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Original Article

R560S: A class II CFTR mutation that is not rescued by current modulators

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ABSTRACT

Background: New therapies modulating defective CFTR have started to hit the clinic and others are in trial or under development. The endeavour of drug discovery for CFTR protein rescue is however difficult one since over 2000 mutations have been reported. For most of these, especially the rare ones, the associated defects, the respective functional class and their responsiveness to available modulators are still unknown. Our aim here was to characterize the rare R560S mutation using patient-derived materials (rectal biopsies and intestinal organoids) from one CF individual homozygous for this mutation, in parallel with cellular models expressing R560S-CFTR and to assess the functional and biochemical responses to CFTR modulators.

Methods: Intestinal organoids were prepared from rectal biopsies and analysed by RT-PCR (to assess CFTR mRNA), by Western blot (to assess CFTR protein) and by forskolin-induced swelling (FIS) assay. A novel cell line expressing R560S-CFTR was generated by stably transducing the CFBE parental cell line and used to assess R560S-CFTR processing and function. Both intestinal organoids and the cellular model were used to assess efficacy of CFTR modulators in rescuing this mutation.

Results: Our results show that: R560S does not affect CFTR mRNA splicing; R560S affects CFTR protein processing, totally abrogating the production of its mature form; R560S-CFTR evidences no function as a Cl^- channel; and none of the modulators tested rescued R560S-CFTR processing or function.

Conclusion: Altogether, these results indicate that R560S is a class II mutation. However, unlike F508del, it cannot be rescued by any of the CFTR modulators tested.

1. Introduction

Cystic fibrosis (CF), the most common life-shortening autosomal recessive disease in Caucasians is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein which plays a major role in chloride (Cl^-) and bicarbonate (HCO_3^-) conductance in epithelia, as well as in regulating other ion channels and transporters [1]. CF-causing mutations prevent the channel from proper functioning, leading to abnormal ion transport and causing dehydration of the airway surface liquid [2]. Most current CF therapies target its symptoms (mucolytics, antibiotics, etc). However, innovative therapies modulating defective CFTR, the basic defect underlying CF, have started to hit the clinic and new ones are in trial or under development [3]. The endeavour of drug discovery to rescue mutant CFTR protein is however, a difficult one since to date >2000 mutations have been reported in the CFTR gene, most of which still with unknown impact, both in terms of their underlying molecular/cellular defect and disease liability [4]. To tackle this very high number of mutations grouping of CFTR mutations into functional classes has been proposed. These original classes are continuously updated [3] especially as they evolve into 'theratypes' [5] since they are becoming increasingly defined by the respective therapeutic strategy designed to rescue the respective molecular/cellular defect. The rationale is that mutations within the same class can be treated by the same therapeutic strategy. Although this classification is helpful to rationalize therapies, the underlying defect (and hence the respective mutation class) is still unknown for the majority of CFTR mutations, in particular for the rare ones. Moreover, there are several examples of mutations within the same class/theratype which however do not equally respond to the same CFTR modulator [6]. It is also often found that patients with the same CFTR genotype (such as those who are homozygous for F508del, the most common disease-causing variant) have significantly different clinical responses to CFTR modulating drugs [6,7]. There is thus an unmet need to test these novel CFTR modulators directly ex vivo on the patient's own tissues/primary cells with robust biomarkers that not only assess modulators efficacy for a given CFTR genotype but also predict clinical benefit for an individual patient.

The p.Arg560Ser mutation (legacy name: R560S) is caused by a A > C transversion at the cDNA nucleotide position c.1680 (legacy: 1812 A

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> C), located in exon 13, leading to the exchange of the amino acid arginine by a serine at codon 560, thus being considered as a missense mutation [8,9]. However, as the c.1680 A > C nucleotide change occurs at the intron11/exon12 boundary (first nucleotide of exon 12), it is plausible to envisage that this mutation may also affect splicing. R560S is described as CF-causing when combined with another CF-causing mutation, in the CFTR2 [www.cftr2.org] mutation database. R560S causes pancreatic insufficiency (PI), when combined with another PI-causing mutation. This mutation was originally reported in the CFTR Mutation Database by B Costes in a Pakistani 11-year old male patient diagnosed at 4 months old, PI, with moderate lung disease and positive sweat chloride, that was homozygous for the mutation. To this date, there are 6 patients reported with this mutation in the CFTR2 database, all of which in compound heterozygosity.

R560 is located in the first nucleotide-binding domain (NBD1) which is involved in ATP binding and plays a role in CFTR channel opening (i.e., gating) [10]. NBD1 is also a "mutational hotspot" for CF, since many CF-causing mutations localize to this domain, including the most frequent one (F508del) which occurs in ~85% of CF patients worldwide and is described to affect CFTR protein processing (class II).

Our aim here was to characterize the rare CFTR-R560S mutation and its response to existing modulators both in a cell line model and in patient-derived materials from one individual with CF who is homozygous for this mutation.

2. Materials and methods

2.1. Chemicals and compounds

All chemicals were of analytical grade. Gentamycin, DMSO, forskolin (Fsk), cysteamine, epigallocatechin gallate (EGCG) and genistein (Gen) were from Sigma-Aldrich (St.Louis, MO, USA). VX-809, VX-661 and VX-770 were from Selleckchem (Houston, TX, USA). VX-809, VX-770, VX-661, Fsk and Gen were dissolved in DMSO. Cysteamine and EGCG were dissolved in water. Unless otherwise stated (see legends of figures), the incubation time was 24 h and the concentrations used were (μM): 3 VX-809, 5 VX-661 and 3 VX-770, which are the standard, established ones and are in agreement with previous usage [11–14]. For cysteamine and EGCG a range was tested: 125–500 μM and 40–160 μM for 24 h, respectively. Fsk was used in a concentration range (0-5 μM) and Gen at 50 μM.

2.2. Subjects and ethics approval

Rectal biopsies from a CF patient homozygous for the R560S CFTR mutation were obtained by small superficial rectal forceps biopsies. The ethical committee of Santa Maria Hospital, Lisboa, Portugal approved this study and the patient signed informed consent forms. The patient genotype was confirmed in the patient-derived materials as the change c.1680A > C, using our previously reported mRNA-based approach to identify CFTR mutations in the complete coding and intronic regions [15] and confirmed that c. 1680A > C/R560S was the only alteration observed (data not shown). The patient was 5 years old at the time of collection and was diagnosed with CF aged 18 months by early on developing typical complications of the CF-disease such as malabsorption, nasal polyps, chronic bronchorrea, positive sweat-test (3 confirming results). At the age of 2.7 years, a first colonisation with pseudomonas aeruginosa was successfully treated (Dr. Christoph Runge, personal communication).

2.3. Crypt isolation and organoid culture from rectal biopsies

Crypt isolation and human organoid culturing were carried out as described previously [16,17]. Briefly, 3–4 superficial rectal mucosa specimens (8 mm in diameter) were recovered with colon forceps and immediately placed into culture medium. Then, the biopsies were washed with PBS and treated with 10 mM EDTA for 90–120 min at 4 °C. Crypts were isolated by centrifugation and then cultured in 50% matrigel (growth factor free, phenol-free, BD Biosciences, Franklin Lakes, NJ, USA), seeded at a density of ~10–30 crypts in 3x10ul matrigel droplets per well, in pre-warmed 24-well plates. The matrigel was polymerized for 10–15 min at 37 °C and surrounded by complete culture medium consisting of: advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, 1× Glutamax, B27 (all from Invitrogen, Carlsbad, CA, USA), 1.25 mM *N*-acetylcysteine (Sigma) and growth factors: 50 ng ml^{-1} mEGF (Invitrogen), 50% Wnt-3a-conditioning medium (WCM), 10% Noggin-conditioned medium, 20% Rspo1-conditioned medium (RCM), 10 mM nicotinamide (Sigma), 500 nM A83–01 (Tocris, Bristol, UK) and 10 μM SB 202190 (Sigma). Antibiotics were added to the growth medium (Primocin, 1:500; Invitrogen), Vancomycin and Gentamycin (Sigma (1:1000)) during the first few weeks of culture. Medium was changed every other day and organoids were passaged after 5–7 days of culturing.

2.4. mRNA and extraction from native cells, cDNA synthesis and transcript analyses

RNA was isolated from rectal biopsies [18] using the NucleoSpin RNAII Kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. CFTR cDNA was generated from 1 μg mRNA using M-MuLV reverse transcriptase (NZYTech, Lisboa, Portugal), as previously [15]. RT-PCR using three different reactions was performed with the following pairs of primers B3R-Ex11L, CF10N-561AL, C1R-CF14a.R as previously described – expected product sizes are 431, 207 and 904 bp, respectively [15]. Products were analysed by agarose gel electrophoresis and identity confirmed by sequencing.

2.5. Cell lines

A novel cell line was generated by lentiviral transduction of the CFBE41o- cell line [19]. For this, site-directed mutagenesis was used to introduce the R560S mutation into CFTR cDNA cloned in the pcDNA5 expression vector (confirmed by sequencing). The cDNA was then re-cloned into the lentiviral expression vector pLVX-Puro that was transfected into the packaging cell line 293 T for production of lentiviral particles. These particles were used to transduce parental CFBE41o- cells (bronchial epithelial cell line) originally isolated from a F508del homozygous patient, but without detectable expression of CFTR [19]. Efficiency of the transduction was assessed by Western blot (WB) to confirm CFTR expression.

2.6. Western blot analysis

For CFTR protein detection, organoids cells were lysed in Laemmli buffer supplemented with complete protease inhibitor tablets (Roche, Basel, Switzerland). Lysates were analysed by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA, USA), using the anti-CFTR monoclonal antibody (mAb) 450, 570 and 596 (CFF) at a 1:3000 dilution [16] and the secondary antibody was horseradish peroxidase-labelled anti-mouse at 1:3000 (Bio-Rad). As a loading control, we used calnexin detected by anti-calnexin antibody (1:3000 dilution) (BD Biosciences, San Jose, CA). Biochemical analysis was also performed in CFBE cells stably transduced with R560S-CFTR cDNA.

2.7. Micro-Ussing chamber recordings

Transepithelial electrical resistance (TEER) of the cells growing on Snapwell inserts was measured with the Chopstick Electrode (STX2 from WPI®). Monolayers with resistance values above 450 $\Omega.cm^2$ were mounted in modified micro-Ussing chambers. Recordings were done as previously described [20,21]. Briefly, the basolateral surface of

CFBE41o- cells was continuously perfused with Ringer solution (NaCl 145 mM, KH_2PO_4 0.4 mM, K_2HPO_4 1.6 mM, D-glucose 5 mM, $MgCl_2$ 1 mM, Ca-gluconate 1.3 mM) and the apical surface with a low Cl^- Ringer solution (NaCl 32 mM; KH_2PO_4 0.4 mM; K_2HPO_4 1.6 mM; D-glucose 5 mM; $MgCl_2$ 1 mM; Ca-gluconate 5.7 mM and Na-gluconate 112 mM). Following a 20-min equilibration period, baseline values were recorded. Subsequently, the cAMP agonist, Fsk (2 μM), the CFTR potentiator Gen (50 μM), and the CFTR channel blocker GlyH101 (30 μM) were added sequentially. Transepithelial resistance (R_{te}) was determined by applying short (1 s) current pulses ($I = 0.5$ μA) and the corresponding changes in transepithelial voltage (V_{te}) were recorded continuously. Equivalent cAMP-stimulated CFTR short-circuit currents ($\Delta I_{eq\text{-}sc}$) were calculated by Ohm's law from V_{te} and R_{te} ($I_{eq\text{-}sc} = V_{te}/R_{te}$). Values for V_{te} were referred to the basolateral side of the monolayers.

2.8. Forskolin-induced swelling (FIS) assay

The Fsk-induced swelling (FIS) assay was carried out as described previously [16]. Briefly, rectal CF organoids from a 5–7-day-old culture were seeded in a pre-warm flat bottom 96-well culture plate (Thermo Fisher Scientific, Rochester, NY) with 4 μl 50% Matrigel containing ~20 organoids immersed in 100 μl complete medium, with or without desired concentration of CFTR modulators as indicated (the concentrations used for the modulators were previously described and are the ones that maximize the dynamic range of the assay [13]). One day after seeding, organoids were incubated with Fsk with or without potentiator(s) at concentrations indicated in the figure legends and live-cell imaging was performed using bright field microscopy (Leica DMI 6000B) and confocal microscopy (Zeiss LSM 800) with the 5× objective for 60 min at 37 °C. Three wells per condition were used and experiments were repeated 3–6 times per donor.

2.9. Quantification of forskolin-induced swelling

FIS was quantified by using Cell profiler and Zen Blue 2 software. The area under the curve (AUC; $t = 60$; baseline = 100%) was calculated using GraphPad Prism version 5.01. A paired *t*-test was used to calculate statistical differences and a p value ≤.05 was considered as statistically significant.

3. Results

3.1. mRNA analysis

Our first aim here was to characterize the molecular consequences of the R560S mutation, in terms of the possible effects of this mutation on mRNA splicing, given that the c.1680A > C nucleotide change occurs at the first nucleotide of exon 12 (see introduction). To this end, we isolated RNA from the patient's organoids which, after cDNA synthesis were analysed by RT-PCR in the region of the mutation, to assess for possible exon skipping of nearby exons (Fig. 1A). Three different PCR reactions were thus used covering exons 8–11, 10–12 and 11–14a, as previously described [15]. Data show that all RT-PCR products have the expected size, thus corresponding to correctly spliced CFTR transcripts for all regions analysed (Fig. 1A), demonstrating that exon skipping is not detected in the cells analysed strongly suggesting that c.1680A > C does not affect splicing.

To assess whether R560S affects CFTR processing, total protein was extracted from rectal organoids and Western blot (WB) was used to assess the presence of immature (band B) and mature (band C) forms of the protein. Results obtained here (Fig. 1B) clearly show that the protein resulting from R560S mutation only appears in its immature (core-glycosylated, band B) form, different from the protein in samples from non-CF individuals that present both the mature (fully-glycosylated, band C) form and some as immature form (band B), similarly to F580del-CFTR (shown also as a control). Altogether, these results indicate that this CFTR mutation causes a defect in CFTR processing. Additionally, organoids from either the R560S homozygous patient or from a patient homozygous for F508del were treated with the corrector VX-809 for 24 h. WB analysis evidences the rescue of F508del-CFTR – through appearance of the fully processed band C (Fig. 1B,C) – a fact that is not observed for R560S-CFTR, as after the treatment only band B is observed.

3.2. Assessment of rescue of R560S-CFTR protein processing by correctors

Next, we assessed the efficacy of different small molecule compounds in rescuing R560S-CFTR. For this, we produced a novel cell line expressing R560S-CFTR by stably transducing CFBE parental cells and the following compounds were tested: VX-809/lumacaftor (corrector drug approved for F508del/F508del patients - in combination with VX-770/Ivacaftor); VX-661/tezacaftor (second generation corrector which already succeeded in plase 3 clinical trials); cysteamine (an FDA-approved drug for the treatment of Cystinosis) alone and in combination with green tea component EGCG, described to rescue F508del-CFTR [14].

Cells were thus incubated for 24 h with: VX-809 3 μM, VX-661 5 μM, DMSO 0.1% v/v as the vehicle control, cysteamine 250 μM alone or in combination with EGCG 50 μM, and at low temperature (27 °C) also described to rescue processing of some class II mutants, namely F508del. In parallel, these compounds were also tested on F508del-CFTR in CFBE cells as a control. As above, we performed WB to assess the maturation status/rescue of F508del and R560S-CFTR (Fig. 2).

As shown in Fig. 2, wt-CFTR is detected in both its immature (band B) and mature forms (Band C). However, R560S-CFTR can only be detected in their immature form (band B) indicating that similarly to data obtained in organoids (Fig. 2B), this protein does not reach the cell surface, similarly to F508del-CFTR. However, in contrast to F508del-CFTR, for which VX-809, VX-661 and low temperature promote the appearance of mature CFTR, for R560S-CFTR, neither treatment nor incubation at low temperature (Fig. 2A,B) has led to the appearance of band C. Treatment with cysteamine however, either alone or when combined to EGCG, did not rescue R560S- nor F508del-CFTR, the latter in contrast to what was previously described [22]. Strikingly, the combined treatment of cysteamine and EGCG leads to the total disappearance of CFTR, in wt-, F508del and R560S-CFTR expressing CFBE parental cells (Fig. 2B,F).

3.3. Assessment of R560S-CFTR function in intestinal organoids and the effect of correctors

Measurements of CFTR-mediated Cl^- secretion in rectal biopsies has been demonstrated to be a robust biomarker, both in the diagnosis and prognosis of CF [23,24] as well as to assess efficacy of in vivo treatment with CFTR modulators, in CF patients being clinically treated with these drugs [25]. However, this approach cannot be used ex vivo, due to poor compound penetration into the tissues. So, in order to assess the efficacy of CFTR modulators (correctors or corrector + potentiators) ex vivo, we used the FIS assay in intestinal organoids (Fig. 3A-D), which has been validated as a robust ex vivo biomarker and a good predictor of clinical benefit for CFTR modulators [16]. To this end, we pre-incubated the R560S/R560S organoids with each of the CFTR correctors (VX-809, VX-661, cysteamine and cysteamine with EGCG) and to activate CFTR channel function, organoids were stimulated with Fsk alone, with potentiators VX-770 (Ivacaftor) or with Gen to further enhance the channel activity. Organoids isolated from the R560S homozygous patient were compared to organoids from a F508del homozygous patient.

Data shows that none of the tested CFTR modulators induced significant Fsk-induced swelling of R560S/R560S organoids, in comparison to the control situation (Fig. 3). In contrast, and as reported previously [16], we could detect Fsk-induced swelling in F508del/F508del organoids when treated with the combination of VX-809 with VX-770

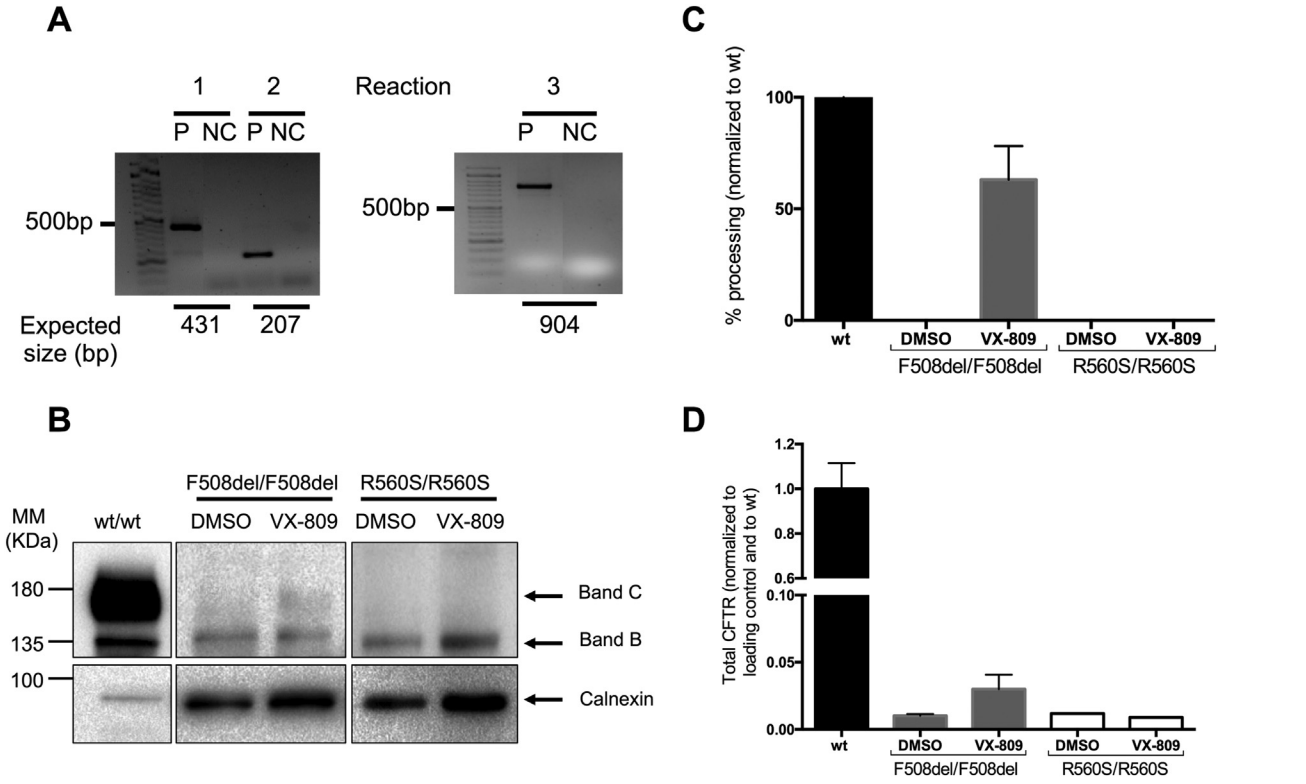

Fig. 1. Impact of the R560S mutation on mRNA splicing and protein processing. (A) RT-PCR analysis of CFTR transcripts extracted from organoids derived from rectal biopsies (RB) from CF patient with the R560S/R560S genotype in the regions between exons 8 and 14a (n = 3). P - RNA from patient (R560S/R560S) organoids, NC - Negative control. Expected sizes – reaction 1: 431 bp, reaction 2: 207 bp, reaction 3: 904 bp. (B) Representative WB analysis of CFTR protein expressed in rectal organoids from a non-CF control (wt), from a F508del homozygous patient or from the R560S homozygous patient. Organoids from CF patients were also analysed following treatment with VX-809 (3 μM) for 24 h. WB was performed using a mixture of anti-CFTR 596, 570 and 450 antibodies. Images were acquired using ChemiDoc XRS+ imaging system BioRad and further processed by Image lab 4.0 software (n = 3). (C) For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalized to the efficiency of processing of wt-CFTR and are shown as mean ± SEM. (D) For each condition, densitometry was used to calculate the total amount of CFTR normalized to the loading control and to the wt control and are shown as mean ± SD (n = 3).

(Fig. 3A, B, C, D). However, we could not detect any swelling for either R560S/R560S or F508del/F508del organoids when treated with cysteamine either alone or in combination with EGCG (Fig. 3E, F).

3.4. Assessment of R560S-CFTR function in cell lines and effect of correctors

To confirm the functional consequences of the R560S mutation, we assessed transepithelial transport in polarized monolayers of CFBE cells expressing this variant when mounted onto Ussing chambers. Activation of cAMP-dependent CFTR-mediated Cl^- secretion by Fsk and 3-isobutyl-1-methylxantine (IBMX) elicited a typical lumen-negative response in CFBE cells expressing wt-CFTR, that was further potentiated under treatment with Gen and inhibited with the CFTR inhibitor GlyH101 (Fig. 4A). A similar approach was used in cells expressing ether F508del- or R560S-CFTR. Whereas in cells expressing F508del-CFTR the absence of response (Fig. 4B) was rescued upon incubation with VX-809 (Fig. 4C), cells expressing R560S-CFTR showed no CFTR-mediated Cl^- secretion with potentiation with genistein (Fig. 4D), and, consistently with WB data, treatment with corrector VX-809 did not produce any detectable CFTR-mediated Cl^- transport (Fig. 4,E).

4. Discussion

The aim of this study was to characterize the rare CFTR-R560S mutation using both patient-derived materials (intestinal organoids) from one CF individual who is R560S-homozygous and a novel bronchial epithelial cell line stably expressing this mutation. Furthermore, we also aimed to assess the responsiveness of both the intestinal organoids and the cell line to existing CFTR modulators.

The R560S mutation exists in a reduced number of patients (6 in total as reported in CFTR2) and is associated with severe CF phenotype, associated with PI when combined with another PI-causing mutation. Reports on this mutation available in the literature describe it in compound heterozygosity with F508del [8,9]. The patients listed in CFTR2 are all compound heterozygotes (4 have F508del on the other allele, 1 has 1199delG and 1 has 3905insT) and are all pancreatic insufficient. The fact that R560S is associated with severe CF in those 6 patients in consistent with the clinical phenotype of the patient described here, and supported by our experimental findings. Furthermore, the mutation was previously studied in Fischer rat thyroid (FRT) cells - a less physiologically relevant cellular model when compared to the CFBE cell line used here – and in such conditions, it resulted in a defect in processing and thus in CFTR function and was described as unresponsive to ivacaftor [26]. Thus, all previous data is suggestive that this mutation is associated with severe CF.

We first assessed whether R560S causes abnormal mRNA splicing given that the c.1680 A > C nucleotide change lies at the intron11/exon12 junction. In fact, other CFTR missense mutations occurring close to splice sites have been reported to alter splicing [27,28]. Our results indicate that R560S does not affect splicing (Fig. 1A). As such observation on splicing pattern is only possible in the presence of introns it would not be detectable in cDNA-based heterologous expression systems, clearly emphasizing the importance of using patient-derived materials for the correct assessment of such a process.

Determination of the levels of immature and mature CFTR by WB in both intestinal organoids and CFBE cells expressing R560S-CFTR showed that the mutation affects CFTR protein processing, totally abrogating the production of its mature form (Figs. 1C and 2), consistently to

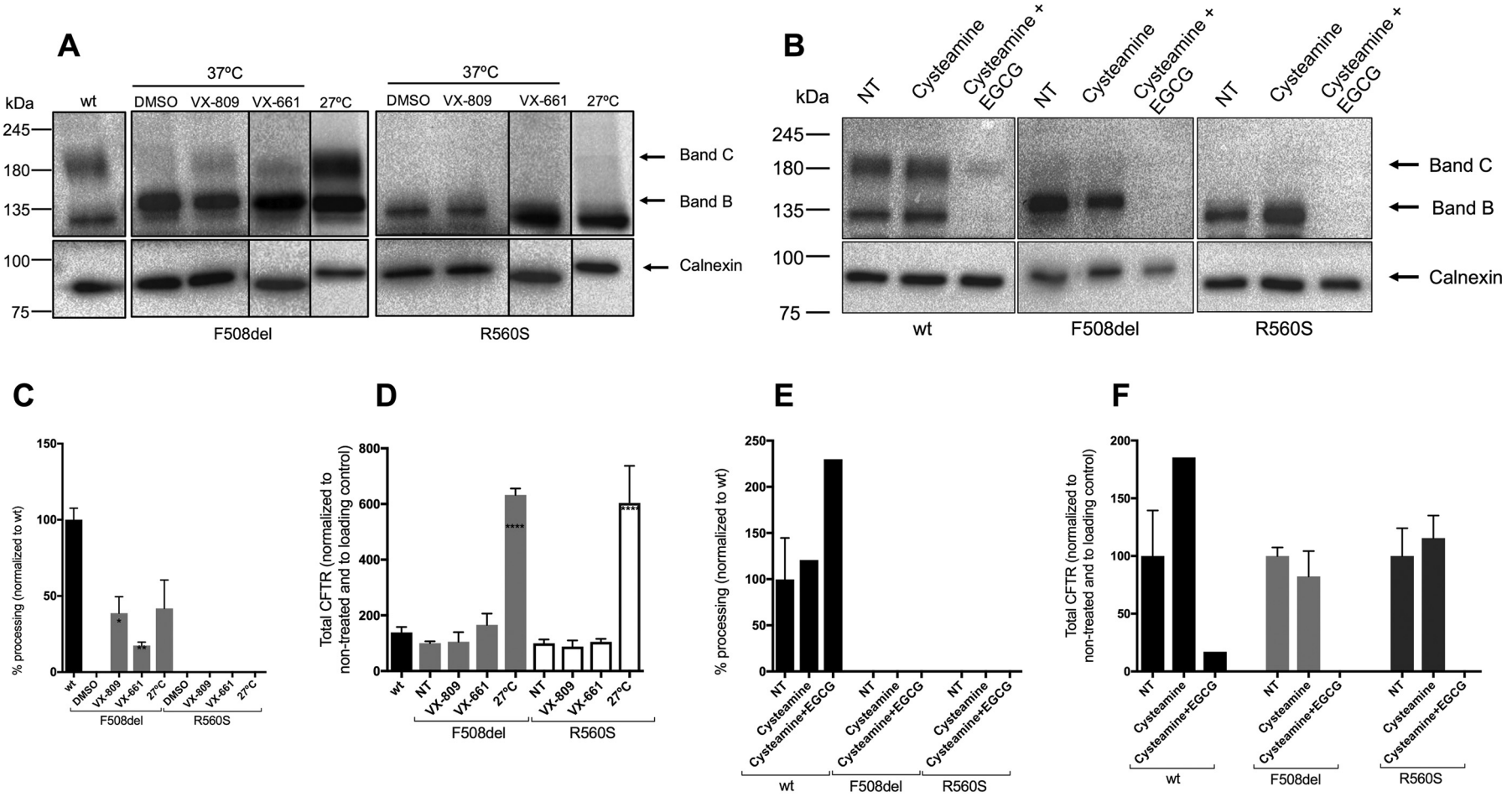

Fig. 2. Effect of correctors on processing of R560S-CFTR and F508del- (control) CFTR protein assessed by WB. (A) Representative WB analysis of CFBE cells stably expressing R560S-, F508del or wt-CFTR, following treatment with VX-809 (3 μM), VX-661(5 μM), DMSO (0.1% v/v) or incubation at 27 °C, for 24 h. (B) Representative WB analysis of CFBE cells stably expressing R560S-, F508del- or wt-CFTR, following the treatment with cysteamine (250 μM) and the combination of cysteamine and EGCG (50 μM) for 24 h, as indicated in above lanes. (C),(E) For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed, following image acquisition as in Fig.1B. Data were normalized to the efficiency of processing of wt-CFTR and are shown as mean ± SEM (D), (F). For each condition, densitometry was used to calculate the total amount of CFTR normalized to the loading control and to the amount of CFTR in the non-treated sample and are shown as mean ± SD (n = 3).

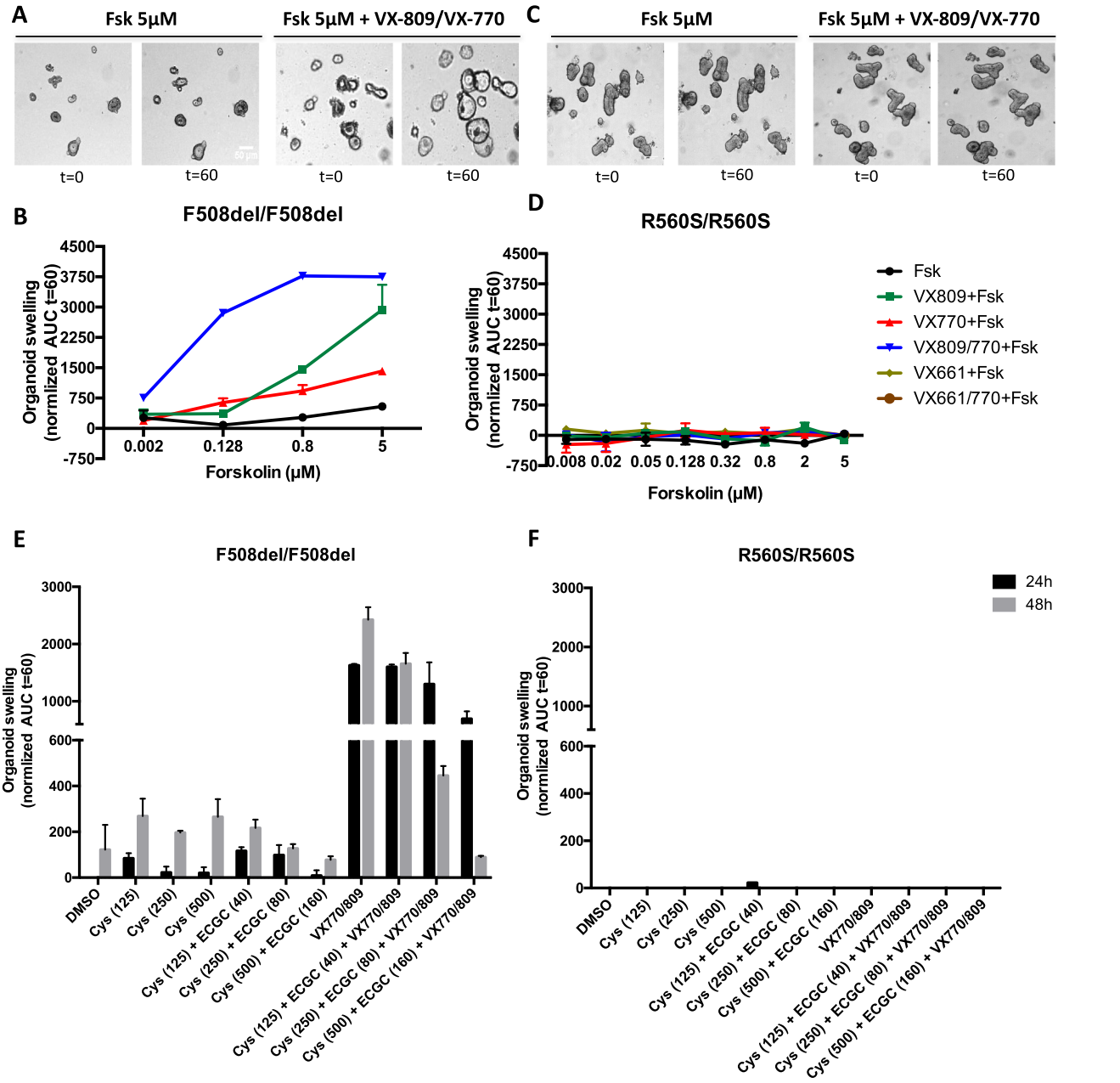

Fig. 3. FIS assay in R560S/R560S and F508del/F508del organoids following treatment with CFTR modulators. Bright-field microscopy images of (A) F508del-homozygous and (B) R560S-homozygous organoids before ($t = 0$) and after ($t = 60$) Fsk addition to determine increase in organoid area (swelling) in the presence of just Fsk or in combination with VX809/VX-770 treatment (both at 3 μM). Graph summarising data of FIS expressed as the absolute area under the curve (AUC) (baseline = 100%, $t = 60$ min) of (C) F508del-homozygous and (D) R560S-homozygous organoids incubated with only Fsk or in combination with VX-809 (3 μM), VX-770 (3 μM), VX-661 (5 μM) or VX-809/VX-770 and VX-661/VX-770 combinations. Bar graphs summarising data of FIS assay expressed as the absolute AUC in organoids isolated from either a F508del-homozygous patient (E) or the R560S-homozygous (F) patient - organoids were incubated with cysteamine (125, 250 or 500 μM) in presence or absence of EGCG (40 or 80 μM) and VX-809 (3 μM), VX-661 (5 μM) and stimulated with Fsk (concentration range 0-5 μM), VX-770 (3 μM) and Gen (50 μM) and performed comparatively in two different time points: at 24 h (black bars) and 48 h (grey bars). Absence of bars represents no swelling. Data are mean ± SD of three experiments. Cys – Cysteamine. The cysteamine and EGCG concentrations (μM) for each bar are shown in brackets.

what was previously shown in FRT cells [26]. Functional analysis both in polarized monolayers of R560S-CFTR CFBE cells and in intestinal organoids evidenced no function of this mutant as a Cl^- channel, most probably due to the absence of mature protein (Figs. 3, 4). These results thus exclude the inclusion of this mutation in classes I, V and VII and suggest that R560S is a typical class II mutation [3].

Other examples of class II mutations include F508del, A561E [29] or N1303K. While the first two mutants are rescued by corrector VX-809 [6,12], N1303K is not [6]. The functional classification of R560S into class II, prompted us to test if the available modulators were able to rescue CFTR bearing this mutation. We assessed the effect of CFTR correctors lumacaftor (approved as Orkambi, when combined with ivacaftor) [12], tezacaftor (which successfully completed Phase III clinical trial) and cysteamine alone or in combination with EGCG [14]. Assessment was done both through detection of mature protein by WB and by of CFTR-mediated Cl^- secretion, determined with FIS measurements in patient-derived intestinal organoids and with transepithelial ion transport in polarized R560S-CFTR CFBE cells. Our results evidenced that, unlike F508del, R560S cannot be rescued by any of the CFTR modulators tested here.

Although absence of response to correctors was previously reported for other class II mutations, including lack of response of N1303 K to VX-809 [6,30] or of G85E to corrector 4a [31], these localize to other CFTR proteins domains (NBD2 and TMD1, respectively). Interestingly, we

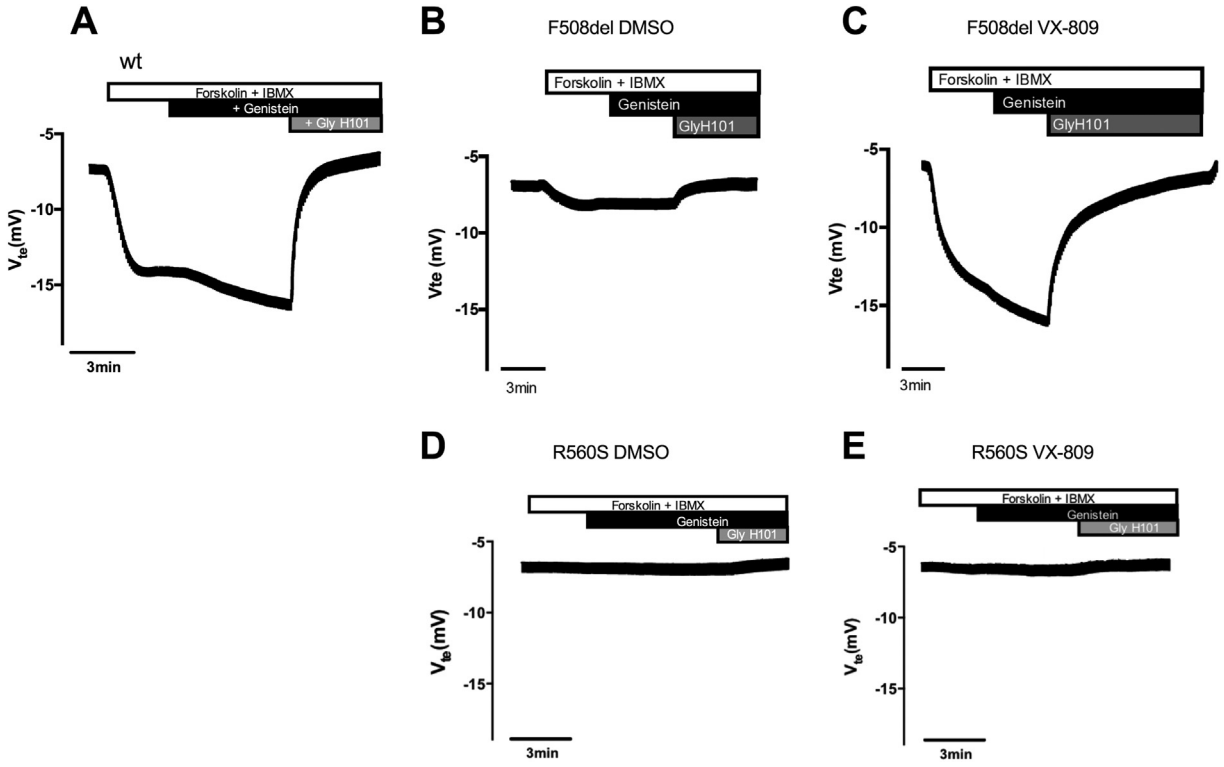

Fig. 4. Original Ussing chamber (open-circuit) recordings showing transepithelial voltage measurements (V_{te}) for CFBE expressing R560S-CFTR. Negative V_{te} deflections are observed in CFBE cells stably transduced with wt-CFTR (A) following the application of luminal Fsk alone (2 μM) or together with Gen, which are reverted by application of the specific CFTR inhibitor GlyH101 (30 μM). CFBE cells expressing F508del-CFTR do not present the same response (B) unless they are previously treated with VX-809 (3 μM) for 24 h (C). The response is not observed in CFBE cells stably transduced with R560S-CFTR either in the presence of Gen (D) alone or in the presence of VX-809 (E).

have previously reported that a mutation occurring at the exact same codon, R560T, is not rescued by incubation at low temperature, an approach which is not mutation-specific but rather kinetically favours protein folding, thus allowing them to overcome the endoplasmic reticulum quality control [32,33]. A third CF-causing mutation occurring at the same codon, R560K, was also reported [34] and associated with pancreatic insufficiency and CF-typical sweat chloride levels. All these three mutations cause severe CF [4], and our results here and previously reported [6] indicate that at least two of these variants are difficult to rescue. The publication of CFTR structure at high-resolution identified these 3 mutations as causing a defect in the interaction between NBD1 and the TMDs [35], being thus plausible to consider this as a very sensitive site for inter-domain folding. This assumption was confirmed by recent work showing that when analysing exonic mutation frequencies in CFTR, R560 is identified as a highly mutated residue [36].

Our results also very strikingly showed a total lack of response for cysteamine, either alone or in combination with EGCG, in contrast to what was previously reported [14]. This was observed for all the conditions tested – organoids and cell lines – for both genotypes – R560S and F508del (homozygous in the patient-derived materials). These observations may suggest that intestinal organoids may not be adequate to detect the previously reported corrective effect of cysteamine/EGCG [14]. However, results from cell lines (directly assessing the presence of CFTR) also failed to detect CFTR for the combined treatment cysteamine/EGCG – even highlighting a potential negative impact of EGCG on CFTR expression/function consistently observed throughout the models used –, suggesting that whatever additional conditions used in the previous studies are not present in our system, and that these compounds thus do not elicit a general correction of F508del-CFTR in all the models/systems.

Altogether, the work presented here evidencing the lack of correction for the R560S mutation, illustrates how difficult it is to rescue all CFTR mutations, even if included in a theratype for which correction is already available for some mutations. In fact, our data show that R560S can be assigned a theratype – i.e. the need for a corrector – but it doesn't respond to the modulators tested. Such observations reinforce the need for continuous effort in searching for better modulators or alternative therapeutic strategies that are able to ameliorate disease phenotype for patients bearing rare mutations.

Conflict of interest statement

There is no conflict of interest related to this work.

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