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Original article

Targeting *G542X CFTR* nonsense alleles with ELX-02 restores CFTR function in human-derived intestinal organoids

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ABSTRACT

Background: Promoting full-length protein production is a requisite step to address some of the remaining unmet medical need for those with Cystic Fibrosis (CF) nonsense alleles. ELX-02 promotes read-through of mRNA transcripts bearing nonsense mutations, including the most common CF nonsense allele *G542X*, in several different preclinical models including human bronchial epithelial cells. Here we evaluate ELX-02 mediated read-through using the CFTR-dependent Forskolin-induced swelling (FIS) assay across a selection of *G542X* genotype patient derived organoids (PDOs).

Methods: CFTR functional restoration was evaluated in ELX-02 treated *G542X* homozygous and heterozygous PDOs in the CFTR-dependent FIS assay. *CFTR* mRNA abundance and integrity were evaluated by qPCR and Nanostring analysis while PDO protein was detected by capillary based size-exclusion chromatography.

Results: PDOs homozygous for *G542X* or heterozygous with a second minimally functional allele had significantly increased CFTR activity with ELX-02 in a dose-dependent fashion across a variety of forskolin induction concentrations. The functional increases are similar to those obtained with tezacaftor/ivacaftor in *F508del* homozygous PDOs. Increased CFTR C- and B-band protein was observed in accordance with increased function. In addition, ELX-02 treatment of a *G542X/G542X* PDO results in a 5-fold increase in *CFTR* mRNA compared with vehicle treated, resulting in normalization of *CFTR* mRNA as measured via Nanostring.

Conclusions: These data with ELX-02 in PDOs are consistent with previous *G542X* model evaluations. These results also support the on-going clinical evaluation of ELX-02 as a read-through agent for CF caused by the *G542X* allele.

1. Introduction

Advances in cystic fibrosis (CF) patient respiratory care, nutrition, and new treatment options in recent years have improved quality of life for many patients. However, the disease modifying therapies available target the protein itself and are therefore not applicable to those individuals with alleles incapable of producing protein at all, including those with nonsense alleles. Nonsense mutations generally yield no functional CFTR, and individuals with these alleles are typically among those with the most severe form of disease. The *G542X* allele is the most common *CFTR* nonsense allele in the CF population and is found in 4.6% of affected individuals according to the Cystic Fibrosis Foundation Registry [1]. The mutation replaces the position 542 glycine codon within the NBD1 domain with an opal (UGA) stop codon, a location close enough to the N-terminus to suggest that even if stable, the truncated product would not function as a channel. Despite this challenge, the *G542X* allele can be rescued through promotion of premature stop codon read-through [2].

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; cAMP, cyclic adenosine monophosphate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DMSO, dimethyl sulfoxide; FIS, forskolin-induced swelling; h, hour(s); min, minute(s); ns, non-significant; PDO, patient-derived organoid; qPCR, quantitative polymerase chain reaction.

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ELX-02, [6′-(R)-Methyl-5-O-(5-amino-5,6-dideoxy-α-L-talofuranosyl)-paromamine sulfate, also referred to as NB124] [3], is a novel small molecule, non-antibiotic aminoglycoside analogue being developed for the treatment of genetic diseases caused by nonsense mutations. ELX-02 is a eukaryotic ribosomal selective glycoside which displays preferential binding to the eukaryotic ribosome and promotes premature stop codon read-through [3]. Evidence of native stop codon read-through has not been detected [4], consistent with conserved cis-element factors that improve native stop codon fidelity. ELX-02 has been evaluated in Phase 1 clinical trials, where it was found to be well tolerated with pharmacokinetic properties consistent with preclinical evaluations [5].

ELX-02 was previously evaluated in FRT cells overexpressing the *G542X* allele and *G542X/F508del*-bearing human bronchial epithelial cells [6]. These studies demonstrated the *G542X* read-through potential of ELX-02, but assessment was limited to a single *G542X* heterozygous patient-derived cell. To evaluate read-through in a larger genotypic cross-section of the nonsense allele bearing CF population, alternative model systems were investigated. Here we expand on these studies by using the CFTR-dependent Forskolin induced swelling (FIS) assay across a selection of *G542X* genotype patient derived organoids (PDOs), which are three dimensional, multi-cellular epithelial structures derived from intestinal stem cells obtained by rectal biopsies. The FIS assay in PDOs is demonstrated to correlate with disease severity and translation potential through positive correlation of assay results with clinical endpoints testing approved therapeutics [7–10]. In addition, PDOs provided the opportunity to evaluate both homozygous and heterozygous *G542X* patient-derived cells which better reflect the genotypic diversity of the target patient population. These studies help evaluate the potential of ELX-02 for *G542X*-mediated CF and future application to other CF nonsense alleles, an area with significant clinical unmet need.

2. Materials and methods

2.1. PDO FIS assay

All intestinal PDO cultures used in this study belong to the HUB CF organoid biobank. Informed consent for the use of all these PDO cultures was obtained from the donating patients before the start of this study. All assays using human PDOs described herein were approved by the ethical committee at University Medical Center Utrecht (UMCU; TcBio #14–008). Informed consent for tissue collection, generation, storage, and use of the PDOs was obtained from the patients at Wilhelmina Children's Hospital (WKZ)-UMCU. All PDO cultures were matched with their original tissue by SNP fingerprinting analysis. Minimal functional classification of CFTR alleles was based upon the allele generating less than 3% of wild-type CFTR activity based on the *CFTR2* database [11].

Collection, isolation, expansion biobanking procedures, and FIS evaluation of PDOs were conducted as recently described [12] with modifications to accommodate the mechanism of action being

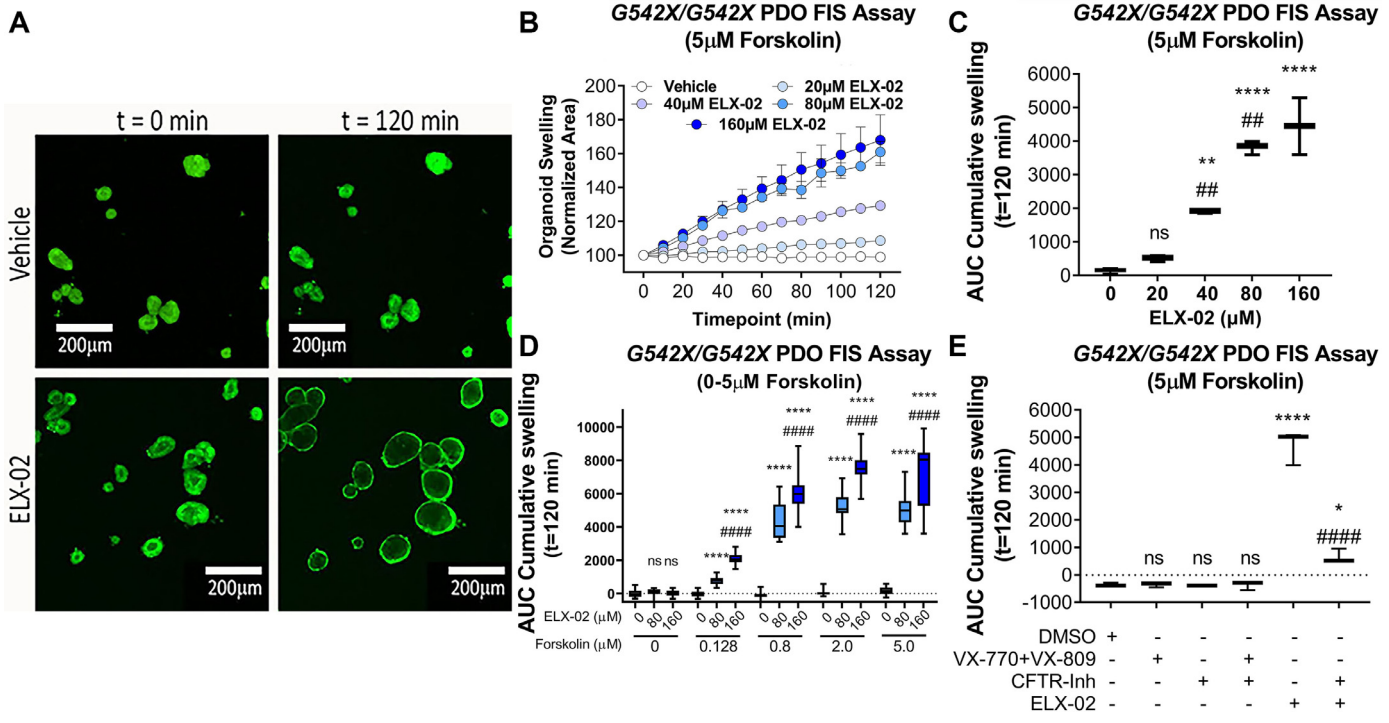

Fig. 1. ELX-02 restores function in *G542X/G542X* PDO. (**A.**) Following induction with forskolin, PDOs treated with either vehicle or ELX-02 were imaged every 10 min for 120 min; representative images demonstrating change in size relative to $t = 0$ min and $t = 120$ min are presented. (**B.**) *G542X/G542X* PDOs demonstrate dose-dependent increase in swelling over time in response to ELX-02. Data are plotted as mean ± SD for $n = 3$ wells organoids per treatment group. For all graphs, when the SD at a given point is sufficiently small, then no error bars are shown for that point. (**C.**) AUC was calculated for the cumulative swelling observed for each organoid in (B.) and the results shown in box plots. ELX-02 concentrations are listed in in µM (based on the free base). One-way ANOVA with Tukey's post-hoc analysis revealed a significant effect of ELX-02 ($p<0.0001$). ** $p<0.005$ vs vehicle, **** $p<0.0001$ vs vehicle, ## $p<0.005$ vs next lower concentration. (**D.**) PDO swelling induced by ELX-02 was observed across a range of forskolin concentrations. Data collected from $n = 9$–33 wells across multiple experiments are presented in box plots. One-way ANOVA with Tukey's post-hoc analysis revealed a significant effect of ELX-02 at each concentration where forskolin was present ($p<0.0001$), but no effect of ELX-02 when forskolin was not included ($p = 0.9445$). **** $p<0.0001$ vs vehicle, #### $p<0.0001$ vs next lower concentration. (**E.**) To confirm that the swelling observed in *G542X/G542X* PDOs induced with 0.8 µM forskolin was CFTR activity dependent and specific to the read-through mechanism of action, 50 µM CFTR channel inhibitor was applied either alone or in combination with either VX-770+VX-809 or 160 µM ELX-02 in $n = 3$ wells of PDOs per condition. One-way ANOVA with Tukey's post-hoc analysis revealed a significant effect of ELX-02 ($p<0.0001$). ** $p<0.005$ vs vehicle, **** $p<0.0001$ vs vehicle, #### $p<0.0001$ vs ELX-02 alone. CFTR channel inhibitor and VX-770+VX-809, either alone or in combination, had no effect on PDO swelling (adjusted $p = 0.9776$).

evaluated. PDOs were plated in three dimensional Matrigel cultures in a 96-well format and treated with either ELX-02, vehicle or a corrector (VX-661 or VX-809, as indicated) for 48 h. While approximately 25–50 organoids were plated per well, each well was treated as a single replicate. Each condition was represented in triplicate across individual experiments and, when available, data are pooled across experiments. After 48 h, PDOs were incubated for 30 min with 10 μM calcein green to fluorescently label the cells for imaging. Following calcein labeling, forskolin was added to stimulate CFTR channel conductance and organoid cross-sectional area measurements by confocal fluorescence microscopy were immediately initiated. Images were taken at 10 min intervals for 120 min. In experiments including potentiator, VX-770, the potentiator was added concurrent with forskolin induction. Forskolin was used from 0–5 μM while VX-770, VX-661 and VX-809 were used at 3 μM each, where indicated. Images generated by Cell Voyager 7000S (Yokogawa) microscope or a PerkinElmer Operetta CLS High-Content Analysis system and automatically analyzed using Fiji (Fiji Life-Line version, 2014 November 25), an open source image processing package based on ImageJ. Individual PDOs were detected and tracked for change in cross sectional area over time (relative to $t = 0$). The average change in PDO size was tracked and represented as a% change from baseline (100%) from which the area under the curve (AUC) was calculated.

2.2. Compound formulation

ELX-02 test article was diluted in Type 1 Milli-Q water to a stock concentration of 20 mg/mL. CFTR inhibitor 172 and CFTR inhibitor-II were sourced from Sanbio and stock solutions were prepared in dimethyl sulfoxide (DMSO). Ivacaftor (VX-770) was supplied by Bio Connect, lumacaftor (VX-809) and tezacaftor (VX-661) were each supplied by Selleck Chem and stock solutions prepared in DMSO. Clinically, Orkambi® is prepared in a dosage form of 200 mg lumacaftor and 125 mg ivacaftor and Symdeko® is prepared in a dosage form of 100/150 mg tezacaftor and 150 mg ivacaftor.

2.3. Protein analysis

PDOs are incubated for 5 days. ELX-02 was added (160 μM free base concentration) during the last 48 h of the assay. At time of sample collection, plates were placed on ice, cell culture media was aspirated, and wells were washed with phosphate buffered saline followed by a depolymerization step with Organoid Harvesting Solution (1 h, 4 °C). Plates were centrifuged at 500 x g for 5 min at 4 °C to pellet the PDOs. Following supernatant aspiration and multiple washes with cold (4 °C) phosphate buffered saline, samples were lysed in radioimmunoprecipitation assay buffer plus protease inhibitors (15 min on ice) then sonicated briefly. Pro-

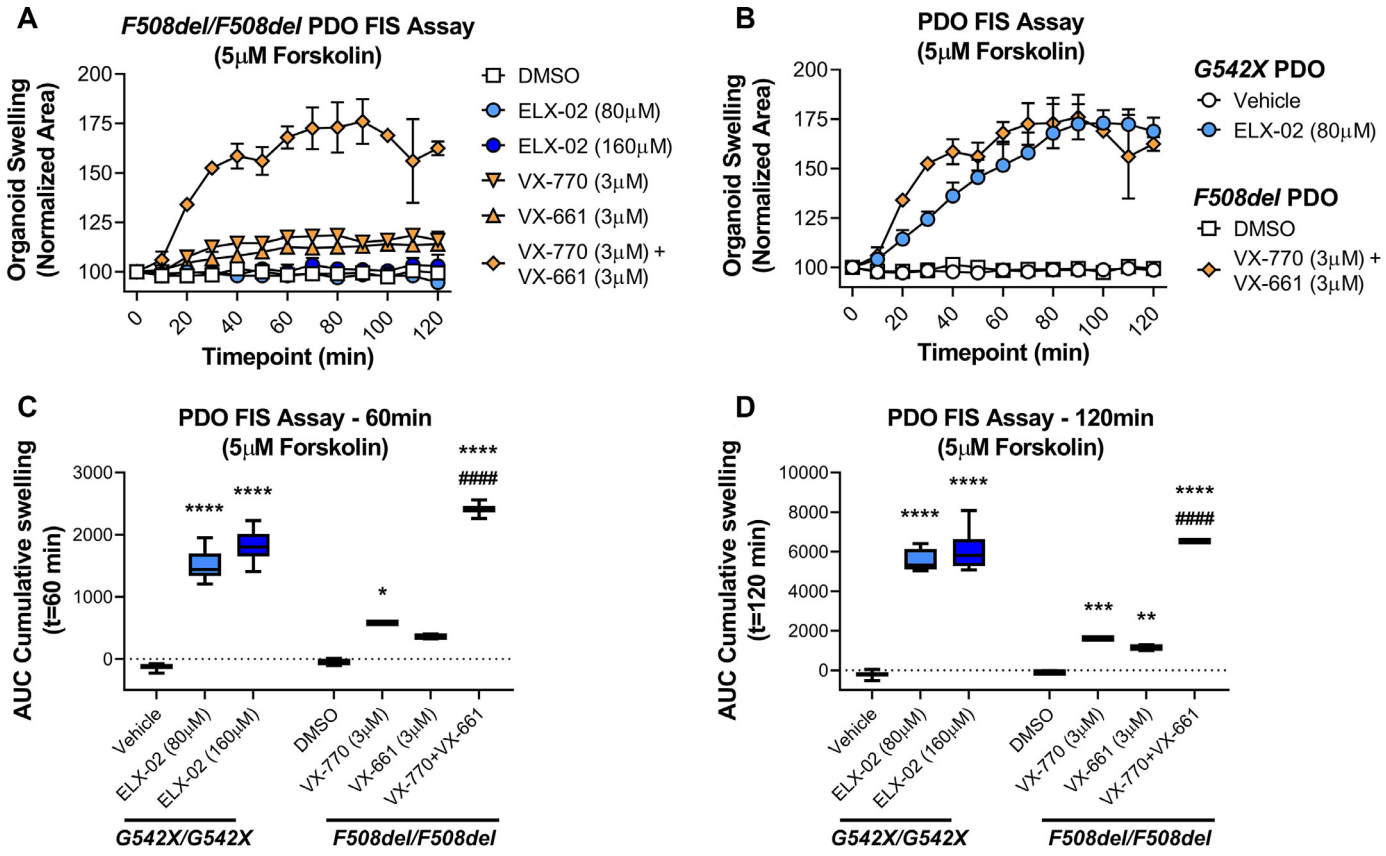

Fig. 2. ELX-02 restores CFTR function in *G542X/G542X* PDOs. (A.) *F508del/F508del* PDO demonstrates CFTR functional restoration in response to VX-770+VX-661, but not to ELX-02. Data are plotted as mean ± SD for $n = 2$ wells of PDOs per treatment group. **(B.)** Comparison of swelling induced by ELX-02 in *G542X/G542X* PDOs and by VX-770+VX-661 in *F508del/F508del* PDOs shows swelling reaching a plateau in the *F508del* PDOs faster than *G542X* PDOs, likely due to increased channel open probability due to the potentiator VX-770. Side by side evaluation of swelling in *G542X/G542X* PDO demonstrates comparable CFTR functional restoration levels in response to ELX-02 treatment ($n = 6$ wells of PDOs per treatment group) when compared to swelling in *F508del/F508del* PDOs treated with VX-770+VX-661 ($n = 2$ wells of PDOs per treatment group). AUC was calculated for the cumulative swelling observed for each PDO after 60 min (**C.**) or after 120 min (**D.**) and the results shown in box plots. One-way ANOVA with Tukey's post-hoc analysis revealed a significant effect of ELX-02 (**** $p<0.0001$). At 60 min, One-way ANOVA with Tukey's post-hoc analysis also revealed a significant effect of VX-770 (* $p<0.05$), a trend for VX-661 ($p = 0.055$), and a significant effect in combination (#### $p<0.0001$ vs individual compounds). At 120 min, One-way ANOVA with Tukey's post-hoc analysis also revealed a significant effect of VX-770 and VX-661, either alone or in combination (** $p<0.005$ vs DMSO, *** $p<0.001$ vs DMSO, #### $p<0.0001$ vs individual compounds).

tein concentration was then determined by bicinchoninic acid assay and then diluted to 1 mg/mL. Samples (5µL/well) were labeled with D6W6L anti-CFTR antibody (Cell Signaling Technology) and subsequently detected using the Simple Western size exclusion chromatography with Compass SW4.0 software for analysis. The CFTR B band was defined in the software to be 160 kDa +/- 2% and the C band was defined to be 189 kDa +/-2%. The study analysis was completed from two independent experiments which included technical triplicates.

2.4. RNA isolation, cDNA preparation and qPCR analysis

For *CFTR* mRNA analysis, PDOs embedded in Matrigel were seeded in a 24-well plate format. After seeding, ELX-02 was added to culture media of appropriate wells and PDOs were incubated for 48 h at 37 °C. RNA was isolated using the RNeasy protocol from Qiagen and the concentration was measured using the Qubit HS RNA assay. cDNA was synthesized using Superscript II with random primers (hexamers) on 200 ng total RNA. The cDNA was then diluted 5-fold, and of this dilution, 1µL was used to run the qPCR in a total reaction volume of 10µL. For every experiment, a standard curve of reference cDNA using 1:5 dilutions were loaded and used for quantification. *CFTR* mRNA was normalized to *GAPDH* expression using the delta-delta Ct method (primers sequences are found in **Supplementary Table 1**). The qPCR reaction was performed in a Quantstudio Real-Time PCR Instrument (ThermoFisher).

2.5. Nanostring analysis

Nanostring PlexSet expression analysis as performed using reagents purchased from Nanostring Technologies, Inc. (catalog# NAA-AKIT-048) and a Probe Pool set generated by Integrated DNA Technologies (**Supplementary Table 2**). First hybridization of RNA samples to TagSets and probes was performed. Briefly, six aliquots of the TagSet were thawed at room temperature, mixed by inversion and collected at the bottom of the tube by brief centrifugation. To generate a Master Mix to the TagSet aliquots, 70μL hybridization buffer and 7μL Probe A working pool were added, mixed and briefly centrifuged. Next, 7μL of Probe B working pool was added to each Master Mix tube, mixed by inversion and collected at the bottom of the tube by brief centrifugation. Next, 8μL of Master Mix and 7μL of RNA sample were added to hybridization strip tubes. Hybridization tubes were then capped, mixed by inversion and samples collected at the bottom of the tube by brief centrifugation. Samples were hybridized at 67 °C for 16 h in a thermocycler and then held at 4 °C until proceeding with Nanostring analysis. Hybridized samples were loaded into prepared Nanostring cartridges, 12 samples per cartridge, and reagents loaded into the Analyzer instrument. Analyzer was run at maximum sensitivity for the data collection cycle using nCounter Software (Version 4.0). Runs were checked for technical errors using spike in internal control counts and image quality. Raw counts of each probe's barcode were assessed and normalized to the geometric mean of the reference probes to account for potential differences in RNA loading. To assess *CFTR* mRNA stability, the normalized values for 3′-most *CFTR* probe (D) was divided by the normalized value for the 5′-most *CFTR* probe (A).

2.6. Statistical analysis

GraphPad Prism 8 software was used for statistical analysis. All data were included for purposes of statistical analysis, including outliers. FIS assay data were tested by ordinary one-way ANOVA with post-hoc Tukey's or Sidak's multiple comparison testing across all subgroups. Testing was performed on transformed data due to unequal variances across groups. qPCR data were evaluated by Student's unpaired *t*-test.

3. Results

3.1. ELX-02-mediated read-through induces human CFTR functional restoration in homozygous G542X PDO

Initially, a homozygous *G542X* PDO was incubated with 0–160μM ELX-02 (free base equivalents) for 48 h, followed by treatment with 5μM forskolin to activate CFTR. Vehicle treated, forskolin-induced organoids demonstrate no swelling, consistent with a complete absence of functional CFTR protein. A dose-dependent increase in swelling was observed over a 120 min pe-

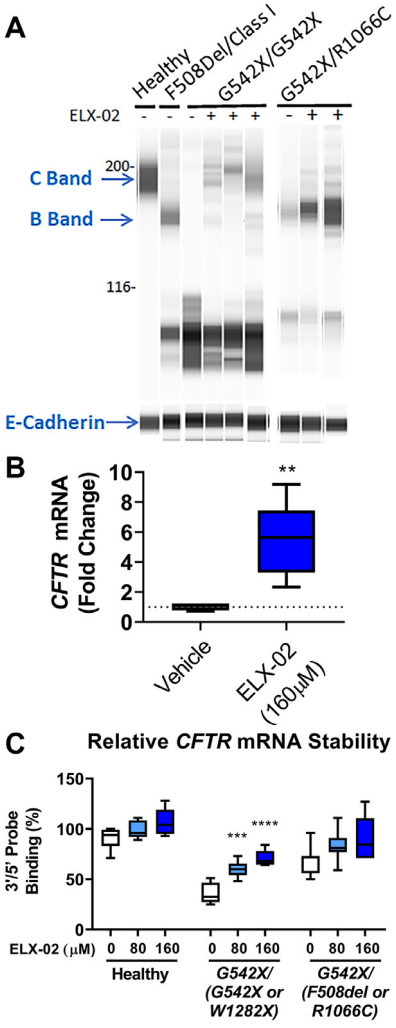

Fig. 3. Effect of ELX-02 on levels of endogenous CFTR protein and transcripts. (**A.**) Wes Protein Simple analysis of CFTR protein in *G542X* PDOs treated with vehicle (-) or 160 µM ELX-02 (+). Healthy and *F508del*/Class I control demonstrate expression of C band CFTR (Lane 1) and B band CFTR (Lane 2) respectively. Analysis included E-Cadherin as a housekeeping gene for normalization. (**B.**) ELX-02 (160 µM) treatment of *G542X/G542X* PDOs results in a 5-fold increase in *CFTR* mRNA compared with vehicle treated PDOs as measured using qRT-PCR (n = 5 PDOs per group). Data were normalized to vehicle treated PDOs. The increase in *CFTR* mRNA by ELX-02 is statistically significant (p = 0.0045; Student's *t*-test). (**C.**) ELX-02 (0, 80 or 160 µM) treatment results in normalization of *CFTR* mRNA as measured via Nanostring in n = 3 PDOs per treatment group. One-way ANOVA with Tukey's post-hoc analysis revealed a significant effect of ELX-02 (p<0.0001) in PDOs containing *G542X* and another nonsense allele (either *G542X* or *W1282X*). *** p<0.0001 vs vehicle, **** p<0.0001 vs vehicle. One-way ANOVA was not significant for ELX-02 effect in either healthy PDOs (p = 0.1097) or PDOs containing *G542X* and either *F508del* or *R1066C* (p = 0.1215).

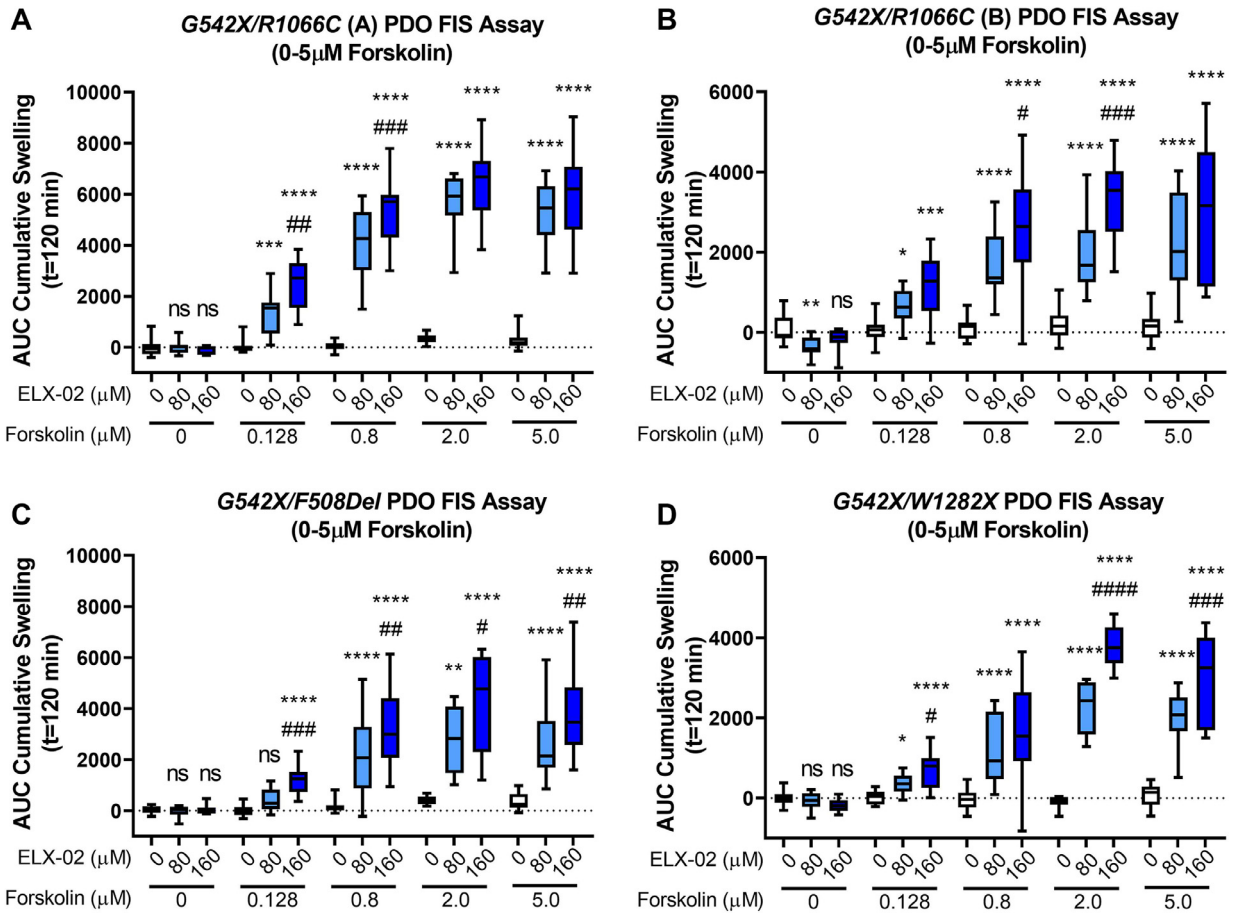

Fig. 4. ELX-02 increases CFTR-dependent swelling in PDOs bearing a *G542X* allele. PDOs derived from different *G542X*-allele bearing patients were evaluated for responsiveness to either 0, 80, or 160 μM ELX-02 using 0–5 μM forskolin induction to initiate swelling. **(A. & B.)** Significantly increased swelling was observed in *G542X* heterozygous PDOs that also have a Class II *R1066C* missense mutation. **(C.)** *G542X/F508del* heterozygous PDOs and **(D.)** *G542X/W1282X* complex nonsense heterozygous PDOs also significantly responded to ELX-02. Data were aggregated across 3 or more independent experiments from n = 9–30 wells of PDOs. One-way ANOVA with Tukey's post-hoc analysis revealed that all data sets with forskolin present reached significance of $p<0.0001$; ns, non-significant, $^{*}p<0.05$ vs vehicle control, ** $p<0.005$ vs vehicle, $^{***}p<0.001$ vs vehicle control, **** $p<0.0001$ vs vehicle control, # $p<0.05$ vs next lower concentration, ## $p<0.01$ vs next lower concentration, ### $p<0.001$ vs next lower concentration, #### $p<0.0001$ vs next lower concentration.

riod across all ELX-02 concentrations (Fig. 1**A,B**). These data, quantified and expressed as an AUC, demonstrate significant increases in swelling at 40, 80, and 160μM ELX-02 (Fig. 1**C**). We next investigated the Forskolin induction dose-dependency in a homozygous *G542X* PDO, recognizing that the Forskolin concentration required for induction could be dependent on the amount of CFTR protein present (Fig. 1**D**). ELX-02 mediated activity was observed at the lowest Forskolin concentration evaluated, 0.128 μM and demonstrated the anticipated dose-dependent effect of Forskolin induction. As anticipated, 1:1 (3 μM each) treatment of VX-770 and VX-809 was not observed to impact the homozygous *G542X* PDO (Fig. 1**E**). Orkambi®, a product approved for homozygous *F508del* patients, is a combination of a CFTR potentiator (VX-770) and a corrector (VX-809) and is not expected to show efficacy against nonsense mutations. ELX-02 potentiated *G542X* PDO swelling is abrogated with co-administration of CFTR channel inhibitors, supporting that the swelling observed is dependent upon CFTR channel function.

Based on the mechanism of action, we hypothesized that the ELX-02 mediated response in the FIS assay was dependent upon the presence of a nonsense allele in *CFTR*. To confirm, a *F508del* homozygous PDO, lacking nonsense mutations, was assessed. Symdeko®, a product approved for the treatment of patients with CF having the *F508del* allele, is a combination of a CFTR potentiator (VX-770) and a corrector (VX-661). Here, these compounds were evaluated individually and in combination at a ratio of 1:1 (Fig. 2**A**). Using 5μM forskolin, the VX-770 and VX-661 combination produced significant increases in swelling, while ELX-02 treatment groups were indistinguishable from vehicle and PDOs treated with VX-770 or VX-661 produced a minimal response. Consistent with previous studies [8], VX-770:VX-661 treated *F508del* PDOs reach swelling plateau at 60-minutes while results with ELX-02 in homozygous *G542X* PDOs demonstrate a swelling plateau at approximately 90-minutes (Fig. 2**B**). These differences may be attributable to the channel potentiating activity of VX-770 altering channel kinetics. Considering this, we evaluated both 60- and 120-minute AUCs (Fig. 2**C,D**) finding the VX-661/VX-770 homozygous *F508del* response to be significantly greater (p = 0.032) than the non-potentiated ELX-02 response in homozygous *G542X* PDOs when comparing 60-minute AUC data and equivalent when comparing 120-minute AUCs.

3.2. ELX-02-mediated impact on CFTR protein and mRNA in G542X *PDO*

We next evaluated endogenous CFTR protein expression in *G542X* PDOs, treated with either vehicle or ELX-02, using a size exclusion chromatography approach to overcome the sample input limitations inherent in 3D culture conditions. Upregulation of both CFTR B- and C-Bands was observed to varying degrees in five *G542X* bearing, ELX-02-treated PDO samples but not in vehicle treated samples, supporting an efficacious functional response

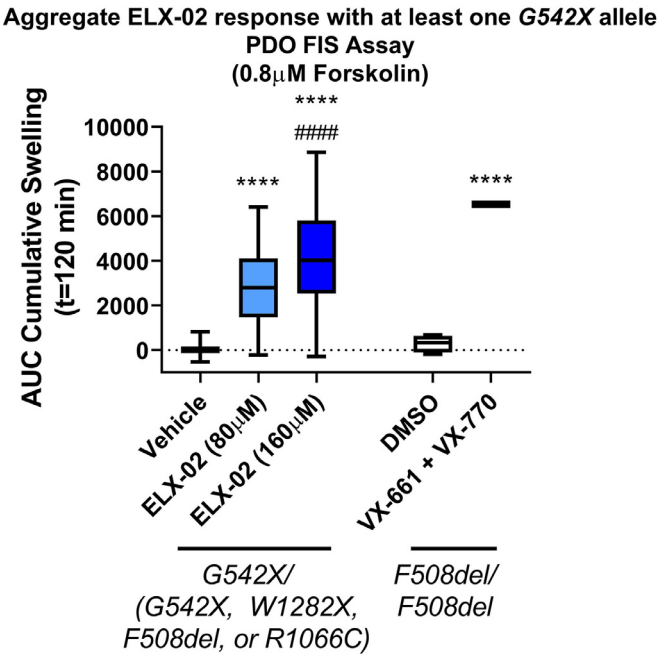

Fig. 5. Aggregate ELX-02 response across PDOs with at least one *G542X* allele. Data represent five independent PDOs evaluated over multiple independent experiments using 0.8 μM forskolin induction (Vehicle $n = 112$, 80 μM $n = 93$, 160 μM $n = 126$). One-way ANOVA with Sidak's post-hoc analysis revealed that all data sets with forskolin present reached significance of $p<0.0001$, **** $p<0.0001$ vs vehicle (water or DMSO) control, #### $p<0.0001$ vs next lower concentration.

(Fig. 3**A**). Levels of total CFTR in the treated PDOs ranged from 1.5–5.2% of the expression found in a *CFTR* wild-type PDO. These results are consistent with previously reported results in the FRT model [6].

To evaluate the impact of ELX-02 on *G542X CFTR* allele mRNA, we evaluated compound treated PDOs by qPCR. This evaluation demonstrated a significant 5-fold increase in steady-state *G542X* mRNA in ELX-02 treated PDOs (Fig. 3**B**). Next, using Nanostring we evaluated the relative integrity of the *CFTR* mRNA pool by tiling 100 base pair probes across the transcript. Transcripts targeted by nonsense-mediated mRNA decay may undergo degradation by XRN1 mediated decapping in a 5′ to 3′ fashion or via deadenylation in a 3′ to 5′ fashion [13]. The *CFTR* transcript has a long 3′ UTR and is subject to microRNA mediated mRNA deadenylation, which has been observed for *F508del CFTR* transcripts in human bronchial epithelia cells [14, 15]. In healthy control PDOs, 5′ and 3′ probe binding of target transcripts was observed in equal abundance. However, *G542X* homozygous PDOs 3′ probe detection was reduced to 47% of that detected by the 5′ probes (Fig. 3**C**). Monitoring the ratio of 3′/5′ binding by pooling *G542X* PDOs with a second nonsense allele (either a second *G542X* allele or a *W1282X* allele) demonstrated a significant dose-dependent increase in 3′ probe detection with 48 h ELX-02 incubation. A trend was also observed in *G542X* heterozygous PDOs (with a second, non-nonsense, disease-causative allele). However, the degree to which 3′ probe binding was reduced in these PDOs was less than that observed for PDOs with two nonsense alleles suggesting that the degree of stabilization is dependent on the nonsense-bearing transcript pool size.

3.3. Meaningful ELX-02-mediated read-through in G542X/*minimal function alleles PDOs*

To understand the impact of genotype heterogeneity on ELX-02 read-through, *G542X* complex heterozygous PDOs were evaluated. PDOs with a second allele with minimal function (including Class 1, Class 2) demonstrated a consistent dose-dependent ELX-02 response (Fig. 4). The degree to which each PDO responds is variable, even in an instance of PDOs having the same CFTR genotype (Fig. 4A and B). This may relate to differences in basal *CFTR* expression or differences in monoallelic gene expression selection. The data reported are AUC values from multiple experiments, supporting the reproducibility across PDOs and across independent experiments. When these data are combined and compared, the response of *G542X* homozygous and *G542X* heterozygous PDOs with a minimal function allele support that the level of function achievable by read-through are equivalent to that achieved by the 1:1 VX-770 / VX-661, potentiator/corrector, treatment of a homozygous *F508del* PDO (Fig. 5), similar to what was shown in Fig. 2B.

4. Discussion

Here we demonstrated ELX-02 mediated *CFTR* mRNA increase, restoration of CFTR protein and CFTR-dependent organoid swelling in PDOs bearing the most common CF nonsense allele, *G542X*. To our knowledge this is the first time that production of functional CFTR protein by a read-through agent suitable for human use was demonstrated in this model system. These data with ELX-02 in PDOs are consistent with previous *G542X* model evaluations including CFTR activity restoration in human bronchial epithelial cells, FRT cells and transgenic mice and support the continued clinical development of ELX-02 for *G542X* mediated CF [6].

Clinical studies evaluating gentamicin, a relatively inefficient aminoglycoside antibiotic read-through agent, delivered via nasal drops or systemic, demonstrated that the read-through mechanism is sufficient to correct CFTR electrophysiology [16–18]. Subsequently, clinical trials with another read-through agent, PTC124 (ataluren), failed to demonstrate efficacy in the primary pulmonary endpoints [19]. Preclinical evaluation of both gentamicin and PTC124 each demonstrated efficacy using a transgenic *G542X* mouse model supporting further pursuit of alternative model systems to evaluate read-through of these alleles [20, 21]. Patient derived models, such as human bronchial epithelial cells and PDOs, provide the opportunity to model the diverse genotypic heterogeneity in this population under native expression conditions. Furthermore, PDOs have demonstrated translational potential by showing positive responses with approved modulators and a lack of activity with PTC124 [8, 9, 22]. With this background in mind, demonstration of ELX-02-mediated *G542X* read-through at concentrations achievable in a clinical setting across a variety of genotypes is a pivotal step in advancing toward clinical assessment.

Evaluation of the translational value of PDOs has advanced considerably over recent years, supporting the FIS assay as an effective means to evaluate CFTR activity. Induced PDO swelling is dependent upon CFTR activity and correlates to disease-relevant measures such as FEV1 and BMI [7, 10]. Moreover, retrospective analyses have evaluated the impact of approved modulators on FIS of PDOs and found it correlates with clinical effect of modulators on sweat chloride levels and pulmonary function [8, 9]. From the practical perspective, biorepository access to a wide range of nonsense genotypes, improved experimental reproducibility and increased signal to noise ratios for cells with low residual CFTR activity has helped address some of the challenges related to direct measure of CFTR activity across a representative sample of CF genotypes in human bronchial epithelial cells. Considering this, the comparison reported here to *F508del* homozygous PDO responses to approved modulators is limited in scope and may not reflect the intra-patient variability in response to VX-661/VX-770. Expanded evaluation of additional PDOs and correlation to clinical responses will provide a platform to interpret potential clinical impact of FIS responses across genotypes and therapeutic approaches. At the

protein level, increased signal of 1.5–5.2% of wild-type was observed. As wild-type levels of CFTR protein may vary and a single wild-type PDO was evaluated, this observation should be considered qualitative until a wider panel is assessed [23]. This degree of restoration should be put into perspective for the minimal starting level of CFTR functional protein inherent in this genotype class and the reported disproportionate clinical impact observed for more modest gains in protein for patients that have severe disease due to a logarithmic relationship between function and clinical features [11].

From the current study it is unclear if, at the individual channel level, the *G542X* read-through products produced as a result of ELX-02 read-through demonstrate function equivalent to each other or native protein. *G542X* read-through products may incorporate cysteine, tryptophan or arginine in place of glycine [24] and single channel recordings demonstrated that an arginine at this site increases channel open probability relative to wild-type [25]. While heterogeneity in *G542X* read-through product is likely at the single cell level, data described here demonstrate the net impact is a CFTR functional response across the evaluated *G542X* PDOs. Future studies are required in order to evaluate the impact of ELX-02 read-through across CF nonsense alleles, to explore how different amino acid substitutions impact CFTR function, and to determine if the relative proportion of these different substitutions demonstrates cell-to-cell variability. Ultimately, measures of CFTR function serves as a practical measure to determine utility of a read-through agent across different nonsense alleles.

In conclusion, ELX-02 produces significant read-through of the *G542X* allele, leading to increased mRNA protein and protein function across multiple *G542X* PDOs. These data support the ongoing clinical development program of ELX-02 for individuals with *G542X* mediated CF.

Credit author statement

All authors: conception and design of experiments, revisions and final approval. J.M., J.P.: data collection. D.K.C., S.L., M.M.G.: data analysis and interpretation. D.K.C. and M.M.G. drafting manuscript.

Declaration of Competing Interest

D.K.C. and M.M.G. are employees of Eloxx Pharmaceuticals, Inc.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2021.01.009.

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