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Functional rescue of c.3846G>A (W1282X) in patient-derived nasal cultures achieved by inhibition of nonsense mediated decay and protein modulators with complementary mechanisms of action

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ABSTRACT

Background: The nonsense mutation, c.3846G>A (aka: W1282X-CFTR) leads to a truncated transcript that is susceptible to nonsense-mediated decay (NMD) and produces a shorter protein that is unstable and lacks normal channel activity in patient-derived tissues. However, if overexpressed in a heterologous expression system, the truncated mutant protein has been shown to mediate CFTR channel function following the addition of potentiators. In this study, we asked if a quadruple combination of small molecules that together inhibit nonsense mediated decay, stabilize both halves of the mutant protein and potentiate CFTR channel activity could rescue the functional expression of W1282X-CFTR in patient derived nasal cultures.

Methods: We identified the CFTR domains stabilized by corrector compounds supplied from AbbVie using a fragment based, biochemical approach. Rescue of the channel function of W1282X.-CFTR protein by NMD inhibition and small molecule protein modulators was studied using a bronchial cell line engineered to express W1282X and in primary nasal epithelial cultures derived from four patients homozygous for this mutation.

Results: We confirmed previous studies showing that inhibition of NMD using the inhibitor: SMG1i, led to an increased abundance of the shorter transcript in a bronchial cell line. Interestingly, on top of SMG1i, treatment with a combination of two new correctors developed by Galapagos/AbbVie (AC1 and AC2-2, separately targeting either the first or second half of CFTR and promoting assembly, significantly increased the potentiated channel activity by the mutant in the bronchial epithelial cell line and in patient-derived nasal epithelial cultures. The average rescue effect in primary cultures was approximately 50% of the regulated chloride conductance measured in non-CF cultures.

Conclusions: These studies provide the first in-vitro evidence in patient derived airway cultures that the functional defects incurred by W1282X, has the potential to be effectively repaired pharmacologically.

Abbreviations: CF, Cystic Fibrosis; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; WT, wild-type; cAMP, cyclic adenosine monophosphate; HEK293, human embryonic kidney 293; HBE, human bronchial epithelial; HNEC, human nasal epithelial cells; FLIPR, fluorometric imaging plate reader; NMD, nonsense-mediated mRNA decay; PTC, premature termination codon; SMG, serine/threonine-protein kinase.

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1. Introduction

Cystic Fibrosis (CF) is an autosomal recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR is an ATP- and PKA-dependent chloride channel, regulating chloride and bicarbonate ion flux across apical membranes of polarized epithelial cells [1–3]. To date, over 2000 mutations have been reported in the *CFTR* gene (CFTR1 database, http://www.genet.sickkids.on.ca). Around 10% of these mutations result in the formation of premature termination codons (PTCs) and prevent synthesis of full length CFTR mRNA and protein.

Aminoglycoside antibiotics (e.g., gentamicin, G418) and the drug PTC-124 (Ataluren; 3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid), developed to treat PTC mutations for Cystic Fibrosis and Duchenne muscular dystrophy (DMD), act by promoting the incorporation of near-cognate tRNAs in place of the termination complex. It has been shown that Gentamicin restores functional protein in short-term using mouse models of DMD, CF, nephrogenic diabetes insipidus, hemophilia, retinal degeneration and APC-mediated colon cancer [4–8]. However, in the setting of CF, Ataluren has not translated into clinical benefits [9,10].

The most prevalent PTC mutation in *CFTR* is c.3846G>A (W1282X), occurring on one or both alleles in about 2.4% of CF patients worldwide (http://cftr2.org). The read-through drug PTC-124, showed no significant improvement in clinical measures in a phase III trial of 238 adult CF patients with nonsense mutations in at least one allele, including 86 harboring the W1282X mutation, however the results may have been confounded by treatment with tobramycin in a subset of the patients [9].

It has been demonstrated that the W1282X mutation generates a truncated CFTR protein that is 1281 rather than 1480 amino acids in length that is capable of channel activity in the presence of CFTR modulators following over-expression in HEK-293 cell or FRT cells [11–14].

Recently it has been shown that ORKAMBI®, a combination modulator therapy developed for the major mutation, p.Phe508-CFTR containing the corrector compound lumacaftor (VX-809) plus the potentiator compound ivacaftor (VX-770), is effective in enhancing the functional expression of W1282X-CFTR when heterologously overexpressed. But, this result could not be replicated in human nasal epithelial cells from a single CF patient bearing the W1282X mutation on both alleles [13], possibly because of its decreased abundance. PTC mutations can result in reduced transcript levels due to nonsense-mediated mRNA decay (NMD) [15]. Recognition of PTC-containing mRNAs and their targeting for degradation requires a set of conserved NMD effectors, which include the Up-frame shift (UPF) proteins UPF1, UPF2 and UPF3B, exon junction complex (EJC) proteins Y14, MAGOH, EIF4AIII and BTZ (MNL51) and the SMG1-SMG9 proteins [16–18]. When the ribosome reaches a PTC, interaction of the release factors eRF1 and eRF3 with downstream EJCs bridged by the UPF proteins triggers the phosphorylation of UPF1 and subsequent degradation of the mRNA [19,20].

SMG-1 kinase inhibitor (SMG1i) is a recently identified inhibitor of the NMD effector protein [21] and has shown modest increases in CFTR mRNA abundance, CFTR expression and channel function in 16HBE14o- cells CRISPR-edited to express the W1282X protein singly or in combination with G418 [22]. Vidaza (5-Azacytidine) is an approved drug for the treatment of myelodysplastic syndrome and myeloid leukemia [23–25], and it has been demonstrated that 5-azacytidine is a potent and specific inhibitor of NMD [18].

The combination of the PTC read-through-promoting compound G418 and the NMD modulator (NMDI14) was shown to stabilize mRNAs with PTCs and restore expression of full-length p53 protein [26]. Recently, it has been demonstrated that the inhibition of NMD (using the small molecule: SMG1i or NMDI-14) or antisense oligonucleotides (ASOs), increased the mRNA and protein expression for W1282X-CFTR in cell lines [22,27,28]. Valley et al. demonstrated that the combination of G418 and SMG1 inhibitor, rescued W1282X-CFTR to ~2.7% of the WT function in 16HBE cell line [22], far less than the degree of rescue (approximately 50%), expected to have therapeutic value [29].

Most recently, Askit and colleagues showed that inhibition of SMG7 by NMDI14 molecule, enhanced the quantity of truncated W1282X message and protein to a level that enabled modulation by the combination of VX-809 plus VX-770 in a recombinant expression system [27]. Unfortunately, the positive effect of this strategy, namely preventing NMD and augmenting protein stability and function with modulators approved for p.Phe508-CFTR was ineffective when studied in patient derived primary tissues, raising questions regarding its efficacy.

In the current work, we tested the hypothesis that, as recently shown for the major mutation; F508del-CFTR [30], a combination of corrector molecules that act via complementary mechanisms will be more effective than a single molecule in supporting the potentiated channel function of the truncated W1282X-CFTR protein. In fact, we found that a specific corrector combination from Galapagos/AbbVie rescued the functional expression of W1282X-CFTR to 50% of normal function in patient-derived nasal epithelial cells as long as the nonsense mediated decay of the W1282X-CFTR transcript was prevented.

2. Material and methods

2.1. Cell culture and transfection

Human embryonic kidney (HEK) 293 GripTite™ cells (HEK293) (a gift from Dr. Daniela Rotin, Hospital for Sick Children, Toronto, Ontario, Canada) were maintained in DMEM (Wisent, St-Bruno, QC) supplemented with non-essential amino acids (Life Technologies, Waltham, MA) and 10% fetal bovine serum (FBS; Wisent, St-Bruno, QC) at 37°C and processed with 5% CO_2 as previously described [31,32].

W1282X homozygote patient nasal epithelial cultures were obtained through the CFIT Program (https://www.lab.resesarch.sickkids.ca/cfit/; [33]). This study was approved by our local Research Ethics Board and all donors or their guardians signed informed consent. The subsequent cell culture was performed as previously described [34,35]. Cells were seeded on collagen coated transwell inserts (6.5 mm diameter, 0.4 µm pore size, Corning, Tewksbury, MA). Once confluent, the cells were cultured for 14 days at an air liquid interface (ALI) with basal differentiation media (PneumaCult™ ALI, StemCell Tech., Vancouver, Canada) [33,34,36].

2.2. Immunoblotting

WT-CFTR and W1282X-CFTR fragments used in this study were transiently expressed in HEK293 cells using PolyFect Transfection Reagent (Qiagen, Hilden, Germany), according to the manufacturer's protocol as previously described [37,38]. HEK293, HBE-W1282X and nasal cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4, 0.2% SDS, and 0.1% Triton X-100) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) for 10 min. Soluble fractions were analyzed by SDS-PAGE on 6% Tris-Glycine gels (Life Technologies). After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and incubated in 5% milk. In HEK293 cells CFTR bands were detected with human CFTR-specific murine mAb 596 (UNC, North Carolina, USA) with 1:5000 dilution and in HBE-W1282X and nasal cultures with 1:500 dilution. The blots were developed with ECL

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(Amersham) using the Li-Cor Odyssey Fc (LI-COR Biosciences, Lincoln, NE) in a linear range of exposure (2–45 min) [39,40].

2.3. Measurement of CFTR fragment stability

Steady-state levels of CFTR fragments, including membrane spanning domain 1 (MSD1) (containing residues 1–380), MSD1 and nucleotide binding domain 1 (MSD1-NBD1) (containing residues 1–646), MSD1-NBD1-R (containing residues 1–845), MSD1-NBD1-R-MSD2 (containing residues 1–1171), A52-tagged MSD2 (containing residues 837–1196) and MSD2-NBD2 (containing residues 850–1480), were determined by Western Blot analysis as previously described [38,39,41]. HEK293 cells were transiently transfected with the plasmids as described above and after 18 h of transfection, the cells were treated with 3 μM VX-809, 0.5 μM AC1 (X281602), 3 μM AC2-1 (X281632), 3 μM AC2-2 (X300549), 10 μM Corr4a (C4) [42] for 24 h at 37 °C. Immunoblot analysis was performed as described above.

2.4. Compound description

Compound AC1 belongs to C1-type corrector targeting early folding of mutant CFTR while AC2-1 and AC2-2 are C2-type correctors whose novel mechanism of action complements C1-type correctors and AP2 is a CFTR potentiator [43].

Corrector AC1 (X281602) is described as compound 72 in a recently published the review by Kym et al. [44]. Corrector AC2-1 (X281632) belongs to the ABBV/GLPG2737 series type C2 correctors belonging to the pyrazolopyridine acylsulphonamide chemical class correctors and is a close analog of compound 93 described in the review by Kym et al. [44] and covered in the patent granted to Galapagos and Abbvie (Patent WO2017060874A1; 2017). Corrector AC2-2 (X300549) belongs to the ABBV/GLPG3221 series type C2 correctors belonging to the pyrrolidine chemical class of correctors and is described as Compound 4 in an Abbvie manuscript [45] and is a close analog of ABBV/GLPG3221 [46]. Potentiator AP2 (X300529) belongs to the sulphonamide-substituted aminopyridines class of potentiators and is a close analog of GLPG2451 [47] and compound 48 described in the review by Kym et al. and covered in a patent granted to Galapagos and Abbvie (Patent WO2016193812A1, 2016).

2.5. qRT-PCR

RNA extraction was performed according to the manufacturer's protocol (Qiagen Micro or Mini Kit). Briefly, cells were lysed, RNA extracted and then RNA concentration was measured using a NanoDrop 2000 instrument. Only samples with a concentration >100 ng/μL were used, with a 260/280 ratio between 1.8 and 2.1. cDNA synthesis was performed using reverse transcriptase (iSCRIPT cDNA synthesis kit, Biorad, Hercules, CA) or without reverse transcriptase (negative control). Quantitative real-time PCR was performed using Eva green (Ssofast Evagreen, Biorad, Hercules, CA) fluorophore in 96 well plates (Biorad, Hercules, CA) and normalized to GADPH. The primers used for amplification are: CFTR forward: 5′-GCATTTGCTGATTGCACAGT-3′, CFTR reverse: 5′-CTGGATGGAATCGTACTGCC-3′, GAPDH forward: 5′-CAAGAGCACAAGAGGAAGAGAG-3′, GADPH reverse: 5′-CTACATGGCAACTGTGAGGAG-3′.

2.6. CFTR channel function in HEK cells

HEK cells were seeded in 96-well plates (black, flat bottom; Greiner). After 24 h the cells were transfected with either W1282X-CFTR construct, and 18 h post-transfection were treated with DMSO, 3 μM VX-809, 0.5 μM AC1 (X281602), 3 μM AC2-1 (X281632), 3 μM AC2-2 (X300549), 0.5 μM AC1+ 3 μM AC2-1 and 0.5 μM AC1+ 3 μM AC2-2 for 24 h at 37 °C. Cells were then loaded with blue membrane potential dye dissolved in chloride free buffer (without CFTR correctors, SMG1i and G418) for 45 min at 37 °C. The plate was then read in a fluorescence plate reader (SpectraMax i3; Molecular Devices) at 37 °C, and after reading the baseline fluorescence (excitation: 530 nm, emission: 560 nm) for 5 min; CFTR was stimulated using forskolin (10 μM; Sigma) and the potentiators VX-770 (1 μM) or AP2 (X300529) (1.5 μM). CFTR-mediated depolarization of the plasma membrane was detected as an increase in fluorescence. Then, CFTR inhibitor (CFTRinh-172, 10 μM) was added to deactivate CFTR. The peak changes in fluorescence to CFTR agonists were normalized relative to fluorescence immediately before agonist (forskolin) addition [36,37,48].

2.7. CFTR channel function in CFF-16HBEge CFTR W1282X cells

16HBE14o- cells genome-edited to produce the homozygous CFF-16HBEge CFTR W1282X cell line were obtained from the Cystic Fibrosis Foundation [22]. Cells were grown at 37°C for 5 days post-confluence submerged on 96 well black well, clear bottom culture plates (Costar) in EMEM media (Wisent BioProducts) with 10% Fetal Bovine Serum (Wisent BioProducts) and 1% Penicillin/Streptomycin (Wisent BioProducts). 24 h before the assay, cells were treated with DMSO, 3 μg/mL G418, 0.5 μM SMG1i, 3 μM VX-809, 0.5 μM AC1 (X281602), 3 μM AC2-1 (X281632), 3 μM AC2-2 (X300549). Cells were then loaded with blue membrane potential dye dissolved in chloride-free buffer (150 mM NMDG-gluconate, 3 mM potassium gluconate, 10 mM HEPES, pH 7.30, 300 mOsm) for 35 min without CFTR correctors, SMG1i and G418. The plate was then read in a fluorescence plate reader (SpectraMax i3; Molecular Devices) at 37 °C (excitation: 530 nm, emission: 560 nm). CFTR was stimulated with 10 μM Forskolin (Sigma-Aldrich) and either 1 μM VX-770 (Selleck Chemicals) or 1 μM AP2. The assay was terminated with 10 μM CFTRinh172 (Cystic Fibrosis Foundation Therapeutics). Changes in membrane potential were normalized to the point before addition of agonist and to the DMSO control response.

2.8. Ussing chamber studies of primary nasal epithelial cells

Primary nasal epithelial cells were grown on transwells at 37 °C and studied in a non-perfused Ussing chamber (Physiologic Instruments, San Diego, CA) [34,36]. The buffer solution (126 mM NaCl, 24 mM $NaHCO_3$, 2.13 mM K_2HPO_4, 0.38 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM glucose) was maintained at pH 7.4 and 37°C and continuously gassed with a 5% CO_2/95% O_2 mixture. 48 h before the experiments, cells were treated with 0.1% DMSO, 3 μM VX-809, 10 μM PTC-124, 0.5 μM AC1+3 μM AC2-1, 0.5 μM AC1+3 μM AC2-2. In some experiments, 0.5 μM SMG1i and or 200 μg/ml G418 were added to the preceding drug treatments. The correctors, SMG1i and G418 agents were not kept in the assay chambers during the functional analyses. The transepithelial potential (Vte) was recorded in open-circuit mode and the baseline resistance (Rte) was measured following repeated, brief short-circuit current pulses (1 μA every 30 s). The results are presented as equivalent transepithelial current (Ieq), which was calculated using Ohm's law. For presentation, raw Vte traces are sampled every 30 s and converted to Ieq traces using the same calculation as above. CFTR function was determined after inhibition of the epithelial sodium channel (ENaC) with amiloride (30 μM, Spectrum Chemical, Gardena, CA) and following cAMP activation with forskolin (10 μM, Sigma-Aldrich, US). CFTR activity was confirmed as Ieq difference following CFTR inhibition with $CFTRInh_{-172}$ (10 μM, EMD Millipore Corp. US).

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2.9. Statistical analysis

Data are represented as mean ± SEM. GraphPad Prism 7.0 software (San Diego, CA) was used for all statistical analysis. Student's *t*-tests, One-way/two-way ANOVA were conducted as appropriate, and *p*-values < 0.05 were considered significant. Data with multiple comparisons were assessed using Tukey's multiple-comparison test with $\alpha = 0.05$. Each experiment is defined as a separate biological study, on independently plated cells.

3. Results

3.1. Novel correctors target distinct regions in the CFTR protein

Previous studies of W1282X-CFTR suggested that small molecule correctors and potentiators were partially effective in rescuing the primary defects exhibited by this truncated protein [13], arguing that read-through agents, which are thought to facilitate production of full length CFTR protein, may not be required to rescue functional expression of W1282X, at least in a heterologous expression system. In that study, W1282X expressed in FRT cells exhibited primary defects in processing and/or stability of the truncated protein as well as defective cyclic AMP-dependent gating. These authors showed that VX-809, developed for correction of the processing defect exhibited by the major mutant, F508del-CFTR, was partially effective in correcting the processing defect exhibited by recombinant W1282X-CFTR. After correction with VX-809, treatment with VX-770 and a second synergistic potentiator, W1282X-CFTR exhibited functional rescue that approached that of Wt-CFTR [13]. These overexpression system results remain to be replicated in patient-derived primary cultures with endogenous W1282X-CFTR expression, where there may be a need to combine CFTR correctors and potentiators with compounds that prevent nonsense mediated decay and/or promote read-through of the premature stop codon in order to observe a significant functional benefit.

The strategy of treating cells with multiple corrector compounds, each interacting with distinct regions of the CFTR protein [37,39,49], has identified combinations that synergistically rescue the processing and functional expression of F508del-CFTR [30,41,50]. VX-809 acts to stabilize the mutant in the early stages of biogenesis, potentially by interacting with MSD1 [41,49,51,52] or NBD1 [53]. Corrector compounds that exert an additive effect in combination with VX-809 likely interact with a different domain at a later stage in biogenesis, potentially with NBD1 or the second half of the CFTR protein [30,54]. Galapagos/AbbVie developed two classes of corrector compounds [41,49,51] to modulate the processing defect of F508del-CFTR. As described in a recent review, corrector AC1 belongs to the same class as VX-809 [44]. A second class of correctors, including AC2-1 and AC2-2, [55,44], acts in a complementary manner to AC-1 to rescue processing of F508del-CFTR. The standard codes for these compounds and their structures are shown in the Material and Methods section and in Figure S1.

In order to better understand the mechanism of action of the second class of correctors: the AC2 compounds, we used the CFTR fragment based approach initially described by Ren et al. [49]. CFTR fragments containing MSD1, i.e. NBD2del-CFTR (1–1171), MSD1-NBD1-R (1–845), MSD1-NBD1 (1–646) and MSD1 (1–380), MSD2 (837–1196) and MSD2-NBD2 (850–1480) were expressed in HEK-293 cells and the cells treated with VX-809 or each of the three novel compounds. As expected from previous studies [41,49], VX-809 pre-treatment for 24 h, enhanced the abundance of the fragments containing MSD1 (Fig. 1). The compound, AC-1, but not AC2-1 or AC2-2 conferred the same effect (Fig. 1), confirming that AC1 acts through a similar mechanism to VX-809 (manuscript submitted).

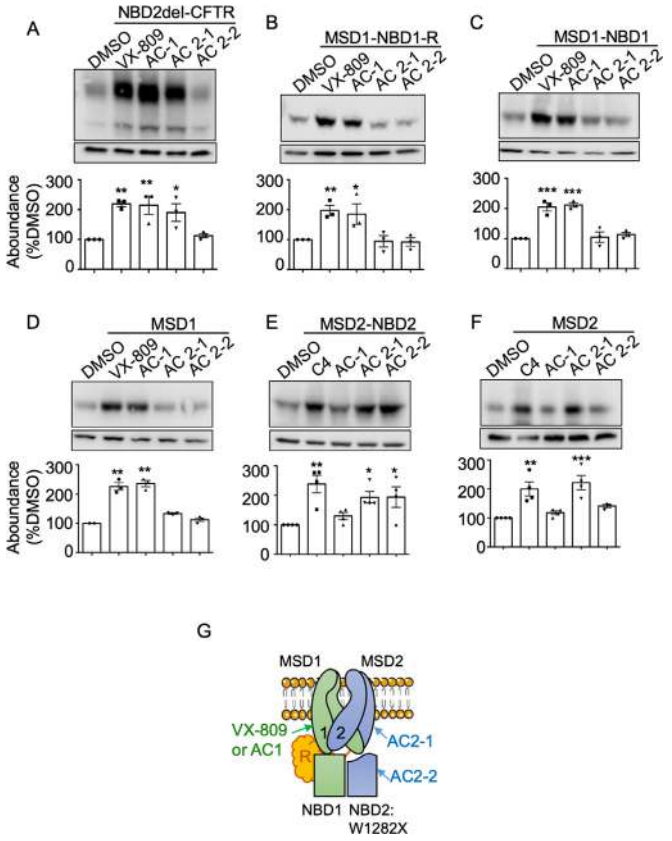

Fig. 1. Novel CFTR modulators stabilize different CFTR domains. (A–F) The effect VX-809 (3 μM), AC1 (0.5 μM), AC2-1 (3 μM) or AC2-2 (3 μM) on the abundance of CFTR fragments expressed in HEK293 cells. Data are representative of three experiments. Bar graphs show the mean (±SEM) of% abundance of CFTR normalized to calnexin loading (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (G) Cartoon of the CFTR structure showing that VX-809 and AC1 stabilize MSD1, AC2-1 stabilizes MSD2 and AC2-2 stabilizes NBD2. Compound AC1 belongs to C1-type corrector targeting early folding of mutant CFTR while AC2-1 and AC2-2 are C2-type corrector whose novel mechanism of action complements C1-type correctors. According to AbbVie, the new standardized nomenclature for Corrector AC1 is X281602, for corrector AC2-1: X281632 and for AC2-2: X300549. Please find the structures for these compounds in Figure S1 and their descriptions in Materials and Methods.

On the other hand, AC2-1 and AC2-2 enhanced steady state abundance of fragments corresponding to the second half of the molecule (Fig. 1). Interestingly, the AC2 compounds are not identical with respect to their mechanism. Similar to the corrector Corr4a (C4) [41], AC2-1 enhanced expression of the isolated MSD2 (Fig. 1(E) and (F)) whereas AC2-2 required NBD2 for its activity (Fig. 1(A) and (F)). Therefore, these data suggest that these AC2-1 and AC2-2 compounds act differently on the CFTR protein (Fig. 1(G)).

3.2. Novel CFTR correctors together with a potentiator are effective in rescuing the functional defect of W1282X-CFTR

Next, we tested the effect of the new modulators relative to VX-809 on the functional expression of W1282X-CFTR, expressed in HEK-293 cells. As for the previous studies with VX-809, the rationale for testing the response to corrector compounds in W1282X, relates to the potential for these compounds to increase the abundance of the mature, properly processed form of the truncated protein on the cell surface. CFTR channel function was measured using a fluorescence-based, membrane potential assay. Interestingly, we found that W1282X-CFTR expressed in HEK cells conferred residual cyclic AMP-dependent channel activity that was augmented

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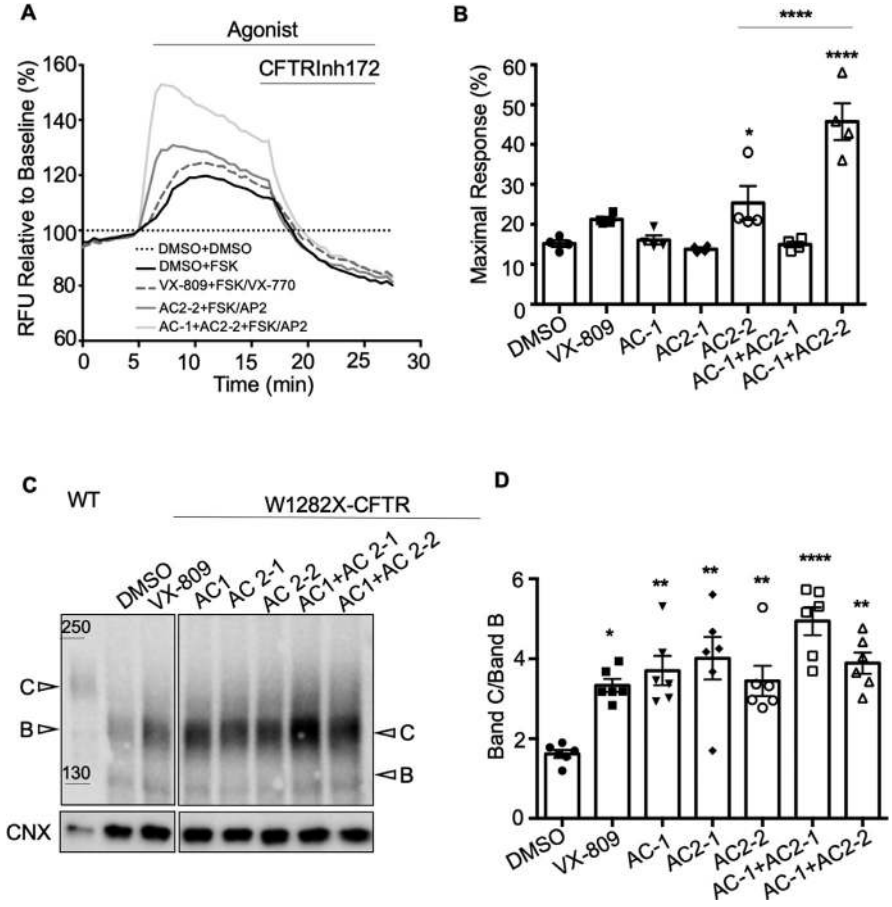

Fig. 2. Novel triple combinations correct W1282X-CFTR in HEK293 cells. (A) Representative traces of W1282X-CFTR-dependent chloride efflux using the fluorometric imaging plate reader membrane depolarization assay in the presence or absence of CFTR correctors (3 μM VX-809, 0.5 μM AC1, 3 μM AC2-1, 3 μM AC2-2). Following 5 min baseline measurement, 10 μM FSK +/− 1 μM VX-770 (for cells pre-treated with VX-809) or 1.5 μM AP2 (for cells pre-treated with AC compounds) were added. After approximately 10 min incubation, CFTR inhibitor (CFTRinh-172, 10 μM) was added to deactivate CFTR, as noted by the change in the slope of the curves. (B) Bar graphs show the mean (±SEM) of maximal activation of CFTR after stimulation by FSK and Potentiators (VX-770 or AP2) ($n = 4$ biological replicates with the symbols being the mean of 4 technical replicates). Biological replicates refer to different cell platings and technical replicate, wells within the plate (C) HEK293 cells were transiently transfected with WT-or W1282X-CFTR in the presence or absence of correctors for 24 h. Mature complex-glycosylated CFTR is denoted by C next to the blot; B, immature core-glycosylated CFTR; CNX is short for calnexin. (D) Bars represent the mean (±SEM) of the ratio of band C/band B ($n = 6$ biological replicates). ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$). Compound AP2 is a potentiator and according to AbbVie, its new standardized nomenclature is X300529. Please find the structure for this compound in Figure S1 and a description in Materials and Methods.

by pre-treatment with AC1 in combination with AC2-2 and potentiation with the AbbVie potentiator AP2 (formally known as: X300529, Figure S1) (Fig. 2(A) and (B)). There was no augmentation observed by pre-treatment with VX-809 and potentiation with VX-770. These findings suggest that the AC corrector combination may be very effective in enhancing the abundance and/or functional competency of the truncated protein. Alternatively, AP2 may be superior to VX-770 as a potentiator for this mutant. AP2 is a close analog of GLPG2451 [47] and like VX-770, increases the open time and reduces the closed time of Wt-CFTR single channel gating. Our studies of Wt-CFTR population channel activity using the FLIPR assay also suggests that VX-770 and AP2 act via similar mechanisms as there was no additivity in their potentiation (Figure S2).

As expected, immunoblotting revealed that the W1282X-CFTR protein was truncated in the HEK expression system (Fig. 2(C)) and further, that all of the corrector modulators enhanced the relative expression of the mature (band C) form of the protein (Fig. 2(D)). Interestingly, the corrector combination that gave rise to superior channel function (AC-1+AC2-2; Fig. 2(B)) was not superior to the other correctors with respect to promoting band C abundance (Fig. 2(C)). Hence, in the case of these studies in HEK-293 cells, increased expression levels of the truncated protein alone do not underlie the superior function mediated by W1282X-CFTR pre-treated with AC-1 and AC2-2.

Since the regulated gating of CFTR requires functional assembly between the first and second half of CFTR, we tested the hypothesis that AC2-2 promotes their interaction. We employed a fragment-based approach and asked if the abundance of MSD1-NBD1, which is not augmented by AC2-2 when expressed alone, will be augmented if the fragment is co-expressed with MSD2-NBD2, the fragment on which AC2-2 can act directly. Indeed the steady-state abundance of MSD1-NBD1 was significantly increased by AC2-2 in this co-expression experiment (Fig. 3(A) and (B)). However, there was no positive (i.e. allosteric) effect of AC2-2 on MSD1-NBD1 when co-expressed with just the MSD2 domain (Fig. 3(C)–(F)). These data suggest that AC2-2 allosterically modifies MSD1-NBD1 through its interaction with NBD2. Since W1282X-CFTR lacks a considerable region of NBD2 (although it retains the Walker A motif), we asked if AC2-2 also allosterically modifies MSD1-NBD1 through its interaction with MSD2-NBD2 (W1282X). As shown in Figure S3, the MSD2-NBD2 (W1282X) fragment is not stably expressed as an isolated polypeptide, so, we could not answer this question definitely. Taken together, our findings in the HEK-293 expression system support the hypothesis that the AC corrector combination may be particularly effective in enabling the

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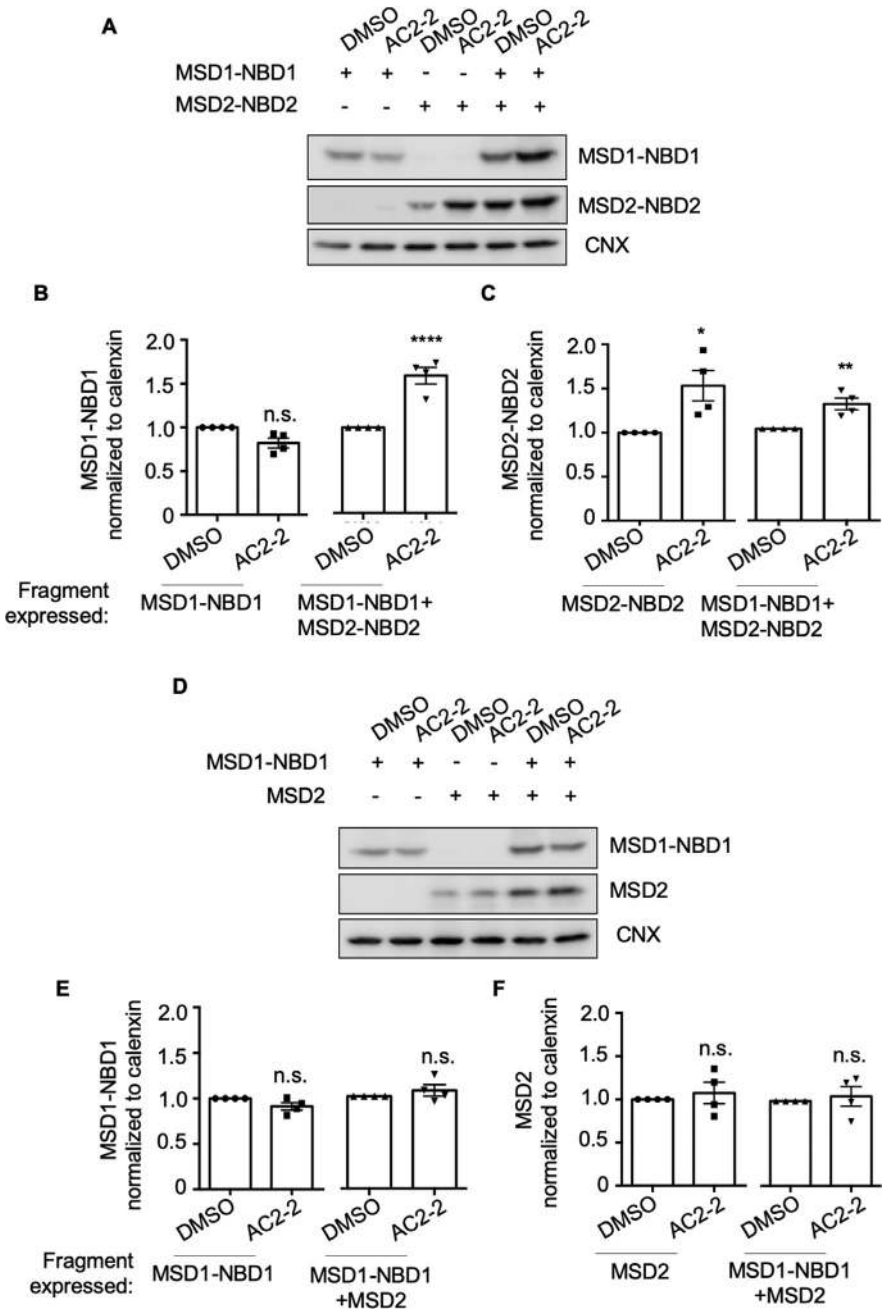

Fig. 3. Novel CFTR modulator, AC2-2, stabilizes MSD1-NBD1 through NBD2. (A) HEK293 cells were transiently transfected with MSD1-NBD1 or MSD2-NBD2, or co-transfected with MSD1-NBD1 and MSD2-NBD2. Cells were treated for 24 h at 37 °C with DMSO or AC2-2 (3 μM). The MSD1-NBD1 fragment was detected with an antibody against the N-terminus of CFTR (i.e. mAb MM13-4) and the MSD2-NBD2 fragment was detected with an antibody against the C-terminus of CFTR (mAb 596) CNX, Calnexin (rabbit Ab, Calnexin) was used as a loading control. (B–C) Bar graphs show the mean (±SEM) of the percentage of the CFTR abundance normalized to calnexin loading. Data are representative of four biological replicates ($^{*}p < 0.05$, $^{**}p < 0.01$; $^{****}p < 0.0001$). (D) HEK293 cells were transiently transfected with MSD1-NBD1 or MSD2, or cotransfected with MSD1-NBD1 and MSD2 domains. Cells were treated for 24 h at 37 °C with DMSO or AC2-2 (3 μM). The MSD1-NBD1 fragment was detected with an antibody against the N-terminus of CFTR (i.e. mAb MM13-4) and the MSD2 fragment was detected with antibody A52) CNX, Calnexin (rabbit Ab, Calnexin) was used as a loading control. (E and F) Bar graphs show the mean (±SEM) of the percentage of the CFTR abundance normalized to calnexin loading. Data are representative of four biological replicates with each replicate indicated as a symbol.

expression of W1282X-CFTR protein with superior functional capacity.

Given the susceptibility of nonsense mutations to undergoing nonsense mediated decay in cells that endogenously express the gene, with the resulting loss of W1282X-CFTR transcript and protein, we asked if the above modulators exert any rescue effect in the context of human airway epithelial cells. We tested the effect of the novel modulators on CFTR channel function in the 14HBEo^{-} cell line (HBE) edited using CRISPR-Cas9 to introduce the W1282X mutation in the context of the complete CFTR gene, rather than the cDNA as in the preceding experiments. This edited cell line was provided by CFFT (HBE-W1282X, Fig. 4) [22]. In contrast to the HEK-293 cell studies, there was no residual cyclic AMP-mediated channel activity conferred by W1282X-CFTR expressed in HBE cells. Further, as shown in Fig. 4(A), there was no functional response, even after pre-treatment with VX-809 and potentiation with VX-770. There was modest response in cultures pretreated with the

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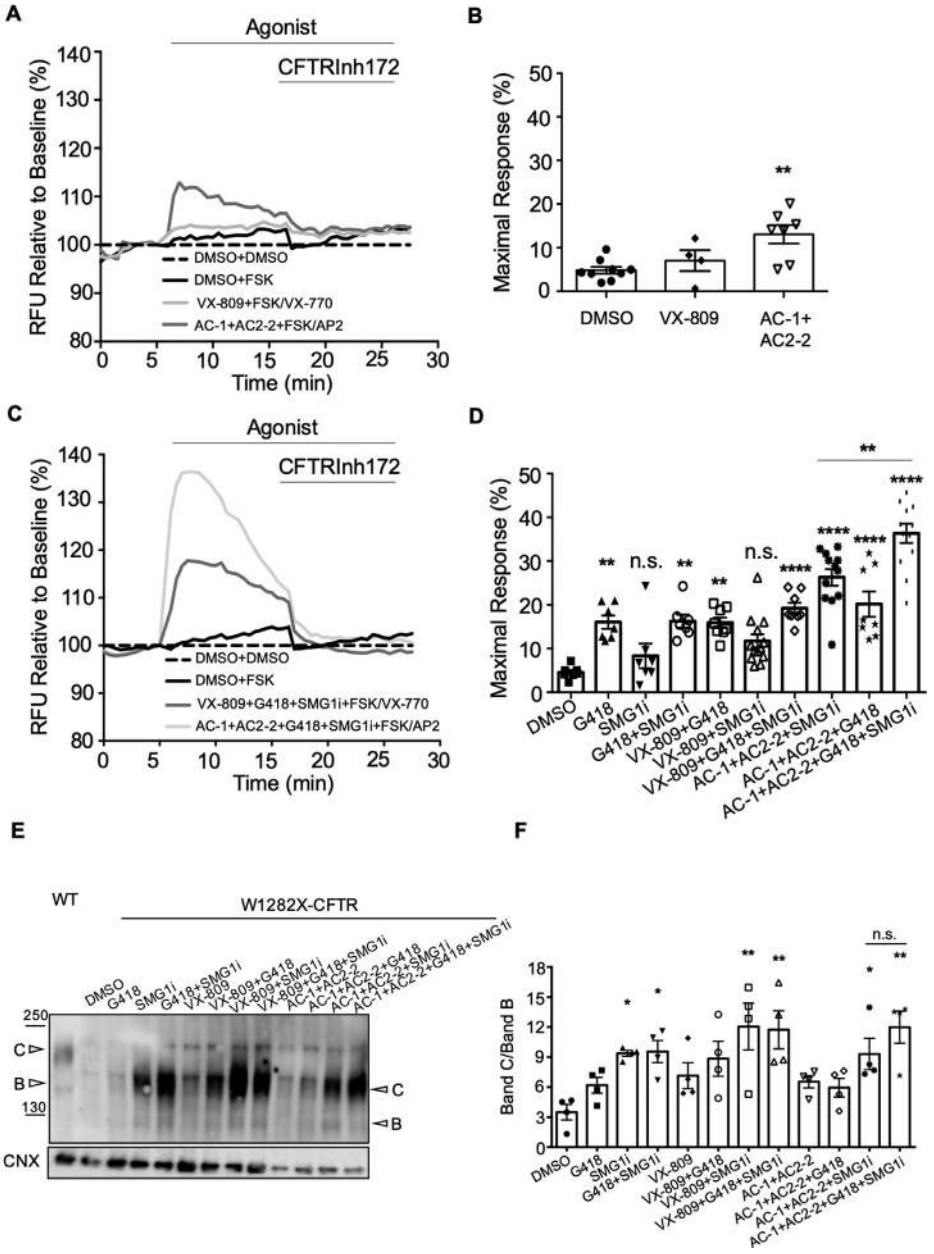

Fig. 4. HBE cells that have been edited by CRISPR/Cas9 to express W1282X-CFTR showed functional rescue with novel F508del-CFTR modulator triple combinations plus NMD inhibitor and readthrough compound. (A) Representative trace of W1282X-CFTR dependent chloride efflux using the FLIPR assay in the presence or absence of CFTR modulators (3 μM VX-809, 0.5 μM AC1 + 3 μM AC2-2). Following baseline measurement, 10 μM FSK +/− 1 μM VX-770 (for cells pre-treated with VX-809) or 1.5 μM AP2 (for cells pre-treated with AbbVie drugs) were added. CFTR inhibitor (CFTRinh-172, 10 μM) was added to deactivate CFTR, as denoted by the change in the curve at approximately 17 min. (B) Bar graphs show the mean (±SEM) of peak CFTR-mediated depolarization response after stimulation by FSK and potentiators (n = 4–9 biological replicates with each symbol being a mean 4 technical replicates). (C) Representative trace of W1282X-CFTR dependent chloride efflux using the FLIPR assay in the presence or absence of CFTR modulators (200 μg/mL G418, 0.5 μM SMG1i, 3 μM VX-809, 0.5 μM AC1, 3 μM AC2-2). (D) Bar graphs show the mean (±SEM) of maximal peak response of CFTR-mediated depolarization after stimulation by FSK and potentiators (n = 7–13 biological replicates with each symbol being a mean 4 technical replicates). (E) Representative blot of CRISPR/Cas9 edited HBE-W1282X after 24 h treatments with CFTR modulators. C corresponds to mature complex-glycosylated and B, immature core-glycosylated. CNX corresponds to the loading control, calnexin. (F) Bars represent the mean (±SEM) of the ratio of band C/band B (n = 4 biological replicate). ($^{*}p < 0.05$; $^{**}p < 0.01$).

AC corrector combination (AC-1+AC2-2) and potentiated with AP-2 (Fig. 4(A) and (B)).

On the basis of studies showing that nonsense mediated decay was limiting to the extent of residual function in the case of nonsense mutations [27], we asked if G418 (geneticin), a small molecule previously shown to cause read-through of PTC mutations, including PTC mutations in CFTR [6,56] would enhance functional rescue of W1282X in this epithelial cell line. HBE cells expressing W1282X were incubated with 200 μg/mL G418 for 24 h alone. Interestingly we did observe a significant increase in potentiated chloride conduction (Fig. 4(C) and (D)), but surprisingly, this was not associated with generation of the full length CFTR protein as expected for a read-through agent (Fig. 4(E)). Similar to previous reports, [21,22], we observed an increase in abundance of the truncated W1282X–CFTR protein after treatment with the NMD inhibitor, SMG1i. However, this single treatment was not associated with a significant increase in potentiated channel function of W1282X-CFTR (Fig. 4(D)).

We reasoned that combinations aiming to promote expression, stability and function will have a more robust rescue effect. Con-

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sistent with this idea, we found that combination of the corrector VX-809 with SMG1i plus G418, led to a significant increase in potentiated channel activity to a level that is approximately 40% of that measured for Wt-CFTR [36] (Fig. 4(D)). Notably, the corrector combination, AC-1+AC2-2 on top of SMG1i or SMG1i plus G418 led to a more robust rescue to 50% and 70% of Wt-CFTR channel activity respectively (Fig. 4(D)). Immunoblotting of W1282X protein expression, confirmed that the SMG1i compound was primarily responsible for augmenting abundance of the truncated mutant protein (Fig. 4(E)–(F)). Furthermore, following pretreatment with SMG1i and G418, we confirmed that the AC combination of correctors (AC1 plus AC2-2) promoted the functional capacity of the mutant protein better than VX-809 in the HBE expression system. With VX-770 as the potentiator used, the functional response was significantly greater for AC1 plus AC2-2 corrected W1282X-CFTR than for VX-809 corrected W1282X-CFTR (Figure S4). Similarly, with AP-2 as the potentiator used, the functional response was greater for the AC1 plus AC2-2 corrected W1282X-CFTR than for the VX-809 modulated W1282X-CFTR (Figure S4).

We compared the relative efficacy of SMG1i to other NMD inhibitors that act via distinct mechanisms (i.e. NMDI-14 that targets SMG7 and its interaction with UPF1 [26] and Vidaza or 5-azacytidine, a compound that modulates nonsense mediated decay in a MYC dependent pathway [18,57] and has been approved as a treatment for myelodysplastic syndrome [58]). Interestingly, as shown in Figure S5A-B, while NMDI-14 is almost as effective as SMG1i in rescuing the potentiated channel function of W1282X when added together with AC1, AC2-2 and AP-2, Vidaza was ineffective.

3.3. Small molecules induced rescue in primary nasal epithelial cells from CF patients homozygous for the W1282X mutation

Our next goal was to determine whether the functional rescue by small molecules observed in HEK293 and HBE-W1282X cells translates to a detectable response in patient-derived tissue. Cultures were generated from nasal epithelial brushings obtained from 4 individuals homozygous for W1282X and differentiated at an air/liquid interface as previously described [36]. Equivalent currents were determined from Ussing chamber studies of small molecule treated nasal cultures as described in the Methods section. Representative traces from cultures from one individual are shown in Fig. 5(A). Consistent with the findings in HBE cells, the novel quadruple combination (AC1+AC2-2) plus SMG1i conferred a significant functional response in these in-vitro studies following acute addition of the potentiator, AP2. The in-vitro response to this combination was close to 50% of the forskolin response measured in non-CF cultures (Figs. 5(B) and S6A). None of the other combinations, including VX-809 plus SMG1i pretreatment with potentiation with VX-770 caused a statistically significant improvement in-vitro response. Interestingly, the addition of G418 to the above pretreatment cocktail decreased the potentiated response size across these patient specific nasal cultures. This additional intervention however, was associated with an increase in the CFTRInh-172 inhibitable current (Figure S6A), an observation for which we do not currently have an explanation.

As shown in Fig. 6(A), we confirmed that SMG1i treatment increased the W1282X-CFTR mRNA up to the mean of WT-CFTR mRNA levels measured in 3 non-CF donors. This robust increase in mRNA abundance following SMG1i treatment also translated to a significant increase in the abundance of the truncated protein (band C/band B) in nasal epithelial cells from CF patients homozygous for the W1282X mutation (Figs. 6(B), (C) and S7). These data argue that the inhibition of nonsense mediated decay was required for the stable expression of the truncated protein.

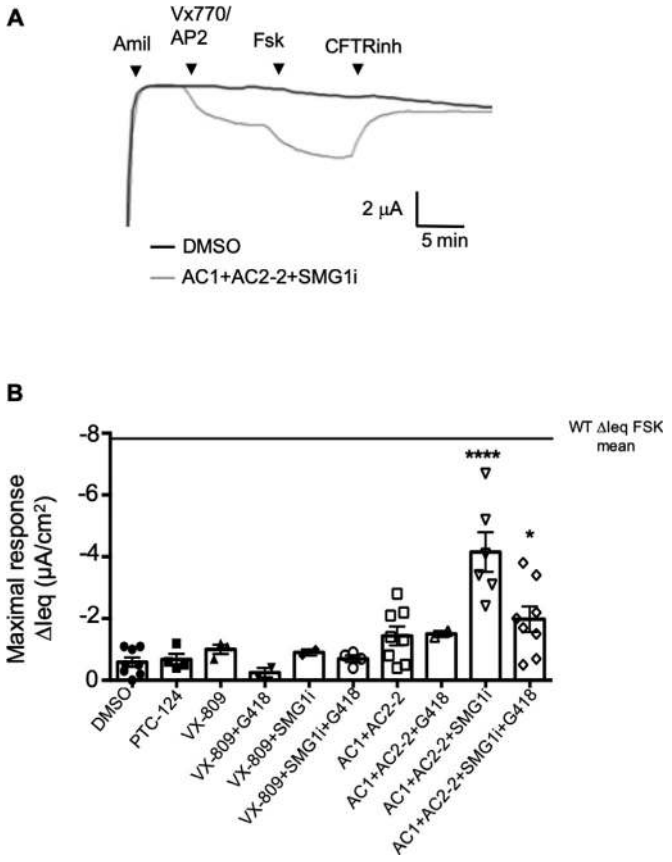

Fig. 5. Nasal epithelial cultures derived from patients homozygous for the W1282X-CFTR mutation exhibit CFTR channel function after rescue by CFTR modulators. (A) Representative Ussing chamber tracings obtained from nasal culture prepared from one patient homozygous for W1282X/W1282X in the absence or presence of small molecule CFTR correctors. Modulators were tested for each patient in 1–2 cultures (peak value for culture shown as dot). Cultures pretreated with DMSO for 48 h typically showed no response to Vx770 and Fsk (forskolin) whereas cultures pretreated with AC1+AC2-2 and SMG1i typically shows a robust response to AP2 and Fsk. (B) Bar graph showing mean peak responses (Ieq (μA/cm^2 ±SEM) to forskolin (10 μM) and VX-770 (1 μM) (for cells pre-treated with PTC-124 and VX-809) or AP2 (1.5 μM) (for cells pre-treated with AC compounds) activated Ieq for nasal cultures from 4 patients after pre-treatment for 48 h with small molecules (200 μg/mL G418, 10 μM PTC-124, 0.5 μM SMG1i, 3 μM VX-809, 0.5 μM AC1, 3 μM AC2-2). ($^*p < 0.05$; $^{****}p < 0.0001$). The symbols show 1–2 technical replicates of nasal cultures studied from 4 patients.

4. Discussion

While the majority of individuals with Cystic Fibrosis have mutations that lead to full length protein that is potentially rescuable with small CFTR modulatory compounds, nonsense mutations result in reduced production of a truncated CFTR protein. Previous studies using heterologous expression systems showed that in the case of W1282X, the truncated protein can be modulated by small molecular correctors and/or potentiators [11,13,59]. Yet, such promising results did not translate to positive findings in preclinical studies on patient derived tissue models, primarily because of nonsense mediated decay and reduced W1282X-CFTR transcript levels. Here, we show in patient derived cells, that a quadruple combination of modulators targeting nonsense mediated decay, different steps along the CFTR biosynthesis pathway and channel gating, is effective in rescuing the functional expression by the nonsense mutation W1282X. This work provides hope for those with certain PTC mutations that small molecules will indeed offer a viable therapeutic option.

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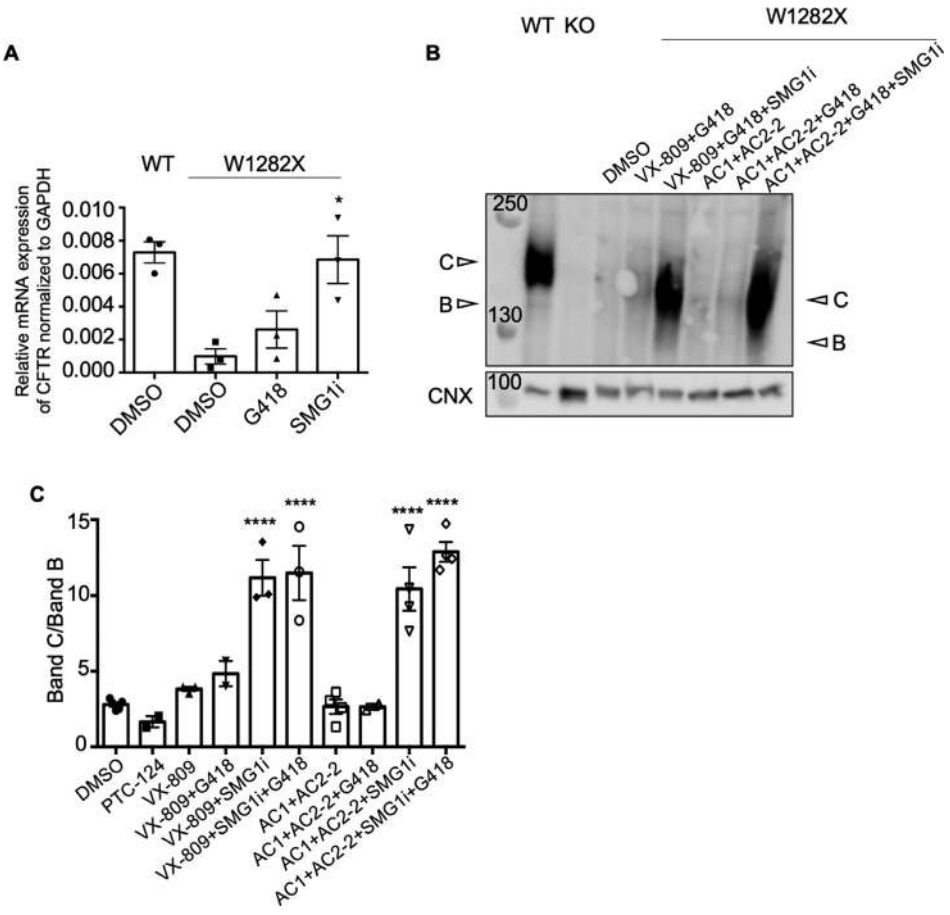

Fig. 6. NMD inhibitor restores mRNA expression of CFTR to the healthy control level. (A) Primary nasal epithelial cells from non-CF donors ($n = 3$) and CF donors (homozygous for W1282X) ($n = 3$) were treated with 0.1% DMSO, 200 μg/mL G418 or 0.5 μM SMG1i for 48 h and *CFTR* message measured using qRT-PCR and normalized to housekeeping gene *GADPH*. Bars represent *CFTR-W1282X* transcript abundance (mean ± SEM) and the difference between DMSO and SMG1i pretreatments was significant (*$p < 0.05$, $n = 3$ biological replicates). (B) Representative W1282X-CFTR protein expression in nasal cultures from a CF patient after 48-h pre-treatment with vehicle (DMSO), VX-809 (3 μM)+ G418(200 μg/mL), VX-809 (3 μM)+ G418(200 μg/mL)+SMG1i (0.5 μM), AC1(0.5 μM)+AC2-1(3 μM), AC1(0.5 μM)+AC2-1(3 μM)+G418(200 μg/mL) or AC1(0.5 μM)+AC2-1(3 μM)+ G418(200 μg/mL)+SMG1i (0.5 μM). Mature, complex-glycosylated CFTR (first lane) and CFTR-W1282X (remaining labeled lanes) is denoted C next to the blot. Note that it is the truncated protein that is produced in nasal cultures from patients homozygous for W1282X, regardless of the intervention. B: immature, core-glycosylated CFTR. CNX, Calnexin was used as a loading control (C) Datapoints represent the mean (±SEM) of the ratio of band C/band B ($n = 2$–4 biological replicates) for each of the interventions.

The use of read through agents in the setting of PTC mutations has been considered as a therapy for Cystic Fibrosis [60–63]. This approach has been used for Duchenne muscular dystrophy (DMD) with some success [62,64]. However, in the setting of CF, Ataluren treatment has not translated into clinical benefit [9,10]. In the current work, we did not observe a positive rescue effect of the putative read-through agents including: PTC-124 or G418 in patient derived nasal epithelial cells. Interestingly, there was a modest positive effect of G418 treatment on potentiated channel activity in an HBE cell line edited to express W1282X-CFTR. However, this effect was not associated with the appearance of the full-length protein in western blot studies. Hence, the molecular basis for its partial rescue effect in this cell line remains uncertain.

We showed that inhibition of the kinase SMG1, a protein that is essential for nonsense mediated decay [4,16,22,28], was effective in augmenting the production of the truncated CFTR (W1282X-CFTR) mRNA and protein in patient-derived tissue. Similarly, the compound NMDI-14 that targets the molecular interaction between SMG7 and UPF1 was effective in enhancing W1282X-CFTR production in the bronchial cell line. Hence there are multiple pathways through which the nonsense-mediated decay of W1282X-CFTR can potentially be ameliorated. However, we showed that such rescue of W1282X-CFTR production is required but not sufficient for functional rescue of this mutant protein in patient derived nasal cultures (Fig. 5). A statistically significant increase in potentiated channel function also required the addition of protein corrector molecules.

We found that addition of modulator compounds was required to render the truncated protein 'channel competent', after augmentation of its production using SMG1i in patient-derived tissues. The novel correctors, AC1 plus AC2-2, were particularly effective in generating a functionally competent form of the truncated protein. In primary human nasal epithelial cultures tested, the correction was approximately 50% of wild type values measured by Ussing chamber. There was variability amongst donor-specific cultures, with cultures from one patient showing correction close to 80% of wild type. Interestingly, enhancement of production with SMG1i and modulation with VX-809 pretreatment failed to yield a functionally competent W1282X-CFTR protein, even with VX-770 potentiation.

Given the recent studies by Haggie and colleagues, showing that the choice of potentiator is important for the functional rescue of the W1282X-CFTR protein [13], we asked if the rescue effects of SMG1i and VX-809 pretreatment were improved if AP2, the potentiator used with the AC modulators, rather than VX-770 was used. Interestingly, in W1282X-HBE studies (Figure S4), we found that

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the substitution of VX-770 with AP2 failed to significantly improve the functional rescue, arguing that, after enhancing mutant protein production, the choice of corrector-like compounds plays a key role in rescuing a functional protein in our cell culture models.

The superior functional rescue mediated by pre-treatment with AC-1 plus AC2-2 relative to VX-809 likely relates to the complementary mechanisms of action of these two compounds. While AC-1 interacts with MSD1, AC2-2 interacts with the second half of the CFTR molecule: MSD2-NBD2 and together, they facilitate the functional interaction of both halves of CFTR. Although AC2-1 also interacts with the second half of CFTR (MSD2), it is ineffective in augmenting the functional expression of the truncated W1282X-CFTR. The superior effect of AC2-2 may relate to its the interaction of AC2-2 with NBD2. Unlike AC2-1, AC2-2 requires NBD2 to enhance expression of MSD2 pointing to a different mechanism of action and/or binding site. Because W1282X-CFTR is lacking a portion of NBD2 and the carboxy terminus, we propose that AC2-2 is effective in promoting the functional expression of this mutant by modulating NBD1:NBD2 interactions via the Walker A motif of NBD2 (including lysine at position 1250) as this motif is still retained in the truncated protein.

The identification of small molecules that mediate effective read-through of premature stop codons would hypothetically obviate the need for nonsense mediated decay inhibitors and appropriate CFTR modulators. Unfortunately, we saw no evidence for PTC read-through (i.e. appearance of the full-length protein) following treatment of cultures with G418 in our studies, supporting the argument that more discovery work is required in this area.

In summary, we showed using multiple cell based models including primary nasal cultures from four patients who are homozygous for W1282X, that the best functional rescue was obtained using a nonsense mediated decay inhibitor plus a combination of complementary protein modulators (that act to stabilize the first and second half of the CFTR protein and potentiate regulated channel function by the truncated protein. This is the first example showing that a cocktail of small molecules can rescue the functional expression of W1282X to therapeutically relevant levels in primary epithelial cultures. Future studies will determine if a similar strategy will be effective for other nonsense mutations that generate mutant proteins, such a W1274X, Y1307X, Q1412X and S1455X.

Declaration of Competing Interest

The authors declare no competing interests.

CRediT authorship contribution statement

Onofrio Laselva: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Paul DW Eckford:** Writing - review & editing. **Claire Bartlett:** Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Hong Ouyang:** Data curation, Methodology, Writing - review & editing. **Tarini NA Gunawardena:** Data curation, Methodology, Writing - review & editing. **Tanja Gonska:** Writing - review & editing. **Theo J Moraes:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Christine E Bear:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2019.12.001.

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