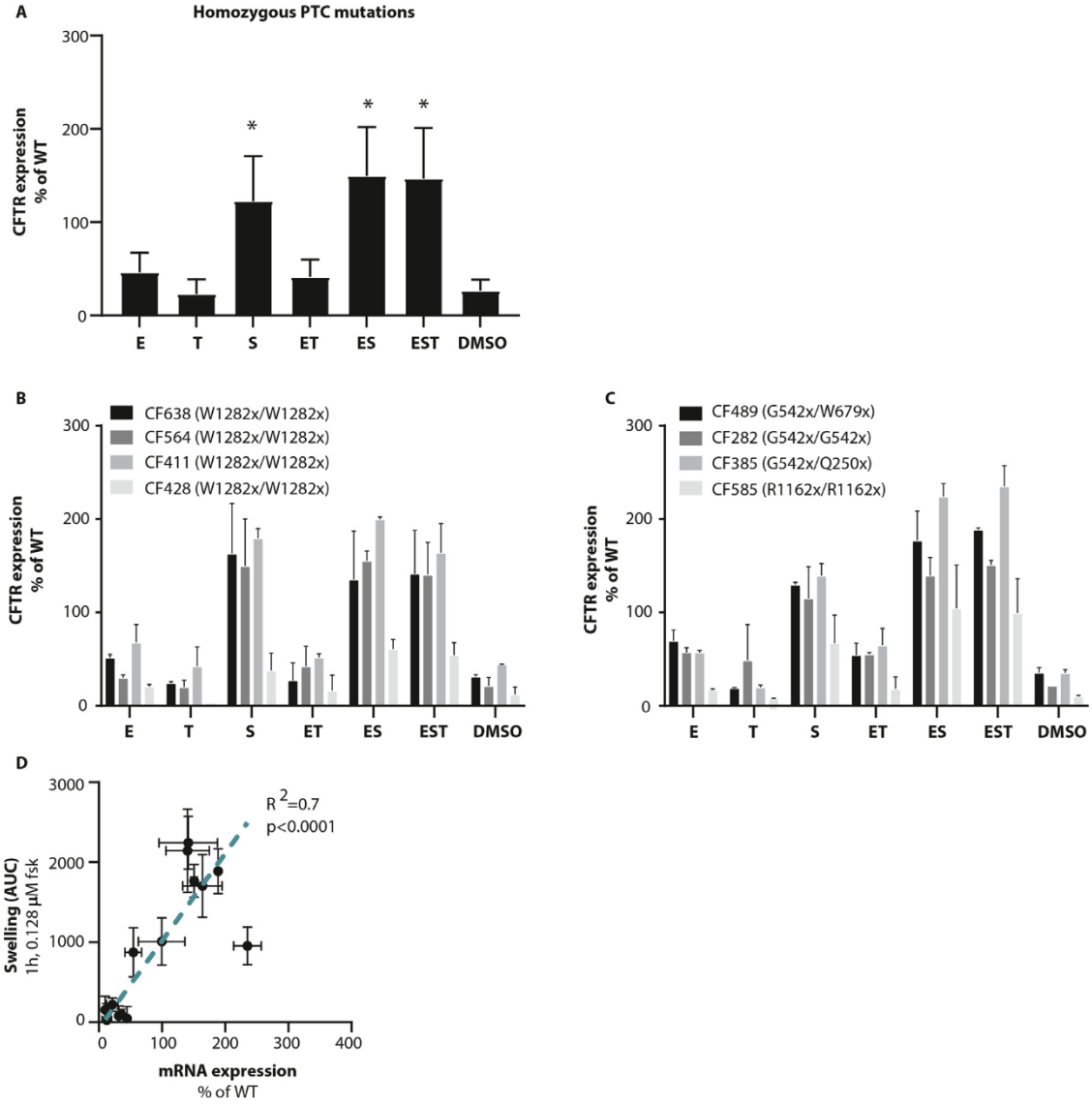

Fig. 4. CFTR mRNA expression of organoid cultures homozygous for PTC mutations upon pharmacotherapy. **(A)** mean mRNA expression level of CFTR, normalized to the reference gene YWHAZ and expressed in % of mean of 5 WT/WT organoid cultures (bars represent mean+SD, n=2). *p<0.05, compared to DMSO. **(B and C)** mRNA expression level of CFTR of idistinct organoid cultures. The organoid cultures and pre-treatments in A and B correspond to those in Figure 3E and 3F, respectively. **(D)** Correlation between swelling (1h, 0.128 μM) and CFTR mRNA expression of untreated (DMSO) organoids or organoid cultures pre-stimulated with EST. Abbreviations: WT = wildtype; E = ELX-02ds (80 μM, 48h); S = SMG1i (0.3 μM, 24h); T = VX-661 (3 μM, 24h) + VX-445 (3 μM, 24h) + VX-770 (3 μM, added simultaneously with forskolin).

to or higher than 100% of WT expression in all organoid cultures. Moreover, the level of mRNA expression increase correlates with the level of CFTR function rescue by EST (R^2 of 0.74, Fig. 4**C**). This indicates that treatment with NMD-inhibitors is required for a high quantity of functional protein following RT, which can be further enhanced with CFTR modulation.

Discussion

In this paper, we hypothesized that CFTR function of CF patient-derived organoids harboring PTC mutations, can be rescued by a combination of pharmacotherapies and investigated the contributions of PTC rescue by molecules that a) amplify CFTR transcription (PTI-428), b) enhance PTC-RT (ELX-02ds), c) inhibit NMD (SMG1i, NMDi-14 and Vidaza), d) stimulate correct CFTR protein trafficking (VX-661 and VX-445) and e) enhance gating activity of CFTR at the cell membrane (VX-770 and ASP-11). We found that a combination of ELX-02ds, SMG1i and VX-661/VX-445/VX-770 resulted in robust CFTR rescue, higher than observed with VX-809/VX-770-treated F508del/F508del organoids. We characterized compound efficacy for a panel of 12 organoid lines harboring various PTC mutations, including the most common W1282X and G542X alleles as well as less prevalent PTC alleles. We observed differences in efficacy between genotypes as well as differences within one genotype.

As the presence of a PTC mutation results in a truncated variant of the protein, efficacy of the RT-agent ELX-02ds was first characterized. In contrast to a recent study [8], ELX-02ds as single agent did not result in AUC values that are comparable to VX-809/VX-770-treated F508del/F508del organoids. Moreover, CFTR mRNA was increased by ELX-02-mediated NMD-inhibition in the recent study, a mode of action we could not fully confirm with our qPCR data. This difference might be contributed to the commercially available ELX-02ds used in this study, versus the ELX-02 from Eloxx Pharmaceuticals itself. Despite this difference, the commercially available version has RT activity that can be strongly enhanced by NMD inhibition and clinically available CFTR protein modulators. Whilst RT is essential for restoring full-length CFTR, the pool of mRNA sus-

ceptible for RT is diminished by NMD. Multiple compounds that inhibit different components of the NMD machinery have been described, but especially SMG1 inhibition has yielded success in various preclinical models [10,14]. We indeed found that SMG1 inhibition resulted in a concentration-dependent elevation of functional CFTR levels. A challenge concerning NMD inhibition however remains that NMD influences multiple cellular pathways. As a result, a high concentration of SMG1i has been associated with in vitro cellular toxicity [17] and caution must be taken with using NMD-inhibitors as a therapeutic compound, which is likely to be the reason only a single NMD-inhibitor has reached the clinic at present-day. The NMD-inhibitor Vidaza (5-Azacytidine) has been approved for the treatment of myelodysplastic syndrome and myeloid leukemia [26–28], however Vidaza therapy is associated with severe side-effects, and showed to be ineffective in our study, which was confirmed by others [10]. Compared to Vidaza or NMDi-14, SMG1i was the least toxic and effectively increased CFTR mRNA expression at a relatively low concentration. SMG1i is therefore an interesting target for further drug development, aiming to develop a safer, yet still effective NMD inhibitor. A strategy to develop more specific NMD inhibitors is to target a different effector protein involved in the NMD machinery. According to our results, targeting SMG7 with NMDi14 or MYC, an endogenous NMD-inhibitor, with Vidaza had no effect on CFTR function rescue. Nevertheless, many other effector proteins remain to be investigated and could potentially be targeted for more selective NMD inhibition.

Theoretically, RT and NMD together could result in normal amounts of full-length CFTR, yet we did not observe high rescue of CFTR function in our FIS assay with this dual pharmacotherapy. This can be contributed to the fact that amino acid incorporation upon RT varies per type of PTC and even per single transcript [16] and may thus not fully recapitulate WT protein function or stability. A previous study has shown that function of such W1282X variants can indeed be enhanced by combining RT agents with conventional CFTR correctors or potentiators [16]. In line with this study, swelling of PTC organoids upon treatment with ELX-02ds/SMG1i and VX-770/VX-661/VX-445 indeed reached AUC levels higher than that of VX-770/VX-809 treated F508del/F508del organoids. The optimal conditions for PTC restoration did not reach efficacies associated with VX-445/VX-661/VX-770 on F508del/F508del organoids. Whilst the results obtained with the ELX-02ds/SMG1i/VX-770/VX-661/VX-445 combination are promising, pharmacokinetic and drug-drug interaction studies will have to further elucidate the feasibility of combining these 5 different pharmacotherapies in vivo. On this note, in this study the effect of VX-445/VX-661/VX-770 was only tested in combination. Future research should investigate whether this is indeed necessary, or whether the combination with ELX-02ds/SMG1i and a single CFTR modulator could result in sufficient functional CFTR rescue.

This report focusses on the use of intestinal organoids as preclinical test model for restoration of PTCs. Organoid FIS is completely CFTR dependent and the relation between in vitro swelling response and *in vivo* drug response and disease severity has been well characterized. Earlier work also found that G418 can induce CFTR function in PTC-containing organoids, but CFTR restoration by PTC-124 was not detected consistent with lack of efficacy in clinical trials with this drug [4,6]. Biobanks of organoids have been established and such infrastructures enable not only large-scale preclinical testing in patient cells but also the recruitment of preclinical responders for clinical trials. Whereas the simple phenotypic swell readout represents one of the strengths of this model, it also represents a limitation as swelling is limited by organoid stretch that limits the dynamic range of the assay at high CFTR function. The data could be further strengthened by protein analysis to demonstrate that ELX-02ds and the various combinations induced full length mature CFTR protein, as recently showed by Crawford et al. [8] in PTC-containing organoids rescued by ELX-02. Contradictory to this study however, Laselva et al did not observe an effect of G418 on CFTR protein level in human nasal cells [10]. Potentially low CFTR levels, below the detection limit of certain assays such as western blot, are sufficient to detect effects on a functional level. This is likely dependent on the exploited phenotypic assay, its sensitivity and the studied cell model. Evidently, comparison of results in different models is valuable. In contrast to the results described in this study, inhibition of nonsense mediated decay by SMG1i with addition of effective protein modulators exerted a dominant functional rescue of W1282X in primary nasal cultures that was not enhanced with the addition of G418. Whether this discrepancy is a consequence of the use of a different in vitro model and cell type, the differences between G418 and ELX-02ds, G418 induced toxicity or even patient to patient variation warrants further investigation. Future research could be conducted to assess whether functional recue of PTC's in nasal or bronchial epithelial cells is achievable with the compounds discussed in this study and whether its efficacy is comparable to the results achieved in intestinal organoids.

Overall, we observed CFTR function rescue in all organoid cultures, yet in between-genotype and within-genotype variation in the level of CFTR function rescue was also observed. RT efficacy has been described to be dependent on the identity of the PTC, from least to most susceptible: UAA<UAG<UGA, yet a donor carrying a UGA mutation on both alleles (R1162X/R1162X-CFTR), showed to be one of the lowest responding donors, indicating the between-genotype variation cannot only be explained by PTC-dependency of RT. While RT-efficacy is also moderated by the local and distant sequence surrounding the PTC [29,30], we did not find additional SNPs in our four W1282X/W1282X organoid cultures which could not explain the observed within-genotype variation. Nevertheless, even the low responding organoid cultures almost reached swelling levels comparable to F508del/F508del-CFTR rescued with VX-770/VX-809, indicating that independent of the PTC mutation, the level of CFTR function rescue has clinical potential.

In conclusion, this proof of concept study shows that truncated, defective CFTR protein harboring PTC mutations can be effectively repaired with a combination of pharmacotherapies. Whilst further studies are necessary to translate these studies to the clinic, we provide a potential mechanism to resolve the unmet need for a therapeutic approach for people carrying PTC mutations.

Author Taxonomy

E.d.P. and **S.S** contributed to the design of the study, the acquisition, verification, analysis and interpretation of the data and have drafted the manuscript. **S.W.F.S., E.K., K.B., S.Y.G., M.A.M., E.J.M.W., M.M.vd.E.** and **G.H.K.** contributed to the acquisition of study data, provided resources and revised the manuscript. **C.K.v.d.E and J.M.B.** have made substantial contributions to the conception and design of the study, interpretation of data and revised the manuscript.

Declaration of Competing Interest

J.M.B. and C.K.v.d.E. are inventors on patent(s) related to the FIS-assay and received financial royalties from 2017 onward. J.M.B report receiving research grant(s) and consultancy fees from various industries, including Vertex Pharmaceuticals, Proteostasis Therapeutics, Eloxx Pharmaceuticals, Teva Pharmaceutical Industries and Galapagos outside the submitted work. C.K.v.d.E report receiving research grant(s) grant(s) from Vertex Pharmaceuticals (money

to institution) outside the submitted work. G.H.K. reports research grants from Vertex Pharmaceuticals, GSK, TEVA, Ubbo Emmius Foundation, European Union, Lung Foundation Netherlands (Money to institution), outside the submitted work. M.A.M. reports research grants and patient recruitment fees for clinical trials from Vertex, for which his institution Charité-Universitätsmedizine Berlin received payment; fees for consulting and advisory board participation from Antabio, Arrowhead, Boehringer Ingelheim, Enterprise Therapeutics, Kither Biotech, Sathera, Sterna Biologicals, and Vertex outside the submitted work. S.Y.G. reports fees for advisory board participation from Chiesi outside the submitted work. All other authors have nothing to disclose.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2021.09.020.

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