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Functional rescue of CFTR in rectal organoids from patients carrying R334W variant by CFTR modulators and PDE4 inhibitor Roflumilast

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ABSTRACT

Background: Many disease-causing variants in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene remain uncharacterized and untreated. Restoring the function of the impaired CFTR protein is the goal of personalized medicine, particularly in patients carrying rare CFTR variants. In this study, functional defects related to the rare R334W variant were evaluated after treatment with CFTR modulators or Roflumilast, a phosphodiesterase-4 inhibitor (PDE4i).

Methods: Rectal organoids from subjects with R334W/2184insA and R334W/2183AA > G genotypes were used to perform the Forskolin-induced swelling (FIS) assay. Organoids were left drug-untreated or treated with modulators VX-770 (I), VX-445 (E), and VX-661 (T) mixed, and their combination (ETI). Roflumilast (R) was used alone or as a combination of I + R.

Results: Our data show a significant increase in FIS rate following treatment with I alone. The combined use of modulators, such as ETI, did not increase further swelling than I alone, nor in protein maturation. Treatment with R shows an increase in FIS response similar to those of I, and the combination R + I significantly increases the rescue of CFTR activity.

Conclusions: Equivalent I and ETI treatment efficacy was observed for both genotypes. Furthermore, significant organoid swelling was observed with combined I + R used that supports the recently published data describing a potentiating effect of only I in patients carrying the variant R334W and, at the same time, corroborating the role of strategies that include PDE4 inhibitors further to potentiate the effect of I for this variant.

1. Introduction

Cystic Fibrosis (CF) is an autosomal recessive inherited disease caused by variants in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene encoding for the CFTR protein [1], an ATP-gated cAMP-regulated chloride (Cl^-) and bicarbonate (HCO_3^-) anion channel expressed at the plasma membrane (PM) of epithelial cells [1–3]. In CF patients, the respiratory tract is mainly involved, but any organ that exhibits significant exocrine function, such as the intestinal tract, can be affected [4,5]. To date, only about 40% of more than 2100 described CFTR variants [6] are classified as disease-causing when present in trans [7], and not all are included in the list of variants eligible for treatment with the currently available CFTR modulators [8]. One of these variants is R334W (c.1000C > T, p.(Arg334Trp)). This variant causes a 'mild' CF phenotype associated with pancreatic sufficiency and is classified as a class IV defect: the protein is properly processed but has a decreased conductance. Described in 408 patients, has an allelic frequency of 0.003%, as reported in the CFTR2 database (accessed in August 2023) [7]. The residue R334 is located within the 6 (TM6) transmembrane segment in the membrane-spanning domain 1 (MSD1) and is a key site for anion binding responsible for maximizing Cl^- flux through the CFTR channel [9,10].

The triple-combination therapy, Elexacaftor-Tezacaftor-Ivacaftor (ETI), has been approved by the FDA for all CF genotypes with at least one F508del copy present or a mutation in the CFTR gene that is responsive based on in vitro data, covering almost 90% of individuals

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with CF in Caucasian populations. Interestingly, for the R334W variant, recent experimental data published in the literature showed that is rescued in vitro by CFTR modulators, independently of the variant in the other allele [9,11]. For this reason, our experiments focused on the effect of combinations based on E, T and I.

Furthermore, it was reported that Roflumilast (R), a phosphodiesterase-4 (PDE4) inhibitor that reduces the rate of cAMP degradation, leads to a significant functional rescue for this variant [12]. Roflumilast is the only PDE4 inhibitor currently approved and used as a second-line drug in a subset of patients affected by severe chronic obstructive pulmonary disease (COPD) with chronic bronchitis, a disease that shares many pathologic characteristics with CF, such as mucus stasis and accumulation [13–15]. According to published data, R increases CFTR activity in vitro in airway epithelial cells, in intestinal monolayers and intestinal segments ex vivo [13,14,16]. The delay in a successful PDE4 inhibitor-based therapy is related to its intolerable adverse effect profile, with nausea, non-infectious diarrhea, abdominal pain, loss of appetite, weight loss, headache, and sleep disturbances as the most commonly observed side effects that could limit its use systemically [17,18]. Being highly expressed in the intestinal epithelium, Lambert and colleagues also hypothesize that R therapy could cause diarrhea by increasing CFTR-dependent fluid secretion [14]. However, identifying a role for this class of drugs might lead to compounds and/or formulations that might be delivered locally, such as aerosol, and thus improve treatment efficacy with CFTR modulators in the airways of patients with CF.

Since no information on the impact of the treatment on protein expression in primary cells is available nor the potential additive/synergistic effect of R to the treatment with CFTR modulators, in this study, we aim to describe the effect of I and ETI combinations using rectal organoids derived from two patients harboring the R334W in trans with a null variant and to evaluate the effect of R treatment on increasing R334W-CFTR conductance. Patient-derived intestinal organoids (PDIOs) are a three-dimensional self-organizing culture system derived from adult stem cells that mimic the architecture of in vivo tissue. For these reasons, PDIOs have been used to model disease phenotypes for drug discovery screening and as a robust predictive tool for drug response in vivo [19].

Our FIS results confirmed the positive effect of I in augmenting R334W-CFTR activity similar to ETI-treated R334W/2184insA and R334W/2183AA > G organoids. Furthermore, treatment with R potentiates the R334W-CFTR channel as well as I and elicits a further increase in the function of the R334W-CFTR channel when used together with I.

2. Materials and methods

2.1. Biological samples

Rectal biopsies were collected from CF participant with R334W/2184insA (n = 1) and R334W/2183AA > G (n = 1) genotype. All participants' subjects provided a written informed consent before colonoscopy examination, according rules of the local ethical committee (CRCFC-CFTR050).

2.2. Clinical data

The patients in this study are two female subjects carrying the R334W variant in trans with the 2184insAA and 2183AA > G CFTR class I variants. The first is a 41-year old woman, presenting with an abnormal sweat Cl-concentration at diagnosis: 96/97 mmol/L; chronic lung infection Pseudomonas Aeruginosa (Pa) and Methicillin-resistant Staphylococcus Aureus (MRSA) and impaired lung function (FEV: 47%) of predicted value. Body Mass Index (BMI) 20.8 kg/m2.

The second is a 36 years old female; she had an abnormal sweat Cl-concentration at diagnosis: 89/96 mmol/L; chronic infection Pseudomonas Aeruginosa (Pa), Achromobacter Xylosoxidans (Ach.Xyl) and Methicillin-Sensitive Staphylococcus Aureus (MSSA) and impaired lung function (FEV: 68%) of predictive value. Body Mass Index (BMI) 23 kg/m2. Both subjects are Pancreatic Sufficient (PS).

2.3. Chemicals

The CFTR modulators VX-661 (Tezacaftor, T) and VX-770 (Ivacaftor, I) were purchased by Selleck Chemicals LLC, Houston, TX, USA, while the modulator VX-445 (Elexacaftor, E) from Med Chem Express. Roflumilast, a potent and selective inhibitor of phosphodiesterase-4 (PDE4), was purchased from Selleck Chemicals (B9302-107).

2.4. Crypt isolation and organoid culture

Human rectal biopsies were treated as previously described [20]. Briefly, biopsies were recovered, stored in surgical medium (cold DMEM/F12 medium from Gibco with antibiotics (50 μg/mL gentamicin and 50 μg/mL vancomycin) and antifungal (2,5 μg/mL amphotericin B, Gibco) and then incubated in 10 mM EDTA for 90–120 min at 4 °C. The isolated crypts, after washing with cold PBS solution, were mixed with Matrigel 3D matrix (Corning, NY, USA) and plated in 40 μL per well in pre-warmed 24-well plates. After Matrigel polymerization, a pre-warmed complete medium consisting of advanced DMEM/F12 (supplemented with 1% penicillin and streptomycin, 0,2% primocin, 10 mM hepes, 1% Glutamax), 1x N2, 1x B27 (all from Invitrogen), 1,25 mM N-acetylcysteine (Sigma), 50 ng mL^{-1} mouse epidermal growth factor (mEGF), 50% Wnt3a-conditioned medium (WCM), 10% noggin-conditioned medium (NCM), 20% Rspo1-conditioned medium, 10 mM nicotinamide (Sigma), 10 nM gastrin (Sigma), 500 nM A83-01 (Tocris), 1 μM SB431542 (Tocris), 10 nM PGE (Sigma) and 3 μM SB202190 (Sigma) was added. The complete medium was supplemented with 10 μM Rho inhibitor (Y27623) and 10 μM GSK3 inhibitor (CHIR-99021) (Sigma) and additional antibiotics 50 μg/mL gentamicin and 50 μg/mL vancomycin) and antifungal (2,5 μg/mL amphotericin B) were used during the first week of culture. Medium was changed every other day and the organoids were expanded 1:3–1:4 times every 7 days.

2.5. Forskolin-induced swelling (FIS assay)

Following published guidelines [21], the organoids from a 7-day-old culture were reseeded in a 96 well plate in 5 μL of Matrigel (Corning) and was added 50 μL of culture medium with Vehicle (DMSO 0,1%) or CFTR correctors: 3 μM VX-661 and 3 μM VX-445. After 24 h of treatment, the steady-state organoids area (SOA area) was analyzed with microscopy by taking a picture at t = 0 hpt (0 h after treatment) and another picture at t = 24 hpt (24 h after treatment) (EVOS® FL Auto Imaging System with EVOS® onstage incubator, Thermo Fisher Scientific, Waltham, MA, USA) with 4× objective, at 37 °C and 5% CO_2. CFTR function recovery was then evaluated by performing a FIS assay, using the CFTR agonist forskolin 0.128 μM, the enhancer VX-770 3 μM or Roflumilast (0-0.5-1-3 μM). The organoids were directly analyzed with EVOS microscopy every 30 min for a total acquisition of 120 min in a time-lapse video. Every condition was analyzed in duplicate (two wells were used to study one condition). The increase in total area (plane xy) relative to t = 0 of forskolin treatment was calculated manually using Image J software (Version 1.53t August 24, 2022). The normalized data are expressed as the total area under the curve (AUC, t = 120 min; baseline, 100%), which was calculated using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA).

2.6. Immunoblotting

R334W/2184insA and R334W/2183AA > G organoids (about 100–150 per well for a total of 5 wells for each condition) were pretreated with VX-661 3 μM and VX-445 3 μM or DMSO 0.1% (Vehicle) for 24h. The Matrigel was removed using cell recovery solution (Corning)

and the pellet was lysed in RIPA lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate) with protease inhibitor cocktail (Roche, Mannheim, Germany) for 30 min in ice. Lysates were separated using SDS-PAGE on 7.5% homemade Tris-Glycine gel at 50 V for 15 min, and then at 100 V until separation was complete. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane via wet transfer at 100 V for 1 h. After blocking with 5% non-fat dry milk – Tris-buffered saline (TBS) –Tween (0.3%) and washing in TBS – Tween (0,3%), CFTR was detected with α-CFTR monoclonal antibodies (mAb) 450, 570, and 596 (CFF, Cystic Fibrosis Foundation) (1:1000 dilution each) at 4 °C overnight in 2% non-fat dry milk – TBS –Tween (0.3%). After the washing steps, the membrane was probed with goat mouse-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell signaling, 1:12,000 dilution in blocking solution). As a loading control, we used β-Actin detected by beta-Actin antibody (1:1000) (Cell signaling, 4970). Protein bands were visualised with ECL Westar Supernova ECL substrate (Cyanagen, Bologna, Italy) on Image-QuantTM LAS 4000 software (GE Healthcare, Chicago, IL, USA). Protein band densitometry was performed using ImageJ software (Version 1.53t August 24, 2022).

2.7. Statistical analysis

Data are represented as mean ± S.D. GraphPad Prism 7 software (San Diego, CA, USA) was used for all statistical analysis. Statistical comparisons between the conditions tested were assessed using one-way ANOVA followed by Dunnett's or Tukey's post hoc tests, and p-values ≤ 0.05 were considered significant.

3. Results

3.1. Evaluation of CFTR modulators effect in protein processing and function for R334W/2184insA and R334W/2183AA > G organoids

The rescue of CFTR function was evaluated using the forskolin-induced swelling (FIS) assay, a reliable ex vivo biomarker and a good predictor of clinical response to CFTR modulators [22]. R334W/2184insA and R334W/2183AA > G rectal organoids were pre-incubated for 24 h with DMSO (vehicle) or with correctors VX-661 (T) in combination with VX-445 (E) and stimulated acutely with forskolin (fsk) and VX-770 (I) as indicated in the figure (Fig. 1A and B). Our data suggest that ET treatment did not recover the steady-state organoids area (SOA area) suggesting that cAMP levels were not sufficient to ensure a detectable increase in swelling in the absence of fsk. Furthermore, the FIS rate for organoids treated with ET was similar to that of the control condition, suggesting that ET treatment did not recover mutant CFTR function. The assay started with the addition of fsk and resulted in a 3-fold increase (AUC value) over the initial area under both conditions (untreated and pretreated organoids). Conversely, stimulation with potentiator I alone resulted in significant organoid swelling when compared with the vehicle-treated organoids and no further increase in swelling was observed with pre-treatment with ET, suggesting a lack of efficacy of the correctors combination (Fig. 1A and B).

We next characterized the impact of R334W on protein maturation status. Our Western blot results showed that both forms of the CFTR protein, immature (band B) and mature (band C), were detected in R334W/2184insA and R334W/2183AA > G organoids under control condition. No additional correction (increase of Band C levels) was observed with pretreatment with ET, in agreement with the FIS results (Fig. 1C and D).

3.2. Roflumilast significantly increases CFTR function in R334W/2184insA and R334W/2183AA > G organoids

Recent works have described the R334W variant as one of the most responsive to PDE4 inhibitors, a family of compounds whose mechanism of action is based on the blocking of the catalytic site of phosphodiesterase enzymes (PDE). The suppression of cAMP degradation results in an increase of PKA activation, subsequently increasing CFTR phosphorylation and function 12,23,24. We therefore sought to confirm these evidences by adding R alone or together with I in FIS assay. We then first evaluated the dose-response of fsk in the presence of 1 μM R (Fig. 2A). A concentration selected after the analysis of a dose response of R (from 0.5 to 3 μM) during organoids stimulation with fsk 0.128 μM (Fig. 2B), in which a maximum swelling was observed at a concentration of 1–3 μM R, resulting in AUC values similar to the treatment of the same OGs with I. Moreover, the combination of I and R significantly improved CFTR function, eliciting a further organoids' swelling for both the genotypes analyzed (Fig. 2C).

4. Discussion

Treating CF patients with the rare CFTR variant is still a challenge due to the lack of eligibility for treatment with approved CFTR modulators. R334W is one of these "untreatable" variants. Here we evaluated the pharmacological response of two CF subjects carrying R334W, in trans with a class I frameshift variant, 2184insA and 2183AA > G, respectively, to CFTR modulators. Phenotypically, these variants are associated with a considerable variability in the respiratory pattern and pancreatic insufficiency when combined with another pathogenic variant that causes pancreatic insufficiency [6,7,25–27]. Combinations in trans R334W/2184insA and R334W/2183AA > G are currently described in the CFTR2 database, respectively, in only one and two pancreatic sufficient patients [7].

Our data show that Ivacaftor is able to rescue CFTR function in rectal organoids compound heterozygous for R334W. In our organoid lines, ET correction does not induce an additional increase in swelling or protein maturation. To our knowledge, CFTR protein expression has never been analyzed in other organoids carrying the R334W variant. Additionally, we confirm that the PDE4 inhibitor R alone significantly potentiates the function of the R334W-CFTR channel, as does I. Notably, the combined treatment I + R added acutely further increase the rescue of CFTR activity, confirming the different and complementary mechanisms that I and R have on CFTR activity of the R334W-CFTR variant.

Previous studies on PDIOs expressing the R334W variant have evaluated the effect of modulators on CFTR functional rescue where a significant increase in channel function has been observed. Ciciriello et al. showed that the ETI combination restored CFTR function in R334W/F508del intestinal monolayers. In addition, a significant clinical response was observed after a few weeks of therapy. But there was no possibility of discriminating which variant effectively responded to the treatment [9]. Restoration of R334W-CFTR activity has also been observed using Ivacaftor alone. A patient with R334W/1677delTA genotype demonstrated an improvement of lung function, following organoid-guided treatment with Ivacaftor with no reported advantage on FIS assay with the use of ET combination [28]. Another result has been observed in both R334W-CFTR expressing CFBE cell line and in PDIOs homozygous or compound heterozygous for R334W, in which the stimulation with ivacaftor resulted in significant organoids swelling. In R334W/R334W genotypes the authors observed no significant increase in functional response following ETI treatment, but a significant increase in swelling was reported for three cases carrying a null variant in the second allele. ETI triple-combination induced a higher level of mature CFTR expression (band C) in CFBE cells suggesting a potential additional rescue of CFTR by the ET correctors [11]. Interestingly de Poel et al., after evaluating the response of the R334W variant to treatment with I and I/L (Lumacaftor), observed that the R334W genotype responds to phosphodiesterases (PDEs) inhibitors [12]. PDE4 is the main PDE variant whose inhibition is related to increased CFTR function and its expression is higher than that of the other PDE variants, as measured in PDIOs [12]. Among the known PDE4 inhibitors Roflumilast, a drug

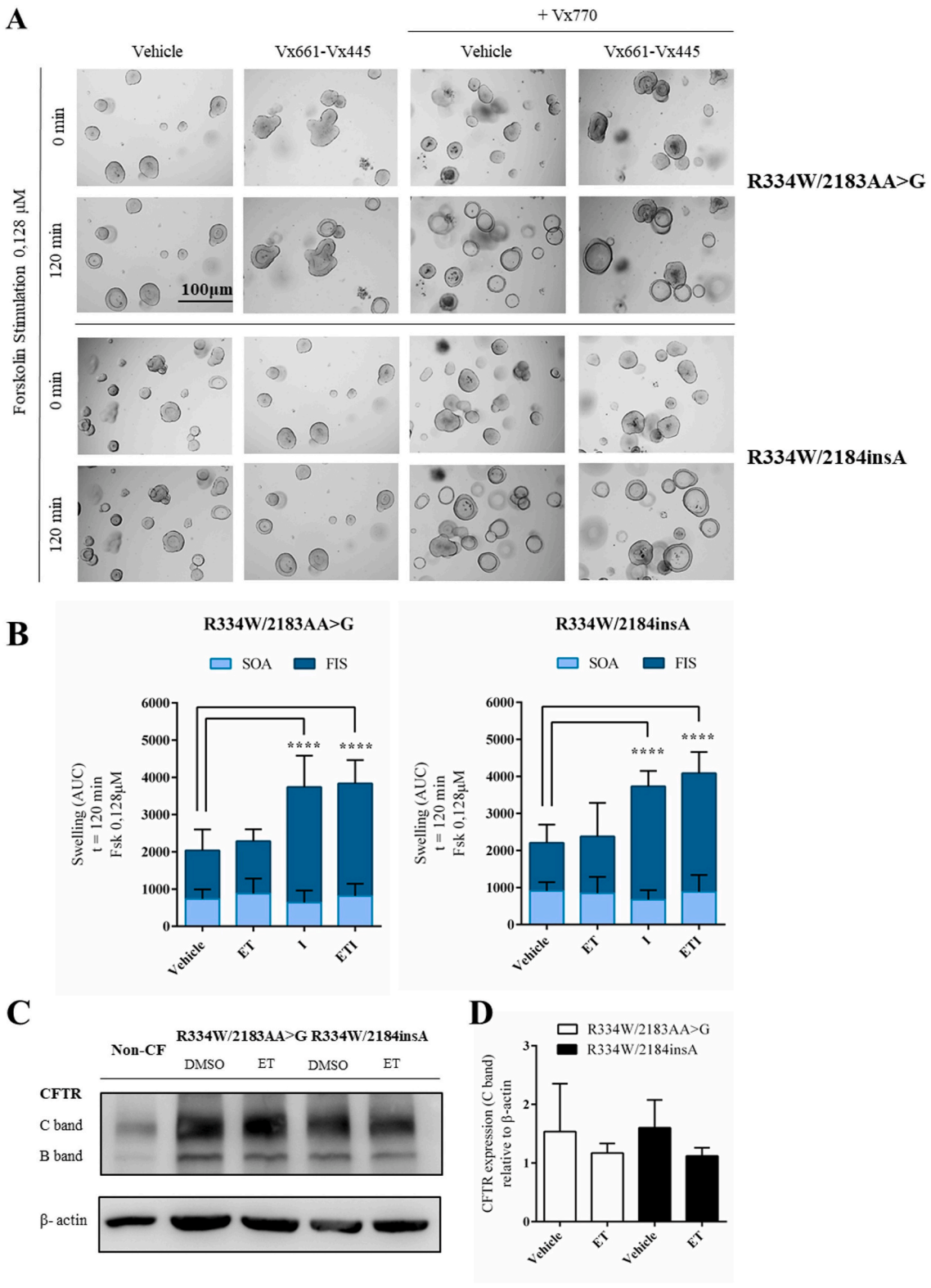

Fig. 1. Pharmacological correction of R334W/2184insA and R334W/2183AA > G on CFTR function and processing. (A) FIS assay: representative brightfield images of rectal organoids with or without VX-770 (I) treatment in VX-661+VX-445 (ET) pretreated organoids (3 µM each). **(B)** Quantification of 120 min of FIS performed on rectal organoids pre-incubated for 24 h with the pharmacological treatments as indicated. Data are mean ± Standard Deviation (SD) from three independent experiments, ****$p < 0.0001$ (Dunnett's test) **(C)** CFTR protein expression by Western blot. The CFTR expression of the C-band in the organoids R334W/2184insA and R334W/2183AA > G (40 µg of protein lysate loaded) remains unchanged by treatment with the correctors VX661/VX445 (ET) compared to control (20 µg of protein lysate loaded). **(D)** Densitometric analysis of Western blot results. Relative band C intensity was normalized to the intensity of the respective β-actin signal. Data are presented as the mean ± SD (n = 2).

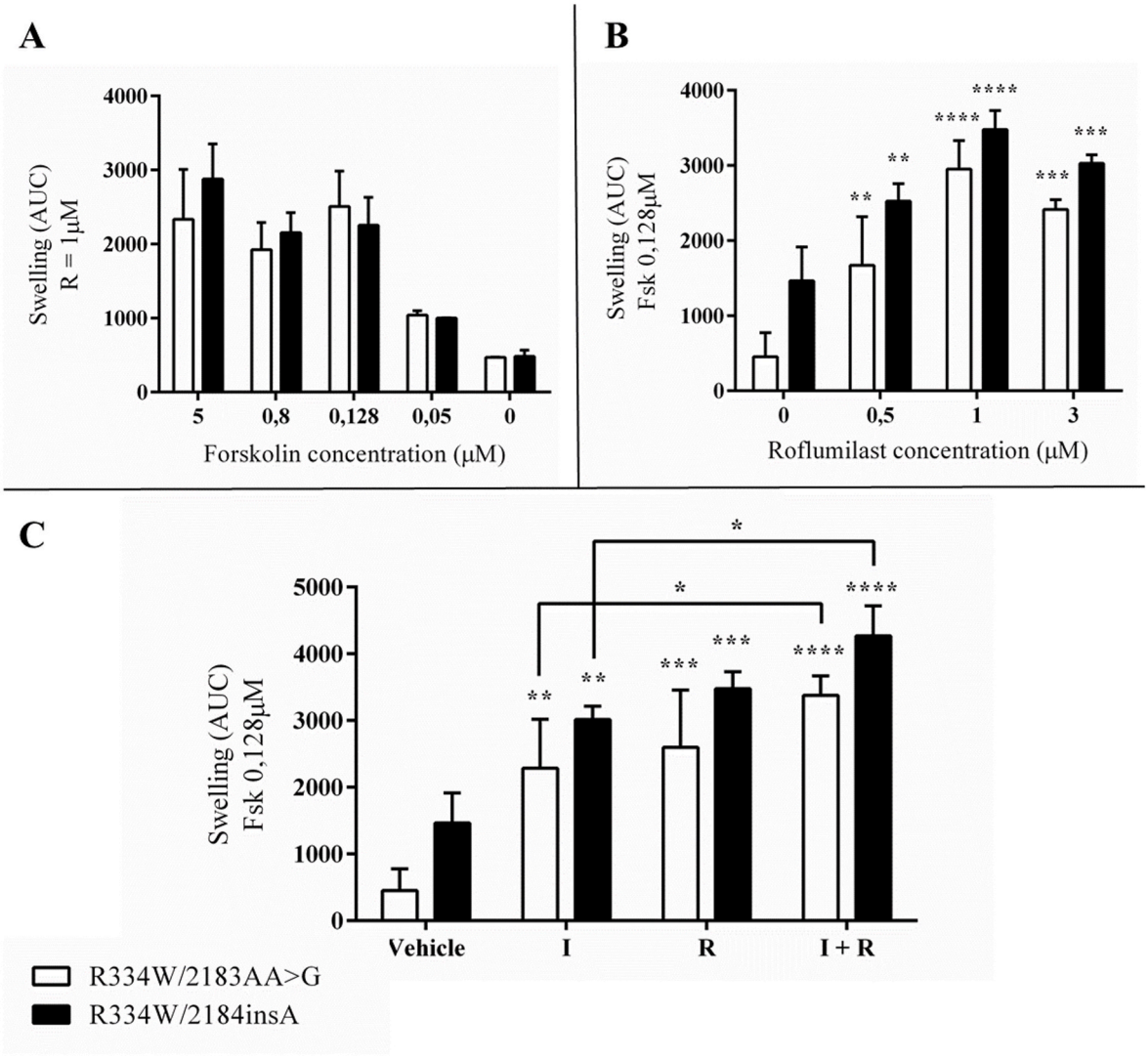

Fig. 2. Roflumilast treatment increases R334W-CFTR function as measured by FIS assay. **(A)** Quantification of FIS rates (AUC levels) on R334W/2184insA and R334W/2183AA > G rectal organoids upon acute treatment of R (1 μM), using different concentration of fsk. **(B)** Organoids swelling in response to fsk 0.128 μM together with acute addition of different concentration of R. Data are presented as mean ± SD. **p ≤ 0,01, ***p ≤ 0,001 ****p ≤ 0,0001 versus Vehicle (R 0 μM) (Dunnett's test) **(C)** FIS rates upon acute treatment of I 3 μM and R 1 μM alone or with their combination. Data are mean ± SD from three independent experiments. **p ≤ 0,01, ***p ≤ 0,001 and ****p < 0.0001 versus vehicle; linked conditions * p < 0.05 I versus I + R (Tukey's test).

approved for the treatment of severe chronic obstructive pulmonary disease (COPD) [29], results in a better increase in CFTR function compared to other compounds in the R334W/R334W genotype tested, and its maximum efficacy is observed at 0.128 μM forskolin. But less effect of R was seen in R334W/R764X genotype. No combination of R with other drugs has been evaluated [12]. Altogether, these data suggest a variable response of the R334W variant to ETI combination, while treatment with I consistently results in an improved CFTR function.

In our study, data obtained from PDIOs carrying the R334W/2184insA and R334W/2183AA > G genotypes show a significant increase in FIS rate following treatment with Ivacaftor alone. The combined use of modulators, such as the ETI combination, did not result in a significant further increase in function nor protein maturation, a result consistent with correct processing of the mutant protein R334W characterized by reduced channel activity [30,31]. Especially, in our experiments, the CFTR expression of the C-band remains unchanged by treatment with the ET combination, compared to the control. As Western blot analysis on CFBE cell line stably expressing R334W-CFTR showed a small but significant effect of ET on protein processing [11], a difference in CFTR processing in native and heterologous systems can be envisioned.

Our results also highlight that treatment with Roflumilast alone significantly increases the FIS response similar to those of Ivacaftor. Indeed, in contrast to R, I potentiates CFTR activation by uncoupling ATPase activity from channel gating [32–34]. This could explain the additive activation recorded. However, both I and R, in the absence of forskolin that acts by increasing the levels of cAMP, are not capable of causing swelling, suggesting the need to reach a threshold level of cAMP inside the cell to detect a functional response, a condition well known in the literature and the basis for the development of the FIS assay.

Since the 2184insA and 2183AA > G variant alleles are class I frameshift variants that result in the absence or extreme reduction of CFTR expression, along with the data already present in the literature, we can conclude that the response observed after treatment with I and with R can be attributed entirely to the variant R334W.

Taken together, our data indicate the potential efficacy of in vivo treatment with Ivacaftor and suggest using PDE4 inhibitor drugs, such as Roflumilast, as a representative member of this class, as potentially beneficial for patients carrying R334W variant. PDE4 inhibitors could be used alone or in combination with modulators either systematically or

locally, even if, in the first case, R-related systemic side effects were reported [14]. Systemic side effects of PDE4 inhibitors include nausea, emesis, gastrointestinal effects, and others, that strongly limit the clinical application of these compounds [17,18,35]. Despite this, PDE4 inhibitors are currently used in patients with inflammatory respiratory disease or, locally, for skin diseases, being Roflumilast, Apremilast, and Crisaborole are the only approved. A specifically formulated inhaled administration of PDE4 inhibitors could reduce systemic exposure and adverse effects for treating respiratory diseases. Several new PDE4 inhibitors are currently under development, among which only inhaled CHF 6001 is advancing through clinical development, showing promising results in phase II clinical trials in asthma and COPD [17,36,37].

5. Conclusions

For carriers of rare variants, PDIOs have the potential to suggest an optimal personalized therapy and, in some cases, can provide the rationale to access modulating drugs used in clinic but not approved for a specific variant. Their support for selecting the most appropriate combination of CFTR modulators is also relevant, as in this case, by providing a rationale for focusing on Ivacaftor as the primary treatment, as ETI combination, apparently is not required for gaining a major additional improvement of CFTR function. In this study we can summarize the new findings as follows: 1) We have analyzed CFTR protein expression in intestinal organoids carrying the R334W variant; 2) we show that the PDE4 inhibitor Roflumilast significantly potentiates the R334W-CFTR channel, as well as Ivacaftor, and how the combined treatment R + I further increase the rescue of CFTR activity; 3) These genotypes were not previously evaluated for their response to CFTR modulators.

Since the ultimate goal of personalized medicine is patient-specific treatment, extensive data on the effectiveness of these pharmacological therapies must be provided before clinical approval. This study supports and extends recently published data indicating that R334W, combined with 2184insA and 2183AA > G frameshift variants, might benefit from combination with other treatments, such as PDE4 inhibitors, that could improve clinical outcomes.

These studies aim to help CF patients with orphan genotypes gain rapid and safe access to available CFTR modulators and possibly support repurposing other drugs.

Availability and requirements

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This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board CRCFC-CFTR050. Informed consent was obtained from the subjects involved in the study.

Declaration of competing interest

P.M. acted as paid expert testimony for Vertex Pharmaceuticals and declares no conflict of interest. R.V.L, M.C., M.B., J.C., K.K. and C.S. declare no conflict of interest.

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