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Early View

Research letter

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The Cystic Fibrosis Airway Milieu Enhances Rescue of F508del in a Pre-Clinical Model

Martina Gentzsch[1,2], Deborah M. Cholon[1], Nancy L. Quinney[1], Susan E. Boyles[1], Mary E. B. Martino[1], and Carla M. P. Ribeiro[1,2,3]
[1]Marsico Lung Institute and Cystic Fibrosis Research Center, [2]Department of Cell Biology and Physiology, [3]Department of Medicine, The University of North Carolina, Chapel Hill, North Carolina, USA

To the Editor:

Cystic Fibrosis (CF) is a life-shortening genetic disease with autosomal recessive inheritance. Most CF morbidity and mortality is associated with pulmonary disease. The cystic fibrosis transmembrane conductance regulator (CFTR) gene, which codes for a chloride channel required for proper hydration of airway epithelial surfaces, is mutated in CF, resulting in airway dehydration. As a result, CF patients suffer from chronic airway infection, inflammation, and overproduction of mucus, which leads to airway obstruction. During the last decade, basic and clinical research has led to strategies for targeted therapies to successfully restore CFTR function in CF patients.

The most common mutated protein in CF, F508del cystic fibrosis transmembrane conductance regulator (CFTR), is retained in the endoplasmic reticulum (ER), which prevents significant amounts of the protein to reach the apical surface of epithelial cells. Because efficient correction of F508del CFTR should benefit most CF patients, it has a high priority in CF therapy. In 2015 the FDA approved Orkambi, consisting of the corrector lumacaftor (VX-809) plus the potentiator ivacaftor (VX-770), for F508del homozygous patients. Phase 2 and 3 clinical trials of Orkambi for these CF patients demonstrated only modest improvements in lung function [1, 2] similar in magnitude to conventional therapies for downstream pathophysiology, e.g., dornase alfa, hypertonic saline, or azithromycin [3]. Comparable outcomes were obtained with the new drug, Symdeko, consisting of the corrector tezacaftor (VX-661) in combination with VX-770 [4]. Hence, there is an unmet need to improve the *in vivo* effectiveness of CFTR-targeting treatments for F508del homozygous patients.

CFTR correctors have not been investigated *in vitro* under the inflammatory conditions found in CF airways *in vivo*. Because inflammation of human bronchial epithelia (HBE) increases the ER protein folding capacity by up-regulating the expression of ER chaperone proteins, folding enzymes, and lipids [5-7], we hypothesized that inflammation of HBE would enhance the correcting effect of VX-809 and, thus, the therapeutic benefit of VX-809 and VX-770 *in vitro*.

To evaluate the action of VX-809 ± VX-770 under conditions that recapitulate the inflammatory status of CF airways, we utilized a pre-clinical model consisting of long-term (30-40 day old) cultured well-differentiated F508del/F508del HBE grown at air-liquid interface [6] and exposed to supernatant from mucopurulent material (SMM) harvested from the airways of excised human CF lungs [5-8]. SMM contains the soluble infectious and inflammatory factors present in CF airways *in vivo* [8], such as bacterial products, neutrophil factors, macrophage and epithelial cytokines, mucins and hundreds of peptides. Its cytokine composition is fairly reproducible from patient to patient [8]. Because the airway epithelia of CF patients are exposed to the combination of all the factors present in SMM, as opposed to a single inflammatory factor, we reasoned that the use of SMM (versus the use of a defined stimulus) would be more relevant to evaluate the impact of the CF airway inflammatory milieu on the efficacy of CFTR modulators. In this study, SMM was pooled from 8 individual human CF lungs.

We utilized a protocol that combines 24 h treatment with VX-809 and acute treatment with VX-770 to provide conditions that maximally enhance CFTR function. This protocol is currently most commonly used in studies on HBE cells conducted by research groups in industry and academia. Well-differentiated F508del/F508del HBE cultures were exposed for 24 h to apically added PBS or SMM [5-8] in combination with basolateral exposure to vehicle or 5 μM VX- 809. Notably, long-term CF cultures do not exhibit a hyperinflammatory phenotype in the absence of SMM [6]. Subsequently, analyses of CFTR were performed based on a) functional ion transport studies utilizing an Ussing chamber protocol that allows sensitive measurements of endogenous F508del activity, which included acute addition of forskolin, VX-770 and CFTR inhibitor ($CFTR_{inh}$-172), b) biochemical evaluation of

protein maturation by Western blots [9-11], and c) evaluation of CFTR mRNA levels by quantitative reverse transcriptase polymerase chain reaction (RT-PCR), utilizing our established method [8].

Figure 1A illustrates that VX-809-treated F508del/F508del HBE cultures exhibited a larger CFTR-mediated response to forskolin and VX-770 vs. vehicle-treated cultures. Surprisingly, these responses were drastically enhanced by the SMM treatment (**Figure 1A**). VX-809-increased forskolin + VX-770-induced Cl^- secretory responses were enhanced by SMM exposure in a dose-dependent manner (data not shown). Notably, SMM-enhanced VX-809-increased forskolin + VX-770 responses were blunted by $CFTR_{inh}$-172 (Figure 1A). The compiled data for the quantification of forskolin + VX-770-induced F508del-mediated responses and inhibition by $CFTR_{inh}$-172 are shown in **Figure 1B**.

To address whether SMM and/or VX-809 treatment affected additional bioelectric responses beyond those mediated by CFTR, we evaluated the baseline currents, amiloride responses, residual/amiloride-insensitive currents, and UTP responses in all groups. The compiled data from these analyses are depicted in **Figure 1C**. Although baseline short-circuit currents (I_{sc}) were not significantly different in any group, there was a trend for basal currents to be higher in SMM-treated cultures. In addition, there was a trend for amiloride responses to be reduced in the VX-809-treated groups, presumably due to restoration of CFTR function and subsequent inhibition of activity of the epithelial sodium channel, ENaC. Residual currents were slightly enhanced in the SMM-treated groups, which may be due to increased baseline function of CFTR (prior to forskolin activation) and/or activation of additional channels, e.g., Ca^{2+}-activated Cl^- channels (CaCC). Indeed, as previously described [8], UTP responses were enhanced in SMM-treated cultures, possibly reflecting an up-regulation of CaCC function. Moreover, we cannot rule out direct or indirect contributions of other channels that may be affected by SMM. Nevertheless, because rescued CFTR responses were the predominant currents affected by SMM and largely inhibited by $CFTR_{inh}$-172, our data indicate the existence of a high selectivity and specificity for the action of SMM on repaired F508del vs. other channels.

In agreement with the functional data, Western blot analyses indicated that mainly the immature ER-residing form of CFTR (band B) was detected in the absence of VX-809 (**Figure 1D**). Notably, while band B was not significantly increased in cultures treated either with SMM or VX-809, it was considerably increased in cultures simultaneously treated with SMM and VX-809 (**Figures 1D and E**, SMM/VX-809). On the other hand, while the mature band C was formed in F508del cultures treated only with VX-809 (PBS/VX-809), the SMM treatment further increased the amount of VX-809-rescued mature band C (**Figures 1D and F**, SMM/VX-809). Utilizing the same experimental conditions, we found that SMM exposure did not significantly increase the mRNA levels of CFTR in the presence or absence of VX-809 (**Figure 1G**). The findings from **Figures 1D, 1E and 1G** suggest that SMM does not significantly increase transcription or translation of F508del. Rather, our data lead to the notion that the mechanism for SMM-enhanced CFTR levels in the presence of VX-809 may be mediated by SMM-induced F508del stabilization, resulting in decreased CFTR protein degradation. Indeed, preliminary data from our laboratories suggest that SMM overcomes destabilization of VX-809-rescued F508del by chronic VX-770 treatment *in vitro* (data not shown).

Contrary to our findings, previous studies suggested that *Pseudomonas aeruginosa* reduces VX-809- and VX-809 + VX-770-stimulated F508del CFTR-mediated Cl^- secretion [12]. However, there are possible explanations for this discrepancy. First, because these studies were conducted 6 h after *Pseudomonas aeruginosa* exposure, we speculate that the time of bacterial exposure might not have been sufficient to expand the ER compartment and increase its protein folding capacity, as we have reported [5], to enhance VX-809-mediated F508del rescue. Second, because *Pseudomonas aeruginosa* constitutes a single stimulus that does not recapitulate all the infectious/inflammatory factors present in CF airways, unlike SMM, it does not likely trigger activation of many signaling pathways necessary to efficiently promote ER expansion in HBE *in vitro*.

Our findings provide a new model for pre-clinical evaluation of CFTR modulators. There are very little data regarding the impact of inflammation on the efficacy of CFTR modulators in human subjects. However, because increased inflammation occurs very early in CF lung disease, the airway inflammation present even in mild disease may be sufficient to trigger the effects noted in this study. The dose-response relationship between CF airway inflammation and efficacy of CFTR modulators should be addressed in future studies. Comparisons between the airway inflammatory status of CF

patients with *in vivo* efficacy of Orkambi or Symdeko, and studies with cells from these patients exposed to their own airway secretions will address this relationship.

The acquired knowledge from our studies may also be applicable for rescuing rare CFTR mutations [13], offering pre-clinical models and precise treatment strategies for all CF patients. Furthermore, the proposed studies may be relevant to other airway diseases, i.e., COPD, where dysfunctional CFTR is emerging as a therapeutic target [14]. Additional studies will be necessary to identify the mechanism underlying the enhancement of VX-809-mediated F508del rescue during inflammation, which may shed light on the lack of benefit of VX-809 as monotherapy in clinical trials. We speculate that the initial enhancement of VX-809-mediated restoration of CFTR activity under inflammatory conditions is counteracted, with time, by its correcting effect (e.g., correction of CFTR function might decrease the airway epithelial inflammatory response, thereby decreasing VX-809 correction). In fact, recent data indicating that restoration of functional CFTR reduces inflammation in CF airways [15] agree with this notion. Notably, the next generation of correctors may have a more significant impact on decreasing airway inflammatory responses of CF patients.

In conclusion, our study indicates that the CF airway infectious/inflammatory milieu has a major impact on the efficacy of CFTR modulators. As more CFTR modulator combinations become available (see https://www.cff.org/Trials/Pipeline), pre-clinical evaluation of the efficacy of modulators under conditions that mimic native inflamed CF airway epithelia may be critical for optimization of CF therapies.

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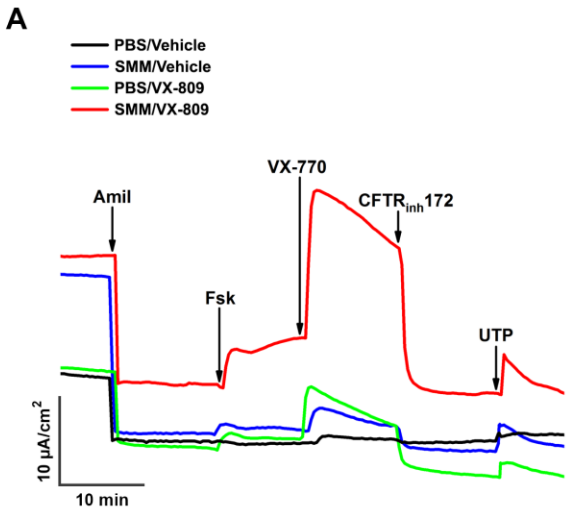
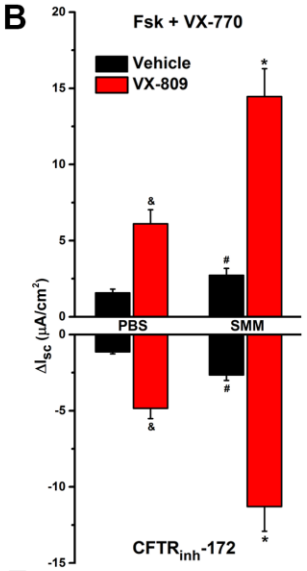
Figure Legend

Figure 1. SMM exposure enhances functional rescue of F508del. F508del/F508del HBE cultures were exposed for 24 h to vehicle or 5 µM VX- 809 in combination with 30 µl mucosal PBS or SMM. **A:** Representative short-circuit currents (I_{sc}, µA/cm^2) recorded in Ussing chambers. **B:** Quantification of F508del-mediated responses to forskolin (Fsk, 10 µM) + VX-770 (1 µM) and $CFTR_{inh}$-172 (10 µM). Data are expressed as mean ± SEM. $^{\&}p<0.05$ VX-809 vs. Vehicle; $^{\#}p<0.05$, SMM vs. PBS; *p<0.05, SMM/VX-809 vs. PBS/VX-809. N = 4 F508del/F508del HBE culture codes, and experiments were performed in 3-4 replicates/code in 14 measurements. **C:** Compiled data from baseline, amiloride-sensitive, residual (amiloride-insensitive) and UTP-dependent Isc responses. **D:** Representative Western blot of immunoprecipitated CFTR and tubulin controls. **E:** Quantification of band B as % values normalized from band B values from control (vehicle- and PBS-treated CF HBE). Data are expressed as mean ± SEM. *p<0.05, SMM+VX-809 vs. VX-809. N = 4 F508del/F508del HBE culture codes. **F:** Quantification of band C as % values normalized from band C values from control (vehicle- and PBS-treated CF HBE). Data are expressed as mean ± SEM. *p<0.05, SMM+VX-809 vs. VX-809. N = 4 F508del/F508del HBE culture codes. **G:** CFTR mRNA levels, determined by RT-PCR. Data are expressed as fold change relative to TATA-box binding protein mRNA. N = 3 F508del/F508del HBE culture codes.





C

Baseline Isc	(μA/cm^2)
PBS/Vehicle	23.23±3.87
SMM/Vehicle	30.90±2.64
PBS/VX-809	20.68±2.86
SMM/VX-809	24.60±2.00
Amiloride	**(μA/cm^2)**
PBS/Vehicle	-22.41±4.49
SMM/Vehicle	-26.77±2.34
PBS/VX-809	-18.23±3.60
SMM/VX-809	-20.70±2.09
Residual Isc	**(μA/cm^2)**
PBS/Vehicle	0.84±0.76
SMM/Vehicle	4.18±1.37
PBS/VX-809	2.46±1.23
SMM/VX-809	3.91±1.24
UTP	**(μA/cm^2)**
PBS/Vehicle	0.85±0.22
SMM/Vehicle	3.21±0.57
PBS/VX-809	1.02±0.45
SMM/VX-809	2.43±0.45