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Potentiator Ivacaftor Abrogates Pharmacological Correction of ΔF508 CFTR in Cystic Fibrosis

Deborah M. Cholon[1], **Nancy L. Quinney**[1], **M. Leslie Fulcher**[1], **Charles R. Esther Jr**[1,2], **Jhuma Das**[3], **Nikolay V. Dokholyan**[3], **Scott H. Randell**[1,4,5], **Richard C. Boucher**[1,4], and **Martina Gentzsch**[1,5,*]

[1]Marsico Lung Institute/Cystic Fibrosis Research Center, University of North Carolina, Chapel Hill, NC 27599, USA.

[2]Department of Pediatrics, Pediatric Pulmonology, University of North Carolina, Chapel Hill, NC 27599, USA.

[3]Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599, USA.

[4]Department of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA.

[5]Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, NC 27599, USA.

Abstract

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[*] To whom correspondence should be addressed: gentzsch@med.unc.edu.

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Author contributions: DMC, NLQ, and MG performed the experiments and analyzed the data. MLF selected and cultured the primary CF and NL HBE cells. MGG, SHR and RCB discussed the overall design of experiments. MGG and DMC wrote the manuscript. CRE measured drug concentrations. JD and NVD determined ΔΔG of the G551D protein. SHR and RCB provided reagents, technical advice and critical evaluation of the manuscript. All authors read and commented on the manuscript.

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Cystic Fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (*CFTR*). Newly developed “correctors” such as lumacaftor (VX-809) that improve CFTR maturation and trafficking and “potentiators” such as ivacaftor (VX-770) that enhance channel activity may provide important advances in CF therapy. Although VX-770 has demonstrated substantial clinical efficacy in the small subset of patients with a mutation (*G551D*) that affects only channel activity, a single compound is not sufficient to treat patients with the more common *CFTR* mutation, *ΔF508*. Thus, patients with *ΔF508* will likely require treatment with both correctors and potentiators to achieve clinical benefit. However, whereas the effectiveness of acute treatment with this drug combination has been demonstrated *in vitro*, the impact of chronic therapy has not been established. In studies of human primary airway epithelial cells, we found that both acute and chronic treatment with VX-770 improved CFTR function in cells with the *G551D* mutation, consistent with clinical studies. In contrast, chronic VX-770 administration caused a dose-dependent reversal of VX-809-mediated CFTR correction in *ΔF508* homozygous cultures. This result reflected the destabilization of corrected ΔF508 CFTR by VX-770, dramatically increasing its turnover rate. Chronic VX-770 treatment also reduced mature wild-type CFTR levels and function. These findings demonstrate that chronic treatment with CFTR potentiators and correctors may have unexpected effects that cannot be predicted from short-term studies. Combining of these drugs to maximize rescue of ΔF508 CFTR may require changes in dosing and/or development of new potentiator compounds that do not interfere with CFTR stability.

Introduction

The most common autosomal recessive genetic disease of the Caucasian population in the United States and Europe, cystic fibrosis (CF), is characterized by abnormal epithelial ion transport. Mutations in the CF transmembrane conductance regulator (*CFTR*) result in loss of CFTR-mediated Cl^- and HCO_3^- transport by secretory and absorptive epithelial cells in multiple organs, including lungs, pancreas, liver, and intestine. In the lung, disturbances of airway surface liquid homeostasis produce thick and viscous mucus that leads to mucus stasis, airway obstruction, persistent infection, inflammation, and a progressive decline in lung function. These features are the hallmarks of CF lung disease and result in limited life expectancy (1–3).

In 1989, the identification of the *CFTR* gene on chromosome 7 and its most common mutation, *ΔF508 CFTR*, raised hope for a cure that would address the underlying cause of CF (4–6). Intense high-throughput screening approaches over the last decade have yielded compounds that modulate mutant CFTR function (7–16). Small-molecule compounds that rescue mutant CFTR can be assigned to 2 groups: 1) “corrector” compounds that promote maturation and delivery of CFTR proteins to the apical surface and 2) “potentiator” compounds that activate apical CFTR by increasing the open time of the channel.

The FDA recently approved the CFTR potentiator compound VX-770 (ivacaftor; trade name Kalydeco) as the first drug that directly restores CFTR activity in CF patients who carry a *G551D* mutation (17–20). G551D CFTR reaches the plasma membrane of epithelial cells, but the protein exhibits a gating defect that abolishes ATP-dependent channel opening and causes severe CF. In patients carrying a *G551D* mutation, VX-770 has proven to be effective in clinical trials (18, 19), in which treated patients exhibited marked improvements in sweat chloride values and pulmonary function. The development of a CFTR-targeted drug that benefits CF patients marked a breakthrough in the treatment of CF. Unfortunately, because less than 5% of the CF population have the *G551D* mutation, this specific therapy helps only a limited number of patients (21, 22). 90% of CF patients carry the Δ*F508* mutation, which produces a protein that does not mature normally and does not traffic to the plasma membrane. VX-770 treatment did not benefit CF subjects with the Δ*F508* mutation (23), likely because this compound only acts on protein that has trafficked to the plasma membrane. Based on these findings, an attractive therapeutic strategy for the Δ*F508* CF patient population is to promote transfer of the ER-retained ΔF508 CFTR protein to the plasma membrane using small-molecule corrector compounds (24–26). Studies have estimated that the extent of correction in Δ*F508* airway epithelial cells must approximate 10–25% of wild-type (WT) CFTR function to provide therapeutic benefit (27, 28). *In vitro* treatment of CF airway epithelial cultures homozygous for the Δ*F508* mutation with the most promising corrector compound, VX-809 (lumacaftor), resulted in CFTR function of ~14% relative to non-CF ("wild-type") human airway epithelial cells (8). However, administration of VX-809 did not provide a significant therapeutic benefit for Δ*F508* CF patients in recent clinical trials, most likely because ΔF508 CFTR correction *in vivo* was less than 10% of wild-type levels, the lower limit of detection, and thus no mature ΔF508 CFTR protein was observed (29). Therefore, a logical next step was to combine corrector and potentiator therapies to rescue ΔF508 and increase protein function (24, 30, 31). One of the most promising current clinical trials designed to optimize ΔF508 CFTR function involved the administration of the corrector VX-809 with the potentiator VX-770. Increases in VX-809-rescued ΔF508 CFTR function have been demonstrated after acute administration of VX-770 in primary human airway epithelial cells from CF patients (8) and human organoids derived from CF (Δ*F508*/Δ*F508*) intestinal tissue (32). Surprisingly, chronic co-administration of VX-809 and VX-770 in Phase 2 and 3 studies produced only small improvements in lung function in CF patients homozygous for the Δ*F508 CFTR* mutation (31, 33).

The aim of this study was to elucidate the molecular mechanism(s) underlying the limited improvement in ΔF508 CFTR function when a corrector, VX-809 and a potentiator, VX-770 were co-administered to CF patients. We therefore investigated whether there were unexpected effects of chronically exposing CF cultures *in vitro* to VX-809 and VX-770, as would be achieved by oral dosing in clinical trials. A combination of CFTR bioelectric and biochemical approaches were utilized to investigate this interaction. Human bronchial epithelial (HBE) cells were used for these studies and exposed for 48 hrs to clinically relevant concentrations of both compounds. In addition, because of the success of VX-770 in CF patients with the *G551D* mutation, it has recently been suggested that treatment with VX-770 may be a pharmacological approach to enhance CFTR function in patients with chronic obstructive pulmonary disease (COPD) (34). Accordingly, similar experimental approaches were utilized to explore the effects of VX-770 on WT CFTR, which matures normally and traffics to the plasma membrane.

Results

Acute and chronic VX-770 treatments rescue G551D CFTR function

It has been recently demonstrated that acute VX-770 administration increased CFTR function in cell lines expressing G551D CFTR and augmented Cl^- secretion in primary human bronchial epithelial (HBE) cells derived from CF patients with the *G551D* mutation on one allele and the *ΔF508* mutation on the other allele (7). To compare the effects of chronic versus acute VX-770 drug administration in CF airway epithelia with a *G551D* mutation, we used well-differentiated primary CF HBE cultures (*G551D/ΔF508*) as a model. Cultures were treated chronically for 48 hrs with VX-770 or vehicle in the basolateral medium, and then transepithelial short-circuit currents (I_{SC}) were measured in Ussing chambers (Fig. 1). Cultures were exposed to amiloride to inhibit the epithelial Na^+ channel (ENaC) and subsequently forskolin to stimulate Cl^- secretion by CFTR. As previously reported, acute administration of VX-770 (aVX770) raised Cl^- secretion after forskolin administration (Fig. 1A). Chronic VX-770 (cVX770) administration raised forskolin responsiveness but eliminated subsequent responses to acute VX-770 administration (Fig. 1A,B,C, Table S1). Cultures chronically treated with VX-770 exhibited total CFTR-mediated responses (Fig. 1D) and inhibition with $CFTR_{inh}$-172 (Fig. 1E) equal to cultures treated with forskolin and acute VX-770.

Chronically VX-770-treated *G551D/ΔF508* HBE cultures also exhibited a decrease in amiloride-sensitive currents (Fig. S1A, Table S2), suggesting decreased ENaC function. This finding is consistent with restoration of CFTR-mediated ENaC inhibitory activity (35, 36) because cleavage of ENaC was diminished in chronically VX-770-treated CF cultures (Fig. S1B). The average UTP responsiveness, an index of Ca^{2+} activated Cl^- channel (CaCC) activity, was reduced with chronic as compared to acute VX-770 administration (Fig. S1C, Table S2).

In sum, these results demonstrate that both acute and chronic treatment with VX-770 improved CFTR function in HBE cells with the *G551D* mutation, consistent with clinical studies.

Chronic VX-770 treatment inhibits functional rescue of ΔF508 CFTR

CF patients harboring the *ΔF508 CFTR* mutation, which produces protein maturation and trafficking defects, have little/no CFTR at the cell surface. Consequently, treatment with a corrector compound, such as VX-809, is required for VX-770 to potentiate surface-localized ΔF508 CFTR. To mimic clinical administration of VX-809 and VX-770 as a combination therapy for the *ΔF508* CF patients, primary CF HBE cultures (*ΔF508/ΔF508*) were treated with both pharmacological agents for 48 hrs and then Ussing chamber experiments were performed to measure ΔF508 CFTR function (Fig. 2, Table S3).

Anion efflux across the apical epithelial membrane of airway epithelia in response to cAMP is mediated by CFTR and was not substantial in vehicle-treated *ΔF508/ΔF508* CF HBE cultures Fig. 2A,B; Vehicle). However, VX-809 administration produced ΔF508 CFTR correction as evidenced by stimulation of Cl^- secretion (Fig. 2). Specifically, correction by VX-809 produced responses to forskolin (Fig. 2A, B; VX809) that were enhanced by acute administration of VX-770 (Fig. 2A,C). These data indicate that acutely applied VX-770 further activated ("potentiated") VX-809-rescued ΔF508 CFTR. However, the CFTR-mediated I_{SC} increase after addition of VX-770 to corrector-rescued ΔF508 CFTR was transient. The I_{SC} decrease over time may be indicative of a rapidly decreasing quantity of functional protein at the apical membrane.

In contrast, rescue of ΔF508 CFTR function was dramatically decreased in cultures that had been chronically treated with VX-809 and VX-770 compared to VX-809 alone (Fig. 2A: VX809 vs. VX809+VX770). This loss of "corrected" function was reflected in reduced Cl^- secretion responses to forskolin (Fig. 2B) and reduced inhibition of stimulated CFTR Cl^- secretion with $CFTR_{inh}$-172 (Fig. 2D). Thus, these data contrast with the significant (P = 0.0177) acute VX-770 responses in VX-809-treated cultures (Fig. 2C). Again, we noticed that the response to UTP-stimulated I_{SC} decreased upon chronic VX-770 treatment (Figs. 2A and S1D).

We also tested the impact of chronic VX-770 treatment on ΔF508 correction in CF HBE cultures (*ΔF508/ΔF508)* by corrector compound VX-661. Similar to VX-809-treated CF cells, VX-661-corrected CF HBE cells showed a drastic reduction in forskolin-mediated CFTR function when VX-770 was chronically added (Fig. S2, Table S4).

Chronic VX-770 administration hinders correction by decreasing the stability of corrected ΔF508 CFTR

To explore the mechanism(s) mediating the VX-770-induced reduction of VX-809-corrected ΔF508 CFTR function, we used Western blotting techniques to analyze protein maturation and turnover. In normal HBE cells, we detected a mature, complex glycosylated form, band C (Fig. 3A, NL: *), with a substantially greater molecular weight than the immature band B (Fig. 3A, NL: ●). In contrast, only band B could be detected in vehicle-treated *ΔF508/ΔF508* CF HBE cells (Fig. 3A, CF). As previously reported, treatment with VX-809 alone resulted in formation of a modest amount of mature band C in CF HBE cultures, which was not present in vehicle- or VX-770-treated CF cells (Fig. 3A, CF). However, when CF cells were treated chronically with both VX-809 and VX-770, the amount of mature ΔF508 CFTR was diminished, and instead the ΔF508 CFTR protein appeared almost exclusively as immature band B. These data suggest that chronic VX-770 treatment impeded correction of ΔF508 CFTR by VX-809. We investigated the impact of chronic VX-770 treatment on protein stability by measuring the turnover rate of corrected ΔF508 CFTR. ΔF508 CFTR was stably expressed in baby hamster kidney (BHK-21) cells and corrected with VX-809 in the presence and absence of VX-770. Rescue with VX-809 was performed at 27°C for 24 hrs because VX-770 prevented VX-809 mediated correction of ΔF508 CFTR at 37°C (Fig. 3A). After rescue, cells were shifted to 37°C and protein biosynthesis was inhibited by addition of cycloheximide. The amount of remaining mature ΔF508 CFTR was then measured after 3 and 6 hrs. The turnover rate of rescued ΔF508 CFTR band C increased and the half-life accordingly decreased by ~2.5 fold in the presence of VX-770 (Fig. 3B,C, Table S5), whereas the decrease in band B levels was not affected by the presence of VX-770 (Fig. 3B). These data clearly show that VX-770 decreased the stability, and thus increased the turnover rate, of VX-809-rescued ΔF508 CFTR.

VX-770 affects correction of ΔF508 CFTR in a dose-dependent relationship

Lower concentrations of VX-770 were chronically administered to *ΔF508/ΔF508* CF HBE cells to study the dose effect on VX-809-rescued ΔF508 CFTR (Fig. 4). To obtain an average measure of CFTR-mediated I_{SC} for the period spanning forskolin stimulation to CFTR inhibition, the area under the curve (AUC) was calculated for this interval. Dividing AUC by time yielded average CFTR ΔI_{SC} between activation and inhibition. When 1 μM VX-770 was administered chronically with VX-809, the forskolin responses of CF HBE cells were intermediate between cells treated with VX-809 alone and cells treated with 5 μM VX-770 and VX-809 (Fig. 4A,B, Table S6). There was a significant reduction in AUC in corrector-treated cells with 1 μM versus 5 μM VX-770 (P =0.0049). Although there was not a significant difference in AUC of VX-809-treated cells with 50 nM VX-770 versus VX-809 alone (Fig. 4B), this low dose of VX-770 caused a rapid decline of the slope after forskolin treatment (Fig 4C, Table S7). Chronic treatment with either 50 nM or 1 μM VX-770 eliminated responses to acute VX-770 (Fig. 4A,C). Western blots to detect mature band C protein in CF HBE cells confirmed that VX-809 rescue was inhibited by VX-770 in a dose-dependent manner. As the concentration of VX-770 increased, the amount of VX-809-corrected ΔF508 CFTR decreased (Fig. 4D, E, Table S8).

Chronic VX-770 treatment decreases function of normal (wild-type) CFTR

To investigate the effects of chronic VX-770 treatment on normal (NL) CFTR, we measured anion secretion of NL primary HBE cultures treated with VX-770 for 48 hrs (Fig. 5). Strikingly, administration of 5 μM VX-770 reduced CFTR-mediated Cl^- secretion, as reflected by decreased forskolin responses (Fig. 5A,B,C, Table S9) and decreased inhibition of CFTR-mediated current by $CFTR_{inh}$-172 (Fig. 5A,D). These functional responses were paralleled by a decrease in CFTR band C (Fig. 5E). In cells chronically treated with VX-770, we also observed a consistent reduction in amiloride-sensitive current (Fig. S3A, Table S2) and a substantial inhibition of UTP-sensitive current (Fig. S3B, Table S2) similar to that observed in CF HBE cells (Fig. S1A,C,D, Table S2).

Chronic treatment with VX-770 does not alter HBE cell integrity or barrier functions

As a test for the specificity of chronic VX-770 effects, we analyzed whether fundamental epithelial parameters were altered in HBE cultures chronically treated with VX-770 (Fig. 6). The morphology of highly differentiated ciliated HBE cultures was identical in cells treated with vehicle (DMSO) or VX-770 (Fig. 6A). Transepithelial resistance (Rt) of primary HBE cultures was also not affected by chronic VX-770 exposure (Fig. 6B, Table S10). The inhibition by VX-770 of both Na^+ absorption (amiloride-sensitive I_{SC}) and Cl^- secretion and currents (CFTR- and CaCC-mediated I_{SC}) raised the possibility that driving forces for ion transport, in part generated by Na^+/K^+ ATPase activity, were perturbed by VX-770. Nystatin, a polyene antibiotic that enables monovalent cations to permeate biological membranes and raise Na^+/K^+ ATPase activity, did not produce significantly different I_{SC} responses when applied to vehicle- or VX-770-treated HBE cultures (Fig. 6C, Table S10), suggesting intact Na^+/K^+ ATPase activity (37). Measurements of intracellular concentrations of VX-809 and VX-770 by mass spectrometry confirmed the presence of these compounds in treated HBE cultures and indicated that cellular VX-809 concentrations were not affected by the presence of VX-770 (Fig. S4, Table S11).

Destabilization by VX-770 is beneficial for G551D, but not for wild-type, or ΔF508 CFTR function

A high C:B band ratio indicates normal CFTR protein maturation. Biochemical analysis by Western blotting showed that chronic VX-770 treatment dramatically reduced the amount of mature CFTR in both NL cultures expressing wild-type CFTR (Figs. 5E and 7A) and VX-809-rescued ΔF508 CFTR in CF HBE cells (Figs. 3A and 4E). Indeed, the C:B band ratio decreased with chronic VX-770 exposure by more than 50% in NL cultures (Fig. 7B, NL, Table S12). In contrast, the levels of mature (band C) G551D CFTR detected in *G551D/ΔF508* CF HBE cultures were not significantly diminished by chronic exposure to 5 μM VX-770, suggesting that G551D was resistant to the destabilizing effects of VX-770 (Fig 7A, G551D/ΔF508).

To explore the relationship between VX-770, VX-809, and CFTR protein stability, we performed calculations of thermodynamic stability of CFTR protein utilizing a structural homology model (38). This model revealed that CFTR amino acid F508 is located at the nucleotide binding domain 1- cytoplasmic loop 4 (NBD1-CL4) interface (Fig. 7C), and therefore participates in important interdomain interactions. Thus, in ΔF508 CFTR, the deletion of amino acid F508 not only reduces the stability of the NBD1 domain, but importantly, may destabilize multidomain assembly of CFTR (39–41). In contrast, in this structural model of CFTR, amino acid G551 is positioned between the 2 NBDs (Figs. 7C, S5), and the *G551D* mutation is thought to contort NBD dimer formation and abolish ATP-dependent channel opening by disrupting the signature sequence in NBD1 (42). To evaluate whether the *G551D* mutation also affects the overall stability of CFTR by inducing conformational restructuring of the protein, we computationally estimated the ΔΔG for G551D CFTR (43, 44). We found that *G551D* had a stabilizing effect on the CFTR protein (ΔΔG = -8.1 kcal/mol).

Discussion

Potentiator compounds act on mutant CFTR channels that are on the surface of epithelial cells. VX-770 has been approved as a pharmacological agent to treat CF patients with at least one copy of the *G551D* mutation. However, the most common mutant protein in CF patients, ΔF508 CFTR, is not found at the cell surface. ΔF508 CFTR has a folding defect and is retained in the ER but can be partially rescued by corrector compounds that promote delivery of a small proportion of mutant ΔF508 proteins to the cell surface. Corrector-rescued ΔF508 CFTR is reported to have a shorter half-life at the cell surface (45–49) and exhibits increased thermal inactivation as well as a gating defect when compared to WT CFTR (47, 50–55).

The efficacy of orally administered VX-770 was established in clinical trials in *G551D* CF patients by multiple outcome measurements (30, 56–58). The clinical benefit of potentiation of G551D function was predicted from the effectiveness of acute administration of VX-770 in Ussing chambers, which measured rates of Cl^- secretion across primary *G551D/ΔF508*

CFTR airway epithelial cultures (7). Our studies confirmed that acute VX-770 administration restored CFTR Cl^- secretion activity in HBE cells from patients carrying the *G551D* mutation. Further, our data demonstrated that *G551D/ΔF508* cultures chronically treated with VX-770 also exhibited increased Cl^- secretion via G551D CFTR, but stimulation with forskolin, which raises intracellular cAMP, was required to activate Cl^- secretion. This result could suggest a benefit from administering cAMP-raising β2-adrenergic receptor agonists as a routine part of the treatment for *G551D* CF patients receiving VX-770. Overall, improvement in G551D CFTR activity with acute VX-770 *in vitro* was also observed with chronic *in vitro* VX-770 administration.

VX-809 appears to restore approximately 15% of normal function in *ΔF508/ΔF508* CF HBE cells (8). However, 10–25% of CFTR function is estimated to be required to overcome CF symptoms (28). Therefore, a combination of VX-809 with a potentiator compound to further enhance ΔF508 function may be necessary. Although acute treatment with VX-770 has been reported to enhance VX-809-rescued ΔF508 activity (8), our data revealed that chronic application of VX-770 in combination with VX-809 or VX-661 did not. Chronic co-administration of VX-770 with either corrector to *ΔF508/ΔF508* CF HBE cultures produced Cl^- secretory responses that were smaller than responses to corrector alone. Our data suggest that the reduced capacity for Cl^- secretion after chronic VX-770/VX-809 exposure reflected an increased turnover rate of corrected ΔF508 CFTR. The VX-770-induced reduction of ΔF508 correction observed in primary CF HBE cells was dose-dependent as measured by functional and biochemical approaches. We did not detect alterations in physiological properties of HBE cells that would suggest that toxic effects contributed to CFTR dysfunction after chronic VX-770/VX-809 treatment. Thus, our studies suggest that data describing the effectiveness of acute addition of VX-770 to VX-809-treated *ΔF508* CF HBE cells do not predict the outcome for chronic VX-770/VX-809 administration.

ΔF508 CFTR has been shown to exhibit an increased thermodynamic instability of NBD1 (51, 52, 59) and improper assembly of NBD1 into a complex with intracellular loop 4 (ICL4) of the second membrane-spanning domain (MSD2) (38). The recently published data on CFTR domain fragments strongly suggest that VX-809 targets MSD1 of CFTR to suppress folding defects of ΔF508 CFTR by enhancing interactions among NBD1, MSD1 and MSD2 (60, 61). Thus, VX-809 is predicted to enhance function of ΔF508 by increasing its stability (Fig. 7D). Importantly, chronic exposure to VX-770 appeared to reverse the stabilization effect of VX-809 in ΔF508 CFTR. Chronic VX-770 treatment resulted in a severe reduction in rescued ΔF508 CFTR protein due to destabilization of rescued protein as reflected by a 2.5× increase in turnover rate. In contrast, G551D CFTR was more resistant to destabilization and loss of mature CFTR protein with chronic VX-770 exposure than WT or ΔF508 CFTR.

Our CFTR computational structural model (38) allows us to speculate how VX-770 may interact with WT or mutant CFTR proteins to alter protein stability. CFTR requires conformational flexibility to function properly (39, 50). The flexibility and stability of the CFTR protein is finely tuned and precisely balanced, which is a requirement for its ability to function properly as a regulated ion channel. The inherent increase in stability of the G551D protein may render it too rigid and inflexible (stable) for proper channel opening under basal cAMP-stimulated conditions. However, a decrease in stability mediated by VX-770 may render the G551D molecule more flexible and allow it to function as a cAMP-regulated Cl^- channel (Fig. 7D). In contrast, destabilization of WT (ideal stability) or VX-809-rescued ΔF508 CFTR (low stability) by VX-770 bound to the CFTR molecule resulted in decreased WT CFTR function and absent ΔF508 CFTR function. These considerations reveal that chemical correction of low stability CFTR mutants is complicated and necessitates precision.

Some airway diseases such as chronic obstructive pulmonary disease (COPD) have been associated with lessened CFTR function and consequently VX-770 has been suggested as a potential therapy (34). In a study that modeled the effects of VX-770 on COPD patients, reduction of CFTR function was achieved by exposing primary HBE cultures to cigarette smoke extract, and these cultures were subsequently exposed to acute administration of VX-770, which led to augmented CFTR-mediated currents (34). The finding that acute VX-770 treatment enhanced WT CFTR function contrasts with our finding that chronic treatment with VX-770 reduced WT CFTR function. In addition, forskolin-stimulated I_{SC} in NL HBE cultures chronically treated with VX-770 was not stable over time, as indicated by the downward sloping trace. These functional data, coupled with our observations that the amount of mature WT CFTR was reduced by the continuous presence of VX-770, do not favor VX-770 as a therapy to enhance CFTR function in COPD.

Although potentiation of more rare CFTR mutants with partial defects in CFTR processing was recently detected upon acute VX-770 treatment in Fisher rat thyroid (FRT) cells overexpressing these variants (62), our data raise the concern that potentiation with chronic VX-770 treatment may not be observed in airway epithelia expressing these rare mutations. To optimize combination therapy for both ΔF508 and rare CFTR processing mutations, minimizing interference of potentiator with corrector activity is required. One approach may be to finely tune the dosing regimens for potentiator compounds. Studies by Van Goor et al. (7) indicate that the EC_{50} for acute application of VX-770 differs remarkably in *G551D/ΔF508* (EC_{50}: 236 ± 200 nM) and *ΔF508/ΔF508* (EC_{50}: 22 ± 10 nM) HBE cultures. These data together with our findings, which demonstrate that inhibition of correction by VX-770 is dose dependent, suggest that drug concentrations are very critical and attempts to optimize potentiator activity on channel function while minimally affecting turnover rate of mutant CFTR should be considered. As a second approach, improved potentiator compounds that do not interfere with ΔF508 CFTR correction and turnover are needed.

After chronic VX-770 treatment, we also observed diminished Cl^- secretory responses to additions of the P_2Y_2 receptor agonist, UTP. UTP-stimulated Cl^- secretion is elevated in CF airway epithelia and may compensate for the lack of CFTR function *in vivo* (63–65). Reduction of UTP-stimulated CaCC activity may constitute a disadvantage in CF airways, particularly if insufficient amounts of CFTR have been rescued. Thus, monitoring CaCC activity may be useful in future clinical corrector/potentiator studies.

We observed a decrease in amiloride responses in the presence of chronic VX-770. While the effects in CF cells can be explained by the restoration of CFTR inhibiting activity (36), the inhibition of ENaC-mediated Na^+ absorption in NL HBE cells raises the possibility that

VX-770 may have off-target effects. Thus, it may be useful to measure ENaC function in future VX-770 clinical trials. Although a decline in ENaC function may be beneficial for CF patients, diminution of Cl^- channels other than CFTR may be a disadvantage for maintaining adequate hydration of CF airways. The recent observation that VX-770 has antimicrobial properties *in vitro* suggests that it may also display off-target effects *in vivo* (66).

A limitation of our studies is that our experiments were performed in primary HBE cultures and not *in vivo*. However, primary HBE cultures are a well-established, near-physiologic system that is the most relevant model for studying CFTR function in airway epithelia, the tissue most affected by CF. Drug concentrations, turnover, and formation of metabolites may also differ *in vitro* and *in vivo*, which are crucial parameters to consider when extrapolating our data to the clinic. We therefore selected *in vitro* doses that mimicked clinically measured drug concentrations. For example, 5-day VX-770 treatments with 150 mg or 450 mg (administered as one dose/day) in patients resulted in VX-770 concentrations in blood plasma of 1.4 μg/ml and 5.5 μg/ml, respectively (67), which are equivalent to ~3.5 μM and ~14 μM. Current clinical trials test VX-770 doses in this range (250 mg taken twice per day). Thus, the concentrations tested in our studies *in vitro* appear relevant to the clinical experience.

We measured intracellular concentrations of VX-809 and VX-770, which revealed that these compounds (particularly VX-770) accumulated in cells and reached much higher concentrations than in the surrounding media. To determine whether the presence of VX-770 might have a negative impact on the intracellular concentration of VX-809, we obtained measurements of VX-809 concentrations in cell lysates with increasing concentrations of VX-770 and observed that the intracellular concentration of VX-809 was not affected by the presence of VX-770. Thus, comparisons of drug concentrations from *in vitro* and *in vivo* tissues may be useful in the future.

Because there are no corrector compounds available that provide sufficient rescue of ΔF508 in CF airways *in vivo* to alleviate symptoms of CF, potentiation of the small amount of corrected ΔF508 CFTR is required. However, combination approaches to restore ΔF508 CFTR function in CF to date have not considered drug-drug interactions of clinically relevant co-administered modulator compounds. Based on our study and the confirmatory data of Veit et al. (68), knowledge of the interactions and interference between corrector and potentiator compounds is essential for successful therapy of the most prevalent mutation in CF patients, most of whom carry at least one allele of the *ΔF508* mutation. Furthermore, understanding the impact that potentiator compounds, such as VX-770, have on the stability of apical WT CFTR may also be important for other airway diseases that would benefit from augmentation of CFTR function.

Materials and Methods

Study design

Simultaneous treatment with small-molecular compounds, VX-809 or VX-661, together with VX-770 (Selleck Chemicals), is currently being examined as therapy for CF patients with the mutation, Δ*F508*. Although *in vitro* studies examining acute treatment with this drug combination have been conducted, we sought to determine the impact of chronic treatment (48 hrs) with these compounds in primary human bronchial epithelial (HBE) cells. Primary HBE cells from normal (NL) or CF patients were obtained from bronchi of human lung tissue, as previously described (69, 70). To evaluate CFTR function HBE cultures chronically treated with compounds were mounted in Ussing chambers to measure short-circuit currents (I_{SC}) (65, 71, 72). We examined differentiated primary CF HBE cells from at least 3 individuals for each genotype. To visualize the amount of mature and immature CFTR protein from HBE cultures, CFTR from whole-cell lysates was immunoprecipitated as previously described (35, 73) and Western blots were performed. Previously created baby hamster kidney (BHK-21) stably expressing ΔF508 CFTR (48), were used in cycloheximide chase studies to examine the rate of protein turnover.

Cell culture

Primary HBE cells were obtained from bronchi of human lung tissue (69, 70) under a protocol approved by the University of North Carolina Medical School Institutional Review Board. Primary NL and CF HBE cells were seeded at passage 2 on collagen-coated Millicell CM inserts (Millipore) and maintained at an air-liquid interface (ALI) at 37°C in 5% CO_2 for 3–4 weeks, which allowed the cells to become fully differentiated.

BHK-21 cells were obtained from the American Type Culture Collection and grown at 37°C in 5% CO_2. BHK-21 cells stably expressing Extope-ΔF508 CFTR were created previously and maintained as described (48).

Immunoprecipitation and Western blotting

Whole-cell lysates were prepared as described previously (74). CFTR was immunoprecipitated as described previously (35, 73) and isolated using Protein A/G PLUS agarose (Santa Cruz Biotechnology). Samples were separated on 4–20% gradient SDS-PAGE gels (Bio-Rad) and then transferred to nitrocellulose. Blots were probed with mouse monoclonal anti-CFTR antibodies and then with IR Dye 680-goat anti-mouse IgG (Molecular Probes). Anti-actin (Cell Signaling) or anti-tubulin (LI-COR) was used as a loading control. Protein bands were visualized using an Odyssey Infrared Imaging System (LI-COR).

Cycloheximide chase to study turnover of rescued ΔF508 CFTR

BHK-21 cells expressing Extope-ΔF508 CFTR were pretreated with compounds (VX-809, 5 μM; VX-770, 5 μM) for 24 hrs at 27°C before treatment with cycloheximide (200 μg/ml; Sigma) in the presence of compounds during chase times at 37°C. Whole-cell lysates were prepared and subjected to Western blotting.

Histology and microscopy

Primary HBE cultures grown at ALI on Millicell inserts were washed in PBS and fixed in 10% neutral buffered formalin prior to being embedded in paraffin and hematoxylin-eosin stained at the UNC CF Histology Core. Slides were viewed on a Leica DMIRB Inverted Microscope with a 40× 1.0 numerical aperture oil objective.

I_{SC} Measurements in Ussing chambers

In Ussing chambers (Physiological Instruments) HBE cultures were equilibrated to 37°C in a bilateral bath of Krebs-bicarbonate-Ringer buffer (KBR; in mM: 140 Na^+, 120 Cl^-, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 2.4 HPO_4^{2-}, 0.4 $H_2PO_4^-$, 25 HCO_3^-, and 5 glucose, pH 7.4) and circulated with 95% O_2-5% CO_2. Short-circuit currents (I_{SC}) were measured as previously described (65, 71, 72). All NL HBE cultures were measured in bilateral KBR. Unless noted otherwise, CF HBE cultures were measured with high potassium, low chloride (HKLC) buffer applied apically and KBR applied basolaterally, creating a Cl^- gradient (5 mM/120 mM). Amiloride (100 μM) was added to block ENaC, followed by forskolin (10 μM), VX-770 (5 μM) and, if applicable, genistein (10 μM) to stimulate CFTR. CFTR was then inhibited with $CFTR_{inh}$-172 (10 μM) and response to UTP (100 μM) was examined. In some experiments, nystatin (40 μM, apical) was added at the end of the measurements. Data were analyzed using Acquire and Analysis (version 2.3) software (Physiologic Instruments). I_{SC} traces were imported to and processed in Origin 9.0.0. (OriginLab Corporation).

Detection of VX-770 and VX-809

Mass spectrometric (MS) methods were developed to detect VX-770 and VX-809 using strategies similar to those previously described (75, 76). VX-770 was detected by monitoring transition of parent to daughter ion of m/z 393.3→171.1 in positive mode MS, with VX-809 detected by monitoring the transition m/z 453.3→197.1. Each compound generated a single peak using previously described liquid-chromatography-tandem MS (LC-MS/MS) methods (75, 76), with run times of 11.1 and 10.8 minutes, respectively. To quantify drug concentrations in epithelial cells, cell lysates were extracted × 2 with equal volume of MTBE (Sigma), which was then lyophilized to dryness under vacuum centrifugation. Lyophilized samples were resuspended in a volume of 20% methanol in water equal to the original lysate volume, extracted, and 5 μl analyzed by LC-MS/MS as above. To control for matrix effects and variable recovery during extraction, untreated lysates were spiked with known concentrations of VX-770 and VX-809 and extracted in parallel. Concentrations in cell lysates were assessed by examining signal relative to the spiked samples.

Computational stability calculation

We computationally estimated the ΔΔG of mutation for *G551D* mutant CFTR using the Eris suite as described previously (43, 44). Eris algorithms re-pack the side chains and evaluate the new free energy according to a physical force field upon the substitution of the relevant residue.

Statistical analysis

Results are presented as means of average response per primary HBE cell donor and error bars are the standard error of the mean (SEM). Statistical analysis was performed by an unpaired two-tailed Student's *t* test in GraphPad Prism version 6.02 (GraphPad Software). *P* values of < 0.05 were considered to indicate statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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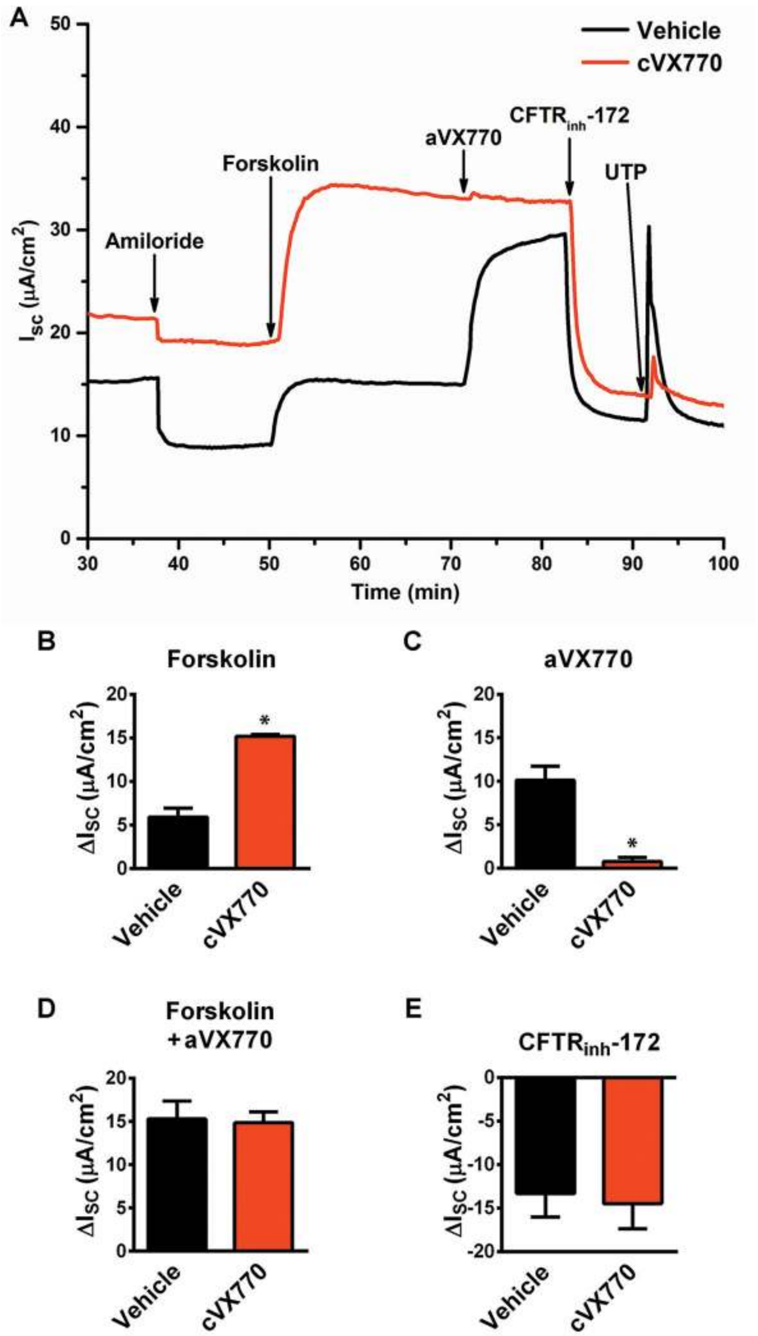

Figure 1. VX-770 treatment restores G551D function

Electrophysiological properties of *G551D/ΔF508* cultures analyzed in Ussing chambers treated chronically with VX-770 (cVX770, 5 μM for 48 hrs) or with vehicle (0.1% DMSO). (**A**) Representative recording of I_{SC} measured in Ussing chambers. Quantification of response to treatment with (**B**) forskolin (significant difference between vehicle and cVX770, $*P = 0.0009$), (**C**) acute VX-770 (aVX770) (significant difference between vehicle and cVX770, $*P = 0.0054$), (**D**) forskolin + aVX770, (**E**) CFTR$_{inh}$-172. Primary CF HBE cultures (*G551D/ΔF508*) were derived from 3 different patients, 3–4 replicates were performed per patient for a total of 10 measurements per treatment.

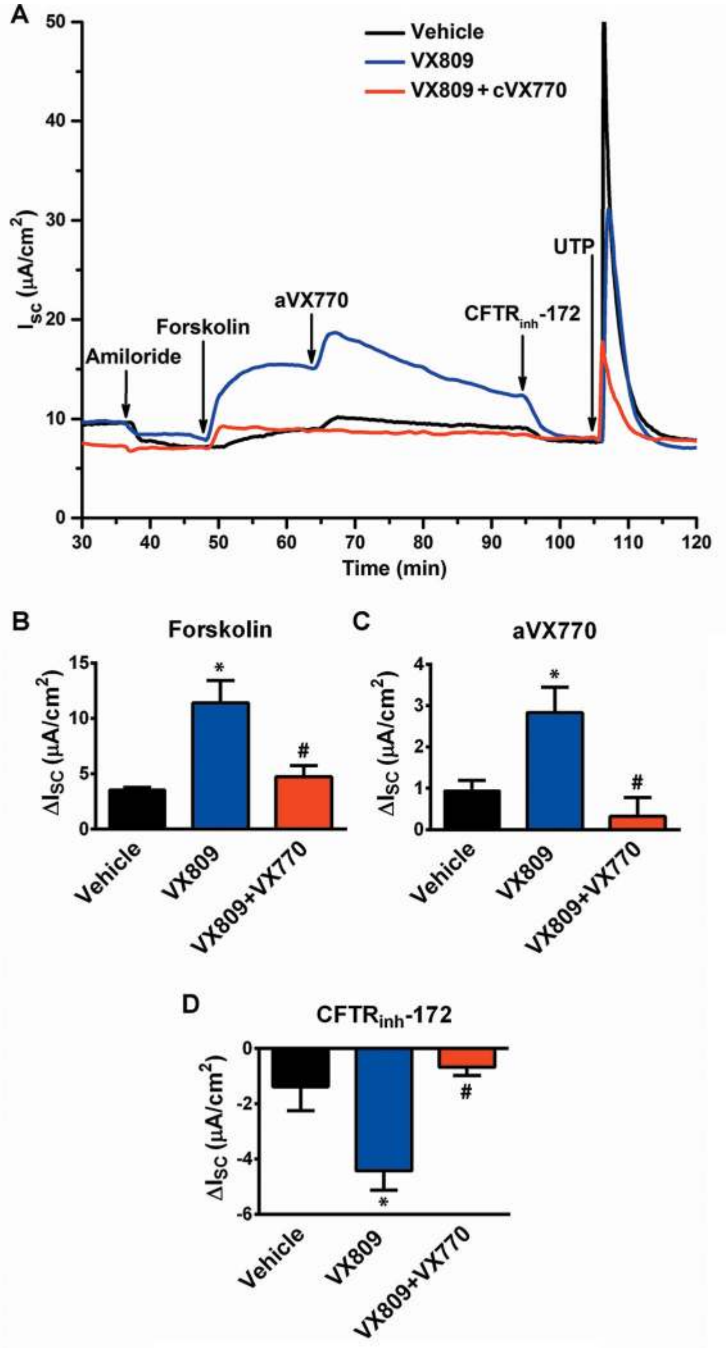

Figure 2. Chronic VX-770 treatment inhibits functional rescue of ΔF508

(**A**) Representative I_{SC} traces of CF HBE cells recorded in Ussing chambers. Primary CF HBE cells (*ΔF508/ΔF508*) were treated with vehicle (DMSO) or VX-809 +/−VX-770 for 48 hrs at 5 μM each. (**B**) ΔI_{SC} response to forskolin observed in VX-809-treated CF HBE cells (*$P = 0.0033$, VX809 vs. vehicle) was prevented by chronic VX-770 treatment and significantly different from VX-809-treated cells (#$P = 0.0147$, VX809 vs. VX809+VX770). (**C**) CF HBE cells treated with VX-809 responded to acute VX-770 exposure (*$P = 0.0177$, VX809 vs. vehicle). This response was significantly abrogated in

VX-809 + VX-770-treated cells (#*P* = 0.0031, VX809 vs. VX809+VX770). (**D**) The response to CFTR$_{inh}$-172 observed in VX-809-treated cells (**P* = 0.0209, VX809 vs. vehicle) was significantly decreased in VX809+VX770-treated cells (#*P* = 0.0006, VX809 vs. VX809+VX770). Primary HBE cultures (Δ*F508/*Δ*F508*) were derived from 6 different patients, 2–4 replicates were performed per patient for a total of 15 measurements per condition.

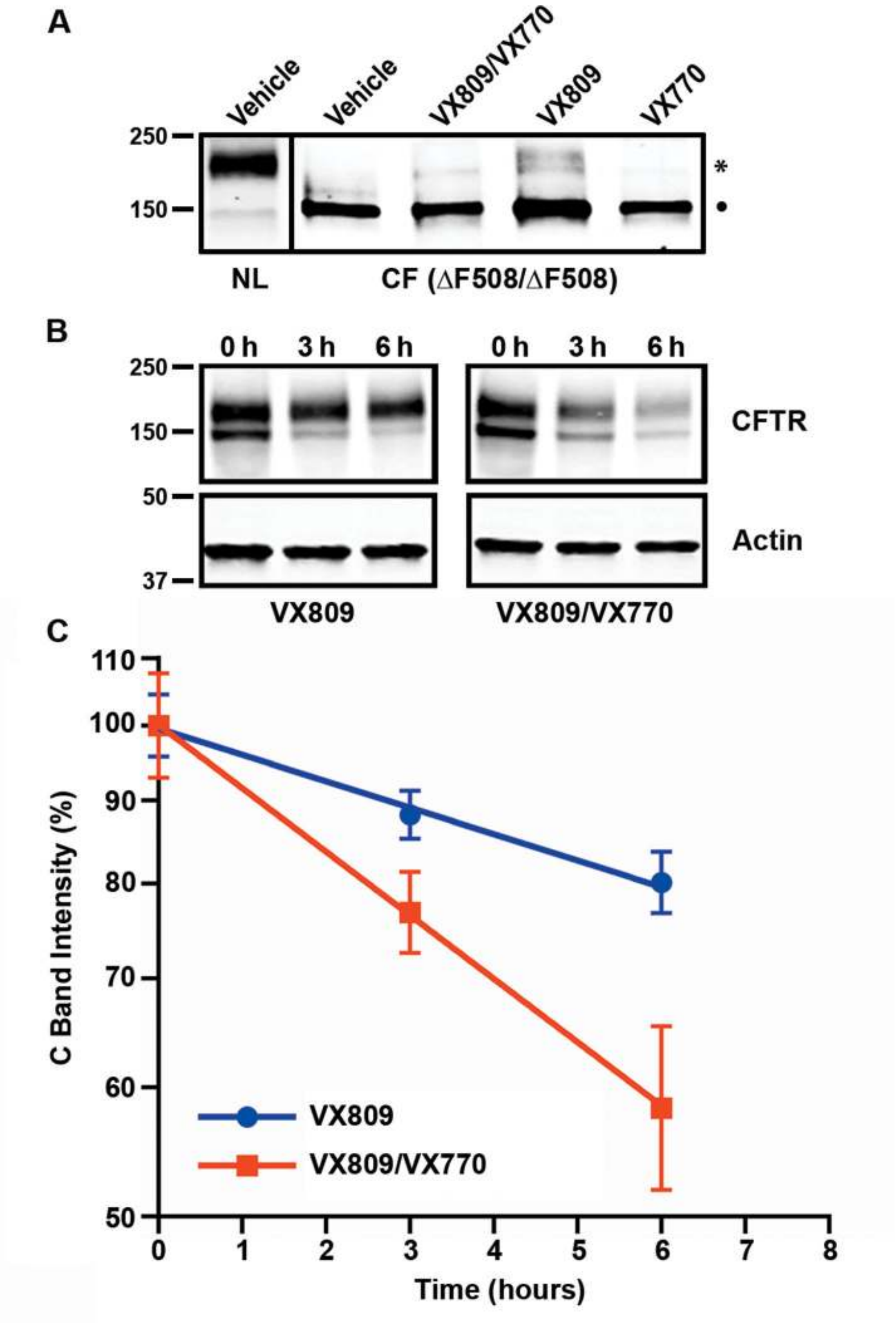

Figure 3. VX-770 diminishes biochemical correction by increasing turnover of corrected ΔF508 CFTR

(**A**) CFTR Western blot of normal (NL) and CF HBE cultures treated with VX-809 (5 μM) +/−VX-770 (5 μM) for 48 hrs. * indicates the mature, complex glycosylated form of CFTR, band C; ● indicates the immature band B. (**B**) Turnover of rescued ΔF508 in BHK-21 cells. ΔF508 was rescued at 27°C in the presence of VX-809 +/− VX-770 for 24 hrs. After adding cycloheximide (200 μg/ml, 37°C) cells were lysed at the indicated times and analyzed by

Western blotting. (**C**) Quantification of remaining band C over time, normalized to actin (n = 3).

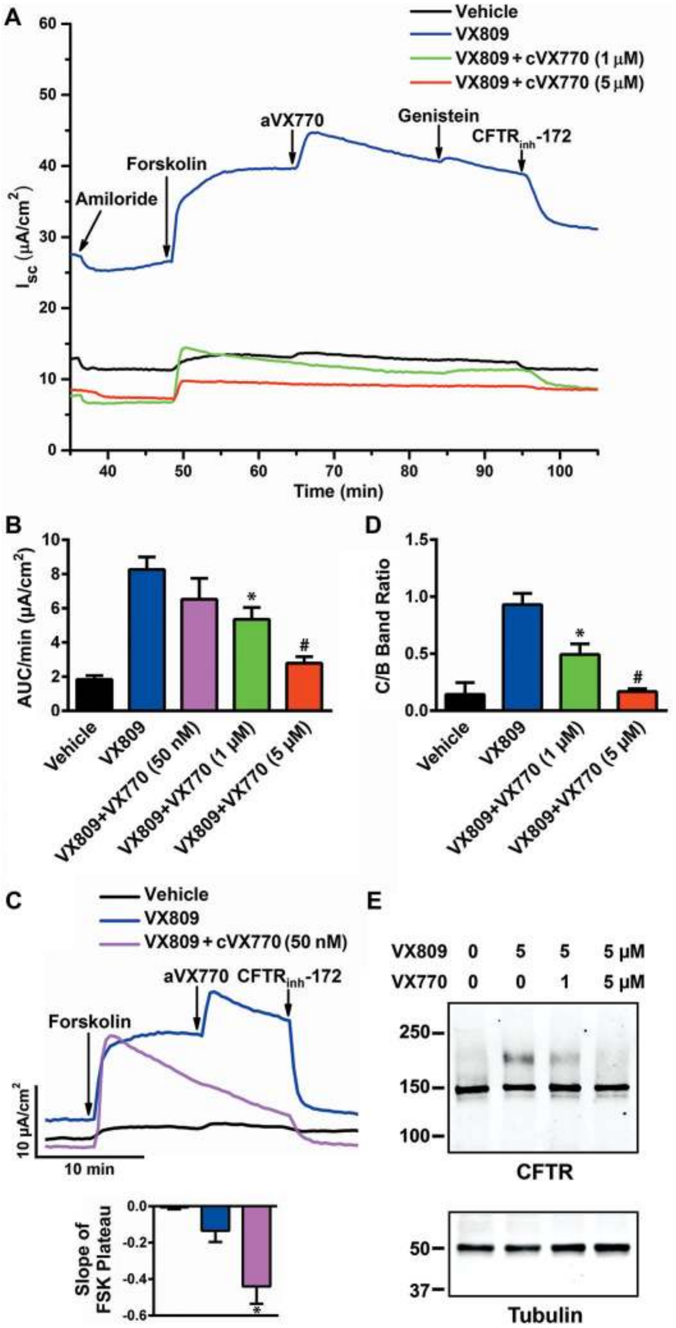

Figure 4. VX-770-induced hindrance of ΔF508 correction is dose-dependent

(**A**) I_{SC} traces of CF HBE cells *(ΔF508/ΔF508*) recorded in Ussing chambers. CF HBE cells were treated as indicated (VX-809: 5 μM, VX-770: 1 or 5 μM) for 48 hrs. (**B**) CFTR function in VX-809-treated cells decreased as chronic VX-770 concentrations increased. Significant reduction of the area under the curve (AUC)/min calculated from the time period between CFTR stimulation by forskolin and CFTR inhibition by $CFTR_{inh}$-172 (yields average ΔI_{sc} ($\mu A/cm^2$)) was observed in CF cells chronically treated for 48 hrs with VX-809 when compared to VX-809 and 1 μM VX-770, (*$P = 0.0352$). A further reduction was detected when the chronic VX-770 concentration was increased to 5 μM (#*P* = 0.0049, VX-809 + 1 μM VX-770 vs. VX-809 + 5 μM VX-770). Primary CF HBE cultures were derived from at least 4 different CF patients; 2–5 replicates were performed per patient for a total of at least 14 measurements per condition. (**C**) In VX-809-corrected CF HBE cultures (Δ*F508*/Δ*F508*), the presence of chronic VX-770 at 50 nM caused a significant decline of the slope after forskolin treatment (**P* = 0.0353, VX-809 vs. VX-809 + 50 nM VX-770). Primary CF HBE cultures were derived from 4 different patients; 3–5 replicates were performed per patient for a total of at least 15 measurements per condition. (**D**) Quantification of C:B band ratio in CF HBE cultures (Δ*F508*/Δ*F508*). CFTR C:B band ratio decreased in CF HBE cells as chronic VX-770 concentrations were increased. The C:B band ratio was significantly reduced in CF cells chronically treated for 48 hrs with VX-809 and 1 μM VX-770 compared to VX-809 alone (**P* = 0.0181), and a further reduction was detected when the chronic VX-770 concentration was increased from 1 μM to 5 μM (#*P* = 0.0151, VX-809 + 1 μM VX-770 vs. VX-809 + 5 μM VX-770). Primary CF HBE cultures (Δ*F508*/Δ*F508*) from 4 different patients were analyzed. (**E**) Representative Western blot of CF HBE cells (Δ*F508*/Δ*F508*) showing decrease of VX-809-corrected ΔF508 as chronic VX-770 concentrations were increased.

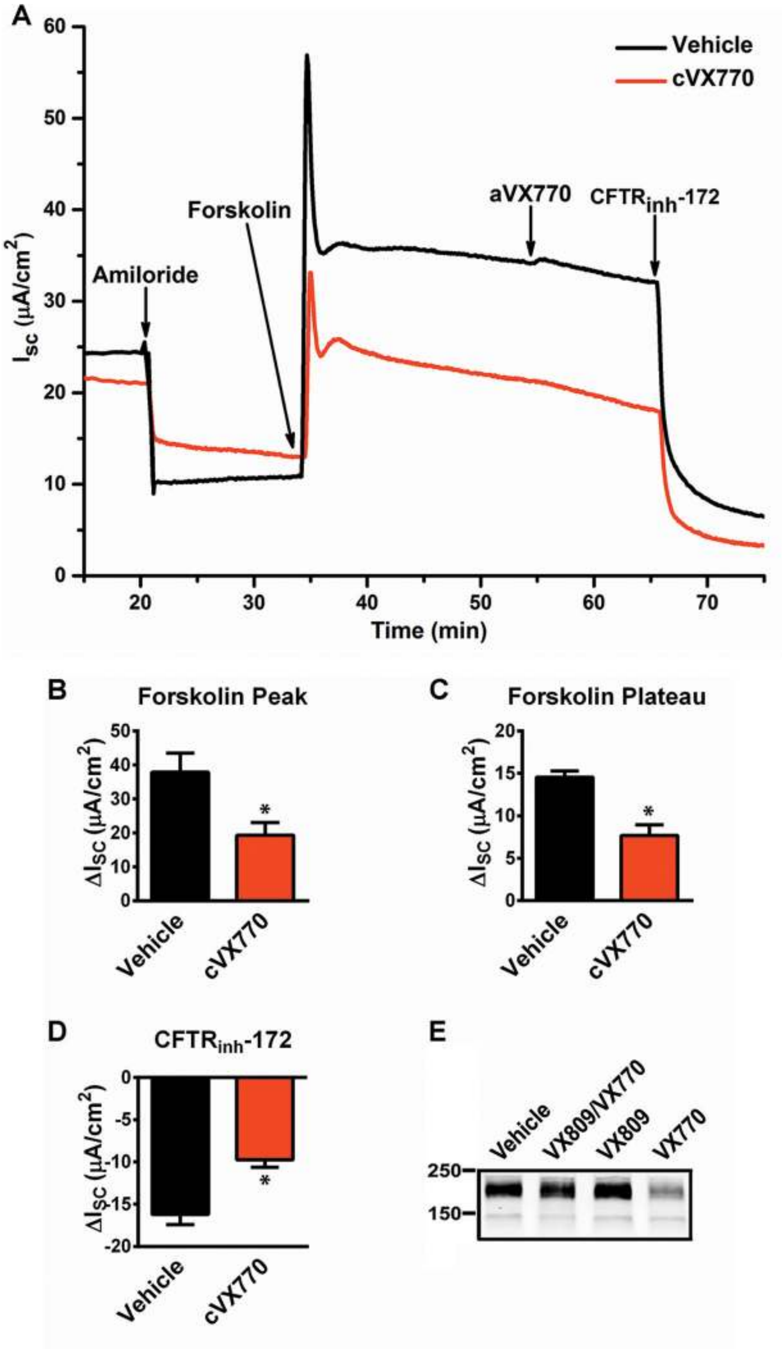

Figure 5. Chronic VX-770 treatment decreases function of wild-type CFTR

(**A**) Representative I_{SC} traces of NL HBE cells recorded in Ussing chambers. Cultures were treated with vehicle (DMSO) or 5 μM VX-770 for 48 hrs. HBE cells that were chronically treated with VX-770 showed significantly reduced response to (**B,C**) forskolin (*P = 0.0198 for forskolin peak and *P = 0.0008 for forskolin plateau) and (**D**) CFTR$_{inh}$-172 (*P = 0.0014). Primary HBE cultures were derived from 6 different individuals, 2–4 replicates were performed per individual for a total of 17 measurements per condition. (**E**) Western blot of HBE cultures treated with VX-809 (5 μM) +/−VX-770 (5 μM) for 48 hrs. Mature CFTR was diminished in HBE cells that were chronically treated with VX770.

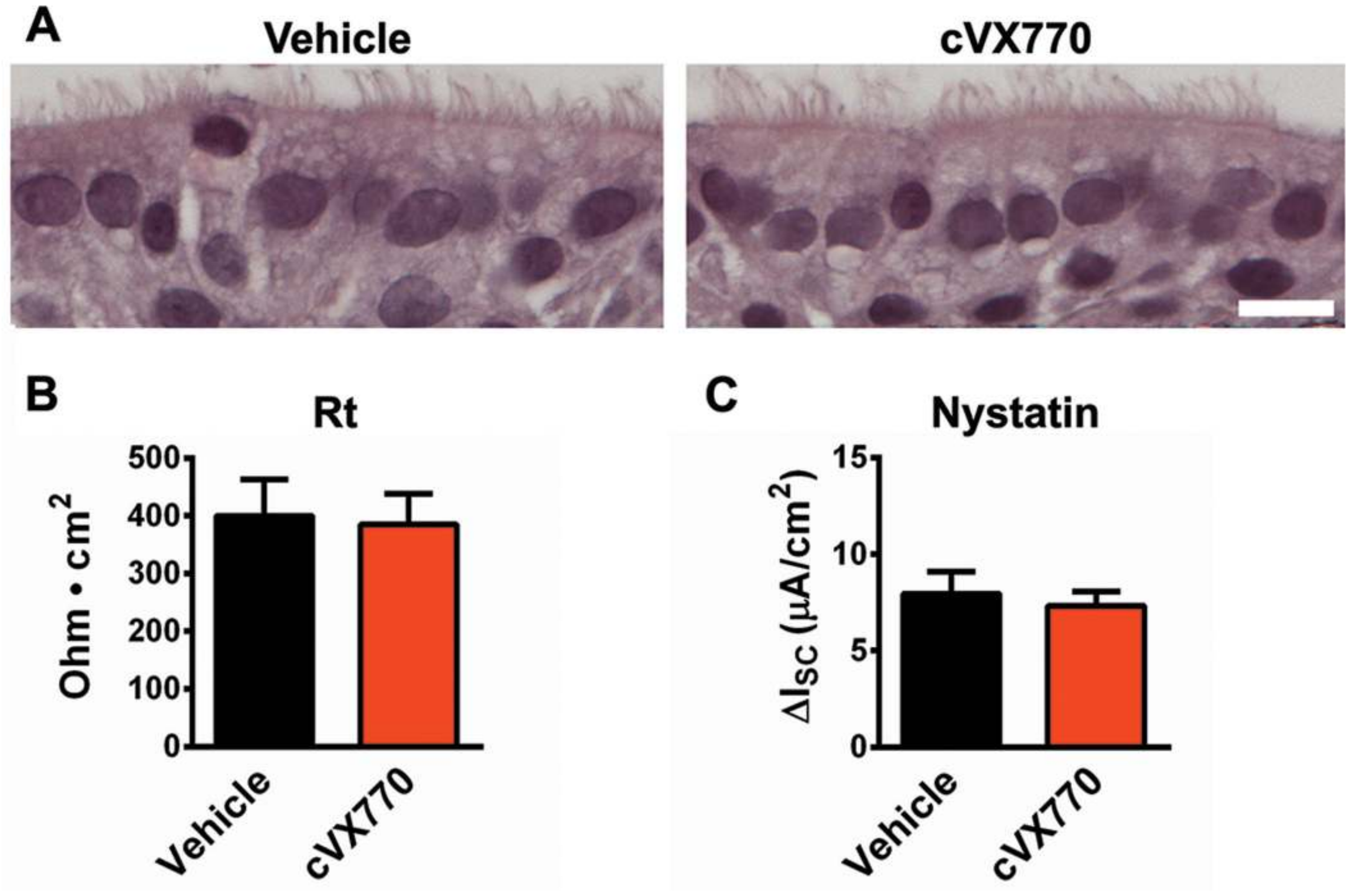

Figure 6. Key physiological properties were not altered in chronically VX-770-treated HBE cultures

(**A**) Microscopy after hematoxylin and eosin (H&E) staining of HBE cultures did not reveal a detectable difference between VX-770- or vehicle-treated cells (bar = 10 m). (**B**) Transepithelial resistance (Rt) of primary HBE cultures was not altered after chronic treatment with VX-770. (**C**) Nystatin responses were not significantly different in primary HBE cultures that were treated with vehicle or VX-770 (48 hrs, 5 μM). Nystatin was added to the apical side in Ussing chambers. Primary HBE cultures were derived from 5 different individuals, and 2–4 replicates per individual were performed.

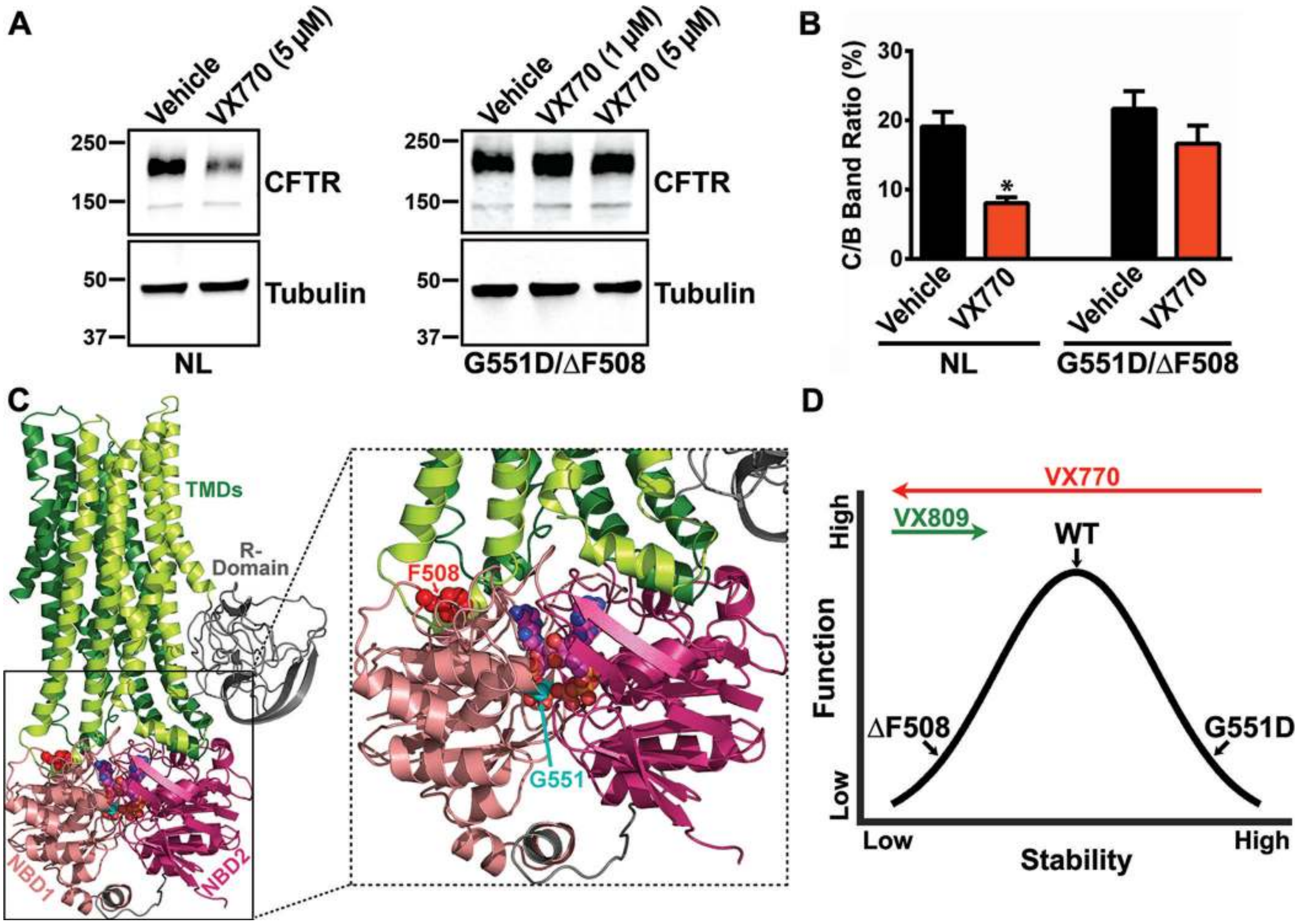

Figure 7. VX-770 reduces stability of CFTR

(**A**) The amount of mature CFTR was reduced when NL HBE cells were chronically treated with VX-770 (48 hrs, 5 μM). G551D is more stable than NL CFTR and the amount of mature G551D protein in CF cultures (*G551D/ΔF508*) was not significantly reduced by 48 hrs treatment with 5 μM VX-770. (**B**) Quantification of C:B band ratio with chronic treatment of VX-770 at 5 μM. C:B band ratio was significantly decreased in NL cells chronically treated for 48 hrs with 5 μM VX-770, (**P* = 0.008) (n = 3, cultures from 3 different NL individuals). The reduction of C:B band ratio in *G551D/ΔF508* cells chronically treated for 48 hrs with 5 μM VX-770 was not statistically significant (n = 3, cultures from 3 different CF patients (*G551D/ΔF508*)). (**C**) Structural model showing positions of G551D and F508 in the CFTR molecule. (**D**) Illustration representing the proposed relationship between function and stability of CFTR variants. Wild-type CFTR has an intermediate stability that allows for optimal function. G551D CFTR is a more rigid protein that exhibits increased stability compared to wild-type CFTR but lacks sufficient function, presumably due to decreased flexibility. VX-770 decreases G551D CFTR stability and renders it a more flexible protein, resembling the stability and function of wild-type CFTR. However, VX-770 causes destabilization of wild-type CFTR and VX-809-corrected ΔF508 CFTR, diminishing their function. VX-809 increases the stability of ΔF508, bringing it closer to resembling wild-type CFTR, but this increased stability is diminished when VX-770 is present.