β_2-Adrenergic receptor agonists activate CFTR in intestinal organoids and subjects with cystic fibrosis

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ABSTRACT We hypothesized that people with cystic fibrosis (CF) who express *CFTR* (cystic fibrosis transmembrane conductance regulator) gene mutations associated with residual function may benefit from G-protein coupled receptor (GPCR)-targeting drugs that can activate and enhance CFTR function.

We used intestinal organoids to screen a GPCR-modulating compound library and identified β_2-adrenergic receptor agonists as the most potent inducers of CFTR function.

β_2-Agonist-induced organoid swelling correlated with the *CFTR* genotype, and could be induced in homozygous CFTR-F508del organoids and highly differentiated primary CF airway epithelial cells after rescue of CFTR trafficking by small molecules. The *in vivo* response to treatment with an oral or inhaled β_2-agonist (salbutamol) in CF patients with residual CFTR function was evaluated in a pilot study. 10 subjects with a R117H or A455E mutation were included and showed changes in the nasal potential difference measurement after treatment with oral salbutamol, including a significant improvement of the baseline potential difference of the nasal mucosa (+6.35 mV, $p<0.05$), suggesting that this treatment might be effective *in vivo*. Furthermore, plasma that was collected after oral salbutamol treatment induced CFTR activation when administered *ex vivo* to organoids.

This proof-of-concept study suggests that organoids can be used to identify drugs that activate CFTR function *in vivo* and to select route of administration.

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Introduction

The *CFTR* (cystic fibrosis transmembrane conductance regulator) gene encodes an apical anion channel and is mutated in subjects with cystic fibrosis (CF) [1]. Subjects with CF have an altered composition of many mucosal surface fluids, leading to dysfunction of the gastrointestinal and pulmonary systems as well as other organs. The most common mutation is a deletion of phenylalanine at position 508 (p.Phe508del; F508del) and is present in ∼90% of subjects with CF, of which ∼65% are F508del homozygotes (www.genet.sickkids.on.ca). CF disease expression is highly variable between subjects due to the complex relations between *CFTR* genotype, modifier genes and environmental factors, which are unique for each individual [2–6].

Approximately 2000 *CFTR* mutations have been described, which are divided into different classes according to their impact on CFTR expression and function [7]. Briefly, class I mutations result in no functional protein (*e.g.* stop codons and frameshifts), class II mutations severely affect apical trafficking (*e.g.* F508del), class III mutations disrupt channel regulation or gating (*e.g.* p.Gly551Asp; G551D), class IV mutations reduce channel conductance (*e.g.* p.Arg334Trp; R334W), class V mutations lead to reduced apical expression of normally functioning CFTR (*e.g.* p.Ala455Glu; A455E) and class VI mutations accelerate CFTR turnover at the plasma membrane. Whereas class I–III and VI mutations are generally associated with no or very limited residual function, some residual function is associated with class IV and V mutations and milder disease phenotype such as CFTR-A455E and CFTR-R117H (p.Arg117His; shared class III and IV) (www.cftr2.org).

Novel drugs are being developed to target mutation-specific CFTR defects. The potentiator VX-770 (ivacaftor) enhances the activity of apical CFTR and was shown to provide clinical benefit for patients with CFTR gating mutations [8–10]. Pharmacological repair of CFTR-F508del has proven more difficult, although encouraging phase III clinical trial results have been reported for CFTR-F508del homozygous subjects treated with a combination of ivacaftor and the corrector lumacaftor (VX-809) [11], which partly restores trafficking of CFTR-F508del to the apical membrane [12]. However, the therapeutic effects of these therapies are variable between subjects, and remain insufficient to fully restore CF and CFTR-related disease markers, indicating that more effective treatments are still required.

Individual CFTR function depends on endogenous signalling pathways that control its channel function. Various endogenous ligands have been identified which activate CFTR in a cAMP/protein kinase A-dependent fashion. Many of these ligands (*e.g.* vasoactive intestinal peptide, prostaglandins and β-adrenergic stimuli) signal by binding to G-protein coupled receptors (GPCRs), which release cytosolic G-proteins that activate adenylyl cyclase to generate cAMP [13–15]. While it is known that tissue-specific activity of CFTR is regulated by diverse ligands, the extent to which CFTR function is limited by cAMP production is not clear.

We hypothesised that cAMP-dependent signalling is a rate-limiting step for CFTR activation *in vivo* and that CF individuals who express alleles associated with residual function might benefit from existing drugs that stimulate cAMP. Therefore, we screened a small chemical compound library of GPCR modulators for their ability to stimulate (mutant) CFTR activity in primary rectal organoids from healthy control and CF subjects. Rectal organoids grow from intestinal stem cells and self-organise into multicellular three-dimensional structures consisting of a single epithelial layer, with the apical membrane facing a closed central lumen [16–18]. Addition of forskolin, which raises cAMP, stimulates CFTR-dependent fluid secretion into the organoid lumen and induces rapid organoid swelling [19, 20]. Here, we provide proof-of-concept that intestinal organoids can be used as tool to identify potential drugs and route of administration for particular CF subgroups.

Materials and methods

Human participants

This study was approved by the Ethics Committee of the University Medical Centre Utrecht and the Erasmus Medical Center Rotterdam. Informed consent was obtained from all subjects. Organoids from healthy controls and CF subjects were generated from rectal biopsies after intestinal current measurements obtained 1) during standard CF care, 2) for diagnostic purposes or 3) during voluntary participation in studies.

Materials

The GPCR compound library, VX-809 and VX-770 were purchased from SelleckChem (Houston, TX, USA). Carvedilol, forskolin, salbutamol, salmeterol, terbutaline, epinephrine, ritodrine, dimethylsulphoxide (DMSO), *N*-acetylcysteine, nicotinamide and SB202190 were purchased from Sigma (St Louis, MO, USA). Formoterol was purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). $CFTR_{inh}$-172 was obtained from CFF Therapeutics (Chicago, IL, USA). Matrigel was purchased from BD (Franklin Lakes, NJ, USA). Calcein AM, supplements N-2 and B-27, Glutamax, advanced Dulbecco's modified Eagle medium/Ham's F-12 (DMEM/F-12), penicillin/streptomycin, HEPES and murine epidermal growth factor (mEGF) were

purchased from Life Technologies (Bleiswijk, The Netherlands). A83-01 was purchased from Tocris (Abingdon, UK). TOPflash and FOPflash were purchased from Millipore (Amsterdam, The Netherlands).

Human organoid cultures

Rectal crypt isolation and organoid expansion was performed with some adaptations of previously described methods [20, 21]. Briefly, rectal biopsies were thoroughly washed with PBS and incubated in 10 mM EDTA for 90 min at 4°C. The crypts were collected by centrifugation and suspended in 50% Matrigel and 50% complete culture medium (advanced DMEM/F-12 media supplemented with penicillin/streptomycin, HEPES, GlutaMax, nutrient supplements N-2 and B-27, *N*-acetylcysteine, nicotinamide, mEGF, A83-01, SB202190, and 50% Wnt3a, 20% Rspo-1 and 10% Noggin conditioned media) that was allowed to solidify at 37°C for 20 min in three droplets of 10 µL per well of a 24-well plate. The droplets were then immersed in pre-warmed complete culture medium and cultures were expanded for at least 3 weeks before assaying CFTR function. Complete culture medium was refreshed three times per week and organoids were passaged weekly. Quality of the conditioned media was assessed by dot blots, ELISA and luciferase reporter constructs (TOPflash and FOPflash) [22, 23].

GPCR compound library

The GPCR small-molecule compound library comprises 61 agonists and antagonists that target a wide range of GPCR families, including adrenergic, dopamine, opioid, serotonin, histamine and acetylcholine receptors. A complete list of chemicals in the library is given in online supplementary table S1.

CFTR function measurement in organoids

Organoids were reseeded 1 day before functional analysis in 96-well plates as described previously [20]. CFTR-F508del organoids were incubated with VX-809 (3 µM) for 24 h, as indicated in text and figure legends. Organoids were stained with Calcein green AM (2.5 µM) 1 h prior the addition of compound and each compound was tested at four different concentrations (10, 2, 0.4 and 0.08 µM). Forskolin (5 µM) and DMSO were used as positive and negative controls, respectively. Organoid swelling was monitored for 1 h using a Zeiss LSM 710 confocal microscope (Zeiss, Jena, Germany). The relative increase in surface area was calculated using Volocity (version 6.1.1; PerkinElmer, Waltham, MA, USA). The area under the curve was calculated as described previously [20]. Carvedilol (10 µM) was incubated for 30 min prior to stimulation and organoids were pre-treated with $CFTR_{inh}$-172 (150 µM) for 4 h to inhibit CFTR-dependent responses.

Halide-sensitive YFP quenching in CFBE41o- cells

CFBE41o- cell lines overexpressing CFTR-F508del or CFTR-WT were grown in α-minimal essential medium containing 8% heat-inactivated fetal calf serum, penicillin and streptomycin at 37°C in a humidified 5% CO_2 incubator as described [24, 25]. CFBE41o- cells were transduced with the ratiometric halide-sensitive pHAGFE2-YFP (46L-148Q-152L)-mKate sensor for measurement of CFTR activity as described previously [26]. Briefly, cells were incubated for 24 h with VX-809 (10 µM). After 20 min stimulation in a chloride-containing buffer, the cells were washed with iodide buffer and the decrease in fluorescence was monitored using a Zeiss LSM 710 microscope for 60 s. The rate of YFP (yellow fluorescent protein)/mKate quenching was calculated using Prism 6 (GraphPad, La Jolla, CA, USA).

Ussing chamber measurements in primary airway epithelial cells

Primary F508del/F508del human bronchial epithelial cells from the Primary Airway Cell Biobank of the CF Translational Research Centre at McGill University were cultured at the air/liquid interface for 3 weeks and pre-treated for 24 h with VX-809 (1 µM). Control monolayers from the same patients were handled similarly but exposed to vehicle (0.1% DMSO) during the pre-treatment period. For electrophysiological measurements, monolayers were mounted in Ussing chambers (EasyMount; Physiologic Instruments, San Diego, CA, USA) and voltage-clamped using a VCCMC6 multichannel current-voltage clamp (Physiologic Instruments, San Diego, CA, USA). The voltage clamp was connected to a PowerLab/8SP interface for data collection (ADInstruments, Colorado Springs, CO, USA) and analysis was performed using a PC as described previously [27]. Solutions were continuously gassed and stirred with 95% O_2/5% CO_2 and were maintained at 37°C by circulating water bath. Ag/AgCl reference electrodes were used to measure transepithelial voltage and pass current. Pulses (1 mV amplitude, 1 s duration) were delivered every 90 s to monitor resistance. A basolateral-to-apical Cl^- gradient was imposed and amiloride (100 µM) was added on the apical side to inhibit the epithelial sodium channel (ENaC) current. Monolayers were exposed acutely to 10 µM forskolin or salbutamol or to the vehicle 0.1% DMSO. After the short circuit current (I_{sc}) reached a plateau, a potentiator (either 50 µM genistein or 100 nM VX-770 as indicated) was added, followed by $CFTR_{inh}$-172 to confirm that the current responses were dependent on CFTR. Salbutamol was also assayed after pre-treatment with the antagonist carvedilol (10 µM) for 30 min as a further test of receptor specificity.

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Pilot study with inhaled and oral salbutamol

In this open-label phase II pilot study, 10 patients were randomly assigned to receive four times daily 200 µg salbutamol per inhalation or four times daily 4 mg salbutamol orally, for 3 consecutive days (www.trialregister.nl/trialreg/admin/rctview.asp?TC=4513). After a wash-out period of at least 4 days, patients received the opposite treatment. We included patients aged ⩾18 years old with a CFTR-A455E or a CFTR-R117H mutation on at least one allele of whom rectal biopsies and organoid cultures showed residual CFTR function in previous studies [20]. Patients were excluded if they had an acute pulmonary exacerbation or an increased risk of side-effects of salbutamol. The primary outcome measures were changes in sweat chloride concentration (SCC) and changes in nasal potential difference (NPD) measurements, which are both *in vivo* biomarkers for CFTR function. The NPD and SCC measurements were performed according to the most recent version of the standard operating procedure of the European Cystic Fibrosis Society Clinical Trials Network (www.ecfs.eu/ctn). The results of these measurements before and after treatments with salbutamol were compared using a Wilcoxon signed-rank test. A secondary outcome measure was the CFTR-activating capacity of the patients' plasma in organoids. Therefore, whole blood was collected in sodium-heparin tubes before treatment and after the last dose of salbutamol, when the maximum concentration of salbutamol in the blood was expected (inhaled salbutamol after 30 min, oral salbutamol after 2 h; www.fk.cvz.nl). Plasma was isolated as described previously [28].

Patient plasma-induced organoid swelling

Patient plasma was collected before and after treatment with salbutamol and incubated (20% and 40% plasma) with organoids derived from subjects with CF with high residual CFTR function (R117H/F508del). Organoid swelling was monitored as described above. Reference values were generated by measurement of spiked salbutamol in 0%, 20% and 40% plasma.

Results

Screen for GPCR modulators of organoid swelling

To identify compounds that can activate CFTR, we assessed CFTR-dependent swelling of organoids in response to 61 GPCR-modulating compounds (figure 1) [20]. As observed previously, forskolin induced rapid swelling of CFTR-WT organoids and, to a lesser extent, of VX-809-treated homozygous CFTR-F508del organoids. As expected, DMSO did not induce swelling (figure 1a). Swelling was expressed as the area under the curve for each specific condition (figure 1a and b). Of the 61 compounds tested, dopamine, epinephrine, ritodrine and salbutamol dose-dependently induced swelling of CFTR-WT organoids, with highest potency for ritodrine and salbutamol, and lowest potency for dopamine (figure 1c and d). Epinephrine, ritodrine and salbutamol are ligands for β_2-adrenergic receptors, and dopamine for the dopamine receptor. At the highest dose, the response to the four compounds was comparable to the forskolin-induced swelling (figure 1d). In VX-809-corrected F508del homozygous organoids, swelling was dose-dependently induced by epinephrine, salbutamol and ritodrine, but not by dopamine (figure 1c and e). The potency was highest for salbutamol and lowest for ritodrine. High levels of salbutamol induced swelling to a similar extent as forskolin (figure 1d). In conclusion, β_2-adrenergic receptor stimulation can potently activate CFTR-WT and drug-corrected CFTR-F508del in organoids.

β_2-Agonists robustly induce organoid swelling

Next, we assessed β_2-adrenergic receptor stimulation by short- and long-acting agonists in organoids with various *CFTR* mutations (figure 2). First, salbutamol- and ritodrine-induced swelling was confirmed in organoids derived from three individual F508del homozygous patients (figure 2a). As expected, robust organoid swelling was only observed after treatment with the CFTR modulators VX-770 or VX-809, and was highest upon VX-770 and VX-809 combination treatment. In line with figure 1, ritodrine was somewhat less potent than salbutamol and forskolin, especially for VX-809-incubated organoids. Both short-acting (ritodrine, terbutaline and salbutamol) and long-acting (formoterol, salmeterol and isoproterenol) β_2-agonists induced fluid secretion. Inhibition by CFTR$_{inh}$172 or carvedilol supported CFTR or β-adrenergic receptor specificity, respectively (figure 2b). Forskolin- and β_2-adrenergic receptor-induced swelling differed between organoids with distinct *CFTR* genotypes: we observed no swelling in organoids expressing two *CFTR-null* alleles (p.Glu60Ter and p.Ile1295fs; E60X and 4015delATTT), some swelling in CFTR-A455E or VX-809-corrected homozygous CFTR-F508del organoids and high swelling in CFTR-R117H expressing organoids (figure 2c). Dose-dependencies of β_2-agonist-induced swelling were independent of the *CFTR* genotype or VX-809-rescued F508del, and indicated that long-acting β_2-agonists were most potent, whereas forskolin was least potent (figure 2d). Representative examples of agonist-induced swelling are indicated in figure 2e. Together, these data demonstrate that various β_2-agonists robustly induce CFTR function in a *CFTR* mutation-dependent manner.

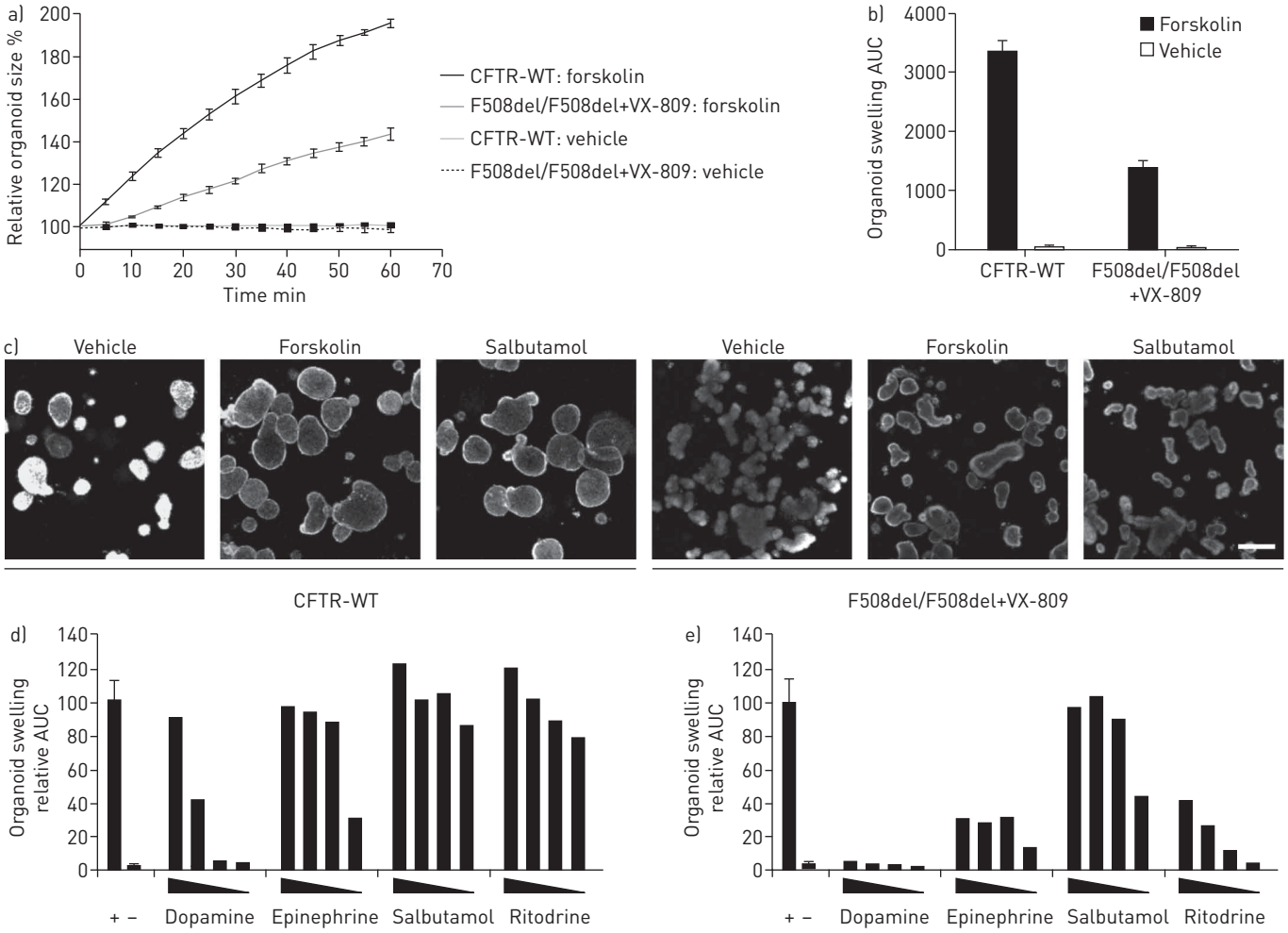

FIGURE 1 G-protein coupled receptor (GPCR) modulator-induced swelling of healthy control and cystic fibrosis transmembrane conductance regulator (CFTR)-F508del organoids. a) CFTR wild-type (WT) and F508del homozygous organoids were stimulated with forskolin (5 μM) or dimethylsulphoxide (DMSO; vehicle, 0.05%) and the relative increase in size was monitored over 60 min. CFTR-F508del homozygous organoids were pre-incubated with VX-809 (3 μM) for 24 h. b) Quantification of the area under the curve (AUC) of a), baseline was set at 100%, t=60 min. c) Representative images of CFTR-WT and VX-809-corrected CFTR-F508del organoids after 60 min of stimulation with DMSO (vehicle), forskolin (5 μM) or salbutamol (10 μM). Scale bar: 200 μm. d) Positive compounds for induction of fluid secretion using CFTR-WT intestinal organoids after screening a GPCR modulator library (61 compounds). Forskolin (+) and DMSO (–) were used as positive and negative control, respectively. GPCR modulators were tested at 10, 2, 0.4 and 0.08 μM. Data are normalised to the forskolin response. e) Same compounds as in d), tested on VX-809 (3 μM)-treated F508del homozygous organoids.

Salbutamol-mediated CFTR activation in bronchial epithelial cells

To confirm that intestinal organoid responses can be relevant for airway epithelial cells, CFTR activation by the β_2-agonist salbutamol was studied in the CF airway cell line CFBE41o- (CFBE) and primary CF human bronchial epithelial cells. First, we studied CFTR-dependent iodide quenching rates in CFBE41o- cells that endogenously express CFTR-F508del and were previously transduced with *CFTR-F508del* (CFBE-F508del) or *CFTR-WT* (CFBE-CFTR-WT) cDNA [24]. To measure CFTR-dependent iodide influx, the cells were stably transduced with a YFP/mKate sensor, as described previously [26]. Quenching of the YFP signal by iodide (indicating CFTR activity) was induced by both forskolin and salbutamol in VX-809 +VX-770-treated CFTR-F508del and CFTR-WT CFBE41o- cells (figure 3a). In addition, Ussing chamber experiments revealed that salbutamol and forskolin induced a CFTR-dependent short circuit current in F508del homozygous human bronchial epithelial cells treated for 24 h with VX-809 and VX-770, but not in primary cultures without CFTR-repairing treatment (figure 3b–d). As expected, the acute response to forskolin and salbutamol was abolished by $CFTR_{inh}$-172 and the salbutamol-induced response was inhibited by carvedilol (figure 3c and d). To conclude, activation of modulator-repaired CFTR-F508del by β_2-agonists was recapitulated in respiratory cell lines and primary airway cultures.

Pilot study with inhaled and oral salbutamol

To evaluate if drugs identified by screens in rectal organoids can be used to modulate CFTR function *in vivo*, 10 CF patients were enrolled in a study, and treated with oral and inhaled salbutamol. One patient

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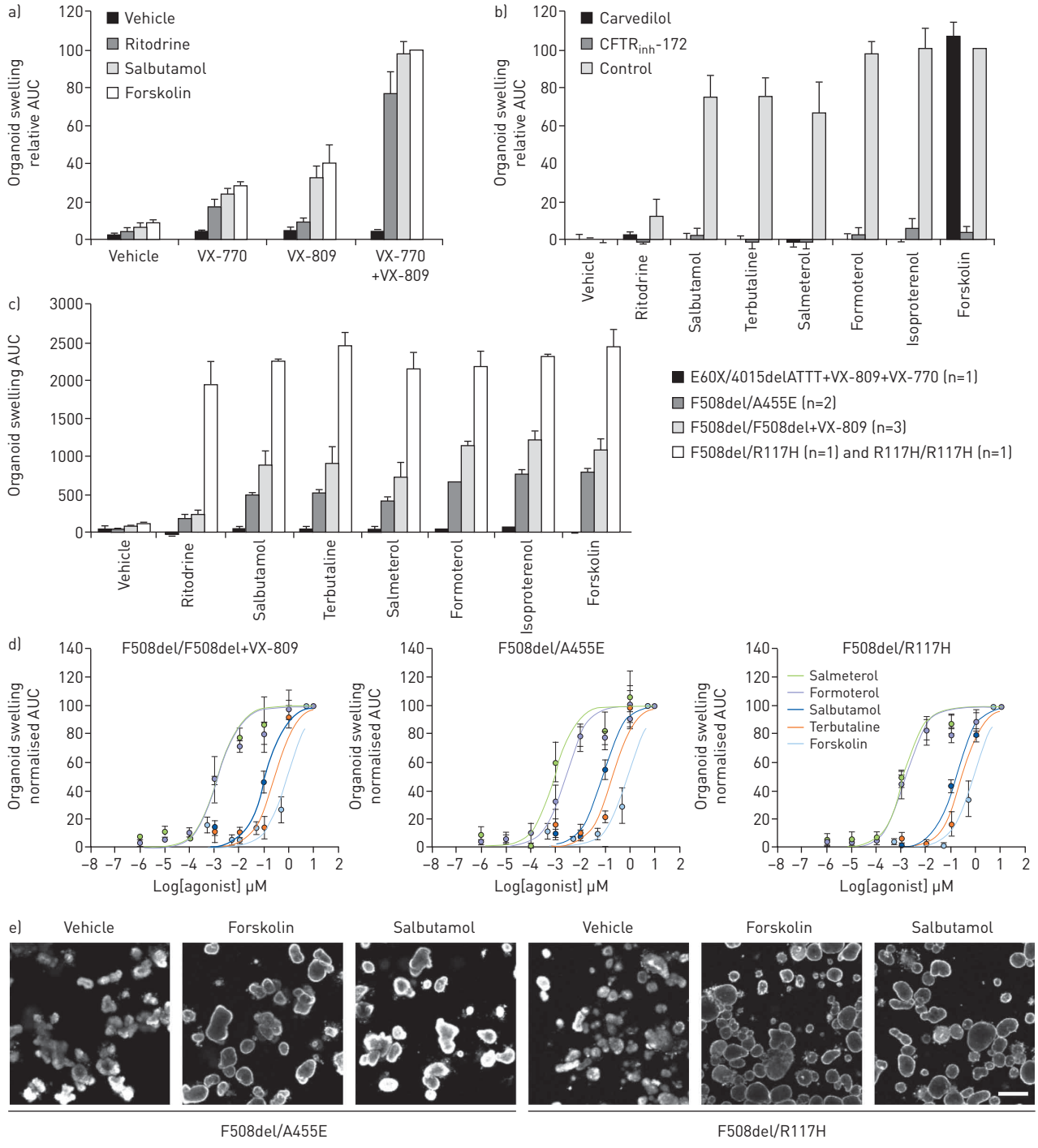

FIGURE 2 β_2-Agonists induce cystic fibrosis transmembrane conductance regulator (CFTR) activity. a) CFTR-F508del homozygous organoids were stimulated with ritodrine (10 μM), salbutamol (10 μM) or forskolin (5 μM). VX-809 (3 μM) was incubated for 24 h prior to stimulation. VX-770 (1 μM) was added simultaneously with the stimulus. AUC: area under the curve. Data were normalised to the combined VX-770+VX-809+forskolin response and organoids from three patients were measured at three independent time points in duplicate. Data are presented as mean±SEM. b) VX-809 (3 μM) treated CFTR-F508del organoids were incubated with $CFTR_{inh}$-172 or carvedilol before stimulation. β_2-Agonists were used at 10 μM and forskolin at 5 μM. All data were normalised to forskolin and represent mean±SEM of three independent measurements in duplicate. c) Organoids derived from patients with different CFTR genotypes were stimulated with β_2-agonists (10 μM) or forskolin (5 μM). n: number of patients, measured at three independent time points in duplicate (mean±SEM). d) Dose–response curves for different β_2-agonists and forskolin in F508del/F508del, F508del/A455E and F508del/R117H organoids. All data were normalised to the highest concentration of stimulus and represent mean±SEM of measurements at three independent time points. e) Representative images of organoids expressing CFTR-F508del and either CFTR-A455E or CFTR-R117H after 60 min of stimulation with dimethylsulphoxide (DMSO), forskolin (5 μM) or salbutamol (10 μM). Scale bar: 200 μm. Vehicle represents DMSO in all cases.

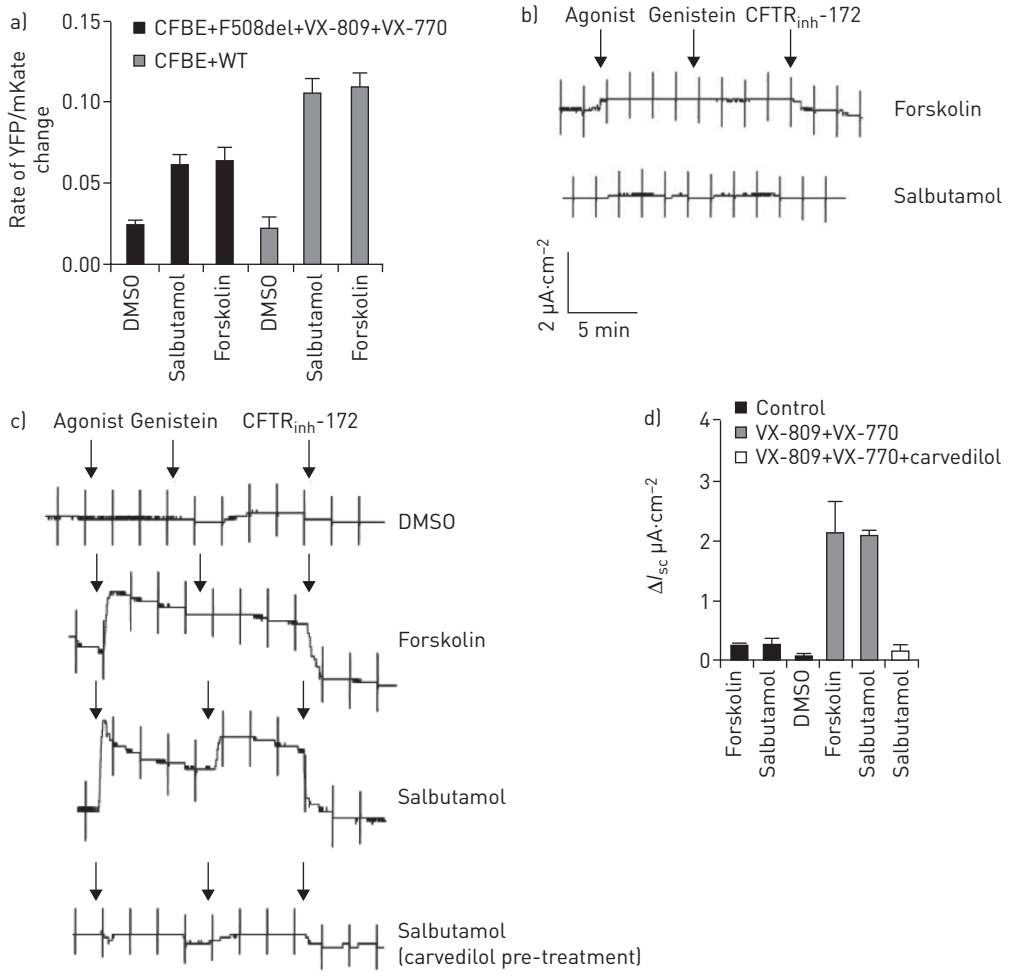

FIGURE 3 β_2-Agonist-induced cystic fibrosis transmembrane conductance regulator (CFTR) activation in bronchial epithelial cells. a) CFTR activity in CFBE41o- cells (CFBE) overexpressing CFTR-F508del or CFTR-WT, and stably expressing YFP (yellow fluorescent protein)/mKate, using a YFP quenching assay. CFBE41o- cells were pre-incubated for 24 h with VX-809 (10 μM), and stimulated with forskolin (25 μM) and VX-770 (10 μM) or salbutamol (10 μM) and VX-770 (10 μM) for 20 min prior to addition of iodide. Data are presented as mean±SEM and are representative of three independent experiments. b) Highly differentiated primary CFTR-F508del bronchial epithelial cells cultured at the air/liquid interface were analysed in Ussing chamber experiments. Representative traces of primary CF cells untreated with CFTR modulator drugs and exposed acutely to forskolin or salbutamol. Constant current pulses used to monitor transepithelial resistance cause the vertical deflections. c) Representative Ussing chamber tracings for VX-809 (1 μM)- and VX-770 (100 nM)-treated primary CF bronchial epithelial cells, stimulated acutely with dimethylsulphoxide (DMSO), forskolin (10 μM) or salbutamol (10 μM). Scaling is identical to b). d) Quantification of b, c). Data are presented as mean±SEM and are representative of three independent experiments.

was only treated with oral salbutamol due to increased asthma symptoms during the wash-out period of salbutamol aerosol between both treatments. The baseline characteristics of the study population are shown in table 1.

To analyse the systemic delivery of salbutamol by inhalation or oral application, we stimulated F508del/R117H and F508del/A455E organoids with plasma collected before and after *in vivo* treatment. Plasma collected after oral salbutamol treatment significantly induced F508del/R117H organoid swelling compared with the plasma collected before treatment or after aerosol administration of salbutamol, indicating that plasma concentrations of salbutamol were highest after oral treatment (figure 4a and b). Spiking of pure salbutamol in pooled plasma of subjects before treatment indicated that active salbutamol levels were below detection levels upon aerosol treatment and detectable but low after oral treatment, amounting to ~5 nM (figure 4c). After correcting for 40% subject plasma samples, circulating salbutamol levels after oral treatment on average reached levels ~12.5 nM.

To monitor *in vivo* modulation of CFTR function, SCC and NPD measurements were performed before and after 3 days of treatment with salbutamol. An overview of the changes in the SCC and NPD values is

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TABLE 1 Baseline characteristics of the 10 subjects enrolled in the pilot study

Age years	38.5 (31.5–49.0)
Male	4 (40)
Body mass index kg·m^{-2}	22.28 (20.38–28.16)
FEV$_1$ % pred (range)	62.0 (44.8–84.8) (31–109)
***CFTR* genotype**	
F508del/A455E	9 (90)
F508del/R117H	1 (10)

Data are presented as median (interquartile range) or n (%), unless otherwise stated. FEV$_1$: forced expiratory volume in 1 s; *CFTR*: cystic fibrosis transmembrane conductance regulator.

given in tables 2 and 3. Consistent with the outcome of the salbutamol bioassay showing low (oral treatment) or undetectable (aerosol treatment) levels of salbutamol in the plasma (nanomolar range), the only significant change in NPD was seen upon oral (but not aerosol) treatment, *i.e.* the median baseline potential changed significantly (by 6.4 mV) in the direction of reduced sodium absorption, indicative of an improved CFTR function (table 2). However, we did not observe any significant changes in other NPD parameters nor in levels of sweat chloride (tables 2 and 3 and online supplementary tables S2 and S3).

Adverse events that were reported during treatment with salbutamol appeared higher for oral salbutamol and are summarised in table 4. The data of this pilot study tentatively indicate that oral, but not inhaled, treatment with β_2-agonists may slightly improve residual CFTR function in nasal epithelium *in vivo*, but failed to further improve CFTR function in the sweat duct.

Discussion

The purpose of this study was to generate proof-of-concept that organoid-based measurements can be used to identify approved drugs that may modulate CFTR function *in vivo*. In this study, CFTR function measurements in organoids were applied to 1) prioritise potential drugs out of multiple candidates, 2) identify and verify subjects with potential responsive CFTR variants, and 3) study the optimal route of administration of the drug. As a whole, the results of the pre-clinical and clinical studies together indicate that organoid-based measurement can aid in designing clinical studies for subjects with CF.

We selected β_2-agonists from 61 compounds that can modulate GPCR signalling, which are known activators of CFTR and anion transport. Surprisingly, the potency of β_2-agonists to stimulate CFTR function was equal or greater than forskolin, which directly stimulates adenylyl cyclase downstream of GPCR, as observed in both primary intestinal and airway cells (figures 2 and 3) [13, 29]. The formation of macromolecular complexes between β_2-adrenergic receptors and CFTR may enable this efficient coupling of signals from β_2-adrenergic receptors to CFTR [30]. The lack of CFTR activation by other compounds in this library most likely reflects the absence of their cognate receptors or their inability to induce sufficient CFTR-activating signals or coupling these to CFTR.

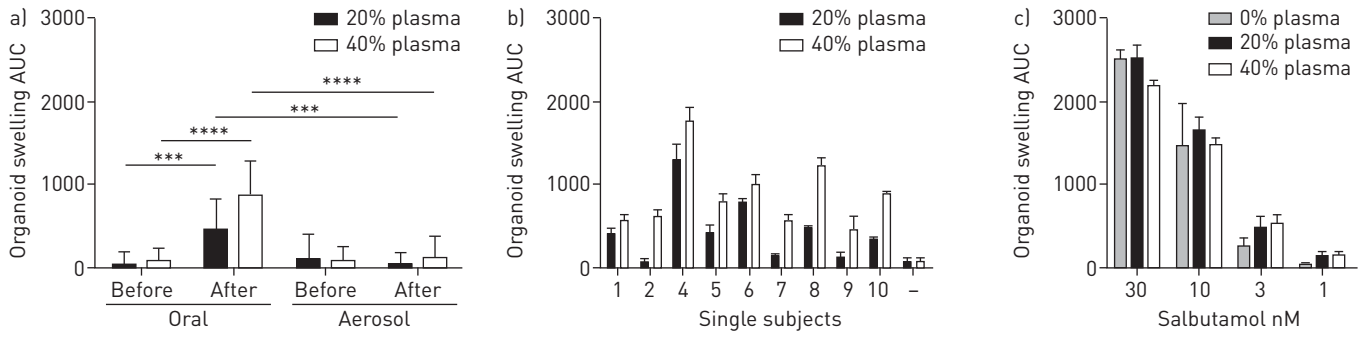

FIGURE 4 Plasma-induced organoid swelling. a) Cystic fibrosis transmembrane conductance regulator (CFTR)-F508del/R117H organoids were stimulated with 20% and 40% plasma of each subject (n=9) both before and after treatment *via* both aerosol and oral administration of salbutamol. AUC: area under the curve (t=60 min). Outcomes of subjects were pooled to compare aerosol with oral administration. Data are presented as mean±SD. Paired t-tests were performed to determine significance. ***: p<0.001; ****: p<0.0001. b) Data from plasma samples after oral treatment of a) are presented per subject. Data are presented as mean±SD. –: pooled plasma of all donors before treatment. c) To quantify the concentrations of salbutamol in the plasma samples of the subjects, F508del/R117H organoids were stimulated with known concentrations of salbutamol spiked in 0%, 20% and 40% plasma.

TABLE 2 Sweat chloride and nasal potential difference (NPD) responses to oral salbutamol

Parameter	Before oral treatment	After oral treatment	Change during oral treatment	p-value
Subjects	10	10		
Sweat chloride mmol·L^{-1}	72.5 (66.8–82.3)	73.0 (67.3–77.0)	+0.5	0.359
NPD measurement mV				
Basal potential difference	−55.2 (−62.0–−43.4)	−48.8 (−57.5–−38.9)	+6.4	0.047#
Δamiloride	38.4 (26.6–44.8)	38.8 (27.8–45.1)	+0.4	0.878
ΔCl$^-$ free	−1.4 (−6.7–5.9)	−5.6 (−10.0–−1.2)	−4.2	0.203
Δisoproterenol	−1.4 (−2.9–2.9)	−0.1 (−2.9–1.7)	+1.3	0.646

Data are presented as n or median (interquartile range), unless otherwise stated. #: p<0.05.

Measurement of *in vivo* CFTR function enhanced by exogenous activators such as β_2-agonists requires a different approach as compared with direct CFTR protein restoring drugs such as VX-770 and VX-809. It relies on the ability of exogenous β_2-agonists to phosphorylate and stimulate CFTR activity beyond levels associated with endogenous conditions. We anticipated that NPD could provide the most promising readout for an improvement of CFTR function, as intranasal infusion with the pan-β-agonist isoproterenol has been shown to further hyperpolarise the nasal epithelium in healthy controls *in vivo* by an average of 6.9 mV under low luminal chloride conditions [31, 32], suggesting that CFTR activity in this tissue is rate-limited by endogenous cAMP signalling. Whereas for diagnostic purposes and direct CFTR protein-restoring drugs, the combined change in NPD after addition of zero chloride solution and addition of isoproterenol is most informative, in patients treated with salbutamol we anticipated to find an enhanced response to low chloride but a reduced response to intranasally applied isoproterenol and no difference in the combined response to low chloride plus isoproterenol as both compounds activate endogenous cAMP. Although there was a tendency to an enhanced hyperpolarising response to zero chloride (−1.4→−5.6 mV) and a decreased response to isoproterenol (−1.4→−0.1 mV) upon oral (but not inhaled) treatment, this difference did not reach statistical significance. The baseline potential difference, which was clearly CF-like in both the A455E and the R117H patients (range −41.6–−55.2 mV; tables 2 and 3) showed a significant but limited increase towards non-CF baseline potential difference values (by 6.4 mV; table 2) in the orally treated patients, but this change was not paralleled by a reduced response to amiloride, an inhibitor of ENaC (table 2). Baseline potential appears to be predominated by ENaC-dependent Na^+ absorption, which is enhanced in CF and modulated through direct CFTR protein-targeting drugs [33–35]. This lack of correlation seems to argue against an inhibitory effect of salbutamol treatment on ENaC activity, although the relatively low power of the pilot study and the large variation in potential difference responsiveness to ENaC does not entirely rule out such an effect. The latter interpretation would be in line with the tentative increase in zero chloride response discussed above and the known inhibitory effect of CFTR (through electrogenic or more complex coupling mechanisms) on ENaC activity in the airways. Alternatively, our data do not exclude the possibility that salbutamol inhibits other electrogenic ion transport pathways in the nasal epithelium, such as apical cation channels different from ENaC, *e.g.* acid-sensitive ion channels [36] or proton channels [37]. Taken together, the outcome of the NPD measurements suggests that oral salbutamol treatment slightly modifies the electrical properties of the nasal CF epithelium towards that of the non-CF nasal epithelium and could therefore be of (limited) benefit for the CF patients.

The lack of robust CFTR activation *in vivo*, in clear contrast with the potent stimulation of organoid swelling by submicromolar levels of salbutamol *in vitro* (figure 2d), is most likely due to the low levels of circulating

TABLE 3 Sweat chloride and nasal potential difference (NPD) responses to salbutamol aerosol

Parameter	Before aerosol treatment	After aerosol treatment	Change during aerosol treatment	p-value
Subjects	9	9#		
Sweat chloride mmol·L^{-1}	67.0 (58.5–71.0)	70.0 (62.5–73.0)	+3.0	0.476
NPD measurement mV				
Basal potential difference	−47.3 (−54.4–−40.9)	−41.6 (−49.8–−37.4)	+5.7	0.123
Δamiloride	29.6 (23.4–41.7)	29.0 (19.8–36.6)	−0.6	0.208
ΔCl$^-$ free	−4.2 (−8.5–3.8)	−1.6 (−8.1–−0.2)	+2.6	0.401
Δisoproterenol	−2.4 (−4.6–−0.9)	−2.0 (−4.7–−0.7)	+0.4	0.878

Data are presented as n or median (interquartile range), unless otherwise stated. #: n=8 for NPD measurement after aerosol treatment.

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TABLE 4 Adverse events during treatment with salbutamol

Adverse event	During oral treatment	During aerosol treatment
Agitated feeling	1	
Palpitations	4	
Cough up more sputum	2	
Dry mouth	1	1
Tremor	5	1
Headache	1	1
Painful breathing		1

Data are presented as n times reported.

β_2-agonist observed upon *in vivo* treatment. In line with the findings in the NPD measurements, we only found a detectable CFTR-activating capacity in blood samples that were collected after treatment with oral, but not with inhaled salbutamol. The latter is consistent with a highly limited systemic delivery by β_2-agonist inhalation [38] (figure 4a). However, even after treatment with oral salbutamol, CFTR-activating levels in plasma were low (figure 4a and b), corresponding with pure salbutamol concentrations in the nanomolar range that can only marginally stimulate organoids (compare figure 4c and 2d). As such, only limited effects *in vivo* might have been expected, as we observed in only one of the NPD parameters. Although plasma levels are not similar to tissue levels, these data suggest that higher dosages may further improve efficacy of treatment, albeit that systemic side-effects of the treatment may limit the feasibility of increasing the dosage.

The lack of response in SCC is also indicative of a limited treatment response, albeit that this parameter needs to be interpreted with care. SCC is a highly sensitive CFTR function parameter, being capable of distinguishing between pancreas-sufficient and -insufficient groups, and pre-treatment SCC in our patient cohort clearly indicates that these patients have significant residual CFTR activity (table 3). Furthermore, in G551D patients this biomarker is also highly responsive to CFTR potentiator treatment [10]. However, isolated sweat glands from subjects also indicate that exogenous β-adrenergic stimuli can only stimulate ~40% of sweat ducts and later studies confirmed a high constitutive cAMP-dependent activation of CFTR in this tissue [39, 40]. This implies that only a limited window for exogenous β_2-agonist stimulation likely exists *in vivo* in this tissue. The lack of treatment response in SCC we observed in this trial was therefore not completely unexpected considering the high constitutive CFTR activation in this tissue combined with the very low levels of circulating β_2-agonists.

Additional clinical studies are required to further validate the effect of long-term treatment with oral β_2-agonists on clinical outcome parameters (*e.g.* percentage predicted forced expiratory volume in 1 s, airway resistance, body mass index, quality of life) in CF patients, as this proof-of-concept study showed a minor but significant impact of treatment on the nasal mucosa, but no significant effect on Cl^- transport in the sweat ducts.

As expected, β_2-agonists stimulate swelling of organoids in a *CFTR* mutation-dependent manner, based on residual function conferred by the *CFTR* genotype or by CFTR-modulating drugs (figure 2). Most subjects included in the study were compound heterozygous for A455E, and their organoids demonstrate residual CFTR function levels between the values seen with F508del and R117H/F508del compound heterozygotes [20]. This appears consistent with the SCC parameters measured in this study and with data from the CFTR2 database (www.cftr2.org). Our NPD data (tables 2 and 3 and online supplementary tables S2 and S3: response to chloride-free and isoproterenol) also showed evidence of residual CFTR function in the A455E patients, with a tendency to increase slightly but not significantly after oral salbutamol treatment. As the VX-770 +VX-809-corrected CFTR-F508del function in organoids is higher than the level of residual function associated with CFTR-A455E [20], β_2-agonists may also have added value for F508del homozygous subjects treated with CFTR-repairing drugs. In this context, cotreatments with β_2-agonists may account for some of the pulmonary heterogeneity between patients that is observed in the response to CFTR modulator treatment [9, 11]. In addition, further stratification for *CFTR* genotypes with higher residual function (*e.g.* CFTR-R117H) may also enhance treatment effects with β_2-agonists.

In conclusion, CFTR function measurements in intestinal organoids were used to screen for CFTR-activating drugs and subjects with CFTR variants that respond to these drugs *in vitro* were selected for *in vivo* treatment. Oral treatment with salbutamol improved some CF characteristics of the nasal mucosa, but treatment efficacy was likely limited due to ineffective dosage, as apparent from measurements of plasma levels of salbutamol in our organoid-based bioassay. The study supports the concept that intestinal organoids are a valuable tool for selecting drugs and route of administration for CF clinical trials.

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